PIERCE'S DISEASE CONTROL PROGRAM

RESEARCH PROGRESS REPORTS

PIERCE'S DISEASE AND OTHER DESIGNATED PESTS AND DISEASES OF WINEGRAPES

~ DECEMBER 2019 ~

cdfa

CALIFORNIA PD/GWSS BOARD
Partnership for Winegrape Pest Solutions

CALIFORNIA DEPARTMENT OF FOOD & AGRICULTURE

Research Progress Reports

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

- December 2019 -

Compiled by:
Pierce's Disease Control Program
California Department of Food and Agriculture
Sacramento, CA 95814

Editor:
Thomas Esser, CDFA

Cover Design:
Sean Veling, CDFA

Cover Photograph:
Photo by David Köhler on Unsplash

Cite as:
Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes. December 2019. California Department of Food and Agriculture, Sacramento, CA.

Available on the Internet at:
https://www.cdfa.ca.gov/pdcp/Research.html

Acknowledgements:
Many thanks to the scientists and cooperators conducting research on Pierce's disease and other pests and diseases of winegrapes for submitting reports for inclusion in this document.

Note to Readers:
The reports in this document have not been peer reviewed.

TABLE OF CONTENTS

Section 1:
***Xylella fastidiosa* and Pierce's Disease**

REPORTS

ABSTRACTS

Section 2:
Glassy-winged Sharpshooter

REPORTS

Section 3:
Other Pests and Diseases of Winegrapes

REPORTS

Section 1:

Xylella fastidiosa

and

Pierce's Disease

ADDRESSING KNOWLEDGE GAPS IN PIERCE'S DISEASE EPIDEMIOLOGY: UNDERAPPRECIATED VECTORS, GENOTYPES, AND PATTERNS OF SPREAD

Project Leader: Rodrigo P.P. Almeida | Department of Environmental Science, Policy, and Management | University of California | Berkeley, CA 94720 | rodrigoalmeida@berkeley.edu
Co-Project Leader: Monica L. Cooper | Cooperative Extension | University of California | Napa, CA 94559 | mlycooper@ucanr.edu
Co-Project Leader: Matt Daugherty | Department of Entomology | University of California | Riverside, CA 92521 | matt.daugherty@ucr.edu
Co-Project Leader: Rhonda Smith | Cooperative Extension | University of California | Santa Rosa, CA 95403 | rhsmith@ucanr.edu

Reporting Period: The results reported here are from work conducted July 2019 to October 2019.

ABSTRACT

In this report we provide a summary of recent research activities. Due to space limitations, not all work that has been done, nor all the results that are available, are included in this report. Note that more detailed reports for each component of this project have previously been submitted to the funding agency.

LAYPERSON SUMMARY

Recent research by our group is aimed at understanding why Pierce's disease (PD) has recently reached historically high levels of prevalence in the North Coast. It is evident that traditional spatial patterns of PD distribution in vineyards continue to occur. However, there are also disease distribution patterns that do not follow expectations. Furthermore, data suggest that there are key components of PD epidemiology that may have changed over time, leading to the large losses due to PD in recent years. The goal of this project is to target three specific topics we have identified as the most urgent current knowledge gaps in PD epidemiology.

INTRODUCTION

In 2014, Sonoma County and Napa County viticulture farm advisors observed an explosion in Pierce's disease (PD) prevalence in vineyards. This regional epidemic was characterized by very large numbers of diseased plants in vineyards throughout California's North Coast, as well as by the presence of disease patterns that did not match epidemiological expectations (i.e., away from riparian zones). In 2015, this project team was awarded a grant from the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board to evaluate factors driving this epidemic. The goals of that project, and of this project, were/are driven by field observations and communications by University of California (UC) Cooperative Extension farm advisors with the industry in consultation with researchers at UC Berkeley and UC Riverside. Our ongoing research has confirmed the observations by growers and farm advisors: PD is leading to large economic losses, and novel spatial disease patterns have been observed, particularly at locations remote from riparian zones. Furthermore, we have also worked with colleagues from other counties who also observed high rates of incidence of PD in their respective regions. Three main questions emerged from our previous research.

First, what is the role of spittlebugs in PD epidemiology? We confirmed that spittlebugs are vectors of *Xylella fastidiosa* (*Xf*) to grapevines (*Vitis vinifera*), that they are present in vineyards, and that field-collected insects transmit *Xf* to grapevines. In addition, as we explored PD infections occurring in an aggregated fashion away from riparian zones, we found and identified multiple, additional species of spittlebugs that may be responsible for the spread of PD in such cases. One of our objectives is to determine the role of spittlebugs on PD epidemiology in more detail, as it appears these insects play a role in PD spread in the North Coast.

The second question is related to the current paradigm that PD "comes" from riparian zones. While it remains true that much of PD is associated with the migration of blue-green sharpshooter (*Graphocephala atropunctata*) vectors from riparian zones to vineyards in the spring, we do not know if those insects acquired *Xf* in vineyards during the previous fall, or from riparian plants during the winter/early spring. The observed increase in PD incidence in the North Coast appears to have developed over years, was not exclusively driven by climate change, and does not fit with the current epidemiological hypothesis, in which primary pathogen spread from riparian zones occurs. An alternate possibility is that spread of PD in the North Coast is dominated by secondary rather than primary spread, that is, spread from grapevine to grapevine, but with a between-year component where vectors overwinter as adults in riparian zones. Because this alternative hypothesis would lead to substantial changes to PD management strategies, we propose to first perform a mathematical modeling exercise exploring the conditions that would result in observed patterns of disease spread.

The third question that resulted from our ongoing research was whether the current PD epidemic was driven by the emergence of a particularly virulent *Xf* genotype. To address this question, we obtained *Xf* isolates from different grape-growing regions in California and sequenced their genomes. Thus far, the results are not consistent with an emerging genotype sweeping through the North Coast. In fact, results demonstrate precisely the opposite; *Xf* from Napa County is distinct from *Xf* from Sonoma County, and from the Temecula Valley, and so on. Our goal in this objective is to determine why different populations of *Xf* exist in different grape-growing regions, what makes these *Xf* populations different, and importantly, if they are biologically distinct. Concurrently, we have inoculated mature commercially-grown grapevines of three varieties in Napa Valley, and we are following disease symptoms and *Xf* genotypic changes in these plants. In these field plots, infected vines developed shriveled clusters just a few months after infection, whereas symptomatic leaves are taking much longer to develop. These findings question our current understanding of disease development after infections, which was believed to start first with leaf symptoms and later with development of shriveled clusters, something we are particularly concerned about as it impacts PD management strategies.

OBJECTIVES

This research project has three objectives, which were identified by stakeholders as pressing issues that need to be addressed to improve our understanding of PD epidemiology:

1. Role of spittlebugs in PD epidemiology.
2. Mathematical modeling of PD spread.
3. *Xf* population genomics.

RESULTS AND DISCUSSION

Objective 1. Role of Spittlebugs in PD Epidemiology

A range of work has been done on spittlebugs as vectors of *Xf*. Here we present some results focusing on a group of poorly characterized vector species whose presence is correlated with areas where PD is present in the absence of detectable blue-green sharpshooters (*Graphocephala atropunctata*; BGSS). To elucidate the life cycle of *Aphrophora* spittlebugs, we have focused on identifying the oviposition and nymphal development hosts during late winter and early spring. We identified four field sites in each county (Napa and Sonoma), and soon after egg hatch marked up to 40 host plants at each monitoring site. Host plants were identified by searching for the presence of spittle masses, which are created by nymphs as they are feeding. On a weekly basis we are characterizing the number of nymphs per host plant and the life stage of each nymph (**Figures 1** and **2**). This will allow us to describe not only which plants are potential hosts, but also give us a measure of host preference by species.

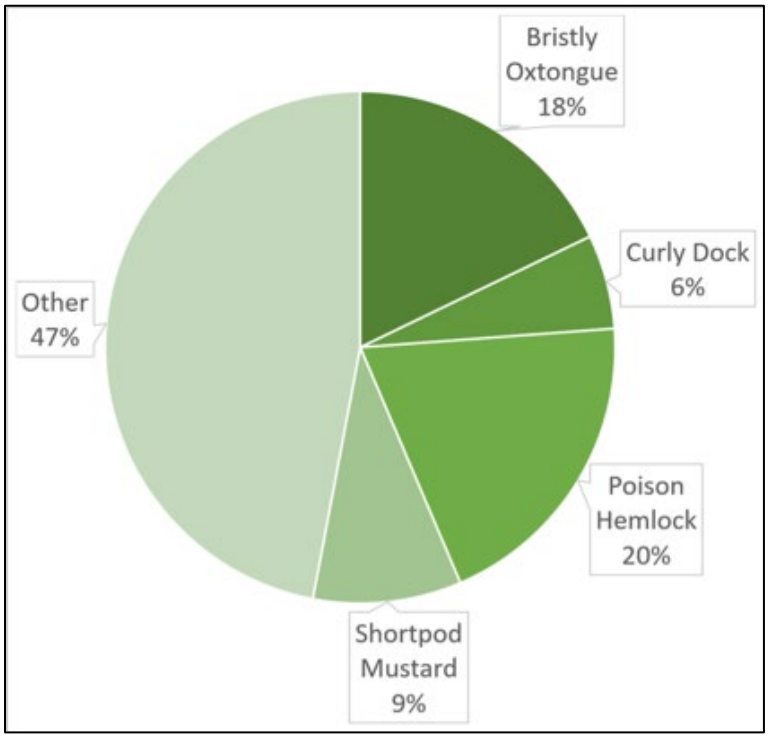
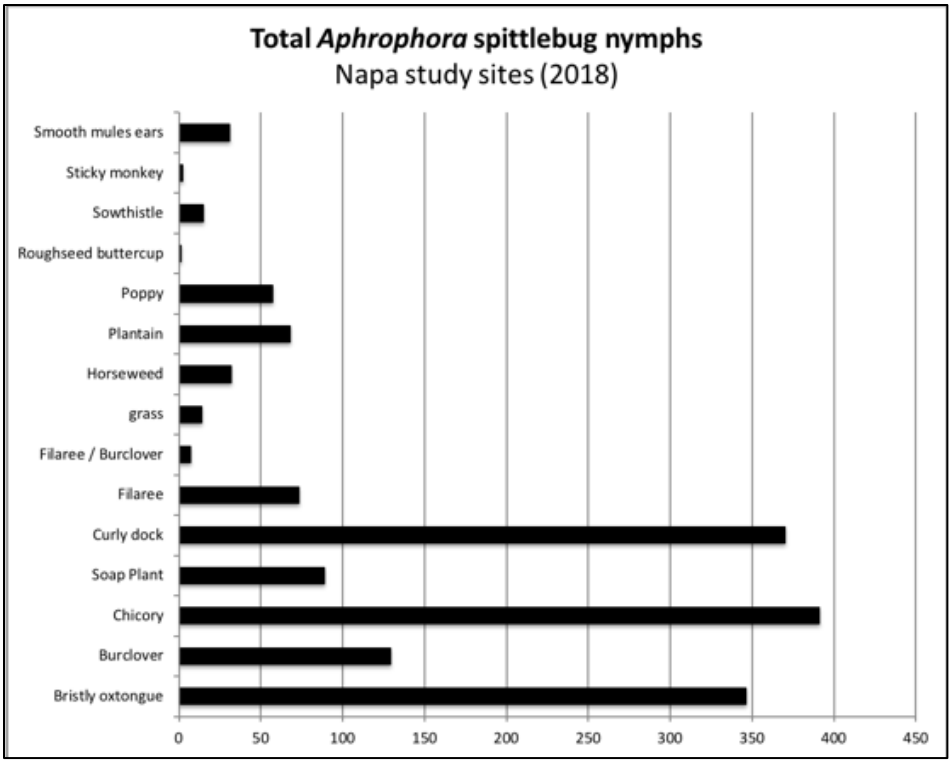


Figure 1. Most common host plants across all eight monitoring sites in Napa and Sonoma counties for *Aphrophora* spittlebugs.



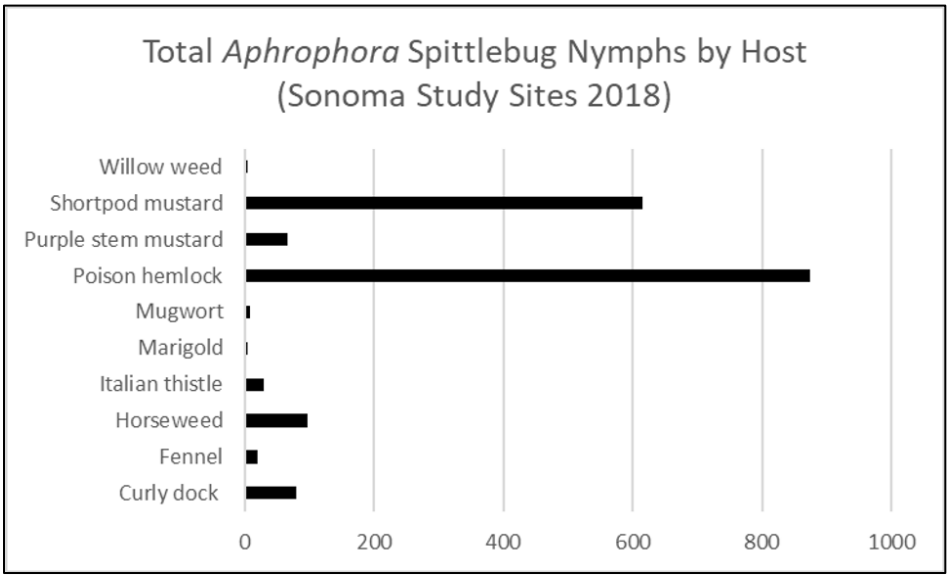


Figure 2. *Aphrophora* spittlebug nymph population levels on host plants at Napa (top) and Sonoma (bottom) study sites.

Objective 2. Mathematical Modeling of PD Spread

We are leveraging a dataset collected by the project team at 32 vineyards in Napa and Sonoma counties between 2016 and 2018 using different types of analyses (one illustrated here). This dataset includes regular sticky-trap monitoring for BGSS, sweep-net monitoring for BGSS and

other vectors in the spring, and PD mapping each fall. The data show substantial seasonal and spatial variability in BGSS abundance at each site, along with the abundance of other sharpshooters, spittlebugs, and other potential vectors (Almeida 2016, 2017). We conducted a hotspot analysis using ArcGIS software; this analysis uses the Getis-Ord Gi* statistic and identifies statistically significant hot and cold spots, given a set of weighted features. Study blocks fell into one of two groups, based on this analysis. One group of blocks showed an expected disease incidence and BGSS relationship (**Figure 3**). These blocks have hotspots of disease incidence at the edges of the block, where BGSS detections also tended to be higher. Another group of blocks had more unexpected hot and cold spot patterns, where hotspots did not appear to be associated with higher counts of BGSS in monitoring traps (**Figure 4**). Subsequent analyses will explore potential relationships between populations of other vectors and incidence of disease (hot and cold spots).

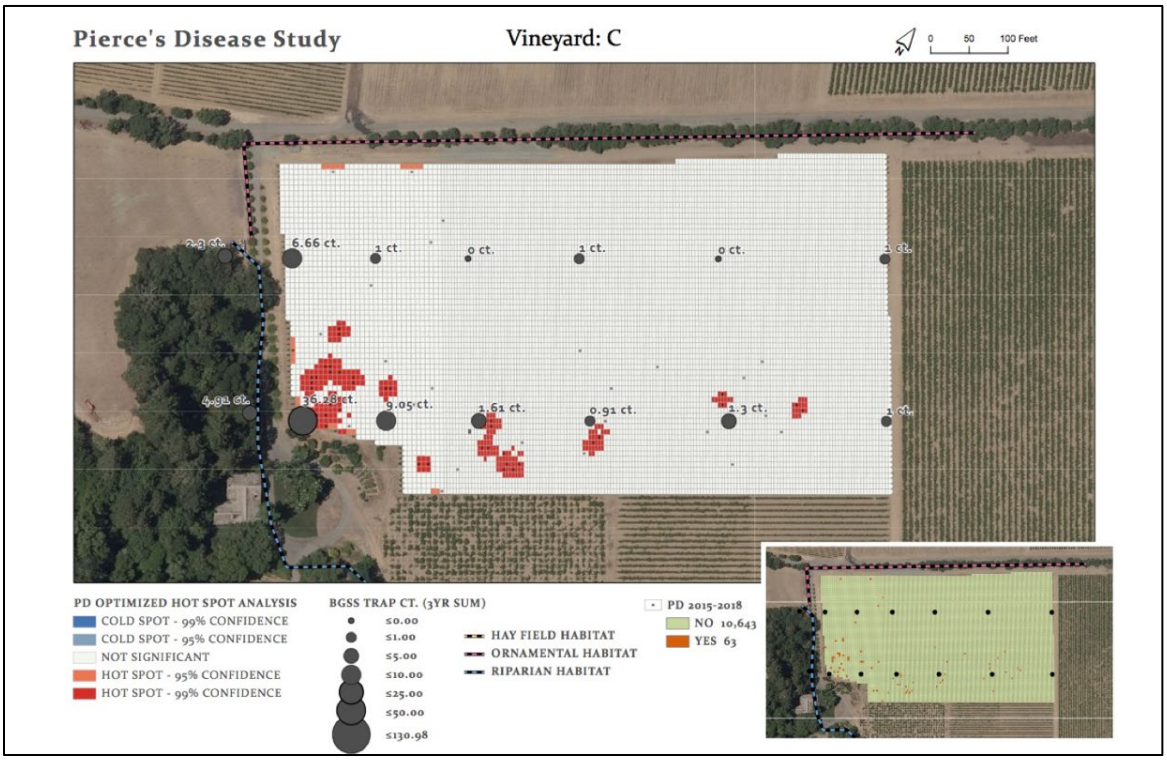


Figure 3. Study site in Napa County, demonstrating an expected pattern where disease hotspots occur at the edge of the vineyard, where BGSS trap counts are higher.

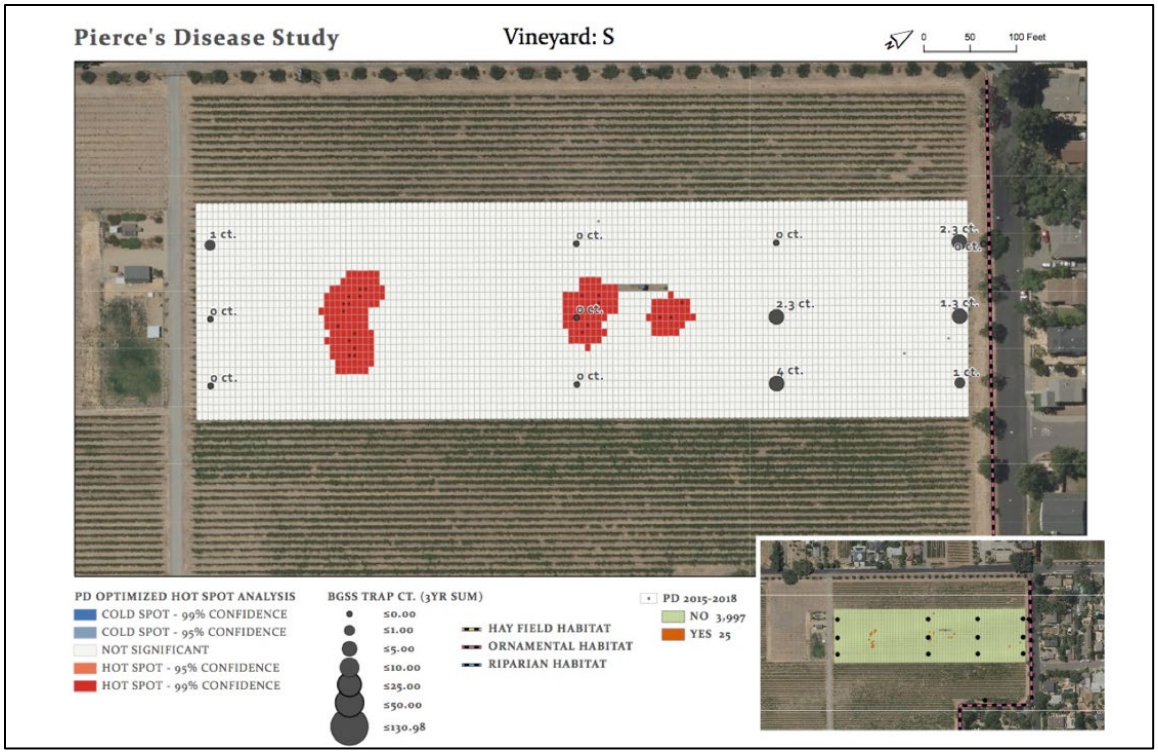


Figure 4. Study site in Napa County, demonstrating an unexpected pattern where disease hotspots do not appear to be associated with higher BGSS trap counts, suggesting the possible importance of another *Xf* vector.

Objective 3. *Xf* Population Genomics

Evaluation of Infection Development in Commercial Vines. A strain of *Xf* subspecies *fastidiosa* was isolated from grapevines in Napa, grown in our lab, and sequenced via both Illumina and Pacbio so as to create a complete genome. In April 2017, 45 grapevines (15 Merlot, 17 Chardonnay, and 13 Cabernet Sauvignon) were inoculated in Napa Valley with this strain, as well as buffer-inoculated negative controls. Since then, *Xf* populations at four or eight locations of each inoculated plant (depending on vine training) have been quantified three times per growing season using quantitative polymerase chain reaction (qPCR). Each year, *Xf* from positive plants were also cultured for genome resequencing, and throughout the growth season, symptoms of PD were quantified biweekly. The symptoms that we focused on as most typical of PD were shriveled clusters, leaf scorch, uneven lignification, match-sticking petioles, stunted shoots, and leaf chlorosis.

Symptoms. In 2017, symptoms correlated with qPCR positives well for all three varietals. Only four inoculated plants that tested positive for *Xf* did not display any symptoms in that first year. Those observed symptoms were predominantly shriveled clusters and leaf scorch. In 2018, the most severe symptom observed in the field was stunting of shoots early in the season for all three varietals, with some leaf scorch later in the season as the plants began to experience more water stress.

Quantitative PCR. While initial inoculation success was high (88% for Chardonnay, 80% for Merlot, and 62% for Cabernet Sauvignon), many of the plants recovered from infection after the first winter (**Figure 5**). During subsequent seasons, there was also trimming done at the vineyards by vineyard managers on some of the more severely symptomatic plants, which influenced our ability to properly monitor both symptom progression and population data.

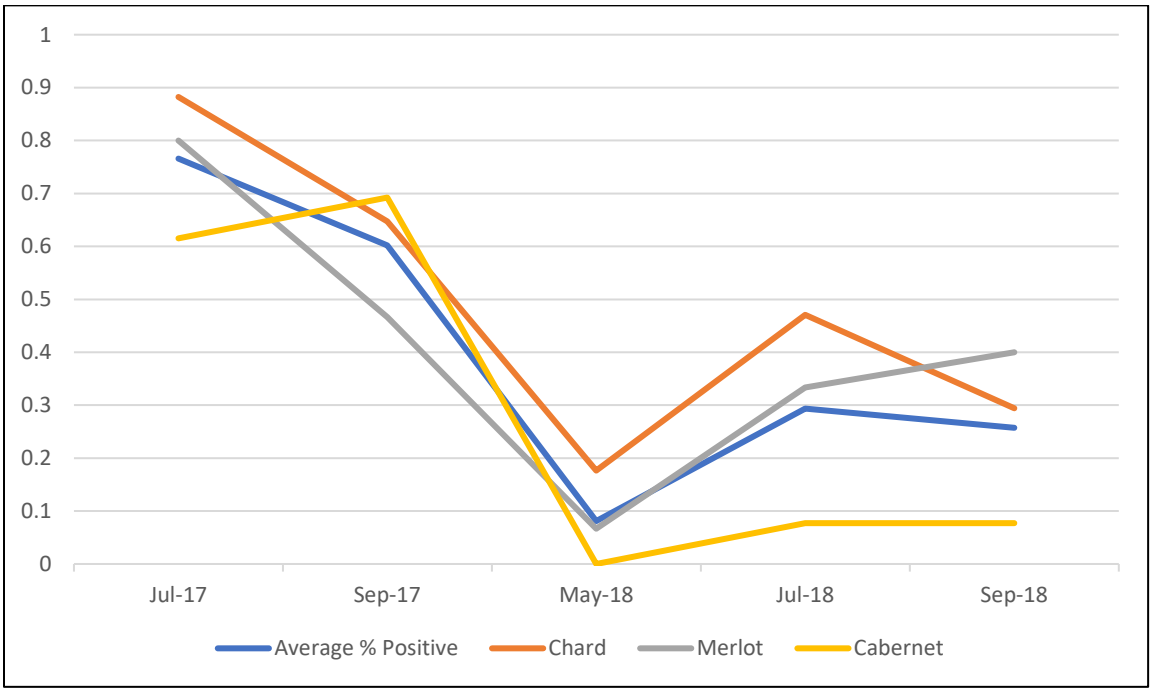


Figure 5. Fraction of inoculated grapevines that tested positive for *Xf* via qPCR from July 2017 through September 2018.

In terms of both percentage of infected plants and population [colony forming units (cfu)/gram] within plants that tested positive for *Xf* at each time point, the two years exhibit different patterns (**Figure 6**). In 2017, post-inoculation, the percentage of infected plants as well as the average populations slightly decreased throughout the season, while in 2018 there is an increase from May to July in the number of plants that tested positive, which stays consistent in September. However, *Xf* populations in plants increased by one order of magnitude between the July and September samplings. We have no final data for 2019.

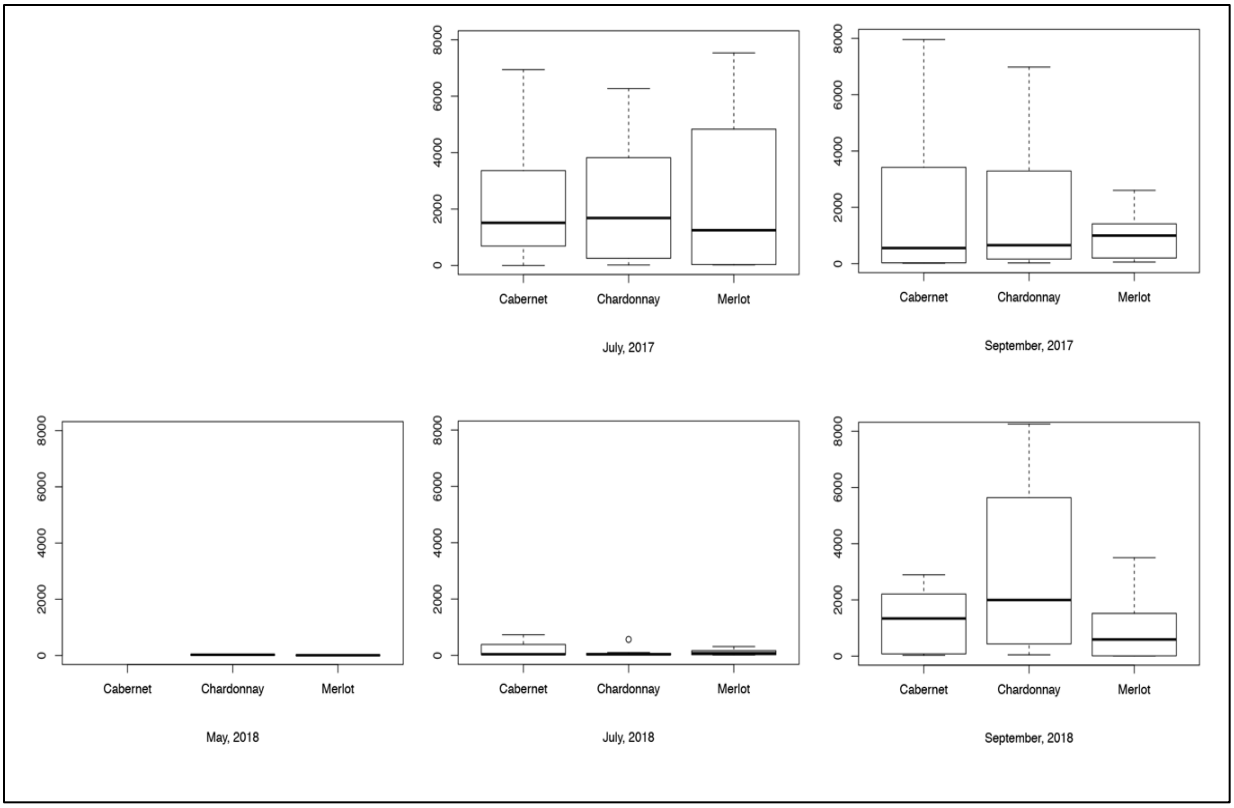


Figure 6. Population sizes in grapevines that tested positive for *Xf* via qPCR from July 2017 through September 2018. Measured in cfu/gram of grape petiole.

PD Population Genomics in California. A summary of our population genomics project is presented here. All raw data have already been made publicly available in the Sequence Read Archive (SRA) database. *Xf* subsp. *fastidiosa* causes PD and has been present in California for over a century. A singly-introduced genotype spread across the state, causing large outbreaks and damaging the grape and wine industry. We used 122 PD-causing isolates from symptomatic grapevines and explored pathogen genetic diversity associated with the disease in California. A total of 5,218 single-nucleotide polymorphisms (SNPs) were found in the dataset. Strong population genetic structure was found; isolates split into five genetic clusters divided into two lineages. The core/soft-core genome constituted 41.2% of the total genome, emphasizing the high genetic variability of *Xf* genomes. An ecological niche model was performed to estimate the environmental niche of the pathogen within California and to identify key climatic factors involved in dispersal. A landscape genomic approach was undertaken aiming to link local adaptation to climatic factors. A total of 18 non-synonymous polymorphisms found to be under selective pressures were correlated with at least one environmental variable highlighting the role of temperature, precipitation, and elevation on *Xf* adaptation to grapevines in California. The data do not support the hypothesis that the recent PD epidemic in California was driven by a novel and particularly virulent genotype of *Xf*. **Figure 7** illustrates the population structure within California, indicating that each grape-growing region has its "own" *Xf* population, with a few exceptions.

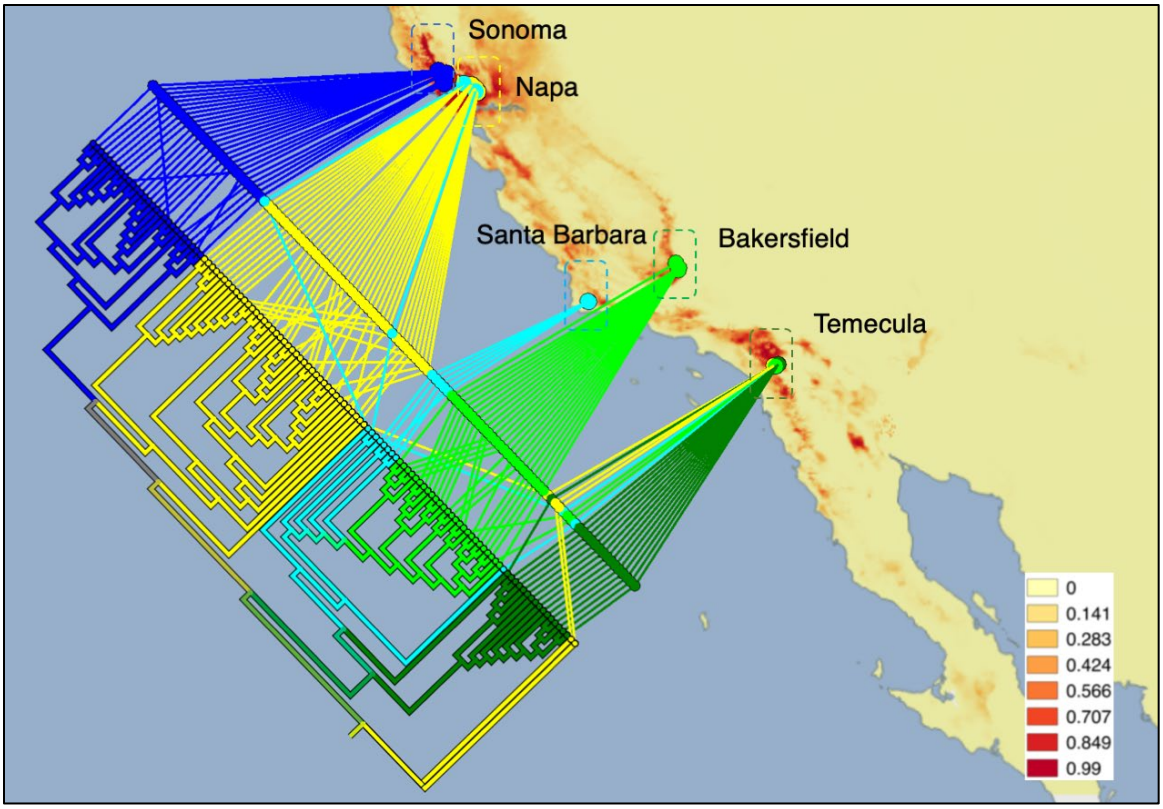


Figure 7. *Xf* population structure within California.

CONCLUSIONS

Ongoing work has shown that spittlebugs are common in North Coast vineyards and they are capable of transmitting *Xf*, but natural infectivity of these insects was only obtained in late summer. As such, it is so far not clear what their role is in spreading *Xf* in the region. The large field-based dataset gathered in a previous project is now being used to parameterize a range of epidemiological analyses. There are no conclusions in this regard, as the work is ongoing. The last component on pathogen population genomics has led to two relevant insights. First, each grape-growing region in California has a unique pathogen population. We are trying to determine the biological meaning of this finding, but its applied relevance is significant. Second, our mechanical infections of commercially-grown vines in Napa led to surprising results in terms of PD symptom development and confirmed preliminary data on vine recovery from infection, as well as the fact that plants have low pathogen populations until late summer. These data are being analyzed quantitatively, therefore conclusions are preliminary. In summary, this work is challenging several established paradigms of PD. Our group is also studying how to present these novel findings to industry stakeholders so that the information provided is accurate and actionable.

FUNDING AGENCIES

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

TESTING OF GRAPEVINES DESIGNED TO BLOCK VECTOR TRANSMISSION OF *XYLELLA FASTIDIOSA*

Project Leader: Rodrigo P.P. Almeida | Department of Environmental Science, Policy, and Management | University of California | Berkeley, CA 94720 | rodrigoalmeida@berkeley.edu

Reporting Period: The results reported here are from work conducted July 1, 2019 to October 15, 2019.

ABSTRACT

This project was initiated in July 2019, as such there are no results to present or discuss. However, we present a summary of the experimental work that has been done during this period, while results require the processing of large numbers of samples in the laboratory.

LAYPERSON SUMMARY

We have proposed an alternative approach to reduce the spread of Pierce's disease by blocking the transmission of *Xylella fastidiosa* (*Xf*) by insect vectors. The initial scientific work necessary to develop the concept of blocking *Xf* vector transmission has been done; candidate peptides worked well when provided to insects in vitro, effectively blocking transmission to plants in the greenhouse. Transgenic plants represented the next logical step to demonstrate that this novel technology continues to be promising. The generation of these plants takes a long time but that has already been done; they carry and express the engineered constructs necessary for our planned experiments. We have propagated this material and initiated experimental manipulations to test various lines. The goal of this project is to test if these transgenic plants block vector transmission of *Xf*, effectively reducing the spread and impact of Pierce's disease of grapevines.

INTRODUCTION

Options to control Pierce's disease of grapevines remain limited, despite many promising approaches that have been under development during the last two decades since the Temecula outbreak in the late 1990s, driven by the glassy-winged sharpshooter. Notably, the recent epidemic of Pierce's disease in Coastal California vineyards, from Santa Barbara to Napa and Sonoma valleys, highlights the need for novel Pierce's disease management strategies. Although this epidemic is still ongoing, the glassy-winged sharpshooter did not drive it, and economic losses have been very large at many vineyards. This proposal focuses on a novel approach to control Pierce's disease; namely, the disruption of *Xylella fastidiosa* (*Xf*) vector transmission by blocking *Xf* -vector interactions. Here we will briefly describe the background of this research line, ignoring other important but not directly relevant literature and data.

Initial studies on *Xf* -vector interactions identified hemagglutinin-like proteins as important in mediating cell attachment to the cuticle of vectors (Killiny and Almeida, 2009a). However, early work with *Xf* gene knockout mutants was challenging as many of these strains were also deficient in plant colonization (consequently reducing vector transmission), potentially limiting the interpretation of results, or requiring substantially more work to provide additional supporting data. The development of an artificial diet system to deliver *Xf* cells to insects, as well as a mutant phenotype required for vector colonization, was an important technical advancement

as it allowed for studies of *Xf* mutant strains without confounding effects of plant-strain interactions (Killiny and Almeida, 2009b).

The artificial diet system led to a number of studies that improved our understanding of *Xf*-vector interactions. Importantly, it became clear that *Xf* proteins were interacting with the chitinous cuticle of sharpshooter vectors. In addition, the approach allowed our team to test if transmission could be disrupted when molecules were provided to insect vectors. We postulated that adding *N*-acetylglucosamine (GlcNAc) to the artificial diet would result in cells binding to that substrate, and not being able to attach to the cuticle of insects. Similarly, we hypothesized that lectins with affinity to substrates similar to the cuticular surface of insects, such as wheat germ agglutinin (WAG), would mask the regions of the cuticle on which *Xf* would bind, effectively blocking transmission. We demonstrated that both of these approaches significantly reduced vector transmission of *Xf* to plants (Killiny et al., 2012). It is important to note, however, that the specific molecules tested in these assays would not be useful for real-world applications. Therefore, we set out to test the same concept with molecules that would be specific and environmentally safe.

That effort led to the determination that a hemaglutinin-like protein (HxfB) previously studied in relation to *Xf* transmission, and a hypothetical protein (PD1764, identified through a custom pipeline), were good candidates for future experimentation. We demonstrated that a domain of HxfB (HAD) and another of PD1764 (LysM), were responsible for blocking *Xf*-vector interactions in vivo, leading to reduced insect spread of *Xf* under greenhouse conditions (Labroussaa et al., 2016). With this information, additional research was performed to generate three constructs capable of blocking transmission to plants when provided to insects in artificial diets: HAD, LysM, and the fusion HAD-LysM (Almeida and Labroussaa, 2014). These three constructs are the focus of ongoing work and this research proposal.

In addition, there are other lines of evidence demonstrating that *Xf* proteins can reduce vector transmission, even those naturally produced while this pathogen colonizes grapevines (Baccari et al., 2014). This is particularly relevant, as there may be multiple technologies that could ultimately be used to block *Xf* transmission using proteins produced by the pathogen. In related but independent work, we have also developed mathematical models to estimate how *Xf* spread may be impacted by the use of transgenic grapevines aimed to reduce the impact of Pierce's disease (Zeilinger et al., 2018).

OBJECTIVES

The objective of this proposal is to test transgenic grapevines with insect-based *Xf* transmission assays to determine if vector spread of the pathogen is blocked. If the experiments lead to blockage of transmission, it is possible that additional experimental research to parameterize and then mathematically model spread of *Xf* with these lines in field conditions would be desirable.

RESULTS AND DISCUSSION

Based on the above described research, we constructed and codon-optimized HAD and LysM domains, and a HAD-LysM fusion proteins for expression in *Vitis vinifera*, and subsequently transformed grapevine cv. Thompson Seedless and rootstock 101-14 to express each protein. The genes were inserted into the binary vector pCB4NN (Harvey et al., 2008; signal peptide to target

the delivery of the peptides to the xylem as in Dandekar et al., 2012), transformed into *Agrobacterium tumefaciens*, and sent to the Ralph M. Parsons Foundation Plant Transformation Facility at UC Davis, generating the following lines (**Table 1**):

Table 1. Generated transgenic plant lines.

	Number of Transgenic Plant Lines		
Grapevine	HAD	LysM	HAD-LysM
Thompson Seedless	18	10*	11
Rootstock 101-14	20	14	10

*Thompson Seedless LysM plants have not yet been received from the UC Davis facility.

Using pressure bomb techniques on transgenic source plants, sap extractions were made for tentative mass-spectrometric analysis of proteins contained therein. We already have data on presence and expression of inserts in these transgenic plants.

Experiment 1. Do Transgenic Plants Block *Xf* Transmission?
Here we tested the hypothesis that peptides expressed in transgenic plants will block later adhesion of *Xf* to vectors (blue-green sharpshooter; *Graphocephala atropunctata*). An important control are insects feeding first on uninfected wild-type plants. Source plants (infected wild-type plant) were inoculated with *Xf* two to three months prior to insect exposure. Insects were allowed to feed for four days on the "transmission-blocking" plants, two days on the infected plants, and four days on the final test plant. When on transgenic/control plants, as well as source plants, insects were transferred in groups (i.e., blocks), while on the last plants (cultivar Cabernet Sauvignon) insects were transferred and kept individually. This experiment was performed; plant and insect samples are stored and being processed in the laboratory.

Experiment 2. Do Transgenic Plants Limit *Xf* Spread?
In practice, while feeding on "transmission-blocking" plants may reduce vector transmission of *Xf*, it is also possible that insects will feed on these plants when they are already infected with the pathogen. This is relevant, as grapevines are perennial plants. Here we are determining if vector transmission is affected when insects feed on *Xf*-infected plants. As a control we will use infected 101-14 rootstock that is not transgenic. Rootstock source plants were mechanically inoculated with *Xf* three months prior to insect access for four days. After insect access insects were transferred to uninfected recipient plants (cultivar Cabernet Sauvignon) for a four-day inoculation access period. As in Experiment 1, plants and insects were stored and will be tested for *Xf.* This experiment was performed; plant and insect samples are stored and being processed in the laboratory.

In addition, two blocks of transgenic rootstock plants, representing our three transgenes and controls, were mechanically inoculated in 2019. These are being followed for symptom development – the same treatments were already done in 2018.

CONCLUSIONS

There are no conclusions, other than the work is being performed as originally proposed and we are now processing samples in the laboratory.

REFERENCES CITED

Almeida RPP, Labroussaa F. 2014. Blocking *Xylella fastidiosa* transmission. *Proceedings of the 2014 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA, pp. 202-210.

Almeida RPP, Purcell AH. 2006. Patterns of *Xylella fastidiosa* colonization on the precibarium of leafhopper vectors relative to transmission to plants. *Annals of the Entomological Society of America* 99: 884-890.

Baccari C, Killiny N, Ionescu M, Almeida RPP, Lindow SE. 2014. Diffusible signal factor-repressed extracellular traits enable attachment of *Xylella fastidiosa* to insect vectors and transmission. *Phytopathology* 104: 27-33.

Dandekar AM, Gouran H, Ibáñez AM, Uratsu SL, Agüero CB, McFarland S, Borhani Y, Feldstein PA, Bruening G, Nascimento R, Goulart LR, Pardington PE, Chaudhary A, Norvell M, Civerolo E, Gupta G. 2012. An engineered innate immune defense protects grapevines from Pierce disease. *Proceedings of the National Academy of Sciences USA* 109: 3721-3725.

Dandekar AM, Jacobson A, Ibanez AM, Gouran H, Dolan D, Aguero C, Uratsu SL, Just R, Zaini PA. 2019. Trans-graft protection against Pierce's disease mediated by transgenic grapevine rootstocks. *Frontiers in Plant Science* doi: 10.3389/fpls.2019.00084.

Harvey JJW, Lincoln JE, Gilchrist DG. 2008. Programmed cell death suppression in transformed plant tissue by tomato cDNAs identified from an *Agrobacterium rhizogenes*-based functional screen. *Molecular Genetics and Genomics* 279: 509-521.

Hill BL, Purcell AH. 1995. Multiplication and movement of *Xylella fastidiosa* within grapevine and four other plants. *Phytopathology* 85: 1368-1372.

Killiny N, Almeida RPP. 2009a. *Xylella fastidiosa* afimbrial adhesins mediate cell transmission to plants by leafhopper vectors. *Applied and Environmental Microbiology* 75: 521-528.

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

Killiny N, Rashed A, Almeida RPP. 2012. Disrupting the transmission of a vector-borne plant pathogen. *Applied and Environmental Microbiology* 78: 638-643.

Labroussaa F, Zeilinger AR, Almeida RPP. 2016. Blocking the transmission of a non-circulative vector-borne plant pathogenic bacterium. *Molecular Plant-Microbe Interactions* 29: 535-544.

Labroussaa F, Ionescu M, Zeilinger AR, Lindow SE, Almeida RPP. 2017. A chitinase is required for *Xylella fastidiosa* colonization of its insect and plant hosts. *Microbiology* 163: 502-509.

Zeilinger AR, Turek D, Cornara D, Sicard A, Lindow SE, Almeida RPP. 2018. Bayesian vector transmission model detects conflicting interactions from transgenic disease resistant grapevines. *Ecosphere* 9: e02494.

FUNDING AGENCIES

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

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

Project Leader: Abhaya M. Dandekar | Department of Plant Sciences | University of California | Davis, CA 95616 | amdandekar@ucdavis.edu
Cooperator: Paulo A. Zaini | Department of Plant Sciences | University of California | Davis, CA 95616 | pazaini@ucdavis.edu
Cooperator: Aaron Jacobson | Department of Plant Sciences | University of California | Davis, CA 95616 | ajacobson@ucdavis.edu

Reporting Period: The results reported here are from work conducted October 2018 to October 2019.

ABSTRACT

This project is a continuation of previously funded projects to evaluate the field efficacy of transgenic grapevine rootstocks expressing a chimeric antimicrobial protein (CAP) or a polygalacturonase inhibitory protein (PGIP) at protecting a grafted scion variety from developing and succumbing to Pierce's disease. A total of 126 independent lines corresponding to seven versions of CAP and five versions of PGIP exploiting components optimized and tested in previously funded projects have been successfully introduced and expressed in Thompson Seedless and the commercially relevant rootstocks 101-14 and 1103. Of these 126 lines, 100 were chosen to progress to the field trial based upon presence of the transgene and protein expression. A total of 379 plants consisting of 60 lines plus wild-type controls, each with six replicates, were planted in the field in August 2018. This summer an additional 240 plants in replicates of six from 38 transgenic lines, along with wild-type controls, were planted in the field, completing our planting. The field is now planted with a total of 100 transgenic lines plus wild-type controls, each with six replicates, for a total of 630 plants. Once expressed in the rootstock these proteins will move into the grafted Pierce's disease sensitive scion variety Chardonnay, and this study aims at evaluating the ability of these proteins to control the development of the disease. These two proteins, CAP and/or PGIP, control the spread and severity of the disease by controlling the bacteria-plant interaction, but do so by disrupting different interacting surfaces. The CAP proteins disrupt the bacterial surface that includes the lipopolysaccharide layer, while the action of PGIP is indirect by preventing/interfering with the disruption of the plant pectin layers found in the plant middle lamella and exposed in pit membranes in xylem tissues. In this project, we will evaluate in the field the effectiveness of rootstocks expressing either of these two proteins in limiting the disease development in the scion while maintaining vine health and productivity. Elite rootstock lines identified in this project will be good candidates for commercialization.

LAYPERSON SUMMARY

This project continues the field efficacy evaluation of standard grapevine rootstocks expressing, individually, seven chimeric antimicrobial proteins (CAP) and five polygalacturonase inhibitory proteins (PGIP) at protecting a grafted scion variety from developing Pierce's disease. Of 126 transgenic lines that were generated, 98 were selected to be tested against infection in the field trial. A total of 630 plants from these lines plus wild-type controls, in replicates of six, have been

grafted with scions of the susceptible cultivar Chardonnay. This summer (2019) we planted the remaining 240 plants from 38 transgenic lines, to join the 379 plants from 60 transgenic lines planted in 2018. Once training of the vines has been completed the vines will be challenged by infection with *Xylella fastidiosa* to identify rootstock lines that can protect the scion from developing Pierce's disease while maintaining their productivity. 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 proteins (CAP; Dandekar et al., 2012a) and polygalacturonase inhibitory protein (PGIP; Agüero et al., 2005, 2006) at providing transgraft protection of a scion grapevine variety against Pierce's disease. A field trial testing four lines of CAP-1 and four lines of PGIP corresponding to PGIP-1, PGIP-2, PGIP-3, and PGIP-4 was recently concluded (Dandekar et al., 2018). Twelve plants corresponding to each of the eight lines (independent transgenic events) were planted in 2011 as transgenic rootstocks grafted to wild-type scions with both rootstock and scion being the Thompson Seedless variety. We had previously demonstrated that both PGIP and CAP-1 are secreted into the xylem, where they were able to protect the vines from developing Pierce's disease (Agüero et al., 2005; Dandekar et al., 2012a). The purpose of the field trial was to evaluate the ability of the transgenic rootstock to trans-graft protect the wild-type scion from developing and/or succumbing to Pierce's disease. The inoculations were performed yearly starting in 2012, and from 2013 until 2015 all 12 replicates of each of the transgenic lines were inoculated only in the grafted scion portion at a point at least 100 cm above the graft union. Disease symptoms, vine death, and other parameters were evaluated each year, and the field trial was concluded in 2017. The data generated over the four seasons of evaluation clearly indicated that both rootstocks (CAP and PGIP) were able to trans-graft protect the scion at a point that was at least 100 cm above the graft union. A significant decrease in vine mortality was observed for vines grafted to transgenic CAP or PGIP expressing rootstock as compared to wild-type rootstocks. Vines grafted to transgenic rootstocks harbored lower pathogen titers compared to those grafted to wild-type rootstocks. Spring bud break, a parameter of vine health, was much higher for vines grafted to either transgenic rootstock, and much lower for the wild-type rootstock (Dandekar et al., 2019). This present study builds on earlier work and incorporates advances in transformation of commercially relevant grapevine rootstocks, as well as incorporates improvements in individual components present in CAP and PGIP constructs. Methods to successfully transform two commercially relevant rootstocks 101-14 and 1103 (Christensen, 2003) were successfully developed (Dandekar et al., 2011, 2012b) and the methods were further improved by David Tricoli in the Plant Transformation Facility at UC Davis. The original CAP-1 construct (Dandekar, 2012a) was improved upon by identifying grapevine-derived components (Chakraborty et al., 2013, 2014b). The surface interacting component (neutrophil elastase) was replaced with P14a protein from *Vitis shuttleworthii* that also displays serine protease and antimicrobial activity (Chakraborty et al., 2013; Dandekar et al., 2012c, 2013) and more recently with PrtA that displays serine protease and antimicrobial activity (Gouran et al., 2016). The antimicrobial peptide component (cecropin B; CB) 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 antimicrobial activity of the selected peptides were verified by their ability to kill cells of *Xylella fastidiosa* (*Xf*) (Chakraborty et al., 2014b). Improvements in the secretion of PGIP were also made based on an earlier study on the

characterization of xylem sap proteins whose signal peptides could be identified and have been used instead of the natural one expressed in the peel tissue of pear fruit (Agüero et al., 2005, 2008). The field introduction of these transgenic 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

The goal of this project is to field test transgenic rootstocks expressing CAP and/or PGIP proteins to determine their ability to trans-graft protect a sensitive scion grapevine from developing and succumbing to Pierce's disease.

1. Develop commercially relevant transgenic rootstock lines expressing CAP and/or PGIP.
2. Field test the efficacy of commercially relevant transgenic rootstock lines expressing CAP and/or PGIP proteins to trans-graft protect a sensitive grapevine cultivar from developing and spreading Pierce's disease.

RESULTS AND DISCUSSION

Objective 1. Develop Commercially Relevant Transgenic Rootstock Lines Expressing CAP and/or PGIP

This objective translates the results of two previously funded projects (CDFA grant numbers 11-0240-SA and 12-0130-SA). Project 12-0130-SA, titled "Building a Next Generation Chimeric Antimicrobial Protein to Provide Rootstock-Mediated Resistance to Pierce's Disease in Grapevines," led to the development of additional CAP proteins with components derived from grapevine and other proteins (Dandekar et al., 2015). Project 11-0240-SA, titled, "Engineering Multi-Component Resistance to Pierce's Disease in California Grapevine Rootstocks," led to the development of a method to transform the commercially relevant rootstocks 101-14 and 1103 (Dandekar et al., 2011, 2013). David Tricoli at the Plant Transformation Facility at UC Davis has further improved upon the grapevine rootstock transformation protocol and carried out all of our transformations. Shown in **Figure 1** are all of the CAP vectors being field tested in this project. CAP-1 is the original vector that was field tested in Thompson Seedless rootstocks, with several lines showing efficacy (Dandekar et al., 2016, 2018). CAP-2 has the original components as described earlier (Dandekar et al., 2012); however, the expression of the CAP has been improved by including a translational enhancer (omega) and an efficient secretion sequence (Ramy3D), and the CAP-2 protein has an epitope tag (FLAG) to enable detection of the protein in transgenic tissues. CAP-3 to CAP-6 are four vector constructs to test the *Vitis*-derived components (**Figure 1**). The CAP-3 and CAP-4 are designed to test the *Vitis* component replacing the protease from CAP-1. The CAP-3 vector, pDP13.35107, tests the VsP14a protein by itself. The VsP14a component is present in *V. shuttleworthii* (*Vs*) and has a similar function to the CAP-1 protease (Dandekar et al., 2014; Chakraborty et al., 2013). Expression of VsP14a by itself confirmed its protease and antimicrobial activity against *Xf* (Dandekar et al., 2014). The fourth vector pDP13.36122 (CAP-4) expressess VsP14a linked to cecropinc B, the antimicrobial peptide domain used successfully in CAP-1 (Dandekar et al., 2012). The fifth CAP-5, pDP14.0708.13 (**Figure 1**), links the VsP14a to a 52-amino acid segment of the HAT protein from *Vitis vinifera* that displays a moderate clearance activity against *Xf* (Chakraborty et al., 2014b; Dandekar et al., 2015). The sixth CAP-6, pDP14.0436.03 (**Figure 1**), links the VsP14a to a 20-amino acid segment of the PPC protein from *V. vinifera* that has very good antimicrobial activity against *Xf* (Chakraborty et al., 2014; Dandekar et al., 2015). The seventh and final

CAP-7, pDG14.01 (**Figure 1**), expresses PrtA, a protease that displayed antimicrobial activity against *Xf* in a tobacco system (Gouran et al., 2016) All of these seven vectors CAP-1 to CAP-7 were transformed in the UC Davis Plant Transformation Facility and transgenic grapevine rootstocks have been obtained.

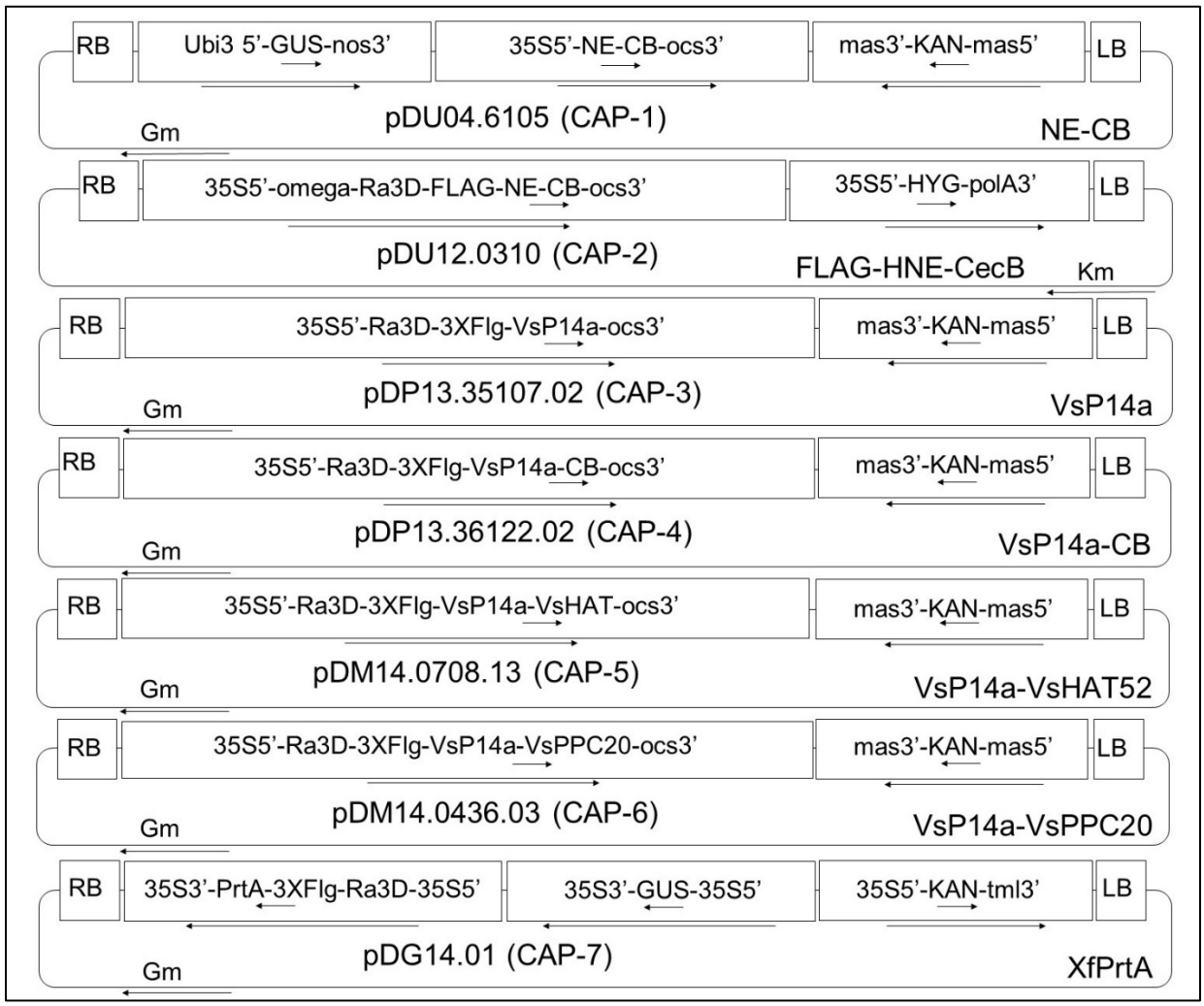


Figure 1. CAP vectors used in this study to develop transgenic rootstocks that will be evaluated in the field.

In addition to the seven CAP constructs, we will also be evaluating the five PGIP constructs shown in **Figure 2**. The PGIP-1, pDU94.0928 (**Figure 2**) construct is the original pearPGIP expressed in grapevine and shown to provide resistance/tolerance to Pierce's disease (Aguero et al., 2005). PGIP-2, pDU05.1002, encodes a pearPGIP sequence with its native signal peptide deleted and is referred to as mPGIP, as it is similar in sequence to the mature form of PGIP found in plant tissues. PGIP-3, pDU05.1910 (**Figure 2**), contains a pearPGIP coding sequence fused to the signal peptide from the nt-protein of grapevine whose sequence was reported by Aguero et al. (2008). PGIP-4, pDU06.0201 (**Figure 2**), contains the mPGIP coding sequence

fused to the signal peptide from the chi protein from grapevine whose sequence was reported by Aguero et al. (2008). PGIP-5, pDA05.XSP (**Figure 2**), contains the mPGIP coding sequence fused to the signal peptide from a xylem abundant protein from cucumber, and PGIP-6, pDU05.0401 (**Figure 2**), links the mPGIP sequence to the Ramy3D signal peptide from the rice alpha-amylase protein.

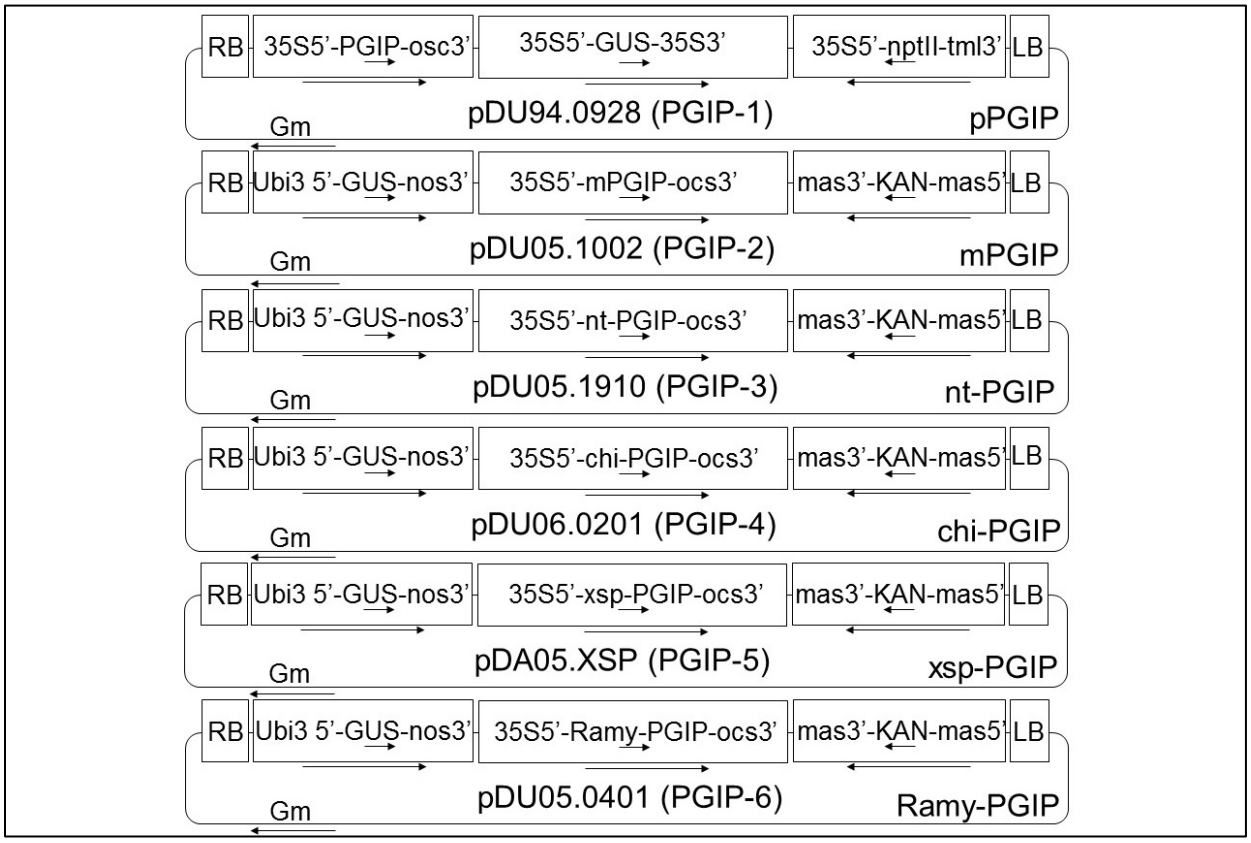


Figure 2. PGIP vectors used in this study to develop transgenic rootstocks that will be evaluated in the field.

The 240 vines that were planted this summer (2019) were clonally propagated using an aeroponic machine and grafted with Chardonnay buds by the Dandekar Lab. In addition to propagating the plants for the 2019 planting, we began training our portion of the 2018 planting in the field trial in a style used by commercial vineyards. While dormant during winter, we pruned the plants to a height of about three inches above the graft union, leaving two or three buds to promote vigorous spring growth. In early July we selected the strongest cane that had pushed from these buds to develop as the trunk of the plant, and removed all other growth to allow its continued development. Suckers from the rootstocks were removed a few times throughout the year to ensure the optimal growth of the scions.

Table 1. List of transgenic lines generated and being maintained as mother plants in the greenhouse.

No	Construct	Binary Vector	Transgene	Number of Novel Lines			No of Events
				101-14	1103	TS	
1	CAP-1	pDU04.6105	NE-CB	6			6
2	CAP-2	pDU12.031	NNE-CB		9		9
3	CAP-3	pDP13.35107	VsP14a	24	1		25
4	CAP-4	pDP13.36122	VsP14a-CB	24	1		25
5	CAP-5	pDM14.0708	VsP14a-VvHAT52	3	4		7
6	CAP-6	pDM14.0436	VsP14a-VvPPC20	7	4		11
7	CAP-7	pDG14.02	XfPrtA	9		14	23
8	PGIP-2	pDU05.1002	mPGIP			4	4
9	PGIP-3	pDU05.1910	nt-PGIP			4	4
10	PGIP-4	pDU06.0201	chi-PGIP			4	4
11	PGIP-5	pDA05.XSP	xsp-PGIP			4	4
12	PGIP-6	pDU05.0401	Ramy-PGIP			4	4
13	WT			1	1	1	3
				Total number of lines			129

Table 2. List of transgenic lines established in the field and derived from vectors shown in **Figures 1** and **2**.

No	Construct	Binary Vector	Transgene	Lines by Pedigree			No of Events
				101-14	1103	TS	
1	CAP-1	pDU04.6105	NE-CB	6			6
2	CAP-2	pDU12.031	NNE-CB		9		9
3	CAP-3	pDP13.35107	VsP14a	16			16
4	CAP-4	pDP13.36122	VsP14a-CB	17			17
5	CAP-5	pDM14.0708	VsP14a-VvHAT52	1	3		4
6	CAP-6	pDM14.0436	VsP14a-VvPPC20	7	4		11
7	CAP-7	pDG14.02	XfPrtA	7		8	15
8	PGIP-2	pDU05.1002	mPGIP			4	4
9	PGIP-3	pDU05.1910	nt-PGIP			4	4
10	PGIP-4	pDU06.0201	chi-PGIP			4	4
11	PGIP-5	pDA05.XSP	xsp-PGIP			4	4
12	PGIP-6	pDU05.0401	Ramy-PGIP			4	4
13	WT			1	1	1	3
				Total number of lines			101

Objective 2. Field Test the Efficacy of Commercially Relevant Transgenic Rootstock Lines Expressing CAP and/or PGIP Proteins to Trans-Graft Protect a Sensitive Grapevine Cultivar from Developing and Spreading Pierce's Disease

This objective focuses on the field testing of all seven CAP and five PGIP lines shown in the last column of **Table 2**. Foundation Plant Services (FPS) helped with creating the grafted plants for the 2018 field planting. First cuttings were harvested from mother plants in the lath house after the plants went dormant. In spring 2018 these cuttings were rooted to make plants that were later budded with the scion variety Chardonnay, creating the vines that were planted in the field. FPS was able to successfully propagate and graft 70% of our lines (**Table 2**); the remaining 30% were successfully propagated and bud grafted by us. On August 1, 2018 we planted the first batch of plants that were grafted with the help of FPS, which constituted 70% of the planting, and on August 19, 2018 we planted the remaining 30% that were grafted by us. All of the 240 plants generated for the 2019 planting were generated by us, and the first 150 were introduced to the field on June 6, 2019. The field infrastructure was not able to accommodate our full set of plants at the first planting, so our remaining 90 plants were planted in late summer on August 20, 2019. We are currently maintaining a stock of back-up plants of all of the lines indicated in **Table 2**, so we can replace any plants that are lost in the field over winter, if any. Cordons should be developed in early summer 2020 from the trunks that were established during the growing season of 2019, and canes will push from those cordons in mid-to-late summer 2020. These canes could be inoculated with *Xf* to begin the evaluation of resistance provided by the transgenic rootstocks, provided funding is granted to conduct the research.

Figure 3. Field planting of test plants after first full year of growth (October 2019). The thickest canes have been allowed to become the trunk of the plant. Cordons will be selected from the most vigorous buds to push during next year's growth.

CONCLUSIONS

The goal of this project is to field test transgenic rootstocks expressing CAP and/or PGIP proteins to determine their ability to trans-graft protect a sensitive scion grapevine from developing and succumbing to Pierce's disease. We have successfully introduced 98 independent events corresponding to 11 constructs of CAP and PGIP, yielding 630 plants. These plants are composed of a transgenic rootstock grafted with a wild-type Chardonnay scion. Sixty independent lines were planted in the field in August 2018 and 38 independent lines were planted in 2019, laid out in 21 rows with 30 plants in each row. The 11 gaps from the 2018 planting were filled during the 2019 planting. We now have all of our lines planted in the field and hope to be able to evaluate their ability to provide protection.

REFERENCES CITED

Agüero CB, Uratsu SL, Greve LC, Powell ALT, Labavitch JM, Meredith CP, Dandekar AM. 2005. Evaluation of tolerance to Pierce's disease and *Botrytis* in transgenic plants of *Vitis vinifera* L. expressing the pear PGIP gene. *Molecular Plant Pathology* 6(1): 43-51.

Agüero CB, Meredith CP, Dandekar AM. 2006. Genetic transformation of *Vitis vinifera* L. cvs. 'Thompson Seedless' and 'Chardonnay' with the pear PGIP and GFP encoding genes. *Vitis* 45: 1-8.

Agüero CB, Thorne ET, Ibáñez AM, Gubler WD, Dandekar AM. 2008. Xylem sap proteins from *Vitis vinifera* L. Chardonnay. *American Journal of Enology and Viticulture* 59(3): 306-311.

Almeida RPP, Purcell AH. 2003. Biological traits of *Xylella fastidiosa* strains from grapes and almonds. *Applied and Environmental Microbiology* 68: 7447-7452.

Chakraborty S, Minda R, Salaye L, Dandekar AM, Bhattacharjee SK, Rao BJ. 2013. Promiscuity-based enzyme selection for rational directed evolution experiments. *Methods in Molecular Biology* 978: 205-216. *Enzyme Engineering: Methods and Protocols.* Samuelson J. (ed.). Springer, New York.

Chakraborty S, Rao B, Dandekar A. 2014a. PAGAL - Properties and corresponding graphics of alpha helical structures in proteins. *F1000Research* 3.

Chakraborty S, Phu M, Rao B, Asgeirsson B, Dandekar A. 2014b. The PDB database is a rich source of α-helical anti-microbial peptides to combat disease causing pathogens. *F1000Research* 3.

Christensen L. 2003. Rootstock Selection. *Wine Grape Varieties in California*. University of California Agricultural and Natural Resources, pp. 12-15.

Dandekar AM, Walker A, Ibáñez AM, Uratsu SL, Vahdati K, Tricoli D, Agüero C. 2011. Engineering multi-component resistance to Pierce's disease in California grapevine rootstocks. *Proceedings of the 2011 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA, pp. 107-110.

Dandekar AM, Gouran H, Ibáñez AM, Uratsu SL, Agüero CB, McFarland S, Borhani Y, Feldstein PA, Bruening G, Nascimento R, Goulart LR, Pardington PE, Chaudhary A, Norvell M, Civerelo E, Gupta G. 2012a. An engineered innate defense protects grapevines from Pierce's disease. *Proceedings of the National Academy of Sciences USA* 109: 3721-3725.

Dandekar AM, Walker A, Ibáñez AM, Tran KQ, Gunawan D, Uratsu SL, Vahdati K, Tricoli D, Agüero C. 2012b. Engineering multi-component resistance to Pierce's disease in California grapevine rootstocks. *Pierce's Disease Research Progress Reports*. December 2012. California Department of Food and Agriculture, Sacramento, CA, pp. 104-108.

Dandekar AM, Ibáñez AM, Gouran H, Phu M, Rao BJ, Chakraborty S. 2012c. Building the next generation chimeric antimicrobial protein to provide rootstock-mediated resistance to Pierce's disease in grapevines. *Pierce's Disease Research Progress Reports*. December 2012. California Department of Food and Agriculture, Sacramento, CA, pp. 89-93.

Dandekar AM, Gouran H, Chakraborty S, Phu M, Rao BJ, Ibáñez AM. 2013. Building the next generation chimeric antimicrobial protein to provide rootstock-mediated resistance to Pierce's disease in grapevines. *Proceedings of the 2013 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA, pp. 89-94.

Dandekar AM, Gouran H, Chakraborty S, Phu M, Rao BJ, Ibáñez AM. 2014. Building the next generation chimeric antimicrobial protein to provide rootstock-mediated resistance to Pierce's disease in grapevines. *Proceedings of the 2014 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA, pp. 99-105.

Dandekar AM, Ibáñez AM, Jacobson A. 2018. Final report: Field testing transgenic grapevine rootstocks expressing chimeric antimicrobial protein (CAP) and polygalacturonase inhibitory protein (PGIP). http://www.piercesdisease.org/projects/388.

Dandekar AM, Jacobson A, Ibáñez AM, Gouran H, Dolan DL, Aguero CB, Uratsu SL, Just R, Zaini PA. 2019. Trans-graft protection against Pierce's disease mediated by transgenic grapevine rootstocks. *Frontiers in Plant Science* 10: 84. doi: 10.3389/fpls.2019.00084.

Gouran H, Gillespie H, Nascimento R, Chakraborty S, Zaini PA, Jacobson A, Phinney BS, Dolan D, Durbin-Johnson BP, Antonova ES, Lindow SE, Mellema MS, Gulart LR, Dandekar AM. 2016. The secreted protease PrtA controls cell growth, biofilm formation and pathogenicity in *Xylella fastidiosa*. *Science Reports* 6: 31098. doi: 10.1038/srep31098.

FUNDING AGENCIES

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

FIELD EVALUATION OF CROSS-GRAFT PROTECTION EFFECTIVE AGAINST PIERCE'S DISEASE BY DUAL DNA CONSTRUCTS EXPRESSED IN TRANSGENIC GRAPE ROOTSTOCKS

Project Leader: David Gilchrist | Department of Plant Pathology| University of California | Davis, CA 95616 | dggilchrist@ucdavis.edu
Cooperator: Abhaya M. Dandekar | Department of Plant Sciences | University of California | Davis, CA 95616 | amdandekar@ucdavis.edu
Cooperator: James Lincoln | Department of Plant Pathology | University of California | Davis, CA 95616 | jelincoln@ucdavis.edu
Cooperator: Bryan Pellissier | Department of Plant Pathology | University of California | Davis, CA 95616 | bpellissier@ucdavis.edu

Reporting Period: The results reported here are from work conducted July 2018 to October 2019.

ABSTRACT

The current project is designed to evaluate expression of dual DNA constructs in adapted rootstocks to effect cross-graft protection of an untransformed Pierce's disease (PD) susceptible scion. This experiment follows a field evaluation, begun in 2010, where plants were transformed with single DNA inserts that had shown potential to suppress symptoms of PD under greenhouse conditions. The disease was successfully introduced into the cordon-trained plants by mechanical injection of *Xylella fastidiosa* (*Xf*) into young stems in the second year after planting. The results of these experiments confirmed that the mechanical inoculations introduced the bacteria into the plants and led to the appearance of classic foliar symptoms of PD and cane death within 24 months in susceptible controls, compared with the suppression of symptoms in the transgenic plants. The current planting, managed to commercial standards, will consist of non-transformed PD-susceptible Chardonnay scions grafted to transgenic rootstocks (1103 and 110-14) expressing the paired constructs. The experimental protocol includes *Xf* mechanical inoculation techniques that were used successfully in past experiments while PD symptoms, bacterial movement dynamics, and fruit yield will be measured during the course of the experiment. All plants were located in a secured, USDA-APHIS regulated area in Solano County.

LAYPERSON SUMMARY

This project will continue to support the field activities related to the evaluation of resistance to Pierce's disease (PD) in transgenic grape rootstocks by expressing dual combinations of five unique transgenes that have shown positive protection against PD under field conditions. The new planting, managed to commercial standards, will consist of non-transformed PD-susceptible Chardonnay scions grafted to transgenic rootstocks (1103 and 110-14) expressing the paired constructs. The field experiment, conducted in a USDA-APHIS regulated site in Solano County, includes *Xylella fastidiosa* mechanical inoculation techniques that were used successfully in past experiments. PD symptoms, bacterial movement, and fruit yield will be measured during the course of the experiment.

INTRODUCTION

This field project began in 2010 to evaluate grapevines expressing potential Pierce's disease (PD) suppressive transgenes from three investigators under field conditions. Each of the plants was transformed with a single DNA insert. All plants were located in a secured, USDA-APHIS approved area in Solano County. The disease was successfully introduced into the cordon-trained plants by mechanical injection of *Xylella fastidiosa* (*Xf*) into young stems in the second year after planting. The plants were monitored regularly for quantity and movement of the bacteria, along with rating inoculated branches for symptoms of PD compared with uninoculated control plants. Thompson Seedless and Freedom rootstock plants expressing transgenes from the Dandekar, Lindow, and Gilchrist projects were compared with non-transgenic PD-susceptible Thompson Seedless and Freedom rootstock plants as controls. The results of these experiments confirmed 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 was no evidence of xylem blockage leading to wilt or epinasty prior to tissue death characteristic of PD foliar symptoms or cane death. There was no evidence of spread of the bacteria to uninoculated susceptible grapevines adjacent to infected plants over the duration of the test, confirming tight experimental control on the pathogen spread. Each of the transgenes tested suppressed the symptoms of PD-inoculated vines to varying degrees (Gilchrist and Lincoln, 2015; Gilchrist and Dandekar, 2016; Gilchrist et al., 2015, 2017, 2018a, 2018b; Lincoln et al., 2018). Consequently, this research has now moved forward to field testing a generation of transgenic rootstocks expressing pairs of the disease suppressive genes in a gene stacking approach, with the genes paired together by differential molecular function. The laboratory confirmed dual construct expressing rootstocks were grafted to susceptible non-transgenic Chardonnay scions to assess potential cross-graft protection against PD in the Solano County field site.

OBJECTIVES

There are four principal objectives:

1. Complete the new planting area within the current USDA-APHIS approved site to contain the second set of lines bearing paired, PD suppressive, DNA constructs, referred to as stacked genes, in two adapted rootstocks (1103 and 101-14). These rootstocks will be grafted to PD-susceptible Chardonnay scions prior to field planting. The goal is to assess the potential of cross-graft protection against PD of a non-transgenic scion. Planting began in 2018 and will be completed by 2019.
2. Train and manage the planting to conform to commercial standards, which will enable collection of fruit yield data as well as collection of disease and bacterial dynamics as the infections proceed. Management will include timely application of pesticides for powdery mildew and insects.
3. Inoculate the individual cordon-trained vines in the second year after the cordons have been secured to the wires. Inoculum will be cells of culture-grown *Xf* confirmed to be pathogenic in the previous field experiments.
4. Quantitative data collection on disease rating and bacterial dynamics (population and movement) beginning in 2019, with fruit yield per plant in the third and fourth years, will be conducted under another CDFA agreement.

RESULTS AND DISCUSSION

Objective 1. Complete the New Planting Area

Rootstocks were grafted to PD-susceptible Chardonnay scions prior to field planting. The first phase of field planting of the stacked gene rootstock combinations was completed on August 1, 2018, with the final planting on August 20, 2019. The development of the stacked gene rootstock transgenics is complete, including molecular analysis of the transgenic rootstock lines released by the Plant Transformation Facility at UC Davis. All field activities will be conducted or coordinated by project leader D. Gilchrist, cooperator B. Pellissier, and Foundation Plant Services (FPS) field personnel.

Objective 2. Train and Manage the Planting to Conform to Commercial Standards

The grafting, planting, and training of the vines was guided by Josh Puckett and Deborah Golino (FPS) working with D. Gilchrist. The FPS crew will continue to provide personnel for trellising and plant management to reflect commercial production standards. The field plot design will enable experimental *Xf* inoculations and pathogen and disease assessments, as well as measures of grape yield. Land preparation and configuration of the experimental area is sufficient to accommodate and manage 900 new plants, including experimental material and control plants. This number includes the dual constructs from the 2018 and 2019 plantings and will accommodate additional plantings established by the Dandekar and Lindow laboratories. Row spacing will be 11 feet between rows with seven feet between plants. This spacing permits 30 plants per row and includes a 50-foot open space around the planted area as required by the USDA-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. A photograph of the completed field planting is provided in **Figure 1**. Total fenced area occupied by plants and buffer zones as required by the USDA-APHIS permit will be approximately 3.4 acres. All plants will be maintained under a drip irrigation system.

Figure 1. Planting configuration for the dual constructs. This image illustrates the new planting of the dual construct transformed rootstocks grafted with an untransformed scion of Chardonnay. This second phase of planting was completed August 2019.

The experimental design will be a complete randomized block with six plants per each of six entries (replications), including all controls. Each plant will be trained as a single trunk with two cordons. 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-inches 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. Irrigation and pest management, primarily for powdery mildew, other foliar pathogens, weeds, and insects, will be coordinated by D. Gilchrist and conducted by B. Pellissier. The field crew utilized to manage the plants to commercial standards will be personnel organized through FPS and coordinated with D. Gilchrist to determine the timing and need for each of the management practices, including pruning and thinning of vegetative overgrowth as necessary (**Table 1**).

Table 1. Schedule for field trial management activities.

2019												
	Jan-19	Feb-19	Mar-19	Apr-19	May-19	Jun-19	Jul-19	Aug-19	Sep-19	Oct-19	Nov-19	Dec-19
Dormant pruning of 2018 planted vines	x											
Collection of dormant material from selections to be included in 2019 planting - GMO treatments	x											
Collection of dormant material from selections to be included in 2019 planting - FPS WT rootstock and Chardonnay 04 material		x										
Root initiation from dormant cuttings (GMO rootstock and WT control selections)				x								
Pruning and training of vineyard					x	x	x					
Chip bud grafting of GMO rootstock and WT control selections with Chardonnay 04 and Thompson Seedless 02A					x							
Maintenance of grafted, container grown vines						x	x	x				
Vineyard planting: organization and implementation									x			
Vineyard rootstock sucker removal											x	
2020												
	Jan-20	Feb-20	Mar-20	Apr-20	May-20	Jun-20	Jul-20	Aug-20	Sep-20	Oct-20	Nov-20	Dec-20
Dormant pruning of 2018 and 2019 planted vines	x											
Pruning and training of vineyard					x	x	x					
Vineyard rootstock sucker removal											x	
2021												
	Jan-20	Feb-20	Mar-20	Apr-20	May-20	Jun-20	Jul-20	Aug-20	Sep-20	Oct-20	Nov-20	Dec-20
Dormant pruning of 2018 and 2019 planted vines	x											
Pruning and training of vineyard					x	x	x					
Vineyard rootstock sucker removal											x	

Regular tilling and hand weeding will maintain a weed-free planting area. Plants were pruned carefully in each spring leaving all inoculated/tagged branches and numerous additional branches for inoculation and sampling purposes in the coming year. All pruning material was left between the rows to dry, then flail chopped and later rototilled to incorporate the residue per requirements of the USDA-APHIS permit. 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 were treated with insecticides when needed. Neither powdery mildew nor insect pressure has been observed in past years with these ongoing practices applied throughout the growing season.

The following protocol, while not part of the field research effort funded by this grant, will be carried out with support from another grant from the CDFA Pierce's Disease and Glassy-winged

Sharpshooter Board and is provided here to illustrate all the research efforts involved with this field experiment, including disease and laboratory analysis. Inoculation of individual canes will follow the same protocol was used successfully in the previous field experiment. Briefly, the protocol is as follows: the Gilchrist lab will produce the bacterial inoculum and the pathogenic cells will be injected by the needle prick method as used in the past to deliver a droplet of 10-20 µl with 2,000 to 20,000 cells per injection site. Each site will be tagged to enable assessment over time. Assistance from the field crew may be used as needed at this step. Both symptom expression and behavior of the inoculated bacteria will provide an indication of the level of resistance to PD infection and the effect of the transgenes on the amount and movement of the bacteria in the non-transgenic scion area. It should be noted that there was no evidence of spread of the bacteria to uninoculated and uninfected susceptible grape plants adjacent to infected plants in the previous five-year experiment in this field area. Hence, the presence and impact of the bacteria is confined to only the mechanically inoculated tissue under these conditions.

Detection of the amount and movement of the bacteria in plant tissues (mainly leaves and stems) by quantitative polymerase chain reaction (qPCR) assays will be done in the Gilchrist lab. The inoculation and analysis will begin in 2020. Evaluation of the experimental plants for plant morphology, symptoms of PD infection, and the presence and movement of the bacteria will continue as in previous years using the same protocols applied successfully in the first generation of field experiments. The symptom assessment will include counting dead or dying buds emerging on inoculated canes that produce tiny leaves but then die quickly, as was recorded previously, and rating of leaf symptom severity as previously done. The assessment format and data collected has been reported annually in the *Proceedings of the Pierce's Disease Research Symposium* (Gilchrist and Lincoln 2015; Gilchrist and Dandekar 2016; Gilchrist et al., 2015, 2017, 2018a, 2018b). Chardonnay fruit yield will be measured after second year fruit set by harvesting fruit from individual plants and combining by genotype.

Research Timetable. Four years beginning with initial planting in the spring of 2018, to be followed by a second planting in spring of 2019. 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 2021-2022 funding cycle and will depend on the results of the field evaluation up to that point.

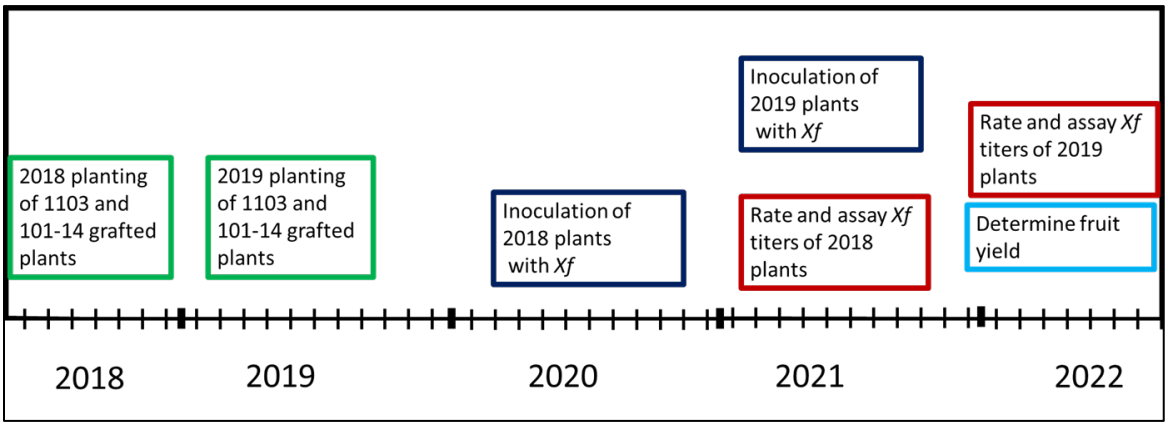


Figure 2. Timeline for field trial.

CONCLUSIONS

The planting of the dual construct transformed rootstocks grafted to untransformed PD susceptible Chardonnay scions was completed in the current USDA-APHIS approved site in 2019. This second set of lines bearing paired, PD suppressive, DNA constructs, referred to as stacked genes, were introduced in two adapted rootstocks (1103 and 101-14). All transgenic rootstocks were verified to contain the requisite pairs of DNA constructs prior to grafting the PD-susceptible Chardonnay scions. The goal is to assess the potential of cross-graft protection against PD of a non-transgenic scion. The experimental protocol includes managing the plants to conform to commercial standards, which will enable collection of fruit yield data as well as collection of disease and bacterial dynamics as the infections proceed. Inoculation of transgenic and control plants will consist of bacterial cells of culture-grown *Xf* confirmed to be pathogenic in the previous field experiments. The individual cordon-trained vines will be inoculated in the second year after the cordons have been secured to the wires. Following inoculation, the plants will be monitored for PD symptoms and the bacteria will be monitored for presence, movement, and concentration. Management will include timely application of pesticides for powdery mildew and insects, along with weed control.

REFERENCES CITED

Gilchrist DG, Lincoln JE. 2015. Field evaluation of grape plants expressing potential protective DNA sequences effective against Pierce's disease. *Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes*. December 2015. California Department of Food and Agriculture, Sacramento, CA, pp. 49-53.

Gilchrist DG, Lincoln JE, Dandekar AM, Lindow S. 2015. Transgenic rootstock-mediated protection of grapevine scions by single and stacked DNA constructs. *Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes*. December 2015. California Department of Food and Agriculture, Sacramento, CA, pp. 59-66.

Gilchrist DG, Dandekar AM. 2016. Field evaluation of cross-graft protection effective against Pierce's disease by dual and single DNA constructs. *Proceedings of the 2016 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA, pp. 54-58.

Gilchrist D, Lincoln J, Dandekar A, Tricoli D, Pellissier B. 2017. Field evaluation of cross-graft protection effective against Pierce's disease by dual and single DNA constructs. *Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes*. December 2017. California Department of Food and Agriculture, Sacramento, CA, pp. 29-34.

Gilchrist D, Lincoln J, Dandekar A, Tricoli D, Pellissier B. 2018a. Transgenic rootstock-mediated protection of grapevine scions by single and stacked DNA constructs. *Proceedings of the 2018 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA, pp. 36-42.

Gilchrist D, Lincoln J, Dandekar A, Tricoli D, Pellissier B. 2018b Field evaluation of cross-graft protection effective against Pierce's disease by dual and single DNA constructs. *Proceedings of the 2018 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA, pp. 30-35.

Lincoln JE, Sanchez JP, Zumstein K, Gilchrist DG. 2018. Plant and animal PR1 family members inhibit programmed cell death and suppress bacterial pathogens in plant tissues. *Molecular Plant Pathology* 19(9): 2111-2123.

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

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

Project Leader: David Gilchrist | Department of Plant Pathology| University of California | Davis, CA 95616 | dggilchrist@ucdavis.edu
Co-Project Leader: James Lincoln | Department of Plant Pathology | University of California | Davis, CA 95616 | jelincoln@ucdavis.edu
Co-Project Leader: Abhaya M. Dandekar | Department of Plant Sciences | University of California | Davis, CA 95616 | amdandekar@ucdavis.edu
Collaborator: David Tricoli | Plant Transformation Facility | University of California | Davis, CA 95616 | dmtricoli@ucdavis.edu
Collaborator: Bryan Pellissier | Department of Plant Pathology | University of California | Davis, CA 95616 | bpellissier@ucdavis.edu

Reporting Period: The results reported here are from work conducted January 31, 2019 to October 15, 2019.

ABSTRACT

This translational research will test for potential cross-graft protection of a Pierce's disease (PD) susceptible Chardonnay scion against the development of PD symptoms by expression of dual combinations of five PD suppressive transgenes in two adapted rootstocks. The protocol includes planting, training, and inoculating to evaluate both disease and yield components specifically in the PD-susceptible scions. It also will enable assessing both potential cross-graft protection of a non-transformed scion and the ability of the transgenes to protect the rootstocks against bacterial movement and death compared to equivalent combinations of non-transformed rootstock/scion control combinations.

LAYPERSON SUMMARY

Xylella fastidiosa (*Xf*) is the causative agent of Pierce's disease (PD) of grapevines. Collectively, a team of researchers (S. Lindow, A. Dandekar, J. Labavitch/A. Powell, and D. Gilchrist) has identified five novel genes (DNA constructs) (**Table 1**) which, when engineered into grapevines, suppress symptoms of PD by reducing the titer of *Xf* in the plant, reducing its systemic spread in the plant, or blocking *Xf*'s ability to trigger PD symptoms. These projects have moved from the proof-of-concept stage in the greenhouse to characterization of PD resistance under field conditions, where current data indicates that each of the five transgenes, introduced as single constructs, reduces the disease levels under field conditions. Importantly, preliminary data indicates that each of the five DNA constructs, when incorporated into transgenic rootstocks, has shown the ability to protect non-transformed scions, with obvious benefit: any of many unmodified varietal scions can be grafted to and be protected by any of a small number of transformed rootstock lines. The ability of transgenic rootstocks to protect all or most of the scions, even at a distance from the graft union, is currently being tested under field conditions regulated by a USDA-APHIS permit. This approach involves "stacking" a combination of distinct protective transgenes in a single rootstock line, which is intended to foster not only durability but also more robust protection of the non-transformed scion against PD.

INTRODUCTION

This progress report will update activities from January 31, 2019 to October 15, 2019 and describe briefly the history, likely function, and impact of transgenes expressed in adapted rootstocks to protect non-transformed Chardonnay scions from the symptoms of Pierce's disease (PD). The test plants are deployed in a USDA-APHIS approved field location, wherein the Chardonnay scions will be mechanically inoculated with *Xylella fastidiosa* (*Xf*) to induce PD. This research began approximately 20 years ago when a team of researchers (S. Lindow, A. Dandekar, J. Labavitch/A. Powell, and D. Gilchrist) identified, constructed, and advanced to field evaluation five novel DNA constructs (**Table 1**) that, when engineered into fully transformed PD susceptible Thompson Seedless grapevines, were shown to suppress symptoms of PD by either (a) reducing the titer of *Xf* in the plant, (b) reducing systemic spread of the bacteria, or (c) blocking *Xf*'s ability to trigger disease symptoms. Each of the five transgenes, when expressed as single genes, reduced the disease levels under field conditions as full plant transgenics. This initial field trial consisting of single gene constructs was begun in 2010 and evaluated until discontinued at the end of the 2016 growing season. The current project, for which the planting was completed August 29, 2019, involves "stacking" paired combinations of the five constructs in each of two adapted rootstock lines. The objective is intended to foster not only durability of potential resistance but also more robust protection of the non-transformed scion through cross-graft protection against PD as described by the Lindow, Dandekar, and Gilchrist labs in previous PD Symposium reports (2, 3, 5).

Table 1. Genes selected to evaluate as dual genes in the second generation field evaluation for suppression of PD in grape.

Gene	Code	Function
CAP	C	*Xf* clearing/antimicrobial
PR1	A	grape cell anti-death
rpfF	F	changing quorum sensing of *Xf* (DSF)
UT456	B	non-coding microRNA activates PR1 translation
PGIP	D	inhibits polygalacturonase/ suppressing *Xf* movement

OBJECTIVES

The primary objective for expressing two genes simultaneously in stacked combinations in two different adapted rootstocks is to explore the potential for cross graft protection of a non-transformed PD susceptible scion. Since several of the five DNA constructs (**Table 1**) have proposed biochemically distinct mechanisms of action, having two differentially acting DNA constructs in a single transgenic rootstock has the potential to 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 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" (6) describes the stacking of several genes for virus resistance in squash. Note, D. Tricoli, the lead author in this paper, has done the stacking transformations in this proposal. Additionally, the Dandekar laboratory has successfully stacked two genes blocking two different pathways synergistically to suppress crown gall in walnut (7).

The specific objectives of this project are:

1. Complete the introduction of pairs of protective paired constructs via the dual insert binary vector into adapted grapevine rootstocks 1103 and 101-14 for a total of 20 independent transgenic lines to be evaluated in the lab, with selections moved later to the field.
2. Conduct extensive analysis, both by Northern analysis and polymerase chain reaction (PCR) and reverse transcription quantitative PCR (RT-qPCR) experiments, of each transgenic plant, to verify the presence of the two stacked genes in the genome, the full RNA sequence, and the expression level of each of the mRNAs expected to be produced by the inserted genes, before they are subjected to grafting and greenhouse inoculation assays for transgene movement and resistance to PD.
3. The second major step in the process, after verification of the genotypic integrity of the transgenic plants, is production of the clonal ramets of each plant line to enable two-cane growth development of the rootstocks and grafting of the Chardonnay scions**.**
4. Establish a new planting area within the current USDA-APHIS approved site to contain a new set of lines bearing paired, PD 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 non-transformed PD-susceptible Chardonnay scion prior to field planting. The goal is to assess the potential of cross graft protection against PD of a non-transgenic scion and determine if the transgenic rootstocks are protected against bacterial movement from the scion to the rootstock, thereby providing protection of the rootstock against *Xf*-triggered death compared to native non-transformed rootstock combinations. Planting was begun in 2018 and completed August 29, 2019, with field inoculations to begin in 2020.

RESULTS AND DISCUSSION

Construction and Analysis of Dual Gene Expression Binaries

The transgenic strategy is to prepare dual-plasmid constructs bearing a combination of two of the protective genes on a single plasmid with single selectable marker as described previously (3). The binary backbone is based on pCAMBIA1300 (8). Binaries were constructed to express two genes from two 35S promoters. Binary plasmids capable of expressing two genes from the same TDNA were constructed by J. Lincoln (3).

All plasmids were transformed into *Agrobacterium* strain EHA105, the preferred transformation strain for grape plants. Extensive analysis of each plasmid before delivery to the UC Davis Plant Transformation Facility confirmed the integrity of the dual binary plasmid, used to transform the grape embryos by D. Tricoli. Each plasmid containing the dual protective DNA sequences was introduced into embryogenic grapevine culture in a single transformation event. At the time this protocol was developed this was a novel attempt to expedite the simultaneous insertions of the two constructs, rather than the traditional, much slower method of using two separate binary transformations. In grape this was a veritable nightmare, since a flowering plant had to be produced from the first transformation. This novel protocol was successful and has been adopted

for future transformation of grape and other plants as a labor and time saving strategy. The new transgenic dual gene expressing grape plant lines exhibit a phenotype indistinguishable from the non-transformed wild-type rootstock used as control. Analysis of the transgenic rootstocks to confirm production in the rootstock and potential movement of the transgene messages or products across a graft union to the untransformed scion will be conducted.

As indicated in Objective 2, one goal is to conduct analysis of the integrity of the insertions in the rootstocks and subsequently begin to develop methods to assess the expression and possible movement of the transgene products.

Analysis of Dual Insertions. This analysis is performed by isolating the RNA from transgenic grape leaves and purifying it using a modification of a cetyl trimethylammonium bromide (CTAB) protocol that includes LiCl precipitation. The RNA is converted to complementary DNA (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 goal is to identify six independently-transformed lines bearing the dual sets of the five transgenes to confirm the genotype of each rootstock to be placed in the field, with six replications of each line. The aforementioned analysis indicated that the successful insertion of two genes into a given transgenic plant was 67 percent of the total plants provided by the Plant Transformation Facility (**Table 2**). This underscores the need for dual transcript verification prior to moving plants forward to grafting and subsequent analysis for product movement across a graft union and symptom suppression of the non-transformed Chardonnay. These assays, while time-consuming and tedious, will ensure that each plant will have a full phenotypic and genotypic analysis prior to inoculating them in the field. D. Tricoli, Plant Transformation Facility manager, confirmed that the aforementioned steps provided the highest success rate in transformation he has experienced with grape.

Table 2. Frequency of dual gene transcripts as confirmed in transgenic plants delivered by the Plant Transformation Facility at UC Davis by reverse transcription and PCR analysis.

Transgene Transcripts	Number of Plants	Percent of Plants
2	230	67
1	99	29
0	12	4

Detection of Transgene Products. Preliminary experiments were conducted to develop protocols for detecting the protein products expressed in both the rootstock tissues and the grafted scion. **Figure 1** shows soluble proteins of the p14 gene isolated from the rootstock and grafted scions. The analysis consists of using p14 antibodies we developed to visualize the presence of the protein by immunological detection using classic Western analysis. A positive detection is revealed by a dark spot, indicating the presence and relative amount of the product isolated from the respective tissues. Lane A shows the p14 protein expressed by an *Escherichia coli* bacterial expression vector using the same coding sequence as was transformed into the grape plants;

lanes B-D show high levels of the p14 protein expressed by each of the three rootstocks; lane E is a sample taken from the Chardonnay scion grafted to rootstock D and indicates presence of the p14 product across the graft union; lane F is a control of wild-type rootstock grafted to the Chardonnay scion, confirming that there is no evidence of the p14 product in either the non-transformed rootstock or scion.

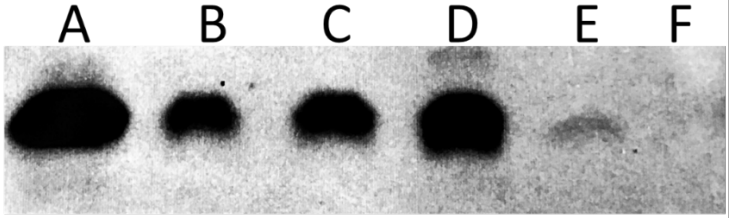


Figure 1. Detection of protein products expressed in the 3 independent transgenic rootstocks and in the untransformed Chardonnay scion. A) E. coli produced p14 (positive control); B-D) 3 independent 101-14 rootstocks expressing p14 in a stem sample from each rootstock; E) Chardonnay scion grafted to plant D; E) chardonnay scion grafted to wildtype 101-14 (negative control)

After verification of dual inserts by RT-qPCR, the selected lines were moved to a lath house (**Figure 2**). Subsequently, the cuttings from the lath house plants were then moved to a greenhouse for final stem development prior to rooting of the transformed rootstocks and grafting of the non-transformed Chardonnay scions (**Figure 3**). The grafted plants listed in **Table 3** were completed successfully and included the non-transgenic controls designed to measure any direct effect of the wild-type 1103 and 101-14 rootstocks. As put forth in the original proposal, the first phase of the field planting was completed in early August 2018, with the final planting completed August 29, 2019 (Objective 4).

Figure 2. Plants selected as rootstock source material. Image shows selected dual construct containing plants in lath house as final site to produce material for rootstock development, for grafting of non-transgenic scions and field evaluation.

Table 3. August 20, 2019 updated list of transgenic paired gene and rootstock combinations including controls in the field

gene code	construct	gene(s)	variety	lines in field	plants in field
AB	[pCA-5oP14HT-5o456]	[456][P14]	1103	6	37
AC	[pCA-5oCAP-5oP14HT]	[CAP][P14]	1103	6	36
AD	[pCA-5oP14HT-5oPGIP]	[P14][PGIP]	1103	6	34
AF	[pCA-5oP14HT-5orpfF]	[P14][rpfF]	1103	3	9
BC	[pCA-5oCAP-5o456]	[456][CAP]	1103	6	33
BD	[pCA-5oP14HT-5o456]	[456][P14]	1103	3	9
BF	[pCK-5o456-5orpfF]	[456][rpfF]	1103	4	14
CD	[pCK-5oCAP-5oPGIP]	[CAP][PGIP]	1103	4	16
CF	[pCA-5oCAP-5orpfF]	[CAP][rpfF]	1103	6	33
DF	[pCA-5oPGIP-5orpfF]	[PGIP][rpfF]	1103	6	31
AB	[pCA-5oP14HT-5o456]	[456][P14]	101-14	6	35
AC	Transformation failed			0	0
AD	[pCK-5oP14HT-5oPGIP]	[P14][PGIP]	101-14	5	20
AF	[pCA-5oP14HT-5orpfF]	[P14][rpfF]	101-14	4	16
BC	[pCA-5oCAP-5o456]	[456][CAP]	101-14	6	22
BD	[pCA-5o456-5oPGIP]	[456][PGIP]	101-14	6	34
BF	[pCK-5o456-5orpfF]	[456][rpfF]	101-14	5	14
CD	[pCA-5oCAP-5oPGIP]	[CAP][PGIP]	101-14	6	21
CF	[pCA-5oCAP-5orpfF]	[CAP][rpfF]	101-14	3	3
DF	[pCA-5oPGIP-5orpfF]	[PGIP][rpfF]	101-14	6	31
AB	[pCK-5oP14HT-5o456]	[456][P14]	TS	3	14
AC	Not attempted			0	0
AD	Not attempted			0	0
AF	[pCK-5oP14HT-5orpfF]	[P14][rpfF]	TS	6	6
ANB	[pCA-5oP14HT-5o456]	[456][P14]	TS	6	33
BC	[pCA-5oCAP-5o456]	[456][CAP]	TS	6	32
BD	[pCA-5o456-5oPGIP]	[456][PGIP]	TS	6	32
BF	Not attempted			0	0
CD	Not attempted			0	0
CF	Not attempted			0	0
DF	[pCK-5oPGIP-5orpfF]	[PGIP][rpfF]	TS	5	21
UP	[pCK-5oP14HT]	[P14]	TS	7	38
456-20	[pCK-5o456]	[456]	TS	1	7
P14-9	[pCK-5oP14HT]	[P14]	TS	1	7
Pcontrol	WT chardonnay scion	1103 rtstk	1103	1	37
Mcontrol	WT chardonnay scion	101-14 rtstk	101-14	1	37
TScontrol	wildtype TS scion	101-14 rtstk	TS	9	9
Totals				**149**	**721**

Figure 4. Planting of the dual constructs. This image illustrates the completed planting of the dual construct transformed rootstocks grafted with a non-transformed clone of Chardonnay. The first phase of the planting was completed August 1, 2018; the final planting of the remaining constructs listed in **Table 3** was completed August 29, 2019.

The Planting and Management Protocols Remain the Same as Originally Proposed

a. The experimental design is a complete randomized block with six plants per each of six entries (replications), including all controls. Individual plants will be spaced nine feet apart in rows on 11-foot centers (11 x 9). Each plant will be trained as a single trunk with bilateral cordons. 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. The plants will be allowed to grow vertically, or close to vertically, 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 and 13-gauge wire used to support the drip irrigation wire, about 18 inches off the ground (**Figure 4**).
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-inches 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. Mechanical inoculation of *Xf* into vegetative shoots will follow the same protocol used to effectively establish the pathogen in the plant tissue and elicit PD symptoms as was done successfully in the previous planting on this field site (3).
d. Grape fruit yield will be measured after the second or third year depending on the fruit set.

e. Evaluation of the experimental plants for plant morphology, symptoms of infection, and the presence of the bacteria will follow past procedures.
f. For the molecular analysis of bacterial dynamics, 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 qPCR assay. The analysis will be done in the Gilchrist lab by the same methods and laboratory personnel as has been done successfully with the previous field planting (5). Stem tissue containing the xylem-based bacteria will be pulverized in liquid nitrogen to preserve the native state of the bacteria in the grape tissue. DNA is isolated by a reproducible CTAB-based extraction method. Quantitative detection of the *Xylella* genome uses specific 16S ribosomal primers. A quantitative qPCR detection method of *Xf* cells in non-transgenic scions and grape rootstocks will be compared with the non-transformed grape scions and grape rootstocks.
g. Both symptom expression and behavior of the inoculated bacteria will provide an indication on the level of resistance to *Xf* infection and the effect of the transgenes on the amount and movement of the bacteria in the non-transgenic scion area and the movement into the rootstocks.
h. In relation to natural spread of the pathogen from infected plants to adjacent plants, there was no evidence of movement between nearby mechanically-infected plants in the previous experiment over a six-year period. This lack of spread of the bacteria from inoculated to non-inoculated plants is an important consideration for the experiments carried out for this project and for the granting of the USDA-APHIS permit. The field area chosen has never had grapes planted therein, which is to avoid any potential confounding problems caused by soil-borne diseases, including nematodes.
i. Irrigation and pest management, primarily for powdery mildew, weeds and insects, will be coordinated by D. Gilchrist and conducted by B. Pellissier, the field superintendent employed by the UC Davis Department of Plant Pathology. The field crew work closely with D. Gilchrist to determine the timing and need for each of the management practices, including pruning and thinning of vegetative overgrowth as necessary by a crew provided by Foundation Plant Services.
j. Regular tilling and hand-weeding will maintain a weed-free planting area. Plants will be pruned carefully in March of each year, leaving all inoculated/tagged branches and numerous additional branches for inoculation and sampling purposes in the coming year. All pruning material will be left between the rows to dry, then flail chopped and later rototilled to incorporate the residue per requirements of the USDA-APHIS permit.
k. 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 has been observed with these ongoing practices throughout the past five growing seasons.

Research Timetable. This project began with an initial planting in 2018 (**Figure 5**) and was followed by additional plantings in 2019 as experimental plants became available in the second year. 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 third and following years will depend on the results of the field evaluation up to that point and will be proposed in the 2020 funding cycle. The field area has been designated legally available

for planting the specified transgenic grapes by USDA-APHIS under permit number 7CFRE340 that is held by A. Dandekar.

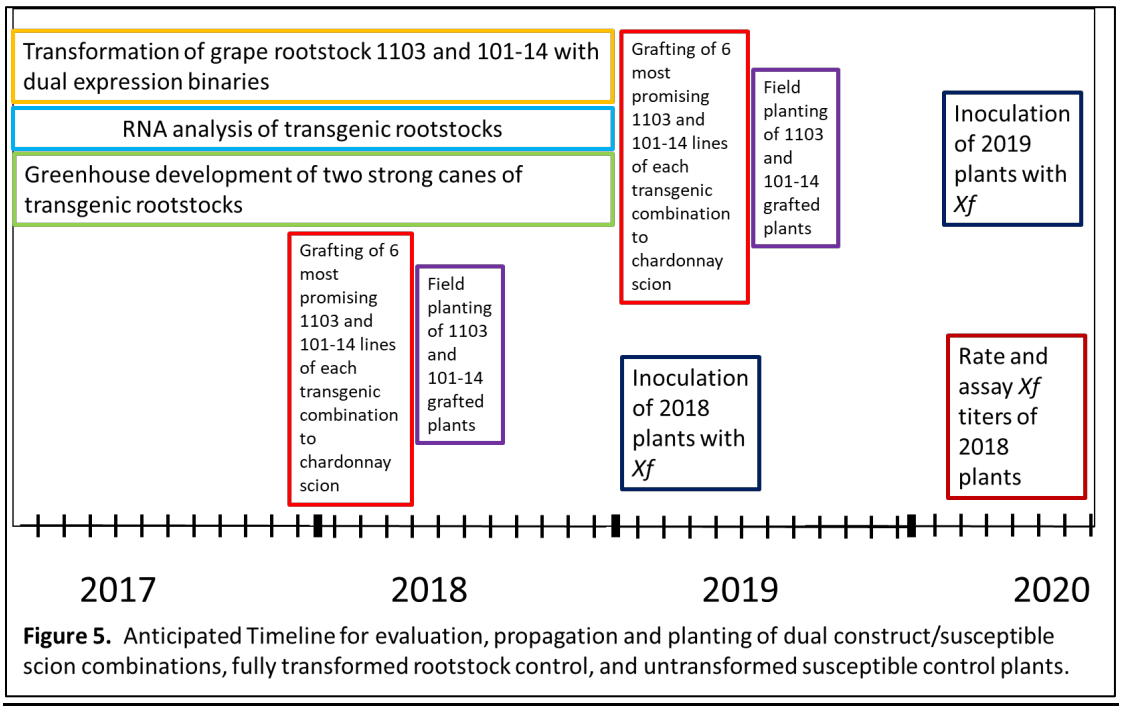


Figure 5. Anticipated Timeline for evaluation, propagation and planting of dual construct/susceptible scion combinations, fully transformed rootstock control, and untransformed susceptible control plants.

CONCLUSIONS

The protocols for managing the existing and the new plantings with the dual constructs have been used successfully over the past five years (3). These protocols include plant management, inoculation with *Xf*, development of classical symptoms of PD 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 PD and/or reduce the ability of the pathogenic bacteria to colonize and move within the xylem of the grape plant. Management of the vines to commercial standards will be directed by D. Golino (UC Davis) and D. Gilchrist. All indicated timelines have been completed within the proposed periods.

REFERENCES CITED

1. Aguero CB, Meredith CP, Dandekar AM. 2006. Genetic transformation of *Vitis vinifera* L. cvs Thompson Seedless and Chardonnay with the pear PGIP and GFP encoding genes. *Vitis* 45: 1-8.
2. Lindow SE. 2013. Continued field evaluation of diffusible signal factor producing grape for control of Pierce's disease. *Proceedings of the 2013 Pierce's Disease Research Symposium.* California Department of Food and Agriculture, Sacramento, CA, pp. 136-144.
3. Gilchrist D, et al. 2016. Transgenic rootstock-mediated protection of grapevine scions by introduced single and dual stacked DNA constructs. *Proceedings of the 2016 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA, pp. 59-66.

4. Lincoln J, Sanchez J, Gilchrist D. 2018. Pathogenesis-related protein PR-1 interferes with programmed cell death and is synthesized under translational control. *Molecular Plant Pathology* 19(9): 2111-2123.
5. Gilchrist D, et al. 2018. Transgenic rootstock-mediated protection of grapevine scion by introduced single and dual stacked DNA constructs. *Proceedings of the 2018 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA, pp. 36-42.
6. Tricoli DM, Carney KJ, Russell PF, McMaster JR, Groff DW, Hadden KC, Himmel PT, Hubbard JP, Boeshore ML, Quemada HD. 1995. 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. *Nature Biotechnology* 13: 1458-1465. doi:10.1038/nbt1295-1458.
7. Escobar MA, Civerolo EL, Summerfelt KR, Dandekar AM. 2001. RNAi-mediated oncogene silencing confers resistance to crown gall tumorigenesis. *Proceedings of the National Academy of Sciences USA* 98(23): 13437-13442.
8. Hajdukiewicz P, Svab Z, Maliga P. 1994. The small versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. *Plant Molecular Biology* 25: 989-994.

FUNDING AGENCIES

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

BIOLOGICAL CONTROL OF PIERCE'S DISEASE OF GRAPE WITH AN ENDOPHYTIC BACTERIUM

Project Leader: Steven Lindow | Department of Plant and Microbial Biology | University of California | Berkeley, CA 94720 | icelab@berkeley.edu
Cooperator: Renee Koutsoukis | Department of Plant and Microbial Biology | University of California | Berkeley, CA 94720 | reneek@berkeley.edu
Cooperator: Clelia Baccari | Department of Plant and Microbial Biology | University of California | Berkeley, CA 94720 | clelia.baccari@berkeley.edu

Reporting Period: The results reported here are from work conducted July 2016 to October 2019.

ABSTRACT

Paraburkholderia phytofirmans strain PsJN was found to be capable of extensive growth and movement within grapevines after both needle or spray inoculation. The population size of *Xylella fastidiosa* (*Xf*) is greatly reduced in plants in which *P. phytofirmans* is either co-inoculated at the same time and location, inoculated at the same time but at other nearby locations, and even inoculated at other locations either three weeks before or after that of the pathogen. The dramatic reductions in population size of *Xf* are observed in all grape varieties tested. Reductions in pathogen population are similarly large when *P. phytofirmans* is inoculated by spraying in a suspension containing 0.2% Break-Thru, an organosilicon surfactant with very low surface tension, as when directly inoculated into plants using a needle. While *P. phytofirmans* can achieve large population sizes in inoculated grapevines within three to four weeks after inoculation, and spread up to one meter away from the site of point inoculation, its population size then often decreases with further time after inoculation. The very large decrease in population size of *Xf* in plants inoculated with *P. phytofirmans* even after that of the pathogen is suggestive of a mechanism by which this antagonistic microorganism sensitizes the plant to the presence of the pathogen, thereby initiating a plant disease resistance reaction. Support for such a model was provided by evidence of up-regulation of the expression of the PR1 and ETR1 genes in grapevines inoculated both with *P. phytofirmans* and *Xf* but not that of the pathogen alone. Substantial control of Pierce's disease in Cabernet Sauvignon, Pinot Noir, and Chardonnay was seen in field trials, with the largest reductions in disease severity observed in plants treated with *P. phytofirmans* applied by droplet puncture or by spray application in a penetrating surfactant either at the same time as, or up to three weeks after, that of the pathogen.

LAYPERSON SUMMARY

A naturally-occurring *Paraburkholderia* strain capable of growth and movement within grapevines has been found that can confer increased resistance to Pierce's disease. We are exploring the biological control of disease using this strain. The growth and movement of *Xylella fastidiosa* (*Xf*) within plants and disease symptoms are greatly reduced in plants in which this *Paraburkholderia* strain was inoculated either simultaneously with, prior to, or 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. Spray application of the bacterium onto leaves with a surfactant that achieves low surface tension appears to be a particularly effective method of inoculation under field conditions. These results

are quite exciting in that they reveal that biological control of Pierce's disease using *P. phytofirmans* is both robust and may be relatively easy to employ by various ways of inoculation.

INTRODUCTION

Much of our previous work on *Xylella fastidiosa* (*Xf*) and the control of Pierce's disease has dealt with a cell density-dependent gene expression system mediated by a family of small signal molecules called diffusible signal factor (DSF) which includes 2-Z-tetradecenoic acid (C14-cis), and 2-Z-hexadecenoic acid (C16-cis). This work revealed that cell density signaling modulated the adhesiveness of cells in the plant, that movement of the pathogen is essential for its virulence, and that artificially increasing DSF levels in transgenic plants greatly increased the resistance of these plants in both greenhouse and field studies to Pierce's disease, by limiting the spread of the pathogen after infection. While endophytic bacteria might be exploited to produce DSF in plants, until recently, no strains capable of growth or movement in grapevines had been found. We found, however, that *Paraburkholderia phytofirmans* strain PsJN was capable of extensive growth and movement within grapevines. Our intention, therefore, was to use such a strain as a surrogate host for the *rpfF* gene from *Xf* that encodes DSF synthase. We found, however, that this *Paraburkholderia* strain itself was capable of mediating very high levels of control of Pierce's disease. Our continuing results from greenhouse studies show remarkable ability of this biological control agent to move within plants and to inhibit the movement of *Xf*, thus achieving very high levels of disease control. The current work is providing a better understanding of the ways in which this biological control agent can be used for disease control, and extensive field evaluations to exploit the information learned from greenhouse studies are underway.

OBJECTIVES

1. Determine how the temporal and spatial interactions of *Paraburkholderia* and *Xf* in grape inoculated in different ways with this biological control agent lead to disease control.
2. Identify the mechanisms by which *Paraburkholderia* confers biological control of Pierce's disease.
3. Evaluate the biological control of Pierce's disease in field trials in comparison with other strategies of pathogen confusion.

RESULTS AND DISCUSSION

Objective 1. Biological Control with *P. 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 Bruce Kirkpatrick, exhibited no ability to grow and move beyond the point of inoculation when re-inoculated. We have recently found that *P. phytofirmans* strain PsJN, which had been suggested to be an endophyte of grape seedlings, multiplied and moved extensively in mature grape plants. Its population size and spatial distribution in grapevines 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 *Paraburkholderia* species, and the genome sequence of *P. 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. Our studies have shown that co-inoculation of *Xf* and *P. 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 *P. phytofirmans* (Baccari et al., 2019).

P. 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 one 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 (Baccari et al., 2019). 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, *P. phytofirmans* conferred a very high level of disease resistance. It thus appears that the beneficial effect of *P. phytofirmans* is not variety specific, and that it should confer high levels of resistant in all grape varieties.

The large reductions in the severity of disease when *Xf* was co-inoculated with *P. phytofirmans* PsJN was associated with the apparent elimination of viable cells of the pathogen both at the point of inoculation as well as at various distances distal to the point of inoculation, either four or eight weeks after inoculation (Baccari et al., 2019). In contrast, the population size of *Xf* increased progressively after its inoculation into grape in the absence of *P. phytofirmans*, reaching population sizes of approximately 10^6 cells/g at all sites within about 60 cm from the point of inoculation, and moved to at least 120 cm from the point of inoculation within eight weeks after inoculation. Such large populations throughout the plant were associated with a high severity of disease, which increased between 11 and 14 weeks after inoculation (Baccari et al., 2019). In contrast, no viable cells of *Xf* were detected at any location in these plants either four or eight weeks after inoculation together with *P. phytofirmans* (Baccari et al., 2019) and no evidence of Pierce's disease was observed even by 14 weeks after inoculation. By four weeks after inoculation, population sizes of *P. phytofirmans* of about 10^4 cells/g were observed at all points up to 60 cm distal to the point of inoculation. Curiously, while readily detected up to 90 cm or more from the point of inoculation when assessed eight weeks after inoculation, *P. phytofirmans* population sizes were consistently about 10-fold lower at a given distance from the point of inoculation than at four weeks. *P. phytofirmans* population sizes were often slightly lower at a given sampling time and location when co-inoculated into plants with the pathogen compared to when it was inoculated alone. Large reductions in population sizes of *Xf*, often to undetectably low numbers, in plants inoculated with *P. phytofirmans* at various times, and in various ways, was always observed in the many experiments undertaken.

To determine whether the inhibitory effects of *P. phytofirmans* on the process of Pierce's disease was dependent on any direct interactions between it and *Xf* that might have occurred because of their mixture together at the point of inoculation, we compared the dynamics of disease process in plants in which the pathogen and strain PsJN were applied as a mixed inoculation in the same site with that in plants in which they were inoculated separately up to 10 cm apart but at the same time. As previously observed, the severity of Pierce's disease in plants in which the pathogen and strain PsJN were applied as a mixed inoculum in the same site in the plant was greatly reduced at a given time after inoculation compared to plants inoculated only with the pathogen

(Baccari et al., 2019). Importantly, disease severity for plants inoculated at the same time but at different locations with these two strains was usually only nearly as low as that in plants receiving a mixed inoculum. Both treatment schemes resulted in very large reductions in disease severity compared to that of control plants inoculated only with the pathogen.

Given that close physical proximity of *Xf* and *P. phytofirmans* at the time of inoculation of the pathogen is apparently not required to achieve large reductions in disease, we explored methods of inoculation of plants with strain PsJN that might ultimately prove more practical for implementation under field conditions than injection into stems. Spray application of bacterial inoculum might readily be adoptable by growers because of similarities in methodology and equipment used in other pest management procedures. Topical application of suspensions of *P. phytofirmans* of 10^8 cells/ml in buffer alone resulted in only very low internalized population sizes of this strain within either petioles or leaf lamina when assessed at different times after spray application. In contrast, the population size of strain PsJN applied in buffer containing 0.2% Break-Thru, an organo-silicon surfactant conferring extremely low surface tension to aqueous solutions (similar to that of Silwet L77), were about 1,000-fold higher than that within leaves sprayed with bacterial suspensions in buffer alone (Baccari et al., 2019). Furthermore, the population size of strain PsJN was about 100-fold higher within the lamina of the leaf compared to that within the petioles. Not only were large internalized populations of *P. phytofirmans* achieved immediately after inoculation ($> 10^3$ to 10^5 cells/g), but these endophytic bacterial population sizes increased with time for at least nine days after spray inoculation. In many other experiments in which strain PsJN was topically applied with 0.2% Break-Thru the population size of strain PsJN within leaves immediately after inoculation was as high as 10^5 cells/g (data not shown). The leaves sprayed with bacterial suspensions containing this surfactant immediately acquired a water-soaked appearance, indicative of water infiltration into the leaf. The number of bacteria introduced into the plant by such sprays appeared to be influenced by the water status of the plant and whether stomata were fully open, both of which influenced the degree of water infiltration. It thus appears that *P. phytofirmans* can be readily inoculated into grape by simple spray application when appropriate surfactants are used.

The severity of Pierce's disease on plants sprayed with *P. phytofirmans* immediately before inoculation with *Xf* was significantly lower than on control plants inoculated with the pathogen alone (Baccari et al., 2019). While disease severity of plants sprayed with *P. phytofirmans* at the same time as inoculation with the pathogen was often slightly higher than that on plants that were puncture-inoculated with this strain at the same time as the pathogen when assessed at a given time, the severity of disease in both treatments was always much less than that of control plants inoculated only with the pathogen, and often did not differ significantly. It appears that topical application of *P. phytofirmans* with a surfactant that allows spontaneous stomatal infiltration is nearly as effective in mediating control of Pierce's disease as direct inoculation of this biological control agent into xylem tissue.

While *Xf* and *P. phytofirmans* apparently do not need to be entirely spatially coincident at the time of inoculation of the pathogen in order to achieve suppression of Pierce's disease symptoms, and substantial disease control was inevitably obtained when the two strains were inoculated at the same time into plants by various ways, insights as to the possible mechanisms contributing to disease control were obtained by inoculating strain PsJN into plants at various

times relative to that of the pathogen. Surprisingly, the extent to which the severity of Pierce's disease was reduced when *P. phytofirmans* was inoculated into plants, either by injection or spray application, four weeks prior to inoculation with *Xf* was invariably less than when the two strains were applied at the same time when made by the same method of PsJN application. For example, in some experiments, Pierce's disease severity in plants treated with *P. phytofirmans* either by needle inoculation or spraying four weeks before that of the pathogen did not differ from that of plants inoculated with the pathogen alone, while simultaneous inoculation with strain PsJN by either method conferred very large reductions in disease severity compared to control plants (Baccari et al., 2019). In other experiments, pre-treatment of plants with *P. phytofirmans*, either by needle inoculation or spraying, conferred significant reductions in disease severity compared to that of control plants, yet the extent of disease control was substantially less than that conferred by needle or spray inoculations applied at the same time as the pathogen (Baccari et al., 2019). Disease severity in plants sprayed with *P. phytofirmans* was, however, consistently less than that in plants to which strain PsJN had been inoculated by puncturing before the pathogen. Even more surprising, however, was the observation that disease control achieved by puncture or spray inoculation of *P. phytofirmans* into plants three to four weeks after inoculation of the pathogen was as great as, and often greater than, that achieved by simultaneous inoculation by a given method. Given that population sizes of *Xf* typically increase and spread extensively in inoculated plants within four weeks, it was remarkable to find, as in other experiments, very low or undetectable population sizes of *Xf* subsequent to such treatments with *P. phytofirmans*, even though it was applied four weeks after that of the pathogen (data not shown).

Insight into the surprising observation that pre-treatment of plants with *P. phytofirmans* inevitably reduced its efficacy in the biological control of Pierce's disease compared to simultaneous or post-inoculation treatments was provided by analysis of the temporal patterns of colonization of plants by strain PsJN. We frequently observed that, while relatively large population sizes of *P. phytofirmans* could be detected throughout grape within a few weeks after inoculation, this population size often subsequently decreased, often dramatically so (Baccari et al., 2019). A more systematic examination of *P. phytofirmans* populations when co-inoculated with *Xf* in grape as a function of time revealed that its population size and distribution distal to the point of inoculation increased for at least three weeks after inoculation, but then started to decrease by five weeks (Baccari et al., 2019). As in most other experiments, viable cells of *P. phytofirmans* often became undetectably low within 10 weeks after inoculation (data not shown). As in all experiments, when inoculated in the absence of *P. phytofirmans* both the population size and extent of distribution of *Xf* distal to the point of inoculation tended to increase with time, while viable cells of the pathogen were not detected at any time or distance from the point of inoculation when co-inoculated with strain PsJN.

Objective 2. Mechanisms of Biological Control

As discussed in Objective 1, it seemed possible that *Paraburkholderia* may alter the behavior and survival of *Xf* by inducing changes in grape plants themselves, such as by stimulating innate plant immunity. Plant innate immunity serves as an important mechanism by providing the first line of defense to fight against pathogen attack. While grape apparently does not successfully recognize and therefore defend against infection by *Xf*, it might be possible that plants could be "primed" to mount a defense against *Xf* by another organism such as *Paraburkholderia*. Certain

beneficial microorganisms such as *P. phytofirmans* PsJN have been shown to prime innate defenses against various pathogens in model plant system such as *Arabidopsis*, and a recent study suggests that it could also do so in grapes. Further, the bacterium induces plant resistance against abiotic stresses, apparently by changing patterns of gene expression in host plants. We thus explored whether the reduced disease symptoms and lower pathogen populations seen in plants inoculated with *Paraburkholderia*, either before or after that of *Xf*, is mediated by the activation of plant innate immunity. To test this hypothesis, we measured the expression of various genes in grapes that are responsible for, or reflective of, responses to pathogens, mechanical, and abiotic stresses in control plants with no treatment, plants injected with the *Paraburkholderia* strain alone, plants injected with both *Paraburkholderia* and *Xf* strains simultaneously, and plants inoculated only with *Xf*.

The abundance of PR1, indicative of induction of salicylic acid-mediated host defenses, JAZ1, indicative of jasmonic acid-mediated host defenses, and ETR1, reflecting ethylene-dependent responses, were determined in RNA isolated from petioles collected from near the point of inoculation of plants by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). The abundance of EF1a, expected to be constitutively expressed, was used as an internal control to account for the efficiency of RNA isolation. The abundance of these indicator transcripts was compared in plants inoculated only with *P. phytofirmans*, *Xf*, or co-inoculated with the pathogen and strain PsJN weekly after inoculation, as well as in mock-inoculated plants. Little expression of JAZ1 was detected in any of the plants, irrespective of the sampling time after inoculation (Baccari et al., 2019). In contrast, some PR1 transcript was seen soon after inoculation of plants only with *P. phytofirmans*, with lesser amounts subsequently detected. Low levels of PR1 transcript were also observed within one week of inoculation of plants only with *Xf*, with reductions thereafter. Most notably, the highest levels of PR1 transcript were observed in plants co-inoculated with *P. phytofirmans* and *Xf*, with the apparent abundance of this transcript increasing with time up to three weeks (Baccari et al., 2019). The abundance of PR1 transcript in these plants decreased rapidly thereafter (data not shown). Very low levels of ETR1 transcript were observed in all plants except those co-inoculated with *P. phytofirmans* and *Xf*. This suggests that an interaction between *P. phytofirmans* and *Xf* induces both the salicylic acid and ethylene-dependent signal transduction pathways in grape to levels higher than that mediated by either strain alone.

Ethylene and salicylic acid mediated host defenses often involve production of reactive oxygen species such as hydrogen peroxide. It is the production of such compounds that is associated with the inhibition of pathogen growth. To move beyond the simple measurement of gene expression changes in grape associated with the presence of both *Paraburkholderia* and *Xf* in the plant, we measured hydrogen peroxide levels in plants that were treated with *Paraburkholderia* immediately before *Xf*. Very low hydrogen peroxide concentrations, similar to those observed in plants inoculated with buffer alone, were observed in plants inoculated with *P. phytofirmans* alone, as well as *Xf* alone (**Figure 1**). Importantly, a much higher concentration of hydrogen peroxide was observed in petioles of plants that had been inoculated with both *Paraburkholderia* and *Xf* (**Figure 1;** note that the smaller value represents a darker image and hence higher concentration of hydrogen peroxide). These exciting results help to confirm that the presence of *Paraburkholderia* is priming grapes for a strong defensive reaction to the presence of the pathogen, whereas the plant appears to have no response to the presence of the pathogen alone.

The induction of reactive oxygen species in such primed plants provides strong support for the observations noted above that viable cells of *Xf* are often not detected in plants co-inoculated with *Paraburkholderia*, and also could explain why the population size of *Paraburkholderia* itself tends to decrease with time, especially when inoculated into plants with the pathogen. This method for detection of reactive oxygen species appears to be one that can be readily scaled up, and we look forward to investigating both the temporal and spatial patterns of reactive oxygen species in plants under field conditions in which *Paraburkholderia* is inoculated into plants at various times relative to that of inoculation with the pathogen.

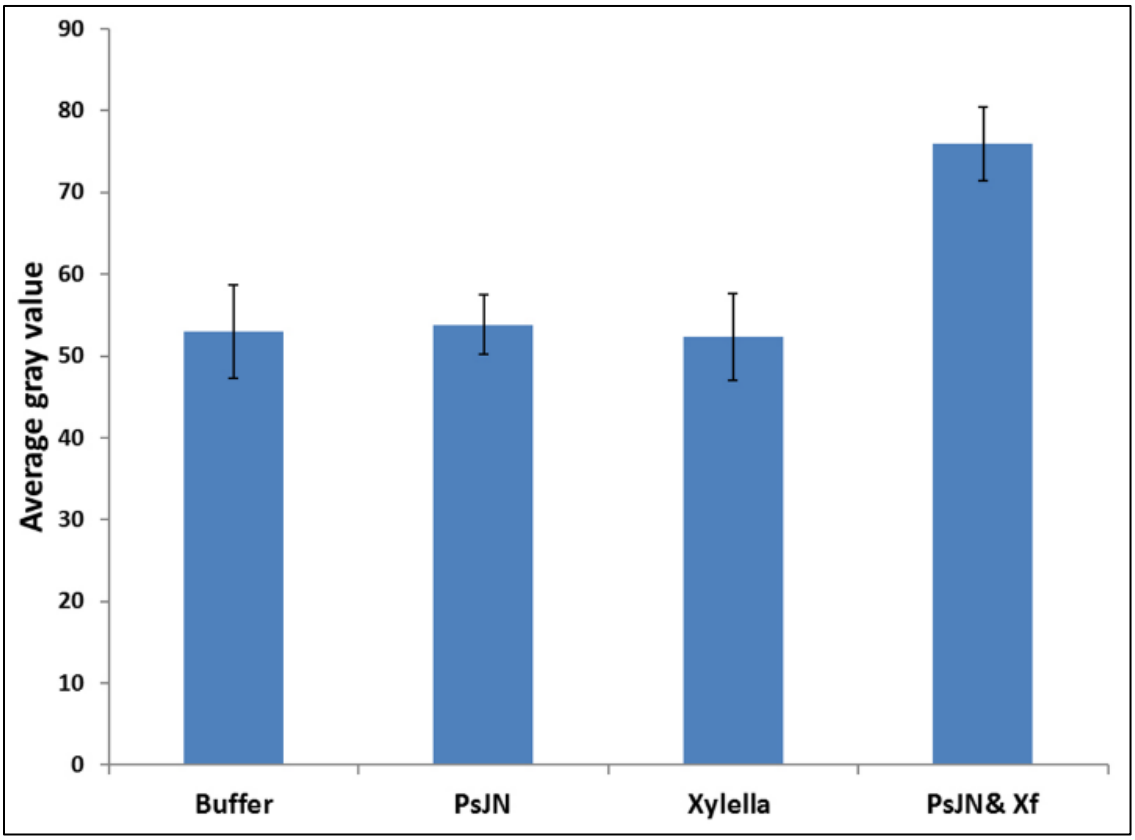


Figure 1. Estimations of reactive oxygen species in plants inoculated with buffer alone, *Paraburkholderia* alone, *Xf* alone, and with both *Paraburkholderia* and *Xf* determined by average of gray scale values for 3,3′-diaminobenzidine (DAB)-mediated tissue printing. The vertical bars represent the standard error of oxidized DAB determined from the analysis of digital images of DAB-treated membranes onto which petioles were pressed.

We have observed in the many experiments in which grapevines have been inoculated with *Paraburkholderia* that population sizes of this biological control agent are maximal in plants within a few weeks after inoculation, but that populations in the plant seem to decrease thereafter. We are continuing work to test the hypothesis that *Paraburkholderia i*s a very efficient colonizer of grape, but one that may be self-limiting. Specifically, we hypothesize that the plant may locally recognize and respond to the colonization of *Paraburkholderia* in a way that leads to a reduction in its own population size. In fact, it may be this response of the plant to

Paraburkholderia that is also responsible for the dramatic reductions in *Xf* populations in plants inoculated with *Paraburkholderia*. If, as we hypothesize, such a host response is relatively local to the plant region colonized by *Paraburkholderia*, the patterns of biological control that we have observed could be explained. Specifically, biological control of Pierce's disease would be expected if *Paraburkholderia* was applied at the same time as or even after that of the pathogen if the rapid movement of *Paraburkholderia* throughout the plant mediated a defensive reaction either before the plant had been colonized by *Xf* or before the pathogen had achieved population sizes sufficient to incite disease symptoms. In this model, the spatial movement and persistence of *Paraburkholderia* in the plant would determine the efficacy of biological control. Our ongoing studies to investigate the spatial movement and temporal persistence of *Paraburkholderia* in plants after inoculation relative to that of the pathogen when inoculated at different times and locations are central to our understanding of how to optimize biological control of Pierce's disease.

Objective 3. Field Efficacy of Biological Control of Pierce's Disease

Large-scale field studies in a replicated field site managed by the Department of Plant Pathology at the University of California, Davis were initiated in 2018 that evaluated the extent to which the factors which we found to control the efficacy of biological control under greenhouse conditions were directly applicable to the control of Pierce's disease in a field setting. The study was also designed to enable us to evaluate the effectiveness of spray applications of *Paraburkholderia* relative to that of direct needle inoculation. The overall experimental design involves the following treatments: (1) Challenge plants with *Xf* relatively soon after needle inoculation or topical treatment with *Paraburkholderia*; (2) Challenge plants with *Xf* several weeks after inoculation of plants with *Paraburkholderia* in different ways; (3) Inoculate *Paraburkholderia* into plants in different ways only after challenge inoculation with *Xf* to assess the potential for "curative effects" after infection has occurred; and (4) Challenge inoculate plants treated with *Paraburkholderia* with *Xf* on multiple occasions, spanning more than one growing season, to reveal the persistence of the biological control phenomenon. Greenhouse studies in our current project have also indicated that topical applications of a DSF-like molecule, palmitoleic acid, with a penetrating surfactant, can also confer disease resistance. This treatment was therefore compared with the various biological control treatments. Each treatment consisted of 10 plants for a given grape variety. For individual vines, one on each of the four cordon arms for a given plant were inoculated. The details of the experimental design are shown in **Figure 2**.

As was observed under greenhouse conditions, topical applications of *P. phytofirmans* with 0.2% Break-Thru to leaves was found to be an efficient way to introduce this bacterium into grapevine tissues under field conditions. Water-soaking was quite apparent within one minute after application to leaves (**Figure 3**). Despite the fact that the water suspensions dried relatively rapidly on the leaves under the relatively warm and often windy conditions in which they were applied, water-soaking was quite extensive and persisted for approximately 15 minutes after inoculation. Large population sizes of strain PsJN were immediately introduced into leaves in this process, and these populations remained high for many days after inoculation (**Figure 3**).

	April	May	June	July	August rating	Sept rating	2019
	Xy STL						
1		Xy B needle					
2		B&Xy mix					
3		Xy B spray					
4	B needle	Xylella					
5	B spray	Xylella					
6		only Xylella (control)					
7	B needle						
8	B spray						
9		UNINOCULATE (control)					
10		Xylella	B needle				
11		Xylella	B Spray				
12		Xylella BREAK					
13		Xy B needle	B needle	B needle			
14		Xy B spray	B Spray	B Spray			
15		Xy B needle	Xy B needle	B needle			
16		Xy B spray	Xy B spray	B Spray			
17		only Xy (control)	only Xy				
18		Xy B TRUNK					
19		Xy & soap	soap	soap			
20		prime with B needle					year 2
21		prime with B spray					year 2

Figure 2. Experimental design and treatment listed for field trials conducted in 2018. Columns represent treatments made at a given time indicated in the headings. Note that on some occasions more than one treatment was applied at a given inoculation time. Unless otherwise noted, all inoculations were made at the base of vines.
Xy or Xylella = inoculation made with *Xf* strain STL via droplet puncture.
B needle = inoculation made with *P. phytofirmans* PsJN via droplet puncture.
B and Xy mix = inoculum of both *P. phytofirmans* and *Xf* were mixed and inoculated as a single droplet puncture.
B spray = inoculation made by spraying *P. phytofirmans* PsJN in 0.2% Break-Thru.
B trunk = inoculation of the trunk of vines (ca. 30 cm from soil level) made with *P. phytofirmans* PsJN via droplet puncture.
Soap = spray application of 2% palmitoleic acid.
Year 2 = challenge inoculation with *Xf* made in spring 2019 in plants that were inoculated in spring 2018 with *P. phytofirmans* in different ways.

Substantial levels of disease control were conferred by application of *P. phytofirmans* PsJN in various ways to Cabernet Sauvignon, either before or after challenge inoculations with *Xf*, in trials in 2018 (**Figure 4**). Similar treatments were evaluated in Chardonnay and Pinot Noir in 2019 (**Figures 5** and **6**). Disease control results in both years for a given treatment were highly reproducible between these varieties. Very high levels of disease were observed in control vines in which *Xf* was inoculated a single time or on multiple occasions, or were treated only with the surfactant Break-Thru after inoculation. Very high levels of disease control were observed in plants treated with *P. phytofirmans* applied in different ways. While the greatest degree of disease control was achieved when both *P. phytofirmans* and *Xf* were co-inoculated together at a

single site into vines, a very high degree of disease control was also observed when *P. phytofirmans* was either injected or sprayed onto plants several weeks after inoculation with *Xf* or inoculated at the same time as *Xf*, but at different locations within a vine. Surprisingly, disease control conferred by a single inoculation of *P. phytofirmans* made after that of the pathogen often provided higher levels of disease control than multiple applications. In contrast to what had been observed in greenhouse studies, injection of *P. phytofirmans* into plants three weeks before they were inoculated with *Xf* also led to high levels of disease control. Given that field-grown plants have a large trunk with cordons from which the vines are borne, we evaluated the direct injection of *P. phytofirmans* into the base of the trunk to determine if systemic and distal effects on disease control could be conferred. Disease reductions from trunk injections were similar in magnitude to those which occurred when *P. phytofirmans* was injected directly into the vines in which *Xf* was inoculated. Repeated topical applications of palmitoleic acid also appeared efficacious for disease control.

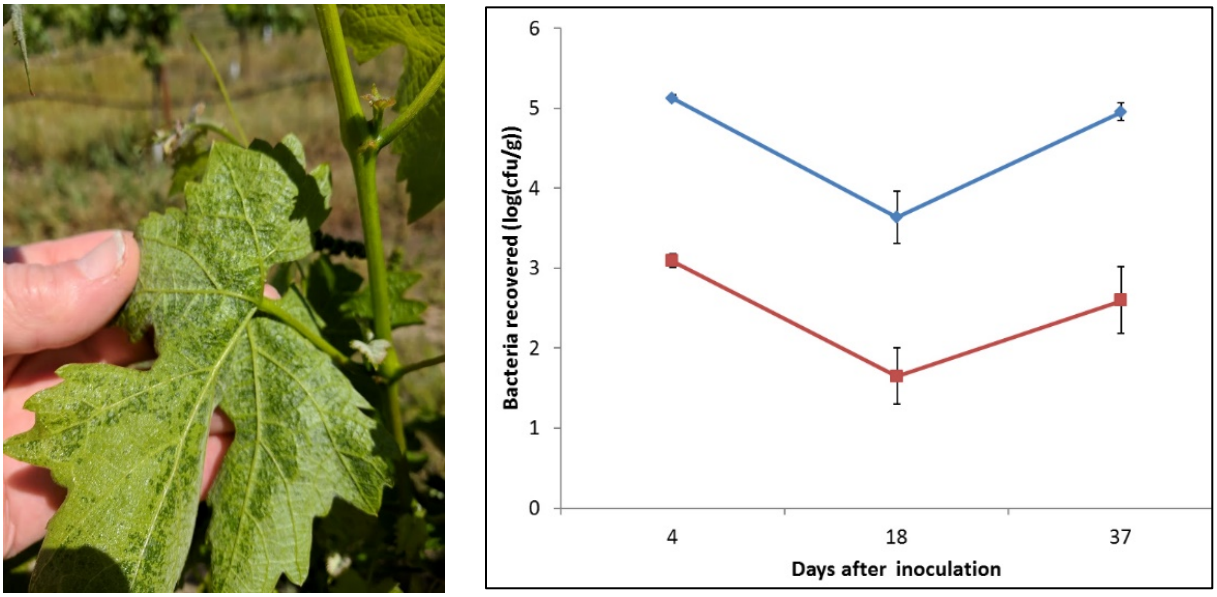


Figure 3. Left: Water-soaking appearance of Cabernet Sauvignon leaves approximately two minutes after topical application of a suspension of *P. phytofirmans* PsJN in 0.2% Break-Thru in a field trial. Right: Population size of *P. phytofirmans* PsJN recovered from surface-sterilized lamina of spray-inoculated leaves (blue line) or surface-sterilized petioles (red line) at various times after spray inoculation. The vertical bars represent the standard error of log-transformed viable bacteria recovered per gram of plant tissue.

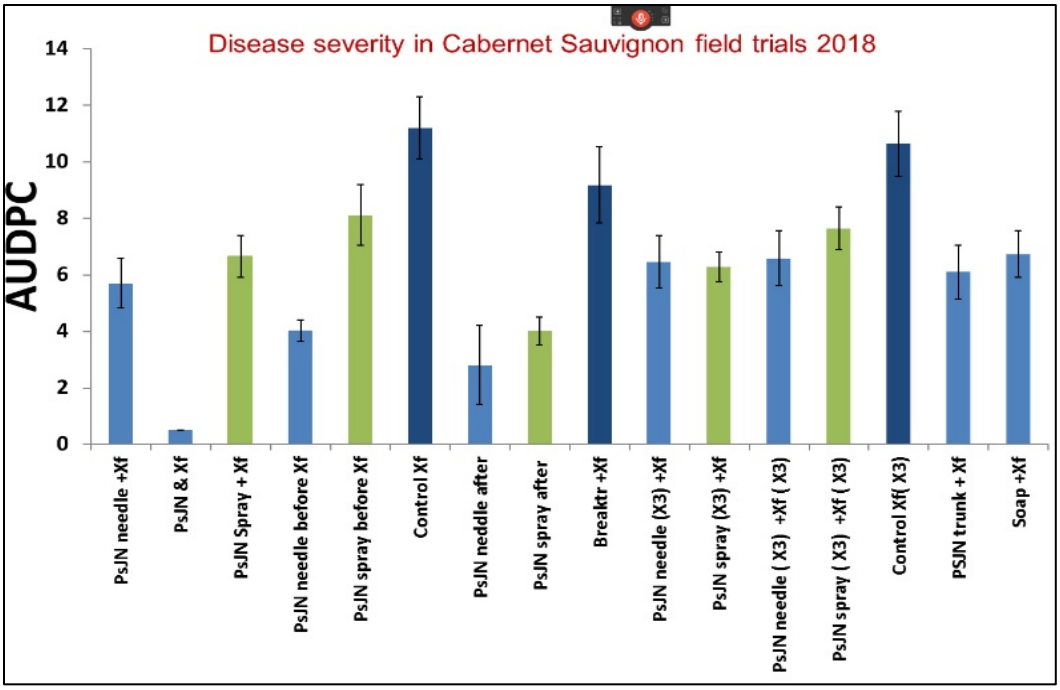


Figure 4. Disease severity of Cabernet Sauvignon grapevines, shown as the area under the disease progress curve for disease assessments made on three occasions in the summer of 2018. The treatments are described in **Figure 2**.

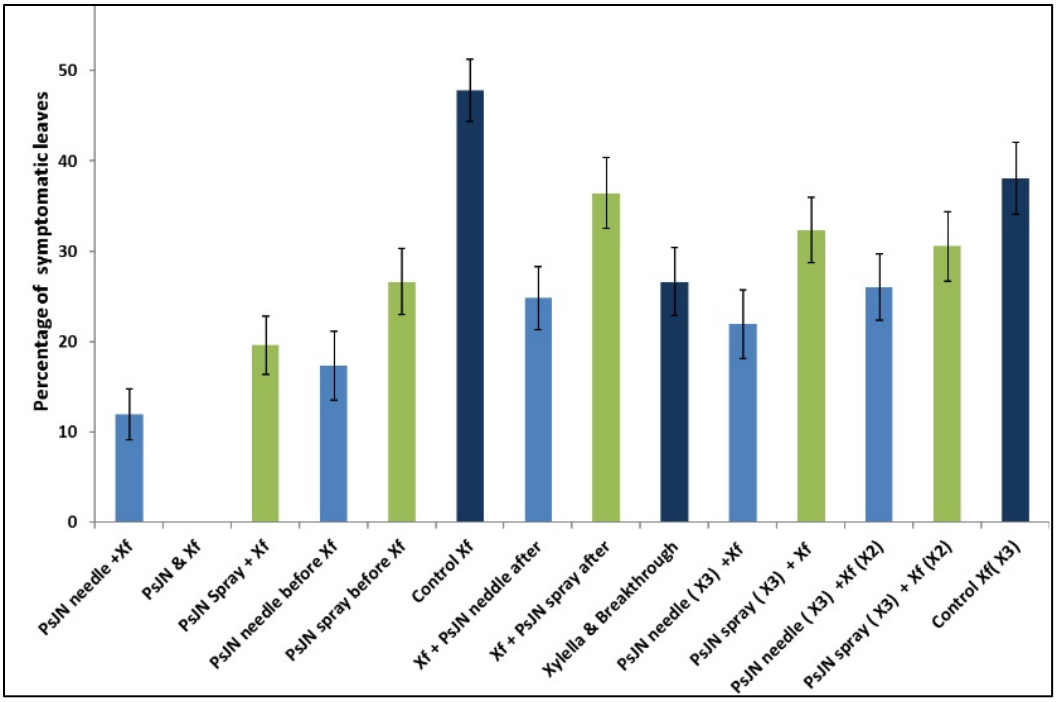


Figure 5. Disease severity of Chardonnay grapevines, shown as the percentage of the leaves on a given shoot that were symptomatic. Shown is the average area disease severity over 40 shoots assessed twice during the 2019 growing season. The vertical bars represent the standard error of the mean. The treatments are described in **Figure 2**.

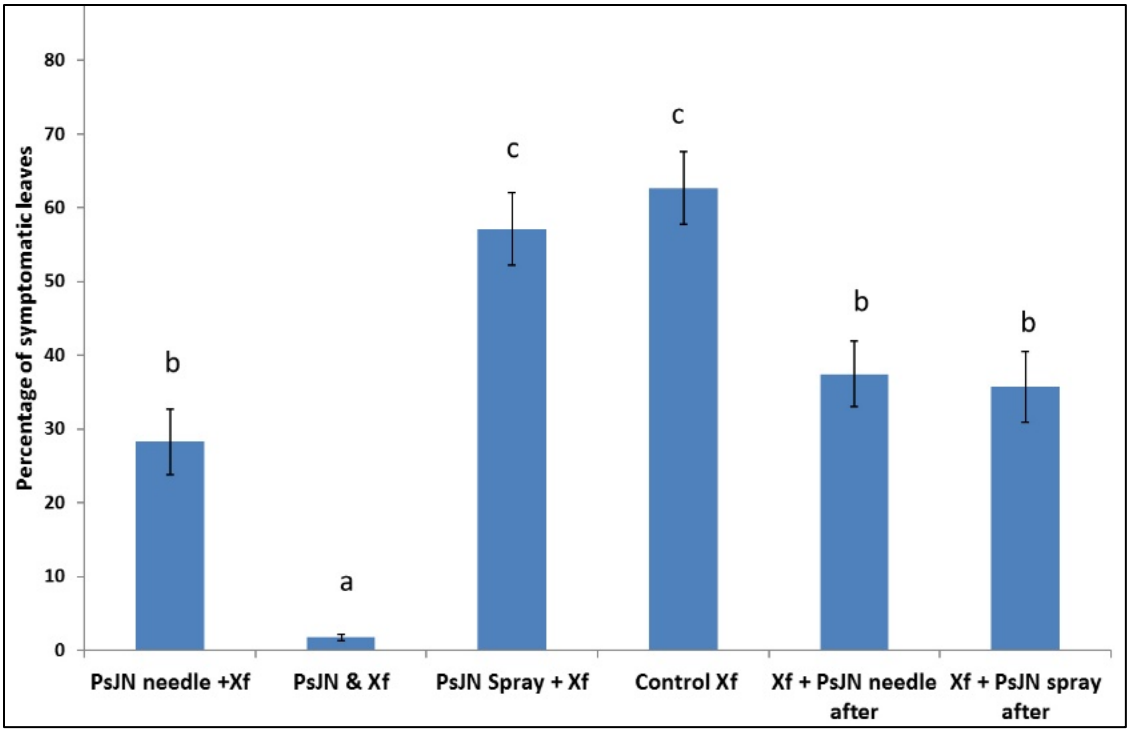


Figure 6. Disease severity of Pinot Noir grapevines, shown as the percentage of the leaves on a given shoot that were symptomatic. Shown is the average area disease severity over 40 shoots assessed twice during the 2019 growing season. The vertical bars represent the standard error of the mean. The treatments are described in **Figure 2**.

In addition to measuring the severity of disease, shown as the proportion of symptomatic leaves on a given inoculated shoot, in the study on Cabernet Sauvignon shown in **Figure 4**, we also assessed the extent to which the pathogen moved from each of the four inoculated shoots on a given plant to infect and cause symptoms on adjacent shoots. We thus counted the number of additional shoots on a given plant that exhibited symptoms of Pierce's disease (**Figure 7**). Even within the short time since plants were inoculated with the pathogen alone, symptoms could be observed on a large number of adjacent shoots on a given plant (**Figure 7**). In contrast, many fewer adjacent shoots exhibited any symptoms of Pierce's disease on plants treated with *P. phytofirmans* in various ways. Generally, those treatments (such as when *Paraburkholderia* was applied soon before or after that of the pathogen) that conferred the greatest reduction in disease severity on inoculated vines also conferred the greatest reduction in spread of disease symptoms to adjacent shoots on a given plant. It was noteworthy that the direct inoculation of *P. phytofirmans* into the trunk of these mature plants also greatly reduced any spread of disease symptoms away from the inoculated shoots, suggesting that the basal inoculation site may have maximized any potential systemic induction of disease resistance that is postulated as a mechanism of action of *P. phytofirmans*. The high levels of disease control seen after inoculation with *P. phytofirmans* are exciting and suggest that even higher levels of disease control could be conferred after further exploration of practical questions of optimum timing and application methods.

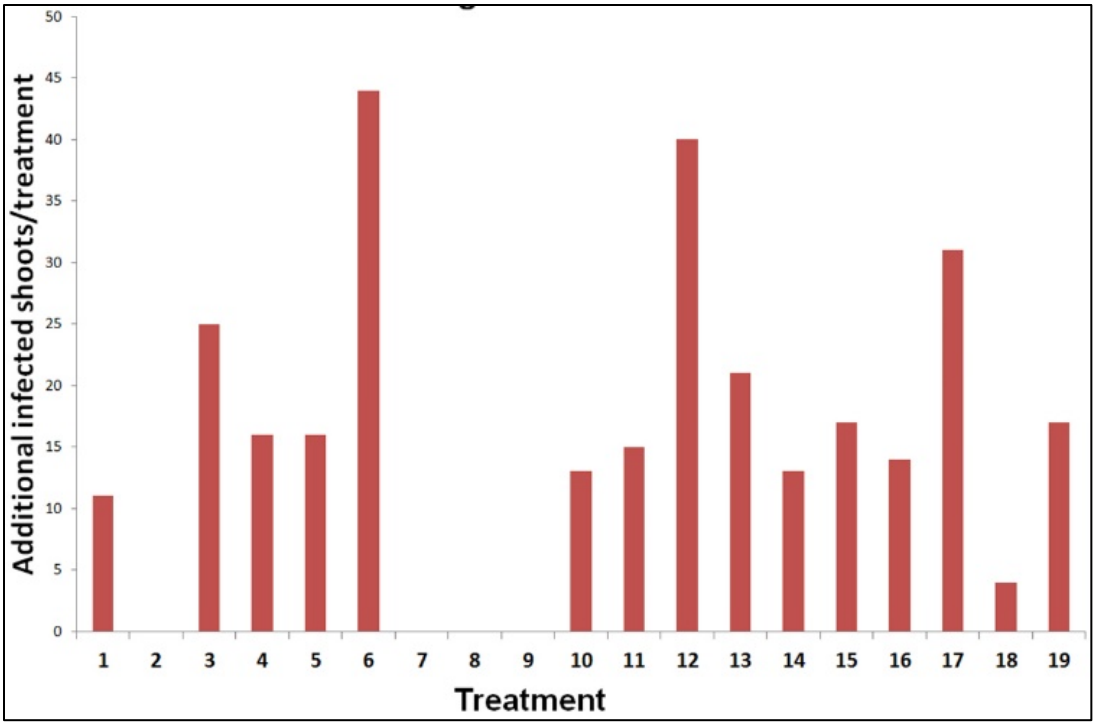


Figure 7. The number of additional shoots on a given plant that were not directly inoculated with *Xf* that exhibited symptoms of Pierce's disease by late September 2018. Shown is the total number of shoots on plants inoculated with *Xf* on the 10 plants receiving a given treatment (described in **Figure 13**) that exhibited symptoms of Pierce's disease.

Since 40 individual shoots on the 10 replicate plants in the field study on Cabernet Sauvignon received a given treatment of both *Paraburkholderia* and *Xf*, it was productive to investigate the patterns of disease that resulted among this large collection of individual shoots. Most commonly, all of the leaves on a shoot that was inoculated only with *Xf* became symptomatic by 14 weeks after inoculation, although a few shoots (< 10%) were unsuccessfully inoculated with the pathogen and a few exhibited high but not 100% disease severity (**Figure 8**). In contrast, a very high proportion of the shoots that were inoculated with both *Paraburkholderia* and *Xf* in various ways exhibited no evidence of disease, with a small proportion of shoots exhibiting some disease (**Figure 8**). That is, inoculation of grapevines with *Paraburkholderia* greatly decreased the probability that inoculation with the pathogen would be successful. *Paraburkholderia* presumably is either eradicating the pathogen before systemic infection could occur, or eradicating infections after they occurred within a given vine and before disease symptoms could result, rather than reducing the severity of symptom development in plants that would have become infected with the pathogen. In other words, inoculation with *Paraburkholderia* in various ways appears to act as an eradicant of *Xf* after it is inoculated into plants, thus preventing successful systemic infection/movement and therefore symptom development. The likelihood that inoculation with *Xf* would lead to infection was therefore reduced four-fold or more, an outcome very distinct from, and much more practical than, simply reducing the level of symptoms that would have occurred in plants that would have become infected. It is very noteworthy that infection can be so dramatically reduced in these plants in the field despite the

fact that they were inoculated with VERY high levels of the pathogen (> 10^7 cells/inoculation site). We presume that viable cells of *Xf* were eliminated in those vines in which disease symptoms did not occur since symptoms never developed, even after prolonged observation throughout the summer. Such a finding is consistent with that of greenhouse studies, which revealed that viable cells of *Xf* were typically not detectable in plants that had been inoculated in a similar manner.

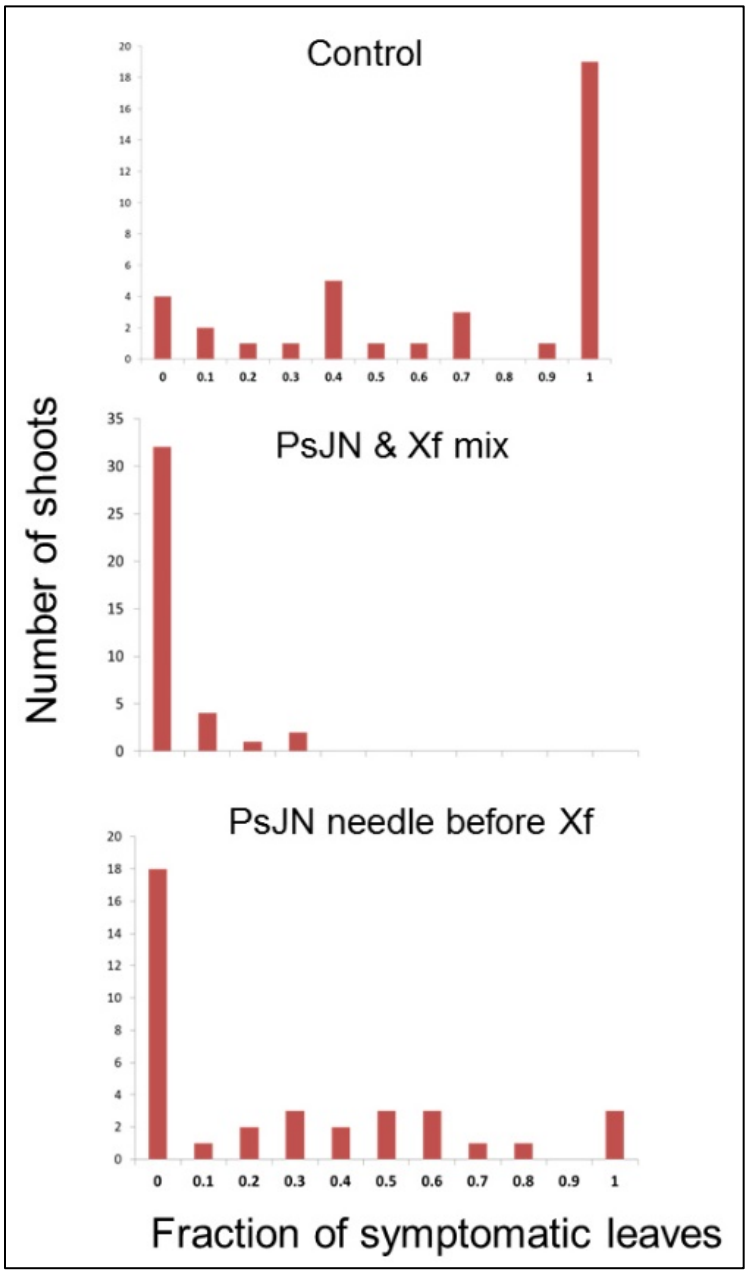


Figure 8. Frequency histogram of the distribution of disease severity observed in 40 individual shoots inoculated only with *Xf* (top panel), inoculated simultaneously with a mixture of *Paraburkholderia* and *Xf* (middle panel), or inoculated with *Paraburkholderia* by droplet puncture three weeks before that of *Xf* (bottom panel).

Further evidence that disease control conferred by treatment of vines with *P. phytofirmans* is an "all or none response," in which vines treated with both the pathogen and *P. phytofirmans* showed either no disease or massive disease, can be seen when the data from the Chardonnay trial shown in **Figure 5** is depicted as the fraction of vines that showed any evidence of Pierce's disease (**Figure 9**). When depicted in this way, it is clear that treatment with *P. phytofirmans* causes a very large reduction in the likelihood that inoculation with the pathogen will be successful in initiating an infection that leads to Pierce's disease. It is clear that inoculation with *P. phytofirmans* reduces the likelihood of infection by greater than three-fold in most cases.

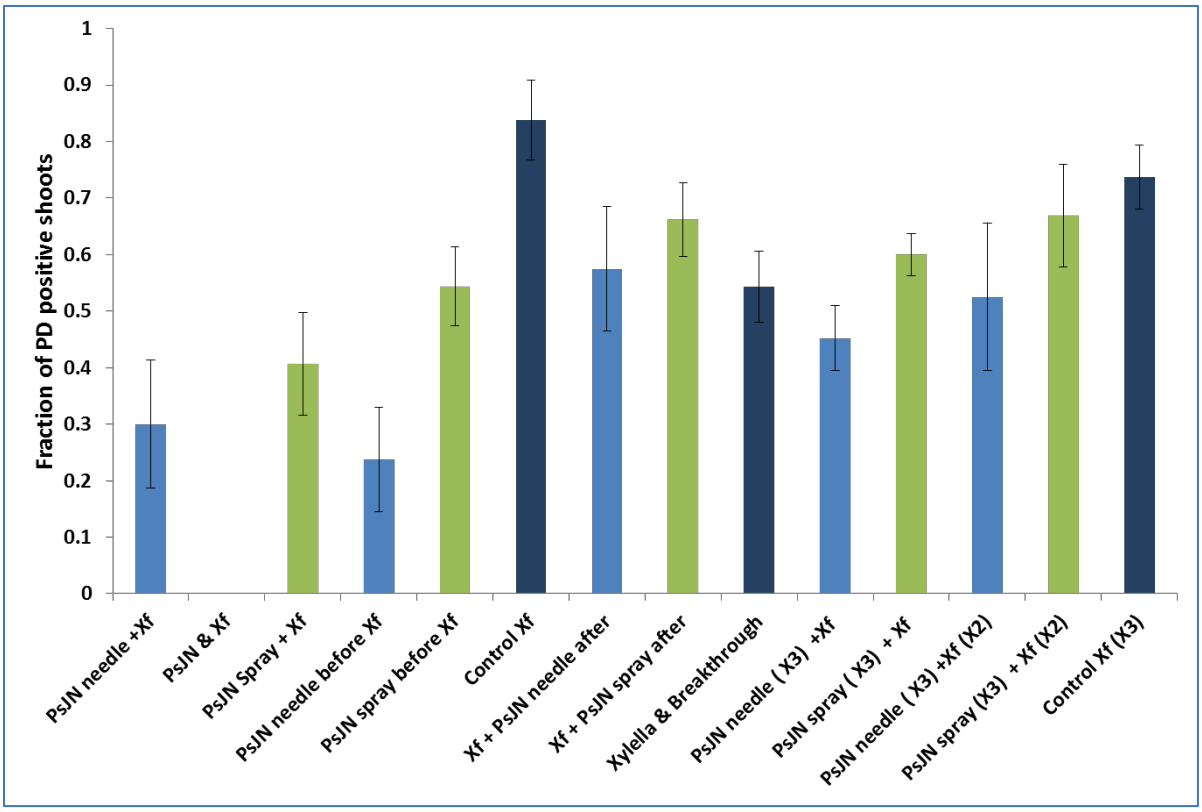


Figure 9. Fraction of shoots of Chardonnay inoculated only with *Xf* or with both *Xf* and *P. phytofirmans* at various times relative to inoculation with the pathogen that show any evidence of Pierce's disease, irrespective of its intensity. The vertical bars represent the standard error of the fraction of the 40 shoots for each treatment that showed evidence of Pierce's disease.

CONCLUSIONS

The studies directly address practical strategies of control of Pierce's disease. Our results reveal that *P. phytofirmans* continues to provide levels of biological control under greenhouse and field conditions that are even greater than what we would have anticipated, and encouraging results were obtained from investigations into practical means of introducing this strain into plants, such as by spray applications. In addition, the fact that *P. phytofirmans* seems to be active even when not co-inoculated with the pathogen is a very promising result suggesting that this method of disease control might also be readily implemented. Both greenhouse and field results indicate

that the biological control agent is highly efficacious and that it could be used in conjunction with other disease control strategies, such as DSF-mediated pathogen confusion in transgenic plants or topical application of signaling molecules, as well as with other resistant plants that are being developed in other laboratories. Given that this well-studied biological control agent is a naturally-occurring strain recognized as a beneficial organism, the regulatory requirements for its commercial adoption should be relatively modest.

REFERENCES CITED

Baccari C, Antonova E, Lindow SE. 2019. Biological control of Pierce's disease of grape by an endophytic bacterium. *Phytopathology* 109: 248-256.

FUNDING AGENCIES

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

OPTIMIZING BIOLOGICAL CONTROL OF PIERCE'S DISEASE WITH *PARABURKHOLDERIA PHYTOFIRMANS*

Project Leader: Steven Lindow | Department of Plant and Microbial Biology | University of California | Berkeley, CA 94720 | icelab@berkeley.edu
Cooperator: Renee Koutsoukis | Department of Plant and Microbial Biology | University of California | Berkeley, CA 94720 | reneek@berkeley.edu
Cooperator: Clelia Baccari | Department of Plant and Microbial Biology | University of California | Berkeley, CA 94720 | clelia.baccari@berkeley.edu

Reporting Period: The results reported here are from work conducted July 2019 to October 2019.

ABSTRACT

Burkholderia phytofirmans strain PsJN was found to be capable of extensive growth and movement within grapevines after both needle or spray inoculation. The population size of *Xylella fastidiosa* is greatly reduced in plants in which *P. phytofirmans* is either co-inoculated at the same time and location, inoculated at the same time but at other nearby locations, and even inoculated at other locations either three weeks before or after that of the pathogen. This strain appears to induce disease resistance in the plant, causing eradication of the pathogen. A variety of studies of the temporal and spatial patterns of movement of the biological control agent within the plant and of the resistance reaction by the plant to the presence of this beneficial bacterium will be undertaken to address the question of when and how a limited number of applications of *P. phytofirmans* might best be applied in field settings to control Pierce's disease. This project has just started and so most objectives have only just now begun. Initial studies, however, reveal that inoculation of plants with *P. phytofirmans* from three weeks before inoculation with the pathogen and up to seven weeks after inoculation with the pathogen all provide equally great reductions in disease severity. The efficacy of disease control decreases abruptly with increasing delay in inoculation time after inoculation with the pathogen after seven weeks. These promising preliminary results suggest that the number of applications of *P. phytofirmans* required for practical control under field conditions may be very limited.

LAYPERSON SUMMARY

A naturally-occurring *Paraburkholderia phytofirmans* strain has been identified that grows and moves extensively within mature grape plants and greatly reduces disease severity when applied to plants at the same time as, or even after, pathogen inoculation in both greenhouse and in large field studies. This strain appears to induce disease resistance in the plant, causing eradication of the pathogen. A variety of studies of the temporal and spatial patterns of movement of the biological control agent within the plant and of the resistance reaction by the plant to the presence of this beneficial bacterium will be undertaken to address the question of when and how a limited number of applications of *Paraburkholderia* might best be applied in field settings to control Pierce's disease. Initial studies reveal that inoculation of plants with *P. phytofirmans* from three weeks before inoculation with the pathogen and up to seven weeks after inoculation with the pathogen provide equally great reductions in disease severity.

INTRODUCTION

This is a follow-on project based heavily on the exciting results we have obtained in the project titled "Biological Control of Pierce's Disease of Grape with an Endophytic Bacterium" (CDFA agreement number 16-0514-SA) which will end June 1, 2020. We found that unlike other bacteria that we recovered from the interior of grapes, the bacterium *Paraburkholderia phytofirmans* is able to grow to large population sizes in and spread extensively within xylem vessels of mature grape (1). Surprisingly, while we considered it a possible surrogate bacterial host for expression of *Xylella fastidiosa* (*Xf*) *rpfF* genes encoding diffusible signal factor production to enable disease control by pathogen confusion, the unmodified strain itself conferred very high levels of disease control when co-inoculated with the pathogen into plants or even when inoculated at different locations.

Almost complete elimination of *Xf* was usually seen in plants inoculated with both *Paraburkholderia* and *Xf,* suggesting that competition or pathogen confusion was not the mechanism by which disease control and reduction of *Xf* populations were achieved. The complete lack of any viable cells of *Xf* in plants that had been inoculated with *Paraburkholderia* was particularly striking given that large numbers of cells of the pathogen had been inoculated, suggesting that it was killed in plants that were also colonized by *Paraburkholderia*. Initial studies in the greenhouse revealed that plant disease resistance genes were induced when both *Xf* and *Paraburkholderia* are present in the plant. Only modest inductions of plant disease resistance genes such as PR1 are seen when only *Paraburkholderia* is inoculated, and little induction is seen when *Xf* alone is inoculated, as has been seen in other studies (12). The presence of *Paraburkholderia* therefore seems to be priming plants for resistance reaction to *Xf* that otherwise would not have occurred. These results are consistent with an observation by the Roper lab who found that (i) the O antigen on lipopolysaccharide (LPS) seems to mask it from perception by the plant, and thus the plant does not actively defend itself against *Xf*, and (ii) that *Xf* is highly susceptible to hydrogen peroxide and other defense chemicals produced by grape (12).

While inoculation of grapevine xylem by puncture inoculation with *Paraburkholderia* either at the same site as or even at different locations than the pathogen is effective in achieving biocontrol of Pierce's disease, large populations within the leaves and petioles and disease control can be achieved by topical application of the biocontrol agent with organosilicon surfactants (1). These surfactants have sufficiently low surface tension that aqueous suspensions of the bacteria can penetrate through stomata directly into plant tissues, with population sizes of the bacteria exceeding 106 cells/gram readily achieved by topical application.

Remarkably, biological control of Pierce's disease can be achieved by inoculation with *Paraburkholderia* at various times relative to that of inoculation with *Xf*. Surprisingly, disease control is much poorer when *Paraburkholderia* was inoculated into the plants three weeks before that of the pathogen, while very good control is achieved when it was inoculated either directly into the xylem or by spray inoculation at the same time as that of the pathogen. The highest levels of control were usually seen when *Paraburkholderia* was inoculated onto plants either directly into the xylem or applied by spray inoculation three weeks after that of the pathogen.

Preliminary results suggest that while *Paraburkholderia* can grow and spread rapidly in grapevine stems within four weeks after inoculation, its population sizes then subsequently decrease, and often become undetectably low by eight to ten weeks after inoculation. It is likely that a plant disease resistance reaction that is induced by *Paraburkholderia* causes it to succumb to the plant defenses. It also would suggest that maximum effectiveness of biological control conferred by *Paraburkholderia* inoculation would occur when it is present in the highest population size and when it was most widely distributed in plants, likely explaining why pre-inoculation of plants with this biocontrol agent is not as effective as simultaneous, or particularly post-inoculation, treatments. A better understanding of the population dynamics of the biological control agent in grapevines under different conditions seems essential to better understand how to optimize biological control.

Initial field studies of *Paraburkholderia* for biological control of Pierce's disease in a large trial conducted at UC Davis have yielded exciting results that largely confirmed the results of greenhouse studies. The efficacy of *Paraburkholderia* for biological control of Pierce's disease in the field was often nearly as good as that seen under greenhouse conditions. As observed in the greenhouse, the highest level of disease control was observed when plants were either co-inoculated with *Paraburkholderia* and *Xf*, and particularly when *Paraburkholderia* was inoculated three weeks after that of the pathogen. The disease severity, shown as the area under the disease progress curve for disease severity assessed as the fraction of leaves on an inoculated shoot that were symptomatic when measured at several times during the growing season, was reduced three-fold to five-fold or more on plants inoculated with *Paraburkholderia* in various ways compared to that of control plants inoculated only with *Xf* one or more times, with disease severity being reduced more than ten-fold on plants co-inoculated at the same site with both the pathogen and *Paraburkholderia*.

Since 40 individual shoots on the ten replicate plants in the field study received a given treatment of both *Paraburkholderia* and *Xf*, it was productive to investigate the patterns of disease that resulted among this large collection of individual shoots. Most commonly, all of the leaves on a shoot that was inoculated only with *Xf* became symptomatic by 14 weeks after inoculation, although a few shoots (< 10%) were unsuccessfully inoculated with the pathogen and a few exhibited high but not 100% disease severity. In contrast, a very high proportion of the shoots that were inoculated with both *Paraburkholderia* and *Xf* in various ways exhibited no evidence of disease, with a small proportion of vines exhibiting some disease. That is, inoculation of grapevines with *Paraburkholderia* greatly decreased the probability that inoculation with the pathogen would be successful, presumably by eradicating the pathogen before systemic infection could occur, or eradicating infections after they had occurred within a given vine and before disease symptoms could result, rather than reducing the severity of symptom development in plants that would have become infected with the pathogen. In other words, inoculation with *Paraburkholderia* in various ways appears to act as an eradicant of *Xf* after it is inoculated into plants, thus preventing successful systemic infection/movement and therefore symptom development. The likelihood that inoculation with *Xf* leads to infection was therefore reduced three-fold or more, an outcome very distinct from, and much more practical, than simply reducing the level of symptoms that would have occurred in plants that would have become infected. It is very noteworthy that infection can be so dramatically reduced in these plants in the field despite the fact that they were inoculated with VERY high levels of the pathogen

(> 107 cells/inoculation site). We presume that viable cells of *Xf* were eliminated in those vines in which disease symptoms did not occur since symptoms never developed, even after prolonged observation throughout the summer. Such a finding is consistent with that of greenhouse studies which revealed that viable cells of *Xf* were typically not detectable in plants that had been inoculated in a similar manner.

More disease control was observed consistently in field studies when *Paraburkholderia* and *Xf* were co-inoculated at the same time at the same site in the plant, compared to that obtained when they were both inoculated simultaneously but at nearby sites on a stem on plants in the field. As we had seen similar efficacy of biological control with both mixed inoculations and nearby inoculations under greenhouse conditions, this result would suggest that either the rate of movement of *Paraburkholderia* in the plant under field conditions was somewhat slower than that under greenhouse conditions, or that plant disease responses to its presence in the plant were either delayed or more spatially restricted than that typically occurring under greenhouse conditions. Studies are proposed in Objectives 2 and 4 to better understand these processes of movement and disease resistance induction in plants under field conditions so as to determine how to achieve the highest levels of disease control with this beneficial bacterium.

The exciting results of biological control of Pierce's disease seen both in the greenhouse and in the field are consistent with a model in which *Paraburkholderia* rapidly multiplies and moves within grapevines after inoculation, thus exposing the plant to features of this bacterium such as LPS and other so-called microbe-associated molecular patterns (MAMPs) that might be perceived as a potential pathogen (5, 10). Immune responses in plants are initiated upon receptor-mediated perception of non-self molecules that are often conserved among both pathogenic and beneficial microorganisms (6, 11,14). *Paraburkholderia* apparently elicits a defensive response, akin to what has been commonly known as induced systemic resistance (ISR) and systemic acquired resistance (SAR). These terms are rather synonymous, although the term SAR is usually used when induced resistance is triggered by a pathogen and is demonstrated to be dependent on salicylic acid (SA) as a systemic signal (11, 14). In contrast, ISR is usually SA-independent and has been examined almost exclusively in small herbaceous plants and in a context in which beneficial bacteria interact with roots (11, 14). *P. phytofirmans* strain PsJN, which we have used in these studies, has been shown to elicit expression of plant disease resistance genes in a variety of plants, although its effect in large woody plants such as grape has received little attention. (3, 4, 7, 8, 9, 13). Given that the inoculation of the foliar parts of grape by *Paraburkholderia* results in alteration in PR1 expression, usually a hallmark of SA-dependent plant resistance responses typical of SAR (2, 6, 11, 13), it appears that it may be inducing a response akin to SAR. There are, however, features of ISR in grapevines inoculated with *Paraburkholderia*, in that there are apparently relatively mild changes in expression of plant disease resistance marker genes in plants inoculated only with this beneficial bacterium, whereas there is a high level of expression of genes such as PR1 and ETR1 three weeks after inoculation in plants inoculated with both *Xf* and *Paraburkholderia*. Such a pattern is characteristic of the process known as "priming," wherein there is a faster and/or stronger activation of plant cellular defenses (11). In both SAR and ISR, MAMPS serve as elicitors responsible for the onset of systemic immunity in the plant (1, 14). Various features of both pathogenic and non-pathogenic microorganisms such as the lipopolysaccharides (LPS) can serve as such elicitors (5, 10) and sensitize the plant to the potential presence of a pathogen. The fact that *Paraburkholderia* and *Xf*

need not be co-inoculated into the same site of the plant (thus assuring spatial coincidence) to achieve disease control suggests that an effect in the plant elicited by the presence of *Paraburkholderia* is causing the death of *Xf* in grapevines even at some distance. For example, it seems extremely unlikely that inoculation of plants with *Paraburkholderia* one month after that of the pathogen could have enabled the distribution of the biological control agent into all of the many xylem vessels that would have harbored *Xf* at such an advanced stage of plant colonization. Results from the Roper lab at UC Riverside reveal that while *Xf* itself does not appear to induce a lethal defensive reaction by grape, it is highly susceptible to death by such defensive reactions if they are induced by another agent (12). For example, *Xf* LPS (when presented in a way in which it was not camouflaged by the presence of the O antigen on an intact cell) was shown to elicit a strong defensive reaction by xylem parenchymal cells in grapevines, resulting in PR1 expression and their release of reactive oxygen species such as hydrogen peroxide (12). *Xf* succumbed to such a defense response in these LPS-treated plants, and disease was therefore greatly reduced (12). These results show both that (i) plants can be induced to mount a strong resistance response to *Xf*, and (ii) such a resistance reaction is operative even within the xylem vessels, apparently because of the toxicity of the hydrogen peroxide and perhaps other molecules released by xylem parenchymal cells in the vicinity of xylem vessels. We presume that a similar phenomenon is occurring in plants that are inoculated with *Paraburkholderia*, either at the same time or even after inoculation with *Xf*. Critical to the success of the apparent induced resistance of grapevines to *Xf* is the rapidity and duration of such an induced resistance reaction conferred by the presence of *Paraburkholderia*, as well as the spatial extent of such a disease reaction. Given such an apparent process, the most effective biological control by *Paraburkholderia* would result when its presence in even a few vessels could induce a disease resistance response throughout an entire plant. Given that we see large, but incomplete, disease resistance in plants inoculated either in the greenhouse or in the field with *Paraburkholderia*, it suggests that such an optimum situation does not always occur. It therefore is important to better understand the processes of colonization of plants by *Paraburkholderia* as well as the resultant plant responses, so as to better understand the conditions under which biological control can be optimized. Our results to date suggest that while *Paraburkholderia* multiplies rapidly and spreads extensively in the plant for a few weeks, its population size subsequently decreases. We lack information on how long it would persist in plants and whether its persistence as viable cells is necessary for a sustained plant disease resistance response. This will be addressed in Objectives 2 and 4. To effectively deploy *Paraburkholderia* in agricultural settings it is also important to know the spatial pattern of induced host disease resistance that occurs in grapevines in its presence. Is there a long-distance systemic response to the presence of *Paraburkholderia* at a given site in the plant, or is the disease resistance response somewhat localized to the vicinity of *Paraburkholderia* itself? While SAR/ISR has been investigated extensively in herbaceous plants, little is known of the process of SAR/ISR in woody plants (11). Such a question is important as it addresses whether broad spatial distribution of *Paraburkholderia* is required for successful induction of host defenses. Equally important is how long *Paraburkholderia*-induced plant disease resistance persists. In some plants, SAR/ISR is a transient event (11). The reduced efficacy of *Paraburkholderia* in conferring disease control when inoculated before the pathogen suggests that the systemic resistance that was induced was sufficiently transient that it was ineffective when the pathogen was inoculated at such a late date. These questions will be studied in Objective 4. A strong transient systemic induction of plant disease resistance appears to be sufficient to yield high levels of disease control by eradicating the pathogen after infection has already occurred. We

presume that such a process explains disease control resulting from application of *Paraburkholderia* three weeks after infection with *Xf*. Understanding whether this phenomenon is indeed occurring in grape has great practical importance, as it would suggest that it would be possible to eliminate Pierce's disease symptoms if *Paraburkholderia* were inoculated into infected plants at any time prior to symptom development. While we have demonstrated the apparent eradication of the pathogen three weeks following inoculation, it seems quite possible that even later inoculations with *Paraburkholderia* would be successful in eliminating Pierce's disease symptoms. Such a strategy might prove to be highly practical under field conditions, as it would minimize the number of applications of the biocontrol agent that might be necessary, perhaps to even a single time during a growing season, even if plants might continually be inoculated by insect vectors. This will be studied in Objective 1. The topical application of *Paraburkholderia* with a penetrating surfactant appears particularly attractive as a means to inoculate grapevines. It is clear that substantial numbers of cells of the biological control agent can be introduced into the apoplast of the plant, with many entering the petiole (1). It is, however, important to know the physical location of *Paraburkholderia* within the plant, particularly whether it needs to enter the vascular tissue and whether such entry is needed in order to achieve disease control. If disease resistance is dependent on the entry of *Paraburkholderia* into the xylem, methods of topical application that improve its entry into the vascular tissue will be explored in an attempt to improve disease control. These issues will be addressed in Objective 3.

These issues are quite practically important because it addresses the question of when and how a limited number of applications of *Paraburkholderia* might best be applied in a field setting where plant inoculation of *Xf* by vectors could occur throughout the summer. If induced disease resistance was somewhat persistent one could imagine a treatment regimen involving a limited number of inoculations of *Paraburkholderia*, beginning early in the season. In contrast, as eradication of *Xf* from infected plants appears possible by inoculations made prior to symptom development, it will be important to know how long infections can develop before eradication is no longer possible. Studies are proposed here to address these important issues.

OBJECTIVES

1. Determine the amount of time after infection of grapevines by *Xf* that inoculation with *Paraburkholderia* can still confer prevention of disease symptoms to identify optimal treatment times and procedures.
2. Determine the persistence and temporal dynamics of *Paraburkholderia* in grapevines in the presence and absence of *Xf* to better determine treatment regimens for disease control.
3. Determine the tissue location and spatial distribution of *Paraburkholderia* in plants as a function of time after topical application with penetrating surfactants.
4. Determine the temporal and spatial patterns of altered grapevine gene expression associated with Pierce's disease resistance conferred by inoculation with *Paraburkholderia* in the presence and absence of *Xf* to better understand and exploit processes leading to biological control of Pierce's disease.

RESULTS AND DISCUSSION

Objective 1. Effect of Inoculation Time on Efficacy of Biological Control

A large number of control and border plants of both Chardonnay and Cabernet Sauvignon from a large field trial established in 2016 by Bryce Falk became available to us at the UC Davis Plant Pathology Research Farm in the spring of 2019. We therefore have taken advantage of this opportunity to conduct an extensive field study to address the relative efficacy of the application of *Paraburkholderia* at various times relative to that of the pathogen when applied either as a foliar spray with 0.2% Breakthru or by direct inoculation by needle puncture. This elaborate trial involved inoculation of five plants of Chardonnay at various times by droplet puncture or Cabernet Sauvignon by topical application. *Paraburkholderia* was applied by these various methods either three weeks prior to that of the pathogen, two weeks prior to that of the pathogen, one week prior to that of the pathogen, on the same day as that of the pathogen, and one, two, three, four, five, six, seven, eight, nine, and ten weeks after plants were inoculated with the pathogen. In this experimental design we therefore have applied *Paraburkholderia* at various times both before and even several weeks after that of the pathogen. The goal of the study is to better determine any reduced efficacy associated with application of *Paraburkholderia* at various times prior to that of inoculation with the pathogen, and particularly to determine whether "eradication" of the pathogen as evidenced by reduced Pierce's disease symptoms can be achieved by application of *Paraburkholderia* even many weeks after inoculation with the pathogen.

P. phytofirmans conferred high levels of disease control when applied topically to Cabernet Sauvignon as well as when directly inoculated into Chardonnay grapevines over a wide range of time relative to that of the pathogen, from three weeks before inoculation with the pathogen to as much as seven weeks after the pathogen was inoculated into the plants (**Figure 1** and **Figure 2**). The efficacy of disease control decreases abruptly with increasing delay in inoculation time after inoculation with the pathogen after about seven weeks. These promising preliminary results suggest that the number of applications of *P. phytofirmans* required for practical control under field conditions may be very limited.

CONCLUSIONS

While this study has only recently begun, continued field trials as a part of Objective 1 reveal a very high degree of efficacy of *P. phytofirmans* for the control of Pierce's disease when inoculated at a wide range of times both before and after the time of inoculation with the pathogen. Furthermore, spray inoculations of the biological control agent continue to be among the best and most practical methods by which it can be applied to plants. Because this strain appears to cause plants to "eradicate" *Xf*, largely irrespective of when the pathogen is introduced into the plant relative to that of the biological control agent, limited numbers of applications of such a biological control agent should be sufficient to achieve high levels of disease control in the field. Given that this well-studied biological control agent is a naturally occurring strain recognized as a beneficial organism, the regulatory requirements for its commercial adoption should be relatively modest.

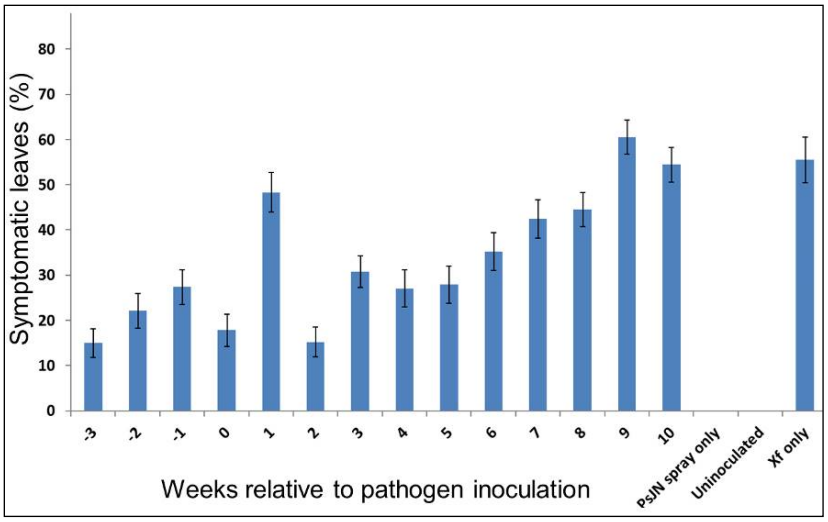


Figure 1. Disease severity of Cabernet Sauvignon grape shown as the percentage of the leaves on a given shoot that were symptomatic on plants that were spray inoculated with *P. phytofirmans* at various times relative to inoculation with the pathogen. Negative values on the abscissa indicate application of the biological control agent before that of the pathogen while positive integers reflect inoculation at the given number of weeks after that of the inoculation with the pathogen. Shown is the average area disease severity over 32 shoots assessed twice during the 2019 growing season. The vertical bars represent the standard error of the mean.

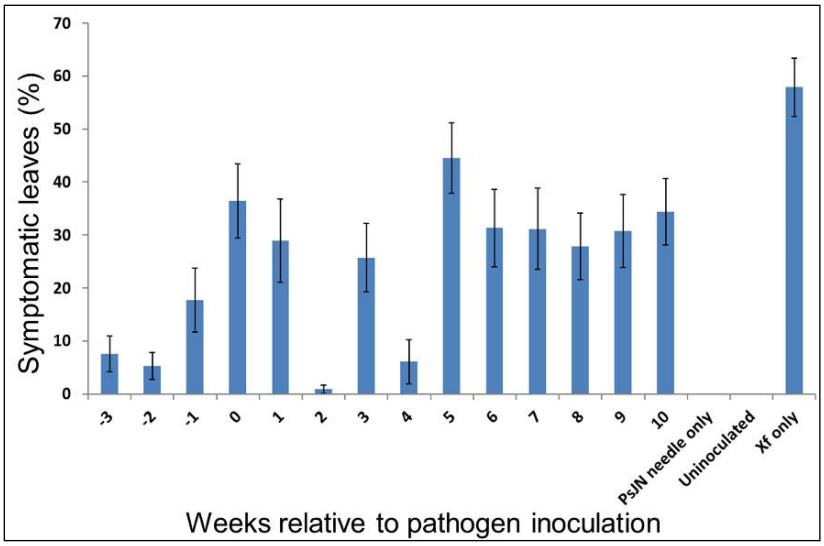


Figure 2. Disease severity of Chardonnay grapevines shown as the percentage of the leaves on a given shoot that were symptomatic on plants that were needle inoculated with *P. phytofirmans* at various times, relative to inoculation with the pathogen. Negative values on the abscissa indicate application of the biological control agent before that of the pathogen, while positive integers reflect inoculation at the given number of weeks after inoculation with the pathogen. Shown is the average area disease severity over 32 shoots assessed twice during the 2019 growing season. The vertical bars represent the standard error of the mean.

REFERENCES CITED

1. Baccari C, Antonova E, Lindow SE. 2019. Biological control of Pierce's disease of grape by an endophytic bacterium. *Phytopathology* 109: 248-256.
2. Chong J, Henanff GE, Bertsch C, Walter B. 2008. Identification, expression analysis, and characterization of defense and signaling genes in *Vitis vinifera*. *Plant Physiology and Biochemistry* 46: 469-481.
3. Compant S, Kaplan H, Sessitch A, Nowak J, Barka AA, Clement C. 2008. Endophytic colonization of *Vitis vinifera* L. by *Burkholderia phytofirmans* strain PsJN: From the rhizosphere to inflorescence tissues. *FEMS Microbiology Ecology* 63: 84-93.
4. Compant S, Reiter B, Sessitch A, Nowak J, Clement C, Barka EA. 2005. Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. *Applied Environmental Microbiology* 71: 1685-1693.
5. Erbs G, Newman MA. 2003. The role of lipopolysaccharides in induction of plant defence responses. *Molecular Plant Pathology* 4: 421-425.
6. Gruau C, Trotel-Aziz P, Villaume S, Rabenoelina F, Clement C, Baillieul F, Aziz A. 2015. *Pseudomonas fluorescens* PTA-CT2 triggers local and systemic immunity response against *Botrytis* in grapevine. *Molecular Plant-Microbe Interactions* 28: 1117-1129.
7. Poupin MJ, Timmermann T, Vega A, Zuniga A, Gonzalez B. 2013. Effects of the plant growth-promoting bacterium *Burkholderia phytofirmans* PsJN throughout the life cycle of *Arabidopsis thaliana*. *PLOS ONE* 8: e69435.
8. Sessitsch A, Coenye T, Sturtz AV, Vandamme P, Barka EA, Salles JF, Van Elsas JD, Faure D, Reiter B, Glick BR, Wang-Pruski G, Nowak J. 2005. *Burkholderia phytofirmans* sp. Nov., a novel plant-associated bacterium with plant beneficial properties. *International Journal of Systematic and Evolutionary Microbiology* 55: 1187-1192.
9. Miotto-Vilanova L, Jacquard C, Courteaux B, Wortham L, Michel J, Clement C, Barka EA, Sanchez L. 2016. *Burkholderia phytofirmans* PsJN confers grapevine resistance against *Botrytis cinerea* via a direct antimicrobial effect combined with a better resource mobilization. F*rontiers in Plant Science* 7: 1236.
10. Newman MA, von Roepenack-Lahaye E, Parr E, Daniels MJ, Dow JM. 2002. Prior exposure to lipopolysaccharide potentiates expression of plant defenses in response to bacteria. *Plant Journal* 29: 487-495.
11. Pieterse CMJ, Zamioudis C, Berendsen RL, Weller DM, Van Wees SCM, Bakker PAHM. 2014. Induced systemic resistance by beneficial microbes. *Ann. Rev. Phytopath.* 52: 347-375.
12. Rapicavoli JN, Blanco-Ulate B, Muszynski A, Figueroa-Balderas R, Morales-Cruz A, Azadi P, Dobruchowska JM, Castro C, Cantu D, Roper MC. 2018. Lipopolysaccharide O-antigen delays plant innate immune recognition of *Xylella fastidiosa*. *Nature Communications* 9: 390.
13. Timmermann T, Armijo G, Donoso R, Seguel A, Holuigue L, Gonzalez B. 2017. *Paraburkholderia phytofirmans* PsJN protects *Arabidopsis thaliana* against a virulent strain of *Pseudomonas syringae* through the activation of induced resistance. *Molecular Plant-Microbe Interactions* 30: 215-230.
14. Van Loon LC, Rep M, Pieterse CMJ. 2006. Significance of inducible defense-related proteins in infected plants. *Annual Review of Phytopathology* 44: 135-162.

FUNDING AGENCIES

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

GEOGRAPHIC DISTRIBUTION OF ISOLATE VIRULENCE IN *XYLELLA FASTIDIOSA* COLLECTED FROM GRAPE IN CALIFORNIA AND ITS EFFECT ON HOST RESISTANCE

Project Leader: Rachel Naegele | San Joaquin Valley Agricultural Sciences Center | USDA Agricultural Research Service | Parlier, CA 93648 | rachel.naegele@usda.gov
Co-Project Leader: Leonardo De La Fuente | Department of Entomology and Plant Pathology | Auburn University | Auburn, AL, 36849 | lzd0005@auburn.edu
Cooperator: Rodrigo Almeida | Department of Environmental Science, Policy, and Management | University of California | Berkeley, CA 94720 | rodrigoalmeida@berkeley.edu

Reporting Period: The results reported here are from work conducted September 2018 to October 2019.

ABSTRACT
Xylella fastidiosa (*Xf*) subspecies *fastidiosa*, the causal agent of Pierce's disease, costs California grape growers an estimated $56 million annually in management costs. Sources of resistance have been identified and a single source from *Vitis arizonica* is being incorporated into new breeding materials for wine, table, and raisin grape markets. This source of resistance has been evaluated against a small set of isolates from California, but its durability has not been evaluated. In California, the genetic diversity of *Xf* is low, but virulence diversity is unknown. Regional differences among isolates appear likely, based on preliminary work. This project is evaluating the variability of *Xf* diversity in California and potential sustainability of Pierce's disease resistant material.

LAYPERSON SUMMARY
Pierce's disease (PD), caused by *Xylella fastidiosa* (*Xf*), seriously impacts California's grape industry. Growers lose an estimated $56 million annually in decreased production and vine replanting. Breeding efforts have resulted in new winegrape cultivars using a single source of PD resistance. This source has been effective against a few strains of *Xf*, but its durability in the field is unclear. The range in virulence (amount of disease a given isolate can cause) of *Xf* in California is not known, and regional differences appear likely. Research is needed to better understand the variability of *Xf* in California and how this might impact PD resistant grape breeding. This work is evaluating *Xf* virulence in grape and tobacco and the sustainability of PD resistant material.

INTRODUCTION
Plant pathogens with broad host ranges, like *Xylella fastidiosa* (*Xf*) if considered at the species level, often rely on multiple virulence and growth factors to colonize their diverse hosts. Though *Xf* was the first plant pathogenic bacterium to have its full genome sequenced, (8, 16, 17) only a small number of studies have looked at virulence variation (3, 5, 7, 10, 13, 14). One small study in alfalfa found significant correlation between genetic relatedness and virulence among 15 strains of *Xf* subsp. *fastidiosa* (3). In grape, virulence studies are lacking, but preliminary data suggest that virulence differences exist in California.

Virulence comparisons among *Xf* strains are also useful for understanding the biology of this pathogen. In tobacco (*Nicotiana tabacum*), different subspecies of *Xf* are capable of colonizing and causing leaf scorch symptoms (1, 10), and show differences in host colonization and symptomatology (9). Tobacco has been used as a model system for understanding changes in host mineral and nutrient composition caused by *Xf* infection (6, 11), bacterial gene function (3, 12), and impact of new DNA acquired from natural competence and recombination (10). Tobacco assays could be a useful tool for predicting isolate virulence on grapevine. Using tobacco to test multiple strains saves considerable greenhouse space and time, as it can take half the time of a grape experiment.

Pierce's disease resistance has been identified in multiple *Vitis* species. How these sources differ in durability (sustainability of resistance when exposed to multiple strains) of resistance is unclear. A single source of resistance, *PDR1*, from a wild southwestern grape (*V. arizonica*) accession has been used to develop high quality winegrapes with Pierce's disease resistance (breeding efforts by Andy Walker, UC Davis). Table grape breeding efforts also use this same source. Plants with *PDR1* have no disease symptoms and low bacterial populations when inoculated with *Xf*. *PDR1* has maintained efficacy in field trials in Texas and northern California, but its durability to individual isolates is unclear. Other sources of resistance or tolerance have been identified, but their efficacy against multiple isolates of *Xylella* has not been evaluated.

OBJECTIVES

The objective of this project is to determine virulence (level of disease caused by a given individual isolate) diversity of *Xf* subsp. *fastidiosa* in California in order to enhance host resistance to Pierce's disease.

1. Evaluate the virulence diversity of *Xf* strains from California.
2. Evaluate known sources of Pierce's disease (PD) resistance against diverse strains of *Xf.*

RESULTS AND DISCUSSION

Objective 1. Evaluate the Virulence Diversity of *Xf* Strains from California

For Objective 1a, greenhouse tobacco infection experiments were performed as previously described (7, 8). Seeds of tobacco (*Nicotiana tabacum*) Petite Havana SR1 (Plant Introduction number 552516) were obtained from the USDA Germplasm Resources Information Network and germinated in SunshineH Mix #8 (Sun Gro Horticulture Canada Ltd., Vancouver, Canada). Greenhouse temperature was maintained between 20 to 25ºC and natural sunlight was used. After one month, 200 seedlings were transplanted into 4.50 square pots. Plants were fertilized three times using Osmocote 19-6-12 (The Scotts Company, Marysville, OH). 30 to 40 days post-transplant, 150 tobacco plants were cut on the top of the stem leaving three healthy adult leaves in the lower portion of the plant. These plants were ready for inoculation and this point was determined as time zero. Inoculum from the different strains of *Xylella* were cultured on periwinkle wilt (PW) solid at 28ºC. The bacteria were scraped off and suspended in two ml succinate-citrate phosphate buffer (PBS). Optical density was adjusted to OD600 = 0.8. A needle was use for inoculation on the base of the petiole of two of the three mature leaves. 20 ml of inoculum per each leaf was applied. 150 plants were infected [(10 plants for each one of the 14 strains used (**Table 1**), plus a PBS-infected control]. After approximately 40 days, symptoms started to appear. The number of plants showing leaf scorch, as well as the number of leaves showing leaf scorch symptoms per plant, were recorded weekly. The percentage of leaves

showing leaf scorch symptoms per plant was considered as measurement of disease incidence, and this was used to calculate the area under the disease progress curve (AUDPC). AUDPC was calculated based on the midpoint rule method (2) as follows: AUDPC = Σ [(yi + yi+1)/2](ti+1 – ti), where i = the number of assessment times, y = disease severity score for each plant at each assessment, and t = time at each assessment. At the end of the symptom evaluation (5 to 6 weeks) samples were collected for further analysis. Greenhouse experiments will be repeated three times independently.

Table 1. Strains used for inoculation and evaluation of symptoms. Most strains were isolated from symptomatic grapevines in California, with the exception of WM1-1 (isolated in Georgia*; 15). California isolates were obtained from Rodrigo Almeida at UC Berkeley.

Strains	Location (CA)*	Grape Variety
E24	Bakersfield	Red Globe
B25	Napa	Merlot
E10	Bakersfield	Scarlet Royal
B13	Napa	Cabernet Sauvignon
E33	Bakersfield	Holiday
1-10	Santa Barbara	Chardonnay
10	Newsome	Malbec
E11	Bakersfield	Flames
F25	Sonoma	Chardonnay
B4	Napa	Merlot
F34	Temecula	Non-available
F18	Sonoma	Gruner Veltliner
Temecula1	Temecula	Temecula
WM1-1	Georgia* (Lumpkin County)	Mourvedre

We observed differences between strains when disease incidence was evaluated and represented as AUDPC. Strains B13, E33, 1-10, F25, and B4 were shown to be slightly more virulent than WM1-1, a previously-described virulent strain (14) (**Figure 1**).

Similar results can be observed on the measurement of incidence performed weekly, where B13, E33, 1-10, and B4 reach 100% of incidence at week six, while strains Temecula1, B25, F34, and E11 were between 60 to 80% at the same evaluation time point (**Figure 2**).

For Objective 1b, virulence assessments in grape were started in a newly established Cabernet Sauvignon field at the USDA Agricultural Research Service's San Joaquin Valley Agricultural Sciences Center (SJVASC) in Parlier, California (**Figure 3**). Donated uber vines were planted in mid-April and inoculations were performed at two times in July (12 and 11 isolates in the first and second sets, respectively). Forty-four strains were received from Leonardo De La Fuente, but only 23 strains were able to be used for inoculations due to contamination issues. Plants were evaluated weekly beginning six weeks after inoculations to detect disease symptoms. A 0 to 5

based scale was used to indicate disease severity, with plant health for each vine with 0 being a dead plant and 5 being a completely healthy plant. Strain differences were observed in vine symptom severity (**Table 2**).

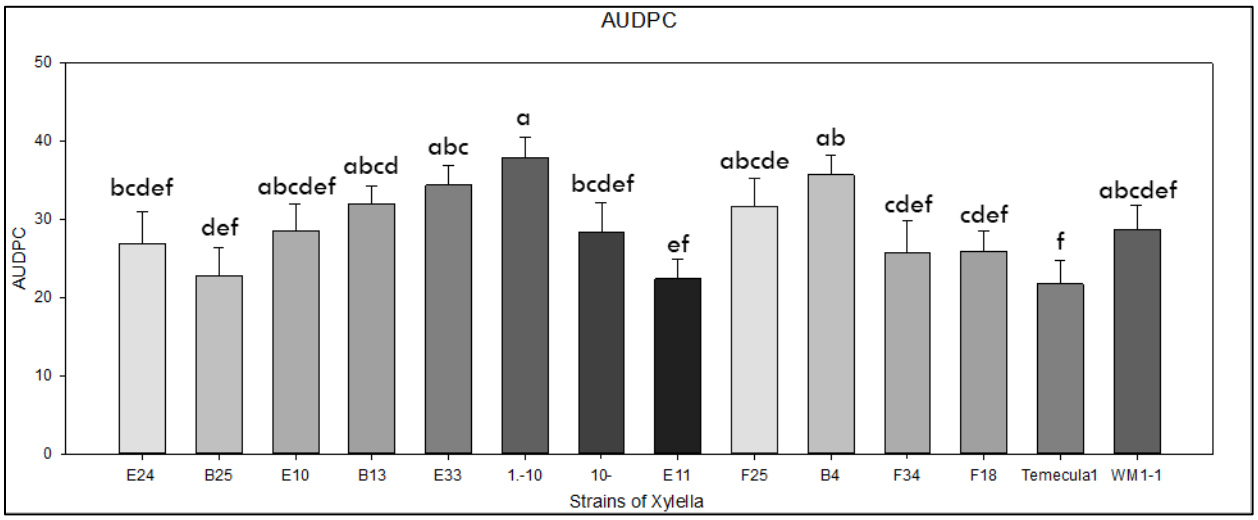


Figure 1. AUDPC for the 14 strains evaluated (see **Table 1**). Significances are marked by letters on top of bars ($p < 0.05$) according to One Way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) (Statistix 8.0).

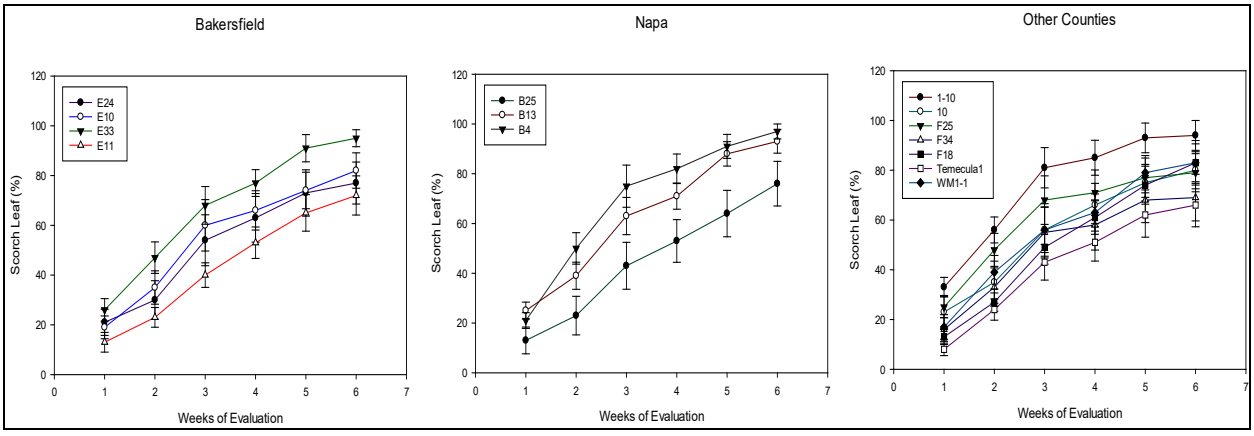


Figure 2. Disease incidence measured by symptom development in tobacco plants. Disease incidence is represented by the percentage of leaves showing scorch symptoms per plant. Ten plants were used for each bacterial strain treatment. Symptoms were evaluated during six weeks, once per week, after first symptoms appeared. For representation purposes the strains were divided into three graphs. The first two graphs from the left are for strains isolated from the same locations in California, while the last graph on the right included the rest of the strains.

Table 2. Least squares (LS) means for plant health/disease scores for Cabernet Sauvignon inoculated with *Xf* strains in early and late July (highlighted in gray).

Xylella Strain	Mean Score	
F46	3.58	D
B16	3.78	CD
B25	3.80	CD
1-19	3.84	CD
F11	3.93	BCD
B13	3.95	BC
F34	3.95	BC
F7	3.98	BC
A5	4.05	BC
F48	4.275	B
F18	3.55	D
B23	3.73	CD
10	3.73	CD
16	3.75	CD
B4	3.78	CD
19	3.78	CD
F39	3.88	BCD
1-17	3.88	BCD
A5	3.88	BCD
E24	3.95	BC
E10	4.05	BC
F36	4.07	BC
PBS buffer control	4.15	B
No buffer control	4.72	A

No buffer control vines (i.e., non-inoculated plants) had significantly fewer symptoms than *Xylella*-inoculated or buffer-inoculated vines. However, significant differences were detected among *Xylella* strains and the buffer-inoculated vines. *Xylella* strains F18 and F46 were the most virulent. Petioles were collected from a subset of inoculated vines to confirm presence of the pathogen with observed symptoms.

Objective 2. Evaluate Known Sources of PD Resistance Against Diverse Strains of *Xf*

Cuttings from resistant material representing four different sources of resistance/tolerance to Pierce's disease were collected and propagated at the SJVASC over the summer. This included material from a new source of resistance, previously identified in a screenhouse trial, that was confirmed as resistant through a second round of the same screenhouse trial funded through a Consolidated Central Valley Pest and Disease Control District proposal this summer (*data not shown*). Additional grating and pots have been ordered for experimental inoculations in spring 2020.

CONCLUSIONS

These data are the first evaluations of *Xf* virulence diversity. Differences in virulence among strains were detected in both the tobacco and grape experiments, but these diferences were not consistent between grapes and tobacco. In grapes, isolates F18 and F46 were the most virulent strains evaluated, yet F18 was not a highly virulent strain in the tobacco study. This indicates that virulence diferences exist among strains and that these diferences are, in part, dependent on host. However, these studies need to be replicated to confirm these results.

REFERENCES CITED

1. Alves E, Kitajima EW, Leite B. 2003. Interaction of *Xylella fastidiosa* with different cultivars of *Nicotiana tabacum*: A comparison of colonization patterns. *Journal of Phytopathology-Phytopathologische Zeitschrift* 151: 500-506.
2. Campbell C L, Madden LV. 1990. *Introduction to Plant Disease Epidemiology*. John Wiley & Sons.
3. Chen H, Kandel PP, Cruz LF, Cobine PA, De La Fuente L. 2017. The major outer membrane protein MopB is required for twitching movement and affects biofilm formation and virulence in two *Xylella fastidiosa* strains. *Molecular Plant-Microbe Interactions* 30: 896-905.
4. Coletta-Filho HD, Bittleston LS, Lopes JRS, Daugherty MP, Almeida RPP. 2015. Genetic distance may underlie virulence differences among isolates of a bacterial plant pathogen. *J. Plant Path.* 97: 465-469.
5. Cruz LF, Cobine PA, De La Fuente L. 2012. Calcium increases *Xylella fastidiosa* surface attachment, biofilm formation, and twitching motility. *Applied and Environmental Microbiology* 78: 1321-1331.
6. Daugherty MP, Lopes JRS, Almeida RPP. 2010. Strain-specific alfalfa water stress induced by *Xylella fastidiosa*. *European Journal of Plant Pathology* 127: 333-340.
7. De La Fuente L, Parker JK, Oliver JE, Granger S, Brannen PM, van Santen E, Cobine PA. 2013. The bacterial pathogen *Xylella fastidiosa* affects the leaf ionome of plant hosts during infection. *PLoS One* 8 (5): e62945.
8. Francis M, Civerolo EL, Bruening G. 2008. Improved bioassay of *Xylella fastidiosa* using *Nicotiana tabacum* cultivar SR1. *Plant Disease* 92: 14-20.
9. Kandel PP, Lopez SM, Almeida RPP, De La Fuente L. 2016. Natural competence of *Xylella fastidiosa* occurs at a high frequency inside microfluidic chambers mimicking the bacterium's natural habitats. *Applied and Environmental Microbiology* 82: 5269-5277.
10. Lopes JRS, Daugherty MP, Almeida RPP. 2010. Strain origin drives virulence and persistence of *Xylella fastidiosa* in alfalfa. *Plant Pathology* 59: 963-971.
11. Lopes SA, Ribeiro DM, Roberto PG, Franca SC, Santos JM. 2000. *Nicotiana tabacum* as an experimental host for the study of plant-*Xylella fastidiosa* interactions. *Plant Disease* 84: 827-830.
12. Navarrete F, De La Fuente L. 2015. Zinc detoxification is required for full virulence and modification of the host leaf ionome by *Xylella fastidiosa*. *Molecular Plant-Microbe Interactions* 28: 497-507.
13. Oliver JE, Cobine PA, De La Fuente L. 2015. *Xylella fastidiosa* isolates from both subsp *multiplex* and *fastidiosa* cause disease on southern highbush blueberry (*Vaccinium sp.*) under greenhouse conditions. *Phytopathology* 105: 855-862.

14. Oliver JE, Sefick SA, Parker JK, Arnold T, Cobine PA, De La Fuente L. 2014. Ionome changes in *Xylella fastidiosa*-infected *Nicotiana tabacum* correlate with virulence and discriminate between subspecies of bacterial isolates. *Molecular Plant-Microbe Interactions* 27: 1048-1058.
15. Parker JK, Wisotsky SR, Johnson EG, Hijaz FM, Killiny N, Hilf ME, De La Fuente L. 2014. Viability of '*Candidatus* Liberibacter asiaticus' prolonged by addition of citrus juice to culture medium. *Phytopathology* 104: 15-26.
16. Rogers EE. 2012. Evaluation of *Arabidopsis thaliana* as a model host for *Xylella fastidiosa*. *Molecular Plant-Microbe Interactions* 25: 747-754.
17. Simpson AJG, Reinach FC, Arruda P, Abreu FA, Acencio M, Alvarenga R, et al. 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. *Nature* 406: 151-157.
18. Van Sluys MA, de Oliveira MC, Monteiro-Vitorello CB, Miyaki CY, Furlan LR, Camargo LEA, et al. 2003. Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. *Journal of Bacteriology* 185: 1018-1026.

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

ACKNOWLEDGEMENTS
The work described in this report was conducted by Laura Gomez Arias, Jeff DeLong, Noor Abdelsamad, Alanna Burhans, and Marcus Merfa e Silva.

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

Project Leader: Caroline Roper | Department of Microbiology and Plant Pathology | University of California | Riverside, CA 92521 | mcroper@ucr.edu
Co-Project Leader: Dario Cantu | Department of Viticulture and Enology | University of California | Davis, California 95616 | dacantu@ucdavis.edu

Reporting Period: The results reported here are from work conducted July 2018 to October 2019.

ABSTRACT
Plants have developed complex mechanisms to defend themselves from constant biotic and abiotic challenges presented by a fluctuating environment. One of these mechanisms, called plant defense priming, is a tool that exploits plant "memory" to counteract pathogens and abiotic stress. Microorganisms have signature molecules called microbe-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs) that can act as stimuli to induce the primed state. This memory allows plants to quickly recognize pathogens and activate strong immune responses that result in disease resistance or tolerance. *Xylella fastidiosa* (*Xf*) is a Gram-negative, xylem-limited bacterium that causes severe diseases in many economically important plants, such as Pierce's disease of grapevine (*Vitis vinifera*). Lipopolysaccharide (LPS) covers most of the cell surface in Gram-negative bacteria and is a well-described PAMP that elicits defense responses in plants. In grapevines, *Xf* LPS-mediated elicitation of plant immunity leads to systemic and prolonged activation of defense pathways related to *Xf* recognition. The goal of this project is to explore the persistence and molecular mechanisms underlying the LPS-mediated plant defense priming phenomenon against *Xf.* We demonstrate that *Xf* LPS treatment primes the immune system and results in reduced symptom development and lower bacterial titer in grapevines inoculated with *Xf*. Interestingly, an additional LPS booster dose enhances this reduced symptom phenotype.

LAYPERSON SUMMARY
Successful plant pathogens, like *Xylella fastidiosa* (*Xf*), must overcome plant immune responses to establish themselves and cause disease. We have shown *Xf* utilizes the prominent O-antigen surface carbohydrate found in its lipopolysaccharide molecule to shield itself from being recognized by the grapevine immune system, effectively delaying its detection by the plant. However, if we isolate its lipopolysaccharide and inject it directly into the plant like a vaccine, it elicits strong immune responses and conditions grapevines for enhanced defense against *Xf.* We will employ this knowledge to better understand the mechanism of this enhanced response, test if we can maintain the primed state, and apply these results to create Pierce's disease resistant grapevines.

INTRODUCTION
Xylella fastidiosa (*Xf*)*,* a Gram-negative fastidious bacterium, is the causal agent of Pierce's disease (PD) of grapevine (*Vitis vinifera*) and several other economically important diseases (Chatterjee et al., 2008). *Xf* is limited to the xylem 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. PD 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 previous study confirmed that lipopolysaccharide (LPS) is a major virulence factor for *Xf (*Clifford et al.,2013; Rapicavoli et al., 2018). LPS comprises approximately 70% of the Gram-negative bacterial cell surface, making it the most dominant macromolecule displayed on the cell surface (Caroff and Karibian, 2003). 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 the *Xf* O-antigen is a linear α1-2 linked rhamnan and compositional alterations to the O-antigen significantly affected the adhesive properties of *Xf,* consequently affecting biofilm formation and virulence (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. We then sought to test our additional hypothesis that the *Xf* LPS molecule acts as a pathogen-associated molecular pattern (PAMP), 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., 2018).

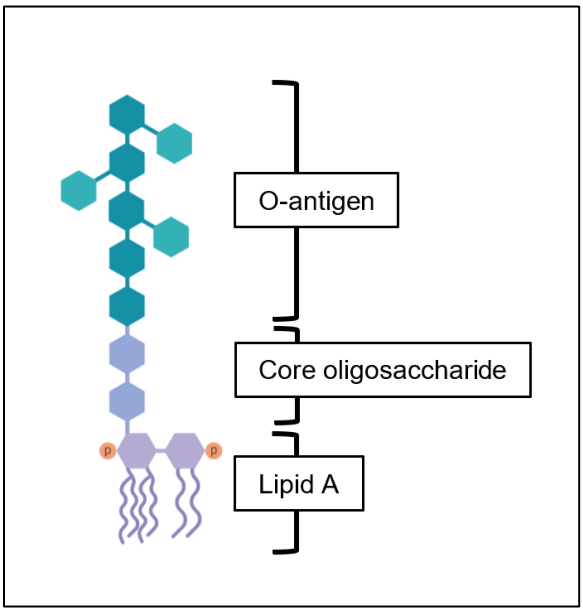


Figure 1. Schematic of a single LPS molecule containing lipid A, core oligosaccharide, and the O-antigen.

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). 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.). 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, we observe profound changes that occur in the xylem that are linked to the presence of *Xf.* These include an oxidative burst and suberin deposition, as well as production of tyloses (Rapicavoli et al., 2018). Interestingly, we also observe significant defense responses to *Xf* in the phloem tissue, a tissue historically overlooked in the context of this xylem-dwelling pathogen, that mainfest in the form of callose deposition. 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 evade the host basal defense response as it successfully colonizes and causes serious disease in grapevine. We tested our hypothesis that 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 (Rapicavoli et al., 2018).

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* (Rapicavoli et al., 2018).

Previously, we completed a global RNA sequencing (RNA-seq)-based transcriptome profile where we sequenced the transcriptomes of grapevines treated with wild-type, *wzy* mutant cells, or 1 x phosphate-buffered saline (PBS) (Rapicavoli et al., 2018). The goal was to identify genes that are differentially expressed when plants are inoculated with either wild-type or the *wzy* mutant while using mock-inoculated plants as the controls. 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). The RNA-seq data demonstrate that the grapevine is activating defense responses that are distinct to each treatment and time point **(Figure 2A)**. 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 2B)**. This included a significant increase in the production of thioredoxins, glutaredoxins, and other ROS-scavenging enzymes involved in antioxidant defense.

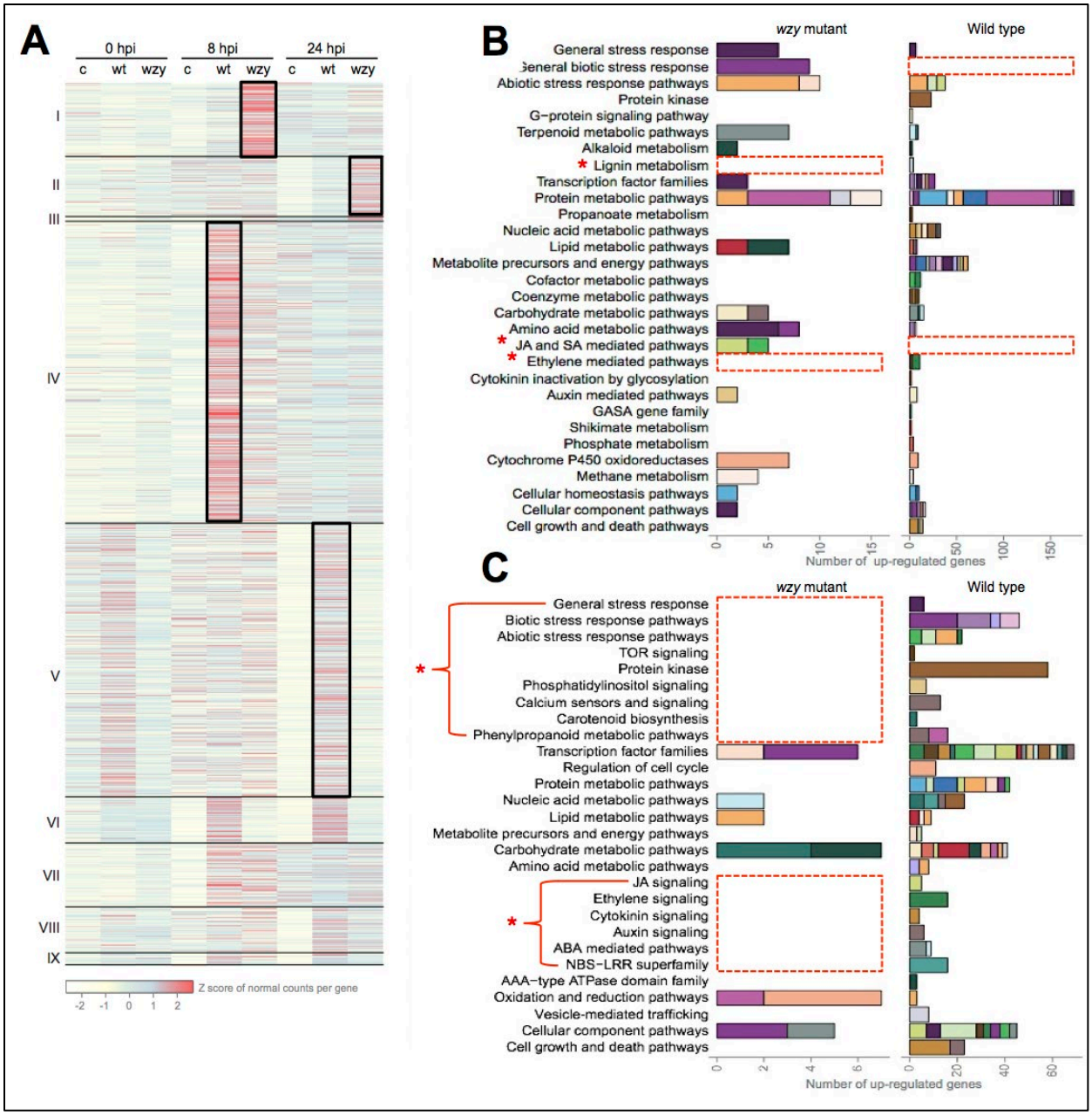


Figure 2. Grapevine responses to early infections by *wzy* mutant and wild-type *Xf*. (A) Upregulated grape genes ($P < 0.05$) in response to *wzy* mutant or wild-type bacteria at eight and 24 hours post-inoculation (hpi) when compared to the wounded control (c). Genes are classified into nine groups (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 upregulated during *wzy* (Group I) or wild-type (Group IV) infections at 8 hpi. (C) Enriched grape functional subcategories ($P < 0.05$) among genes upregulated during *wzy* (Group II) or wild-type (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.

In addition, there was high expression of genes involved in the production of phytoalexins (e.g., stilbene synthase), antimicrobial peptides (e.g., thaumatin), and pathogenesis-related (PR) genes. In contrast, wild-type-responsive genes in this time point were enriched primarily in response to abiotic or general stresses (i.e., drought, oxidative, temperature, and wounding stresses) and were not directly related to immune responses **(Figure 2B)**. 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 2C)**. We hypothesize that at eight 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 damage-associated molecular patterns (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 as a shield to delay host recognition, allowing the bacterial cells to establish an infection (Rapicavoli et al., 2018). Complete RNA-seq data can be found in the supplementary information in Rapicavoli et al., 2018.

In addition to exploring early defense response, we also characterized the transcriptional response at systemic locations distal to the point of inoculation (POI) and at longer time points: 48 hours, one week, and four weeks. This tested 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. Local tissue of *wzy*-infected plants induced genes enriched in cell wall metabolism pathways, specifically pectin modification, at four weeks post-inoculation **(Figure 3A)**. This is a stark contrast with wild-type-inoculated vines, in which these pathways were upregulated as early as eight hours post-inoculation. This likely explains why this pathway is not enriched in local tissues 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 (EDS) 1 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 upregulation 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 3A)**.

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 3B**). 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 3B)**. Furthermore, genes enriched in ethylene response factor (ERF) transcription factors were upregulated 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 upregulated in systemic tissue, and expression continued to increase at four weeks post-inoculation. This consistent enrichment and upregulation 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 upregulated 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 upregulation of these genes (beginning at 24 hours post-inoculation) establishes this structural barrier as an important plant defense response to *Xf* infection. Overall, the RNA-seq 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.

In addition to the role of LPS in promoting bacterial infection, pre-treatment of plants with LPS can prime the defense system, resulting in an enhanced response to subsequent pathogen attack. This defense-related memory is called plant defense priming and stimulates the plant to initiate a faster and stronger response against future invading pathogens (Conrath, 2011; Newman et al., 2000). We demonstrate that pre-treatment with LPS isolated from *Xf* would result in an increase in the grapevine's tolerance to *Xf* by stimulating the host basal defense response. Our ex vivo data showing that both wild-type and *wzy* mutant LPS elicit an oxidative burst, an early marker of defense that can potentiate into systemic resistance, in grapevine leaf disks supports this hypothesis. To determine if the primed state affects the development of PD symptoms, we documented disease progress in plants that were pre-treated with either wild-type or *wzy* LPS and then challenged with *Xf* either four or 24 hours later. Notably, we observed a decrease in PD severity in vines pre-treated with *Xf* LPS and then challenged with *Xf* **(Figure 4) (**Rapicavoli et al., 2018).

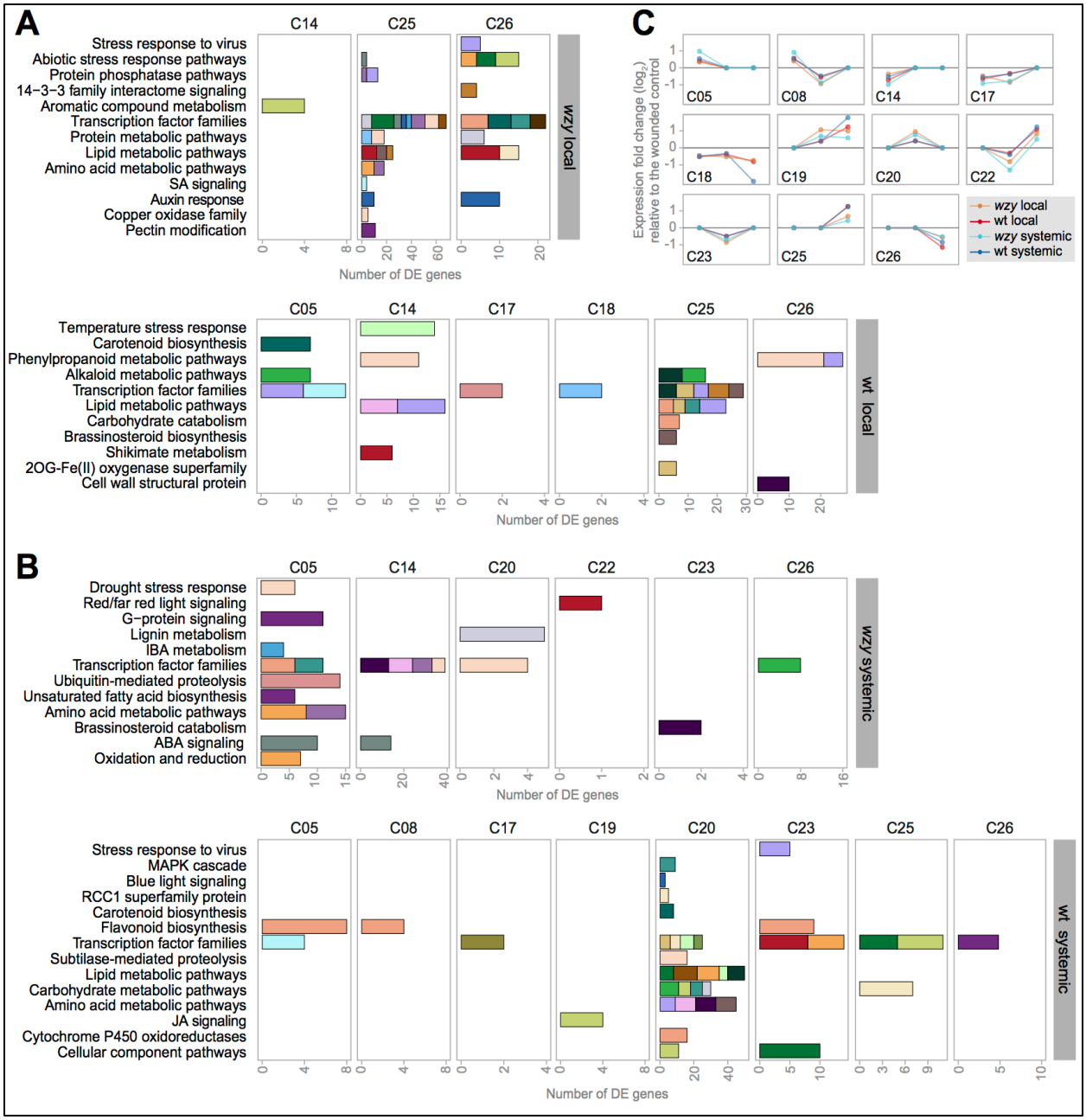


Figure 3. Transcriptomic analysis of late grapevine responses to *Xf* wild-type and *wzy* mutant strains in local and systemic tissue. Enriched grape functional pathways ($P < 0.05$) in differentially expressed (DE) gene clusters representing local (A) or systemic (B) responses to *Xf* inoculation. Only 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 (log2) at a given time point post-inoculation (in order: 48 hours, one week, and four weeks) when compared to the wounded control.

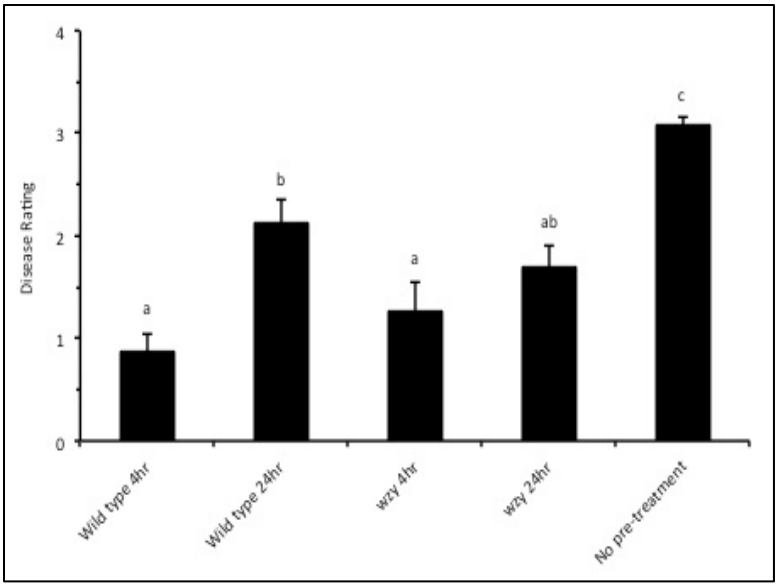


Figure 4. PD symptom severity in grapevines primed with purified *Xf* LPS. Average disease ratings of *V. vinifera* Cabernet Sauvignon grapevines pre-treated with wild-type or *wzy* mutant LPS (50 µg/ml), then challenged at four hours or 24 hours post-LPS treatment with *Xf* cells. Disease ratings were taken at 12 weeks post-challenge. The LPS pre-treated plants are significantly attenuated in symptom development, compared with plants that did not receive pre-treatment ($P < 0.05$). Graph represents the mean of 24 samples per treatment. Bars indicate standard error of the mean.

Our previous findings indicate *Xf* LPS-mediated elicitation of the basal defense response in grapevines leads to systemic and prolonged activation of defense pathways related to *Xf* perception. Additionally, we showed treating grapevines with LPS before inoculating with *Xf* reduces PD symptoms observed at a single time point (12 weeks post-inoculation) (Rapicavoli et al., 2018). To evaluate overall disease severity, we monitored our grapevines for PD symptom development over the course of 18 weeks and collected plant tissue from these plants to investigate transcriptional changes in plants treated with LPS and challenged with *Xf.*

OBJECTIVES

1. Characterize the temporal aspects of the primed state in grapevine.
2. Characterize the molecular mechanisms underlying the grapevine immune response to *Xf.*

RESULTS AND DISCUSSION

Objective 1. Characterize the Temporal Aspects of the Primed State in Grapevine

We previously showed treating grapevines with LPS before inoculating with *Xf* reduces PD symptoms at 12 weeks post-inoculation (**Figure 4**) (Rapicavoli et al., 2018). To explore if the primed state can be extended over time, we have tested if an additional LPS application following elicitation of the plant defense priming can increase PD tolerance. Grapevines were treated with wild-type LPS (50 µg/ml) and challenged with *Xf* four hours later. After 48 hours or one week, grapevines received an additional LPS treatment (50 µg/ml). Appropriate controls received diH_2O instead of LPS and 1 x PBS instead of *Xf* cells. All plants were scored for PD symptom development using a disease rating scale of 0 to 5, where 0 is a healthy vine and 5 is a

dead vine (Guilhabert & Kirkpatrick, 2005). To assess the overall performance of the grapevines during the disease development trial, we used the area under the disease progression curve (AUDPC) method as a measure of disease development. The p-values for the one-week LPS application AUDPC scores and titer did not state any significant difference between an additional LPS application or water treatments. The AUDPC values for primed vines that received an additional LPS application at 48 hours were significantly lower than primed vines that did not receive an additional LPS application (**Figure 5A**). It appears that an additional LPS dose at 48 hours increases the reduced symptom phenotype observed in primed plants. However, no significant difference was found between titer values of these two treatments (**Figure 5B**). The petioles sampled to measure *Xf* titer were collected 13 weeks post-inoculation. It may be that collecting petioles at this point is too late to observe any significant titer differences between treatments.

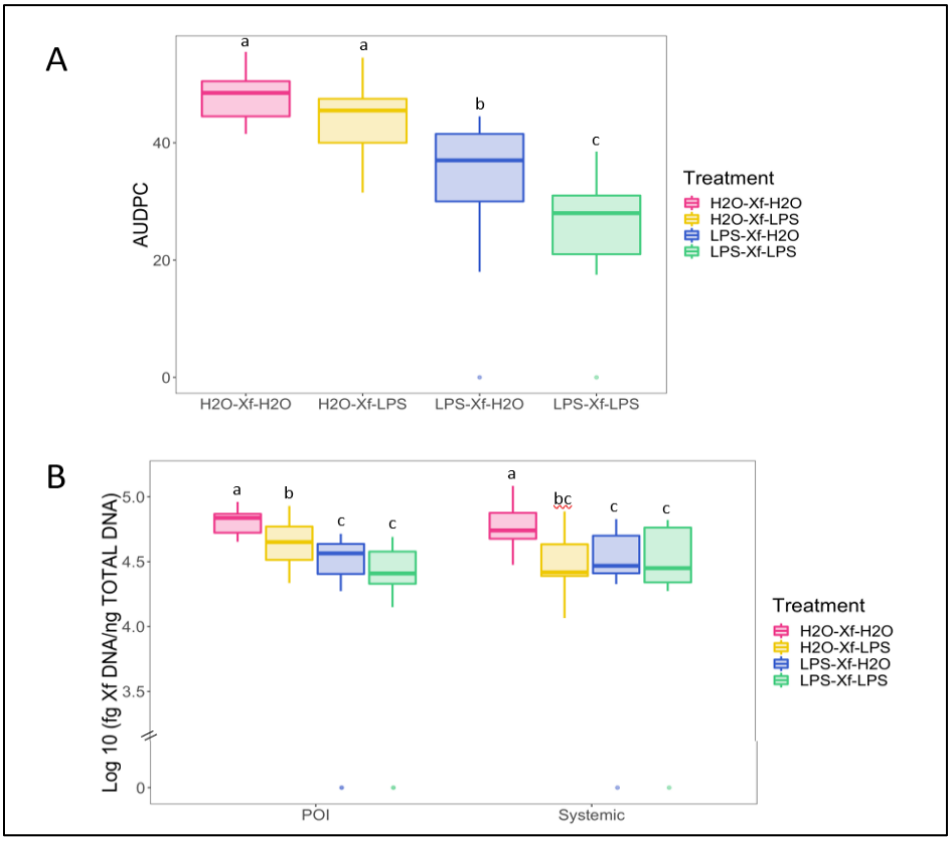


Figure 5. An additional LPS application enhances the reduced symptom phenotype observed in primed vines. *V. vinifera* Cabernet Sauvignon grapevines received an additional LPS application 48 hours after being inoculated with *Xf* wild-type LPS (50 µg/ml) or water and challenged with *Xf*. (A) AUDPC values for primed vines (vines that received LPS pre-treatment) and naive vines (vines that received water instead of LPS) treated with an additional LPS application. Grapevines were scored on a weekly basis using a PD rating scale of 0 to 5 ($P < 0.05$, Wilcoxon test, n = 13). (B) Quantification of *Xf* DNA (Log10 fg *Xf* DNA per ng of total DNA) in petioles of primed and naive grapevines with an additional LPS dose at the POI and 20 nodes above (systemic) using quantitative polymerase chain reaction (qPCR) ($P < 0.05$, Wilcoxon test, n = 13).

Objective 2. Characterize the Molecular Mechanisms Underlying the Grapevine Immune Response to *Xf*

The molecular mechanisms underlying defense priming and its importance in enabling heightened immunity to counteract pathogens are poorly understood. To better understand the changes occurring in gene expression patterns that potentiate the priming phenotype in grapevine, we will perform a series of RNA-seq experiments that will highlight genes and pathways induced during priming in both local and systemic tissue. We showed treating grapevines with LPS before inoculating with *Xf* reduces PD symptoms at 12 weeks post-inoculation (Rapicavoli et al., 2018). We repeated this experiment and monitored plants for PD symptom development, and harvested petioles from these plants for RNA-seq analysis and targeted defense metabolite analysis. Grapevines were treated with wild-type LPS (50 µg/ml) and challenged with *Xf* cells four hours later. Petioles for this experiment were harvested at four hours, 24 hours, and 48 hours post-*Xf* challenge from the POI and 20 nodes above the POI. RNA-seq differential expression analysis and high-performance liquid chromatography (HPLC) are currently in progress. To assess the overall performance of vines treated with LPS over the entire disease development trial, we used the AUDPC method as a measure of disease development. The AUDPC values showed primed plants are significantly lower in disease severity compared to the naive plants which did not receive LPS. Our results indicate LPS treatment primes the immune system before *Xf* challenge, leading to reduced symptom development and significantly lower bacterial titer in grapevines (**Figure 6)**.

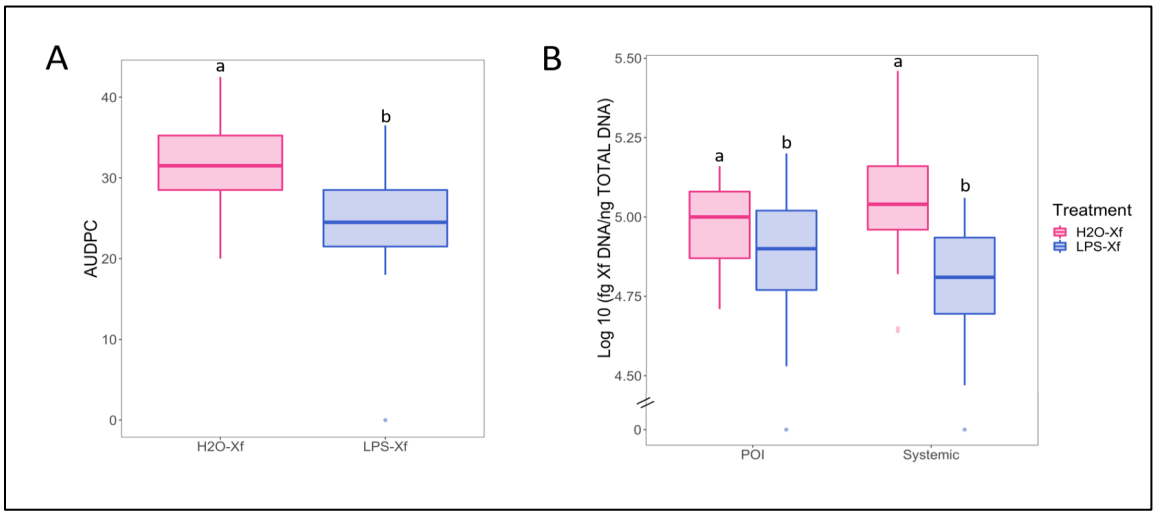


Figure 6. Pre-treatment with LPS reduces PD symptom development and bacterial titer in grapevines infected with *Xf*. *V. vinifera* Cabernet Sauvignon grapevines were inoculated with *Xf* wild-type LPS (50 µg/ml) or water four hours prior to inoculation with *Xf*. (A) AUDPC values for primed vines (vines that received LPS pre-treatment) and naive vines (vines that did not receive an LPS pre-treatment and received water instead). Grapevines were scored on a weekly basis using a PD rating scale of 0 to 5 ($P < 0.0001$, Wilcoxon test, n = 26, n = 25). (B) Quantification of *Xf* DNA (Log10 fg *Xf* DNA per ng of total DNA) in petioles of primed and naive grapevines at the POI and 20 nodes above (systemic) using qPCR. Appropriate controls received water instead of LPS and 1 x PBS instead of bacterial cells and showed no symptoms, and no *Xf* was detected. (POI: $P < 0.05$, Systemic: $P < 0.0001$, Wilcoxon test, n = 26, n = 25).

CONCLUSIONS

We previously demonstrated treating grapevines with LPS before inoculating with *Xf* reduces PD symptoms observed at a single time point (12 weeks post-inoculation) (Rapicavoli et al., 2018). To evaluate overall disease severity, we monitored our grapevines for PD symptom development over the course of 18 weeks. AUDPC and titer values showed primed plants were significantly lower in disease severity and *Xf* presence compared to the naive plants (non-LPS treated), indicating a priming effect in plants treated with LPS. Our ongoing work demonstrates that *Xf* LPS primes the grapevine immune system and elicits an immune response. This results in reduced disease severity in primed plants inoculated with *Xf.* Interestingly, an additional LPS dose enhances this reduced disease severity. Finally, to better understand the molecular mechanisms underlying the LPS-mediated plant defense priming phenomenon against *Xf*, we have collected petioles from primed plants for transcriptome analysis. The overall outcome will result in fundamental knowledge about grapevine immune responses at the molecular level that we will utilize to test novel gene targets for creating PD-resistant grapevines.

REFERENCES CITED

Caroff, et al. 2003. *Carbohydrate Research* 338: 2431-2447.
Chatterjee S, et al. 2008. *Annual Review of Phytopathology* 46: 243-271.
Clifford JC, et al. 2013. *Molecular Plant-Microbe Interactions* 26: 676-85.
Conrath U. 2011. *Trends in Plant Science* 16: 524-531.
Dow M, et al. 2000. *Annual Review of Phytopathology* 38: 241-261.
Guilhabert MR, Kirkpatrick BC. 2005. *Molecular Plant-Microbe Interactactions* 18: 856-868.
Newman KL, et al. 2004. *Proceedings of the National Academy of Sciences USA* 101: 1737-1742.
Newman MA, et al. 2000. *Molecular Plant Pathology* 1: 25-31.
Newman MA, et al. 2002. *Plant Journal* 29: 487-495.
Newman MA, et al. 2007. *Journal of Endotoxin Research* 13: 69-84.
Nicaise V, et al. 2009. *Plant Physiology* 150: 1638-1647.
Rapicavoli JN, et al. 2018. *Nature Communications* 9: 390.
Rapicavoli JN, et al. 2015. *Applied and Environmental Microbiology* 81: 8145-8154.
Silipo A, et al. 2005. *Journal of Biological Chemistry* 280: 33660-33668.
Sun QSY, et al. 2013. *Plant Physiology* 161: 1529-1541.
Tao Y, et al. 2003. *The Plant Cell* 15: 317-330.
Whitfield C. 1995. *Trends in Microbiology* 3: 178-185.

FUNDING AGENCIES

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

ACKNOWLEDGEMENTS

We thank Christopher Wallis at the USDA-ARS Crop Diseases, Pests, and Genetics Research Unit in Parlier, California for performing HPLC analysis on secondary metabolites in LPS-primed grapevines.

GENERATING PIERCE'S DISEASE RESISTANT GRAPEVINES USING CRISPR-CAS9 AND TRADITIONAL TRANSGENIC APPROACHES

Project Leader: Caroline Roper | Department of Microbiology and Plant Pathology | University of California | Riverside, CA 92521 | mcroper@ucr.edu
Co-Project Leader: Dario Cantu | Department of Viticulture and Enology | University of California | Davis, CA 95616 | dacantu@ucdavis.edu

Reporting Period: The results reported here are from work conducted July 2019 to October 2019.

ABSTRACT

Using a combination of global transcriptomics (RNA sequencing), functional genomics, analytical biochemistry, and disease phenotyping, we have discovered a *Xylella fastidiosa* (*Xf*) cell surface molecule, lipopolysaccharide, that acts as a potent elicitor of the grapevine immune system. We capitalized on this robust immune response to tease apart early elicitation of the basal defense response that leads to systemic and prolonged activation of defense pathways related to *Xf* perception in grapevine (Rapicavoli et al., 2018). Using this information about immune responses to *Xf*, our experimental objective is to generate grapevines that can effectively defend themselves against *Xf* infection. Thus, we will utilize our collection of data on grapevine immune responses to *Xf* to produce Pierce's disease resistant vines through both (1) a traditional transgenic approach and (2) a new clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 approach.

LAYPERSON SUMMARY

Xylella fastidiosa (*Xf*), a bacterial pathogen, causes Pierce's disease of grapevine and poses a serious threat to the viticulture industry. We identified an entity that is an integral piece of the bacterial cell surface that acts as a potent elicitor of the grapevine defense response. Using this elicitor, we have stimulated the grape immune system and determined how this subsequently imparts protection against future encounters with the *Xf* pathogen. This protection results in significantly less bacterial colonization and significantly less disease in vines. The goal of the proposed work is to use this knowledge of the grapevine immune response to *Xf* to generate Pierce's disease resistant vines.

INTRODUCTION

We have identified grapevines genes that, when induced, lead to prolonged and systemic immune elicitation following *Xylella fastidiosa* infection and are thus potentially related to Pierce's disease (PD) resistance. We have also identified those related to PD susceptibility. Using the transgenic approach, we will generate *Vitis vinifera* grapevines that overexpress immune-related genes that we have found to be linked to effective immune elicitation against *Xf* (Rapicavoli et al., 2018). The goal of the transgenic approach is to enhance immunity through overexpression of these selected genes. In contrast, using the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 approach, we will also generate grapevines that contain gene deletions in genes we have identified that are related to PD susceptibility. The goal of the CRISPR-Cas9 approach is to edit out susceptibility factors that would result in a resistant vine. Vines generated by CRISPR-Cas9 are not considered to be genetically modified organisms.

Once we have generated grape lines by either approach, we will determine their relative resistance levels to PD in robust and established greenhouse bioassays that test for PD severity levels as well as bacterial colonization levels (Roper et al., 2007; Clifford et al., 2013). We have also structured in experiments that include microscopic phenotypic assessments of how these vines respond to *Xf* infection in the vasculature. These will include studies on the amount of tyloses produced in the xylem of wild-type parental vines as compared to the amount in the vines produced by the transgenic approach or the CRISPR-Cas9 approach. Tyloses (outgrowths of xylem parenchyma cells into the xylem) are well established to be one of the major consequences of *Xf* infection that result in exacerbated PD symptoms (Sun et al., 2013) and we hypothesize that preventing or curbing tylose development in *V. vinifera* will result in PD resistance. In support of this hypothesis, we have shown that vines that have early induction of specific salicylic acid (SA)-mediated defense responses produce few to no tyloses and exhibit few to no PD symptoms (Rapicavoli et al., 2018) (**Figure 1**). In addition, we will conduct transcriptional profiling experiments that interrogate how these newly generated grape lines respond at the transcriptional level to *Xf* as compared to their wild-type parents (Rapicavoli et al., 2018) to further characterize the nature of the defense response to *Xf*.

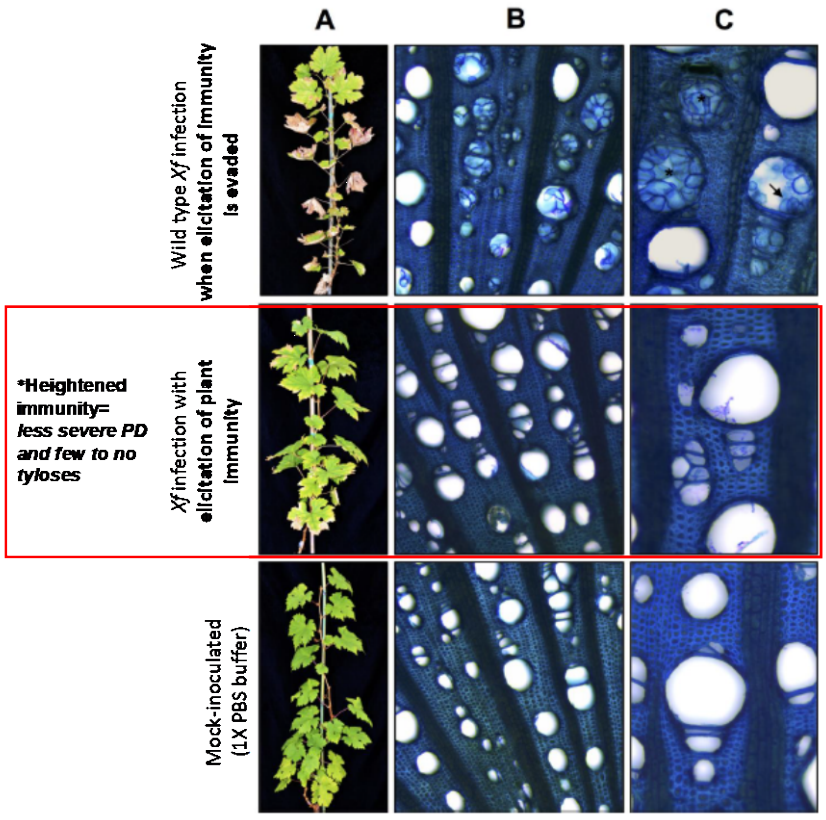


Figure 1. Elicitation of sectors of the grapevine immune system affects PD severity and tylose development. (A) PD severity at the whole vine level. **(B)** Ultrastructural study showing tylose production in cross sections of grapevine xylem (brightfield stained with Toluidine Blue O). **(C)** Close-up of xylem vessels showing complete occlusion with multiple tyloses (*) in wild type-inoculated vines. **Tyloses were largely absent in the xylem vessels of vines with heightened immunity to *Xf* *(Red box)*.** No tyloses were present in the stems of 1X PBS-inoculated vines.

This project will generate vines that are resistant to PD. This project addresses a key aspect of the interaction of *Xf* with the grapevine host immune system and capitalizes on data we have collected to date to create vines that are resistant to PD. Using a combination of global transcriptomics (RNA sequencing; RNA-seq), functional genomics, analytical biochemistry, and disease phenotyping, we discovered a *Xf* cell surface molecule (lipopolysaccharide; LPS) that acts as a potent elicitor of the grapevine immune system. We used derivatives of this molecule as a tool to tease apart immune responses to *Xf*, and from those experiments we learned that early elicitation of the basal defense response leads to systemic and prolonged activation of defense pathways related to *Xf* perception in grapevine (Rapicavoli et al., 2018). Furthermore, we have demonstrated that elicitation of defense pathways associated with a reactive oxygen species (ROS) burst and a SA-mediated defense response results in significant reductions in bacterial titer and overall reduced disease when these induced plants are challenged with live *Xf* cells (Rapicavoli et al., 2018) (**Figure 2**). These are exciting and compelling findings that have identified key sectors of the grapevine immune system involved in the response to *Xf* infection.

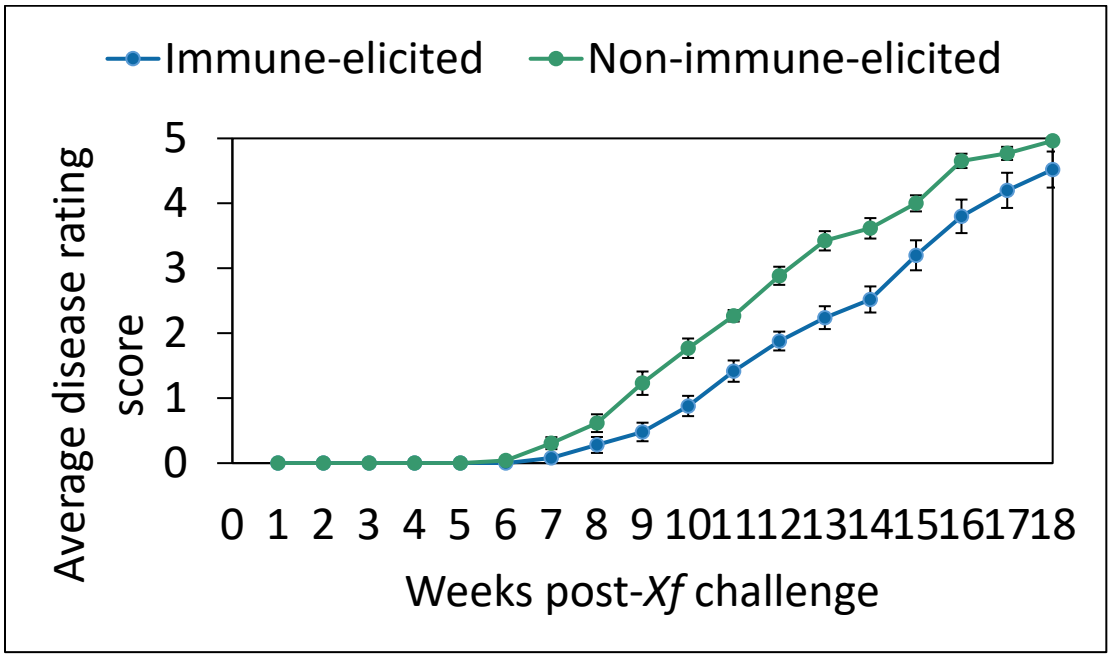


Figure 2. PD symptoms are significantly less severe in immune-elicited plants. Grapevines were pre-treated with the *Xf* LPS elicitor that elicits sectors of the grapevine immune response and then challenged with *Xf* cells four hours post-LPS treatment. PD symptoms were significantly less severe in elicited vines. Our goal in this project is to over-express genes underlying this phenotype and use these for disease control. Graph represents the mean of n = 25 and n = 26 samples per each treatment. Bars indicate standard error of the mean.

We are now poised to test our hypothesis that specific key components of grapevine immunity involved in host recognition of *Xf* can be exploited to develop grapevines that are resistant to PD. We are defining resistance as the ability of a plant to hinder or overcome, to some degree, the effects of an invading pathogen. To do this, we will generate vines that we will test for PD

resistance by two methodologies using (1) a transgenic approach that generates grapevines that overexpress genes that we have identified as related to an effective immune response to *Xf* (Rapicavoli et al., 2018), and (2) a CRISPR-Cas9 approach that will generate vines with gene deletions in genes that are linked to PD susceptibility (Rapicavoli et al., 2018) (Objective 1). We will then phenotype these plants using our established disease phenotyping assays and also examine their responses to *Xf* infection at the ultrastructural level in the vasculature (Objective 2). Embedded in Objective 2 are experiments to further characterize the grapevine lines we generate by performing global transcriptomic studies to compare how these new lines respond at the transcriptional level to *Xf* compared to the wild-type parental grape line.

OBJECTIVES

1. Functional genomics of grapevine immune responses to *Xf* using transgenesis and CRISPR-Cas9 mediated gene editing.
2. Evaluation of PD resistance: Disease phenotyping and characterization of defense responses to *Xf* challenge.

RESULTS AND DISCUSSION

Objective 1. Functional Genomics of Grapevine Immune Responses to *Xf* Using Transgenesis and CRISPR-Cas9 Mediated Gene Editing

Objective 1a. Overexpression of Genes Associated with Elicitor Activation of Defense Against *Xf*. The genes of interest we will target come from a selection of genes from our recent study that we found to be significantly upregulated early in the infection process in LPS-mediated elicited grapevines as compared to wild-type inoculated plants (Rapicavoli et al., 2018). This includes two Class III peroxidases. These enzymes are among the enzymes that accumulate in abundance in xylem sap during colonization by vascular pathogens (Yadeta and Thomma, 2013; Chakraborty et al., 2016). Their functions include roles in defense against pathogen infection, such as enhanced production of ROS (as signal mediators and antimicrobial agents) and enhanced production of phytoalexins (Hiraga et al., 2001). Most importantly, the upregulation of these peroxidase genes corroborates our phenotypic data of enhanced and dynamically different production of ROS in the xylem of LPS-mediated elicited plants (Rapicavoli et al., 2018).

Genes encoding key facets of SA-mediated signaling pathways [enhanced disease susceptibility 1 (*EDS1*) genes] uniquely expressed in local tissues of plants with LPS-mediated elicited immunity to *Xf* had steady expression over time and in systemic tissue. *EDS1* genes encode proteins associated with the SA pathway and have been implicated in grapevine defenses against powdery mildew. In addition, overexpression of *EDS1* in *Arabidopsis thaliana* confers pathogen resistance against biotrophic pathogens (Cui et al., 2016). We will overexpress *EDS1*. We will also overexpress one of the several pathogen resistance (*PR-1)* precursor genes found to be significantly upregulated as early as eight hours into the infection process (Rapicavoli et al., 2018). PR-1 proteins are known markers of the SA-mediated defense pathway, which further supports the role of SA in activating defenses when grapes have the ability to perceive *Xf* attack. A gene encoding a thaumatin protein was also significantly upregulated in our immune stimulated grapevines that we found to be tolerant of *Xf*. Thaumatins are PR proteins that exhibit antimicrobial activities during some host-pathogen interactions (Kuwabara et al., 2002). Interestingly, Kirkpatrick (2009) found a thaumatin-like protein at elevated levels in the xylem sap extracted from vines that had been cured of *Xf* following cold treatment, further reiterating

the link between thaumatins and defense against *Xf*. We will overexpress the thaumatin encoding gene (*VIT_02s0025g04290*).

Overall, we will be generating and testing five overexpression lines. In all cases, the resulting transformants will be multiplied and propagated into full-grown plants and will be ready for virulence testing in year 2. Plants will be tested for resistance to PD by needle inoculating them at the base of the plant with 40 µl of a 10^8 colony-forming units (cfu)/ml *Xf* inoculum suspension or 1x phosphate-buffered saline (PBS) buffer only, as described below in Objective 2a. Control plants (non-transgenic Thompson Seedless vines) will be used as positive controls for the experiment. Plants will be assayed for PD symptom development, *Xf* population numbers, and *Xf* movement as described below in Objectives 2a and 2b.

Thus far, eight constructs have been made to transform Thompson Seedless grapevine. Four constructs are in modified pCambia1302:

- p35S:EDS1-gDNA (VvCabSauv08_P0022F.ver1.0.g361850)
- p35S:EDS1-CDS (VvCabSauv08_P0022F.ver1.0.g361850)
- p35S:CP1 like-gDNA (VvCabSauv08_P0022F.ver1.0.g361850)
- p35S:CP1-CDS (VvCabSauv08_P0022F.ver1.0.g361850).

We initially tested these constructs in *A. thaliana Col* wild-type to determine transformation efficiency and any potential lethal or growth effects that overexpression of these genes may have in plants. We opted to test them in *A. thaliana* first before embarking on transformations in *V. vinifera* (Thompson Seedless) because of the time it takes to generate grape transformants (up to six months). All four constructs have been successfully transformed into *A. thaliana Col* wild-type. Transformants have been obtained and homozygous lines will be generated and evaluated for growth. The T0 transformants do not appear to have any growth defects, so all four constructs have been sent to the UC Davis Plant Transformation Facility and have been transformed into *V. vinifera*. We are awaiting the results on those transformations.

In addition, four constructs are in modified pCambiaK-APS (pCK4):

- pCK4-EDS1-gDNA (VvCabSauv08_P0022F.ver1.0.g361850)
- pCK4-EDS1-CDS (VvCabSauv08_P0022F.ver1.0.g361850)
- pCK4-CP1 like-gDNA (VvCabSauv08_P0022F.ver1.0.g361850)
- pCK4-CP1-CDS (VvCabSauv08_P0022F.ver1.0.g361850).

All of these constructs have been transformed into *Agrobacterium tumefaciens*. As described above, these will initially be transformed into *A. thaliana* Col wild-type and then sent to the Plant Transformation Facility at UC Davis to be transformed into *V. vinifera* (Thompson Seedless).

Objective 2. Evaluation of PD Resistance: Disease Phenotyping and Characterization of Defense Responses to *Xf* Challenge

Objective 2a. Disease Phenotyping. We will mechanically inoculate the grapevines arising from transgenesis or the gene-editing protocol using the pin-prick method (Hill and Purcell, 1995). We will inoculate 15 plants/treatment (*Xf* cells or 1x PBS buffer) per transgenic or gene-edited line and 15 plants/treatment (*Xf* cells or 1x PBS buffer) of the wild-type non-transgenic/non-edited Thompson Seedless vines. All plants will be visually examined for PD symptom development

over a period of 12 weeks and rated on a disease rating scale of 0 to 5, where 0 = healthy and 5 = dead or dying (Guilhabert and Kirkpatrick, 2005). All experiments will be replicated three times to allow for robust statistical evaluation. These experiments cannot be completed until we have generated transgenic vines and clonally propagated enough for a full virulence experiment.

Quantification of *Xf* Titer in Grapevines. We will quantify bacterial populations in both local and systemic petioles when the positive control grapevines (wild-type parental Thompson Seedless inoculated with *Xf*) begin rating a 1 to 2 on the disease scale. We will also quantify bacterial titer at a second time point later in disease progress when the control grapevines are rating a three to 4 on the disease scale. Petioles will be surface sterilized and ground in two ml of sterile 1x PBS. The resulting suspension will be diluted and plated on solid PD3 medium and colonies will be counted and normalized to tissue weight to obtain quantitative data on cfu/gram of petiole tissue.

Objective 2b. Ultrastructural Characterization of the Vasculature in Transgenic and Gene-Edited Plants. One of the significant findings we found in immune-elicited grapevines is that they contained significantly fewer tyloses (**Figure 1**) in the xylem. Tylose formation is a consequence of *Xf* infection, and the presence of tyloses makes PD symptoms worse (Sun et al., 2013). Thus, preventing this host defense response may reduce or completely prevent PD symptom occurrence. Stem sections of *V. vinifera* cultivar Thompson Seedless (transgenics, CRISPR-Cas9 gene-edited, or wild-type vines) will be harvested at 12 weeks post-inoculation with *Xf* wild-type or a 1x PBS negative control. Tissue will be fixed in 80% ethanol prior to histological examination. Freehand sections will be made of approximately 100 µm, stained with toluidine blue O (0.05%), and observed using a brightfield microscope (Zeiss, Axio Observer 5).

Interestingly, we also observed defense-related responses in the phloem, a vascular tissue typically overlooked in the PD pathosystem. This included callose deposition in *Xf*-infected vines (Rapicavoli et al., 2018). Because of the striking amount of callose deposition in sick vines, we will continue exploring the role of callose in PD etiology. Stem sections of *V. vinifera* cultivar Thompson Seedless (transgenics, CRISPR-Cas9 gene-edited, or wild-type vines) will be harvested at 12 weeks post-inoculation with *Xf* wild-type or a 1x PBS negative control and examined for callose deposition.

Objective 2c. Global Transcriptome Analysis of Transgenic and CRISPR-Cas9 Generated Vines. To better understand the molecular processes that potentiate effective immune responses to *Xf* infection, we will perform a series of global transcriptomic, RNA-seq experiments that will illuminate genes and pathways induced following *Xf* infection in both local and systemic tissue in our transgenic and gene-edited lines as compared to wild-type parental Thompson Seedless vines. Our experiments will be performed at graduated time points in vines that either receive or do not receive a *Xf* challenge. In addition, these data will complement the information we already have regarding the transcriptional responses to *Xf* whole cells (Rapicavoli et al., 2018). We will validate the RNA-seq findings using quantitative PCR to monitor expression of genes we found to be differentially regulated from the RNA-seq analysis.

CONCLUSIONS

Using the information we have gleaned from the grapevine defense response to the elicitor LPS we have created eight constructs to transform Thompson Seedless grapevines through the Plant

Transformation Facility at UC Davis thus far. Once transformed, these grapevines will be assessed to determine their ability to protect themselves against bacterial colonization and express significantly less disease symptoms. This research will result in the development of PD-resistant vines and contribute to the knowledge about overexpressing and silencing genes to produce disease-resistant vines.

REFERENCES CITED

Agüero CB, et al. 2006. *Vitis* 45 (1): 1-8.
Almeida R, et al. 2018. *Proceedings of the 2018 Pierce's Disease Research Symposium.* California Department of Food and Agriculture, Sacramento, CA.
Anders S, et al. 2010. *Genome Biology* 11: R106.
Blanco-Ulate B, et al. 2014. *Frontiers in Plant Science* 5.
Blanco-Ulate B, et al. 2013. *Frontiers in Plant Science* 4: 142.
Cantu D, et al. 2008. *Proceedings of the National Academy of Sciences USA* 105: 859-864.
Cantu D, et al. 2009. *Plant Physiology* 150: 1434-1449.
Cantu D, et al. 2011a. *PLOS One* 6.
Cantu D, et al. 2011b. *BMC Genomics* 12.
Cantu D, et al. 2013. *BMC Genomics* 14: 270.
Chakraborty S, et al. 2016. *PeerJ* 4: e2007.
Cho SK, et al. 2006. *FEBS Letters* 580: 3136-3144.
Clifford JC, et al. 2013. *Molecular Plant Microbe Interactions* 26(6): 676-685.
Cui H, et al. 2016. *New Phytologist*. doi: 10.1111/nph.14302. (epub ahead of print).
Di Genova A, et al. 2014. *BMC Plant Biology* 14: 7. doi: 10.1186/1471-2229-14-7.
Dobin A, et al. 2013. *Bioinformatics* 29: 15-21.
Grabherr MG, et al. 2011. *Nature Biotechnology* 29: 644-52.
Guilhabert MR, et al. 2005. *Molecular Plant Microbe Interactactions* 18: 856-868.
Hill B, et al. 1995. *Phytopathology* 85: 1368-1372.
Hiraga S, et al. 2001. *Plant and Cell Physiology* 42(5): 462-468.
Jaillon O, et al 2007. *Nature* 449(7161): 463-467.
Kirkpatrick B. 2009. *Proceedings of the 2009 Pierce's Disease Research Symposium.* California Department of Food and Agriculture, Sacramento, CA.
Kuwabara C, et al. 2002. *Physiologia Plantarum* 115: 101-110.
Lloyd G, et al. 1981. *International Plant Propagators' Society Proceedings* 30: 421-427.
Murashige T, et al. 1962. *Physiologia Plantarum* 15: 473-497.
Ookawara R, et al. 2005. *Annals of Botany* 96: 693-702.
Patel S, et al. 2018. *BMC Genomics* 19(1): 57. doi: 10.1186/s12864-018-4434-2.
Pérez-Donoso AG, et al. 2007. *Plant Physiology* 143(2): 1024-1036.
Perring TM, et al. 2018. *Proceedings of the 2018 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA.
Priyam A, et al. 2015. bioRxiv 033142. doi: https://doi.org/10.1101/033142.
Rapicavoli JN, et al. 2018. *Nature Communications* 9: 390.
Redak R, et al. 2018. *Proceedings of the 2018 Pierce's Disease Research Symposium.* California Department of Food and Agriculture, Sacramento, CA.
Roper MC, et al. 2007. *Molecular Plant Microbe Interactions* 20: 411-419.
Skinner ME, et al. 2009. Genome Research 19: 1630-1638. doi: 10.1101/gr.094607.109.
Sun Q, et al. 2013. *Plant Physiology* 161(3): 1529-1541.

Tapia E, et al. 2009. *Journal of Biotechnology* 139: 95-101. doi: 10.1016/j.jbiotec.2008.09.009.
Xing HL, et al. 2014. *BMC Plant Biology* 14(1): 327. doi: 10.1186/s12870-014-0327-y.
Yadeta KA, et al. 2013. *Frontiers in Plant Science* 4.
Zottini M, et al. 2008. *Plant Cell Reports* 27: 845-853. doi: 10.1007/s00299-008-0510-4.

FUNDING AGENCIES

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

GRAPE PROTOPLAST ISOLATION AND REGENERATION OF PLANTS FOR USE IN GENE EDITING TECHNOLOGY

Project Leader: David Tricoli | Plant Transformation Facility | University of California | Davis, CA 95616 | dmtricoli@ucdavis.edu

Reporting Period: The results reported here are from work conducted September 6, 2018 to October 23, 2019.

ABSTRACT

Genome editing technology allows for precise alterations in plant genomes, facilitating targeted changes of the genetic material of crop plants. Protoplast culture provides one of the best avenues for producing non-chimeric gene edited plants for clonally propagated species such as grape (*Vitis vinifera*). This report describes the successful isolation, purification, culture, cell division, mini-calli formation, and whole plant regeneration from protoplasts isolated from embryogenic cultures of Thompson Seedless and Merlot grapes. Isolated protoplasts are encapsulated in calcium alginate beads and cultures with cell suspension feeder cultures, which were previously acclimated to grow in high osmotic medium containing 0.4 M mannitol. Protoplasts were observed dividing at approximately day 14. The addition of antioxidants and the inclusion of the polyamine spermine greatly enhanced the percentage of protoplasts that divided and the speed of mini-callus formation. Embryo formation could be observed from mini-callus colonies while still encapsulated in the calcium alginate beads. Calcium alginate beads were dissolved in 300 mM KH_2PO_4 releasing the developing embryos and mini-calli, which could be grown into complete plants on agar-solidified medium.

LAYPERSON SUMMARY

Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 is a gene editing technology that allows one to make precise changes in a plant's genetic blueprint. There are a number of methods for delivering CRISPR-Cas9 into the animal cell. However, unlike animal cells, plants cells are encased in cell walls that prevent easy introduction of DNA into the cell. This makes the utilization of CRISPR-Cas9 or other gene editing approaches more difficult for plant cells. Protoplasts are plant cells which have had their cell walls removed. These cells are very delicate and require careful manipulation of the solution in which they are grown. If the pressure of the solution outside the protoplast is not adjusted to match the pressure of the conditions within the cell, the protoplasts will implode or burst. However, if protoplasts can be stably maintained in solution, they allow for gene editing delivery techniques used with animal cells to be employed for plant cells. We have developed a robust method to generate protoplasts from grape embryo suspension and stimulate the protoplasts to reform a cell wall and divide. We have successfully stimulated the small cell colonies to form into embryos and germinate into plants. These techniques will provide a valuable tool for deploying gene editing techniques to produce non-chimeric gene edited plants.

INTRODUCTION

Genome editing technology allows for precise alterations in plant genomes, facilitating targeted changes of the genetic material of crop plants. In seed-propagated crops, gene editing can be introduced into the plant cell using *Agrobacterium tumefaciens* or biolistic-mediated DNA

delivery systems. Once gene editing has been accomplished, the editing sequences, such as the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 insert, can be segregated out of the population in the seed generation and the null segregants, containing only the desired gene edit, advanced using traditional plant breeding. However, for clonally propagated plants like winegrapes (*Vitis vinifera*), it is not possible to use breeding to eliminate the CRISPR-Cas9 sequences and still maintain the fidelity of the clonal germplasm. A limited number of grapevine clones have been used for many decades to produce high quality wine. These clones are maintained by vegetative propagation to preserve the intrinsic quality of this material. Therefore, the implementation of genome editing technology to introduce new traits into existing *Vitis* cultivars without altering their essential characters and identity is crucial.

Protoplast culture provides one of the best avenues for producing non-chimeric gene edited plants for clonally propagated species. CRISPR-Cas9 has been introduced into plant protoplasts using PEG or electroporation and expressed transiently without integration of the CRISPR-Cas9 DNA. Cell walls re-form on protoplasts in 48 to 72 hours and the edited cells can be stimulated to form mini-callus colonies. However, routine regeneration of whole plants from protoplasts has not previously been achieved in grape. The UC Davis Plant Transformation Facility has previously developed extensive cell biology capability in grape, which includes the establishment of suspension cultures, formation of somatic embryos from those cultures, and regeneration of whole plants from somatic embryos. We have utilized these advances to develop a method to isolate plant protoplasts from grape embryogenic cultures, generate mini-callus colonies from the protoplasts, and regenerate whole plants from the callus.

The development of a system that allows the isolation of grape protoplasts, formation of mini-calli, and regeneration of protoplast-derived plants has significant relevance to the Pierce's disease research community and the winegrape industry. It provides an excellent vehicle for deploying non-*Agrobacterium*-mediated, non-integrating gene editing technology for fundamental research and product development. It will allow for the production of non-chimeric gene edited plants, which is critical for clonally propagated crops such as grape.

Protoplast technology was actively researched in the 1980s and early 1990s, but the advent of transgenic technology resulted in this cell culture technique falling out of favor. Although there are published reports in the literature demonstrating successful isolation of protoplasts from grapes, production of mini-calli from grape protoplasts has historically proven to be inefficient, with less than 5% of the isolated protoplasts forming calli (Xu et al., 2007). In addition, to my knowledge, regeneration of grape plants from protoplasts has not yet been achieved. In this report, we demonstrate that encapsulating protoplasts isolated from rapidly dividing grape embryogenic cultures in alginate beads and culturing them in conditioned nurse cultures stimulates protoplast division, mini-calli formation, and plant regeneration.

OBJECTIVES

1. Develop protoplast isolation techniques for grape using actively dividing grape suspension cultures.
2. Culture grape suspension protoplasts in calcium alginate beads and stimulate the formation of mini-calli.
3. Stimulate plant regeneration from protoplast-derived mini-calli.

RESULTS AND DISCUSSION

Objective 1. Develop Protoplast Isolation Techniques for Grape Using Actively Dividing Grape Suspension Cultures

This past spring, we successfully established new somatic embryogenic cultures for Merlot, Chardonnay, and Thompson Seedless from anther filaments harvested from immature flowers collected from the Foundation Plant Service's vineyards. We will use these somatic embryo cultures to establish new 2019 embryogenic suspension and callus cultures for isolation of protoplasts.

Protoplast Isolation and purification. Aliquots of cell suspension of Merlot or Thompson Seedless were plated onto agar-solidified plates containing Lloyd and McCown Woody Plant Medium (WPM) supplemented with 20 g/l sucrose, 1 g/l casein, 1 mM 2-(N-morpholino) ethanesulfonic acid (MES), 500 mg/l activated charcoal, 10 mg/l Picloram, and 2.0 mg/l thidiazuron (TDZ) (Pic/TDZ). Embryogenic callus that developed on these plates were harvested and treated in an enzyme solution consisting of filter sterilized 0.5% Onozuka Cellulase R10, 0.25% pectinase, 0.25% macerozyme R10, 0.4 M mannitol, 5 mM $CaCl_2$, 10 g/l bovine serum albumin (BSA), and MES. We subjected the cells to infiltration under house vacuum for three, 2-minute exposures and incubated the solution in the dark at 25ºC on a platform shaker at 50 rpms. After approximately 16 hours incubation, we filtered the protoplast solution through a 40 um screen and collected the protoplasts by pelleting via centrifugation at 2,000 rpm for 10 minutes. We washed the protoplasts twice in an osmotically adjusted wash solution containing 0.4 M mannitol, 2 mM $CaCl_2$, 1g/l BSA, and 1,191 mg/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). We purify protoplasts using a dextran gradient consisting of 2 ml of a 13% dextran solution, overlaid with 1.5 ml of 0.4 M wash solution. We can readily harvest the protoplast band from the interface between the dextran and 0.4 M wash solution composed of 72.87 g/L mannitol, 222 mg/l $CaCl_2$, 1 g/l BSA, and 1,191 mg/l HEPES and transfer them to a 60 x 15 mm petri dish using a Pasteur pipette. Yields of protoplasts from 500 mg fresh weight of embryogenic callus range from 2.5 to 8 x 10^6 cells per ml **(Figure 1)**.

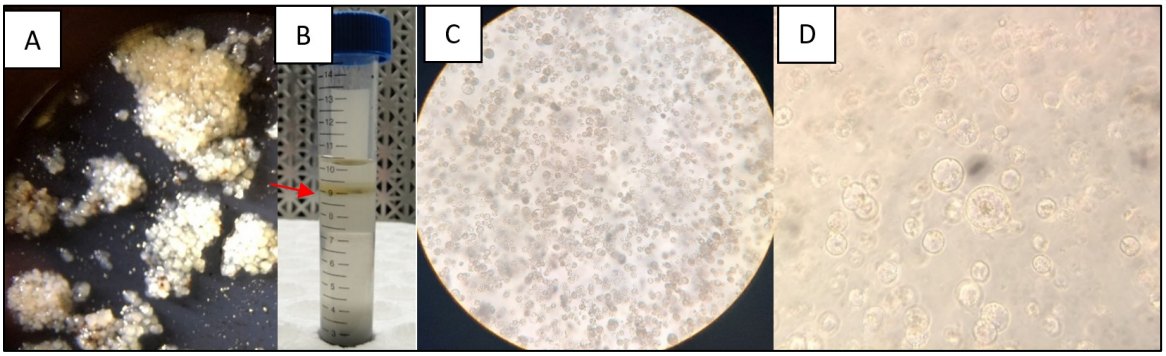


Figure 1. (A) Grape cell suspension-derived embryogenic callus used as the tissue source for protoplast isolation; (B) dextran gradient separates protoplast from debris (red arrow indicates protoplast band); (C) and (D) harvested grape protoplast prior to encapsulation in calcium alginate beads.

Objective 2. Culture Grape Suspension Protoplasts in Calcium Alginate Beads and Stimulate the Formation of Mini-Calli

The Plant Transformation Facility has developed a method for encapsulating and culturing protoplasts in alginate beads with an osmotically conditioned feeder suspension culture. The feeder suspension is used to stimulate the protoplast to divide to form mini-calli even at low protoplast culture density. This system was demonstrated to be efficacious in soybean (Tricoli et al., 1986) and lettuce protoplasts (Tricoli, unpublished). We generated osmotically conditioned grape feeder suspensions of Thompson Seedless and 1103P by gradually increasing the osmotic potential of the suspension medium over time. During bi-weekly subcultures of the suspension cultures, we removed one-half of the suspension and replaced it with grape suspension medium containing WPM medium supplemented with 20 g/l sucrose, 10 mg/l Picloram, 2.0 mg/l TDZ, 72.87 g/L mannitol, 222 mg/l $CaCl_2$, 1 g/l casein, 1,191 mg/l HEPES, and 2g/l activated charcoal, pH 5.7. During the subsequent bi-weekly subculture, we again removed one-half of the old suspension and replaced it with an equal volume of medium containing 72.87 g/l mannitol, 1,191 mg/l HEPES and 1g/l BSA, pH 5.7. We repeated this process bi-weekly so the cells gradually acclimated to the high osmotic medium over time.

In order to generate the protoplast containing alginate beads, we adjusted the protoplast density to two times the desired final density with 0.4 M mannitol/wash solution. We mixed the protoplast solution with an equal volume of 3.2% Na alginate solution composed of 72.87 g/l mannitol, 222 mg/l $CaCl_2$, 1,191 mg/l HEPES, and 3.2 g/l sodium alginate (adjusted to pH 5.7). Beads are formed by drawing up the solution into a 12 ml sterile syringe and expelling the solution dropwise through a 23-gauge needle into an osmotically adjusted 50 mM $CaCl_2$ solution composed of 72.87 g/l mannitol, 222 mg/l $CaCl_2$, 1 g/l BSA, and 1,191 mg/l HEPES (pH 5.7). After 30 minutes in the $CaCl_2$ solution, we rinse the beads one time in 0.4 M mannitol/buffer wash solution **(Figure 2).**

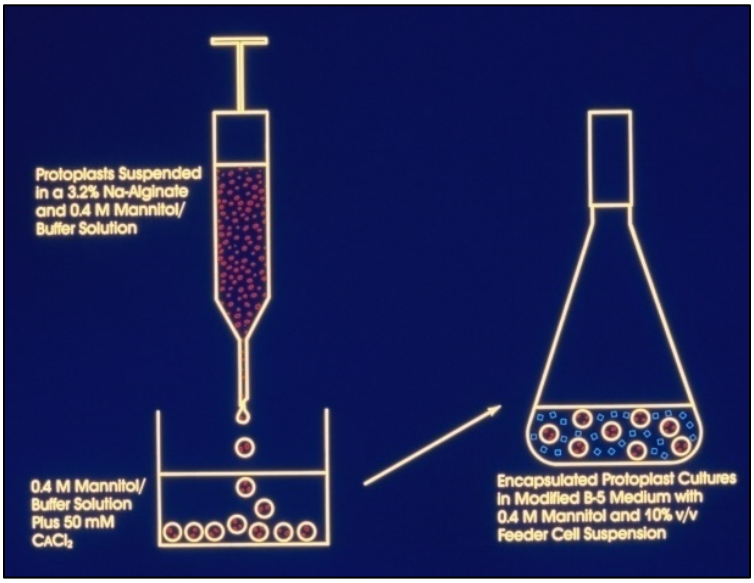


Figure 2. Diagram of the production of protoplasts encapsulated in alginate beads and cultured in conditioned medium.

In addition to allowing one to test various media formulations, embedding protoplasts in calcium alginate beads also ensures that each protoplast derived callus colony is from single cell descent. This will be important for gene editing experiments since, if protoplasts are not fixed in a matrix, they will rapidly clump together, making determining single cell descent impossible. Normally when cultured at low density, protoplasts fail to divide. However, culturing embedded protoplasts in conditioned media or with feeder suspensions has been shown to stimulate protoplast division in other species, even at very low cell densities. Since the alginate matrix is permeable to nutrients, the conditioned medium or feeder suspensions serve as a nurse culture. We have successfully embedded grape protoplasts of 1103P, Thompson Seedless, and Merlot in calcium alginate beads, and they have survived the embedding process **(Figure 3).** We initially cultured the embedded protoplasts in 24-well plates, which allowed us to test multiple hormone and media formulations for their ability to stimulate cell division, using a factorial design. We placed one ml of medium osmotically adjusted with 0.4 M mannitol in each well along with 10-20 beads and incubated the plate in the dark on a platform shaker at 50 rpms. After 14 days, we added 1 ml of medium of the same formulation but lacking mannitol to each well, thereby reducing the osmotic of the medium to half. We monitored protoplasts for cell wall formation and division over a four-to-six-week period. We found that the best-conditioned medium for stimulating protoplast cell division was Pic/TDZ, and this formulation was used in studies involving cell suspension feeder cultures in 125 ml shake flasks or 60 x 15 mm petri dishes.

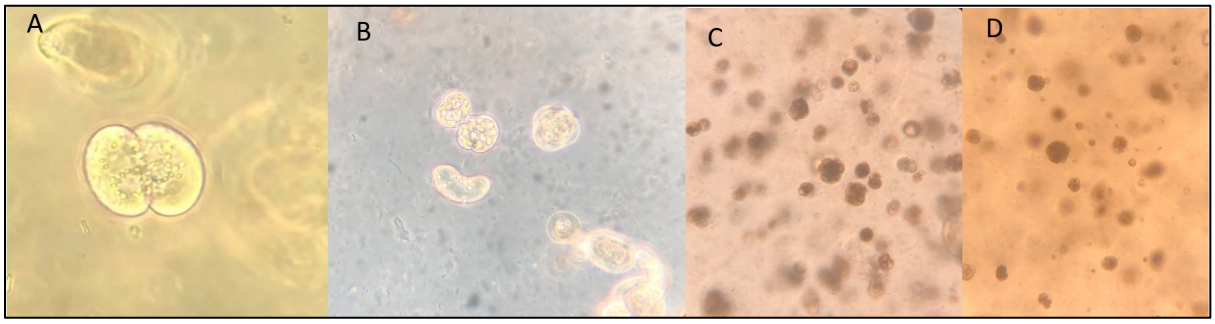


Figure 3. (A) First cell division of a Thompson Seedless protoplast embedded within a calcium alginate bead; (B) multi-cell stage; (C) Thompson Seedless and (D) Merlot protoplasts forming mini-callus colonies.

The system has also allowed us to rapidly test non-hormonal medium addendums including putrescene, spermidine, pluronic F68, resveratrol, citric acid, ascorbic acid, L-cysteine, and reduced glutathione, either alone or in various combinations. Although this 24-well format allows us to observe protoplast viability, cell wall formation, and the first few cell divisions, the number of protoplasts that divide is low. In addition, although cells underwent a few divisions in the 24-well plates, they failed to advance beyond the four-to-eight-cell stage. Still, this 24-well format allowed us to determine which salt formulations, hormone combinations, and non-hormone addendum to advance to feeder suspension studies.

Antioxidants. Callus colonies that developed in alginate beads often became discolored due to phenolic production. We were concerned that these compounds might be toxic to the growth and development of the protoplast-derived callus. We have developed and tested an antioxidant

solution consisting of 100 mg/l ascorbic acid, 150 mg/l citric acid, 30 mg/l reduced glutathione, and 100 mg/l L-cysteine. We tested the effect of this antioxidant addendum on protoplast viability and division of Thompson Seedless and Merlot protoplast cultures. Protoplasts were isolated and encapsulated in 1.6% calcium alginate beads. Calcium alginate beads from the same protoplast preparation were randomly transferred to shake flasks containing conditioned 1103P feeder cell suspensions with or without the addition of 1x or 5x of the antioxidant solutions. After 14 days, the osmotic strength of the feeder suspension was reduced from 0.4 M to 0.2 M mannitol. At day 21 for Thompson Seedless and at day 71 for Merlot, we randomly harvested five beads per treatment and counted the number of mini-calli per bead. For both Thompson Seedless and Merlot protoplasts, a significantly higher percentage of callus colonies were observed developing in beads grown in the suspensions containing the antioxidant solution (**Table 1**). Merlot protoplasts tended to produce more phenolic than Thompson Seedless protoplasts, and the antioxidant addendum had a much more profound effect on the division of the Merlot protoplasts than the Thompson Seedless protoplasts. Without the addition of the antioxidant mixture, no mini-calli were observed in Merlot, whereas an average of 168 mini-calli were produced per bead from protoplasts grown in feeder cultures with the antioxidant addendum (**Table 1**). We also observed increased Thompson Seedless protoplast viability and increased callus development with increasing antioxidant concentration (**Table 2**).

Callus colonies continued to develop within the calcium alginate beads and often grew large enough that they could be seen rupturing through the surface of the beads (**Figure 4**).

Table 1. Addition of antioxidant solution consisting of 100 mg/l ascorbic acid, 150 mg/l citric acid, 30 mg/l reduced glutathione, and 100 mg/l L-cysteine enhances protoplast-derived callus formation.

Number of Callus Colonies Per Bead				
Bead	TS wo/Anti-Oxidants	TS w/ 1x Anti-Oxidants	Merlot w/o Anti-Oxidants	Merlot w/ 1x Anti-Oxidants
1	13	45	0	199
2	42	52	0	182
3	27	37	0	159
4	30	36	0	160
5	30	69	0	139
Average	28.4	47.8	0	167.8

Table 2. Increasing the concentration of the antioxidant formulation consisting of 100 mg/l ascorbic acid, 150 mg/l citric acid, 30 mg/l reduced glutathione, and 100 mg/l L-cysteine from 1x to 5x enhances protoplast-derived callus formation in Thompson Seedless protoplasts.

Number of Callus Colonies Per Bead		
Bead	TS w/ 1x Anti-Oxidants	TS w/ 5x Anti-Oxidants
1	2	112
2	6	114
3	10	130
4	10	118
5	13	125
Average	8.2	119.8

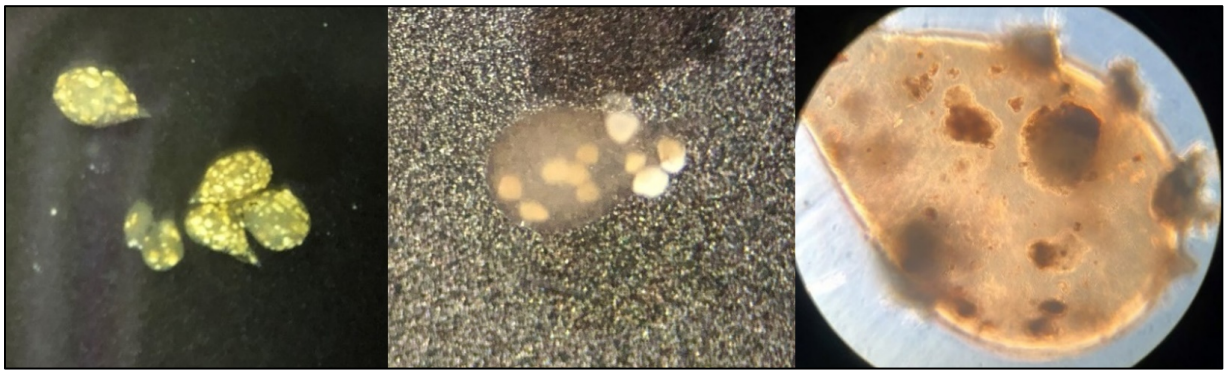


Figure 4. Left: Mini colonies from Thompson Seedless protoplasts encapsulated in calcium alginate beads and grown in conditioned cell suspension of 1103P. Middle: callus colonies growing out of the alginate matrix. Right: close-up of an individual bead developing callus colonies six weeks after encapsulation.

Polyamines. Polyamines are polycationic compounds which affect many aspects of growth and stress responses in plants including cell division, embryogenesis, organogenesis, floral, fruit, and pollen development, and senescence. The major polyamines in plants include putrescine (PUT), spermidine (SPD), spermine (SPM), and cadaverine (CAD). We are testing the ability of these polyamines to increase the plating efficiency of encapsulated protoplasts as well as increase the rate of cell division. Initially we tested 1.0 mM putrescine in combination with 0.0 or 0.1 mM spermidine since we have previously found these levels to be beneficial in other plant species. We found the inclusion of 1.0 mM putrescine stimulated callus colony formation but the addition of 0.1 mM spermidine did not enhance the response. We also tested 1.0 mM putrescine in combination with 0.0, 0.1, and 1.0 mM spermine and 0.0, .01, or 1.0 mM spermidine. Interestingly, we have found that the addition of 1.0 mM spermine to the medium enhanced the number of protoplasts that divided and the size of the mini-calli that developed irrespective of the spermidine concentration (**Table 3** and **Figure 5**).

We also tested cadaverine at 0.0, 0.1, or 1.0 mM in combination with 1.0 mM putresciene, 0.1 mM spermidine, and 0.0, 0.1, or 1.0 mM spermine. Cadaverine does not seem to enhance cell division or cell growth from grape protoplasts. However, we again saw a significant enhancement in callus colony growth with treatments that contained 1.0 mM spermine after only 21 days of culture, regardless of the level of CAD tested **(Table 4** and **Figure 5).**

Objective 3. Stimulate Plant Regeneration from Protoplast-Derived Mini-Calli

Once protoplasts developed into callus colonies of approximately 16 to 32 cells within the alginate beads (approximately day 50), the beads and conditioned feeder suspension were transferred into a 100 x 20 mm petri dish. Using forceps, individual beads were manually transferred to a 100 x 20 mm petri dish containing 40 ml of Lloyd and McCown minimal organics medium supplemented with 20 g/l sucrose, 1 g/l casein, and 222 mg/l CaCl2, without hormones or activated charcoal. This transfer/washing process was repeated two more times to eliminate any of the feeder suspension cells. Beads were then transferred onto agar-solidified 100 x 20 mm feeder plates containing 40 ml of Lloyd and McCown minimal organics medium supplemented with 30 g/l sucrose, 50 g/l sorbitol,1 g/l casein, 1 mM MES 0.5 mg/l benzylaminopurine (BAP), and 0.1 mg/l napthaleneacetic acid (NAA) onto which 1.0 ml of a 1103P grape suspension culture is plated. The grape suspension culture medium consisted of WPM medium supplemented with 20 g/l sucrose, 10 mg/l Picloram, 2.0 mg/l TDZ, 72.87 g/L mannitol, 222 mg/l $CaCl_2$, 1 g/l casein, 1,191 mg/l HEPES, and 2 g/l activated charcoal at pH 5.7. An 85 mm Whatman filter paper was placed over the plated suspension and a 70 mm Whatman filter paper placed on top of the 85 mm filter. Beads were placed on top of the 70 mm filter paper. One ml Lloyd and McCown minimal organics medium supplemented with 30 g/l sucrose, 1 g/l casein, 1 mM MES, 10 mg/l Picloram, and 2.0 mg/l TDZ was added to each plate to prevent desiccation. Cultures were incubated at 26ºC in the dark. After approximately three to four weeks, mini-calli developed into somatic embryos within the beads **(Figure 6**). If the beads are held on medium containing sorbitol they will not germinate further and become dormant **(Figure 7).**

Table 3. Mini-callus colony formation 14 days after encapsulation of Thompson Seedless protoplasts in calcium alginate beads and grown in conditioned 0.4 M Pic/TDZ 1103P feeder cell suspensions containing 1 mM putrescine and varying levels of SPD and SPM.

Number of Colonies Per Alginate Bead Sampled								
Bead		1	2	3	4	5	Total	Average
mM Polyamine								
SPD	SPM							
0.0	0.0	28	49	48	39	25	164	32.9
0.1	0.0	91	93	120	110	93	507	101.4
1.0	0.0	81	97	79	125	89	471	94.2
0.0	0.1	96	81	79	59	84	399	79.8
0.1	0.1	135	119	79	130	85	548	109.6
1.0	0.1	147	107	103	137	90	584	116.8
0.0	1.0	275	126	238	169	221	1029	205.8
0.1	1.0	219	168	154	84	188	813	162.6
1.0	1.0	212	262	196	220	162	1052	210.4

Table 4. Mini-callus colony formation 14 days after encapsulation of Thompson Seedless protoplasts in calcium alginate beads and grown in conditioned 0.4M Pic/TDZ 1103P feeder cell suspensions containing 1.0 mM PUT, 0.1 mM SPD, and varying levels of SPM and CAD.

Number of Colonies Per Alginate Bead Sampled								
Bead		1	2	3	4	5	Total	Average
mM Polyamine								
SPM	CAD							
0.0	0.0	99	103	116	67	73	458	91.6
0.1	0.0	68	79	64	65	86	362	72.4
1.0	0.0	133	121	115	133	98	600	120
0.0	0.1	100	115	92	104	95	506	101.2
0.1	0.1	90	85	71	66	79	391	78.2
1.0	0.1	154	155	106	105	101	621	124.2
0.0	1.0	104	82	87	92	73	438	87.6
0.1	1.0	66	125	88	60	79	424	84.8
1.0	1.0	141	114	132	11	127	633	126.6

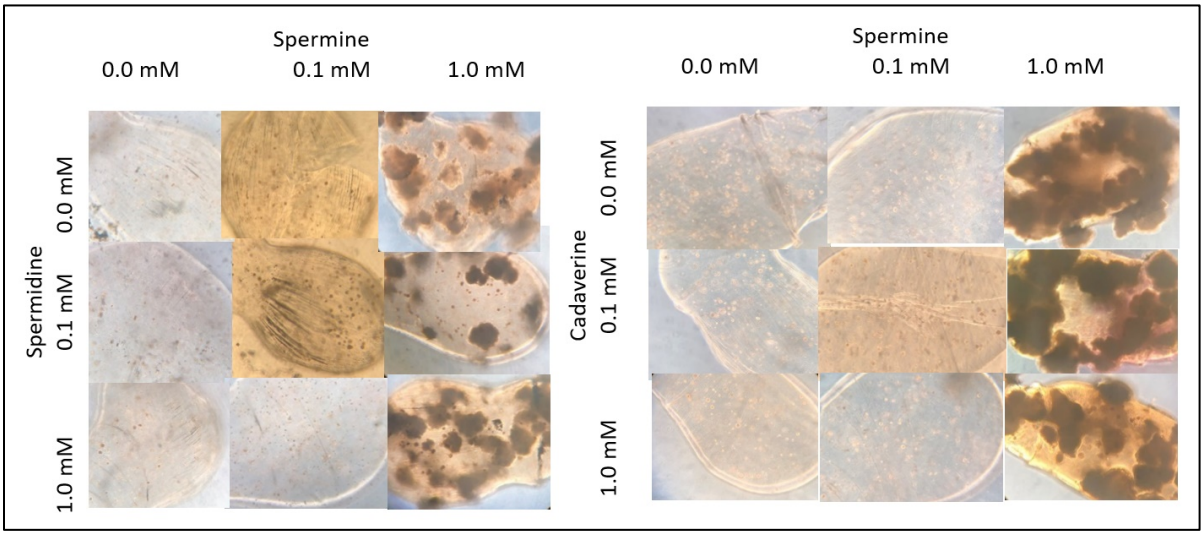


Figure 5. Enhanced growth of Thompson Seedless mini-calli when grown in the presence of 0.0, 0.1, or 1.0 mM SPD in combination with 0.0, 0.1, or 1.0 mM SPM (left) or 0.0, 0.1, or 1.0 mM CAD in combination with 0.0, 0.1, or 1.0 mM SPM 21 days post encapsulation (right). All treatments also contained 1.0 mM PUT and 5x antioxidant solution.

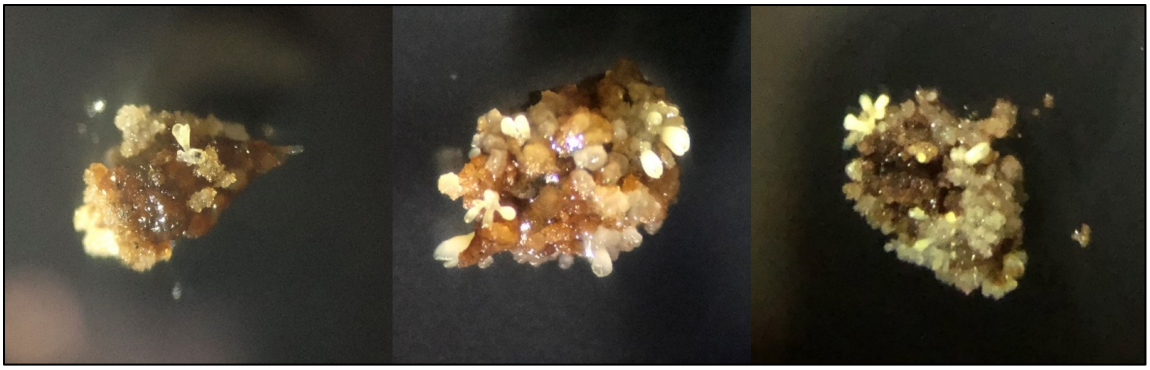


Figure 6. Somatic embryos developing from protoplast-derived callus encapsulated in calcium alginate beads.

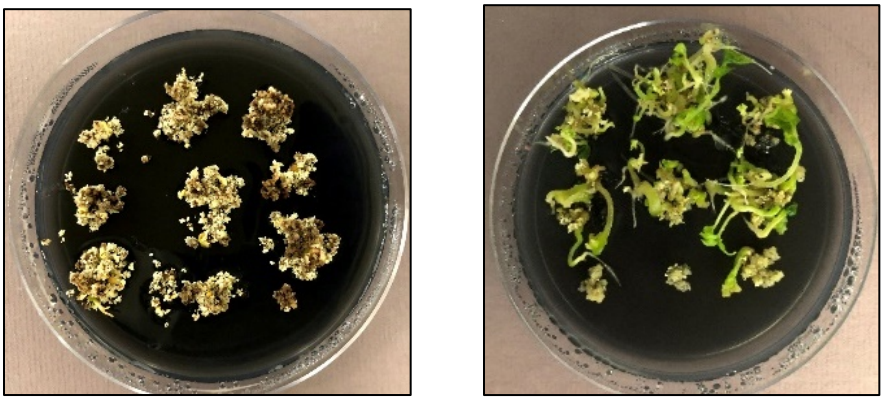


Figure 7. Somatic embryos developing from protoplast-derived callus encapsulated in calcium alginate beads and maintained on high sorbitol containing medium remain dormant (left) until transferred to medium without sorbitol (right).

Once mini-calli and embryos formed within the beads, the beads are transferred to agar solidified WPM supplemented with 20 g/l sucrose, 1 g/l casein, 1mM MES, 500 mg/l activated charcoal, 0.5 mg/l BAP, 0.1 mg/l NAA without sorbitol, and 7 g/l agar and transferred to continuous light, which induced germination of the developing somatic embryos and production of plants (**Figure 8**).

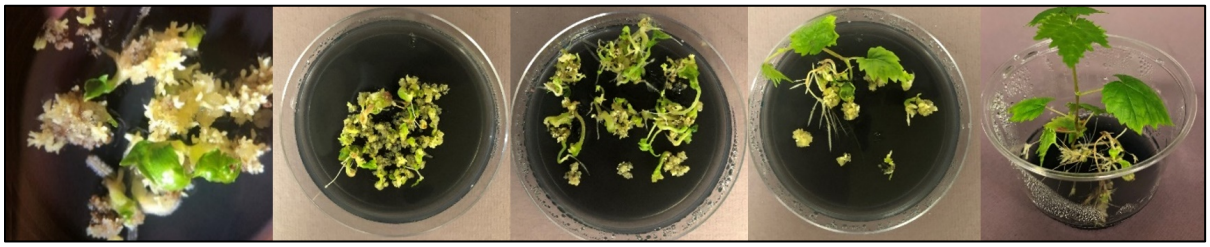


Figure 8. Thompson Seedless grape embryos and plants developing from protoplast-derived callus encapsulated in calcium alginate beads from experiment.

We have recently repeated this procedure for the wine genotype, Merlot. Although regeneration frequencies were much lower than for Thompson Seedless, we are seeing embryo development and germination from isolated protoplasts **(Figure 9).**

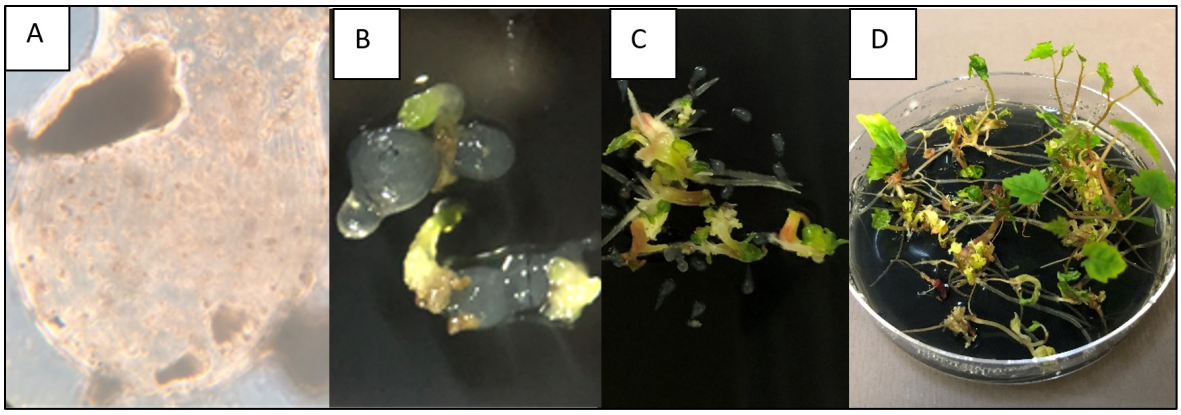


Figure 9. (A) Merlot embryos developing from encapsulated protoplasts within a calcium alginate bead; (B) embryos emerging out of calcium alginate beads; (C) embryos germinating on solidified WPM supplemented with 20 g/l sucrose, 1 g/l casein, 1 mM MES, 500 mg/l activated charcoal, 0.5 BAP, and 0.25 NAA; (D) rooted protoplast-derived plants.

Alternatively, intact calcium alginate beads containing well-developed embryos could be dissolved by transferring them to 125 ml shake flasks containing 300 mM KH_2PO_4 and culturing them overnight at 100 rpms. The matrix will still be partially intact the next morning, but can be dissolved by pipetting the solution up and down several times through a 10 ml pipette. The embryos, mini-calli, and KH_2PO_4 solution are transferred into a 15 ml centrifuge tube and centrifuged at 1,000 x g for 5 minutes. All but 1 ml of remaining supernatant is removed with a pipette and the remaining one ml, containing the embryos and mini-calli, is plated onto agar-solidified plates containing agar-solidified WPM supplemented with 20 g/l sucrose, 1 g/l casein, 1 mM MES, 500 mg/l activated charcoal, 0.5 BAP, and 0.25 NAA, where they grow into whole plants.

CONCLUSIONS

During the past year, we have successfully isolated protoplasts from embryogenic grape callus. When encapsulated in calcium alginate beads and grown in combination with osmotically adjusted grape suspension cultures, the protoplasts undergo cell division and form mini-calli within the calcium alginate bead. We have observed that the addition of antioxidants and polyamines significantly enhances the number and size of protoplast-derived calli developing within the matrix. When the intact beads with mini-calli were transferred to Lloyd and McCown minimal organics medium supplemented with 30 g/l sucrose, 50 g/l sorbitol, 1 g/l casein, 1 mM MES, 0.5 mg/l BAP, and 0.1 mg/l NAA, somatic embryos develop. Once somatic embryos formed, the beads were transferred to the medium of the same formulation but lacking sorbitol, and the somatic embryos germinated into rooted plants.

REFERENCES CITED

Grosser J, Gmitter F. 2011. Protoplast fusion for production of tetraploids and triploids: Applications for scion and rootstock breeding in citrus. *Plant Cell Tissue Organ Culture* 104: 343-357.

Lloyd G, McCown BH. 1981. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by shoot tip culture. *Proceedings of the International Plant Propagation Society* 30: 421-427.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497.

Nitsch JP, Nitsch C. 1969. Haploid plants from pollen grains. *Science* 163: 85-87.

Tricoli DM, Heins MB, Carnes MG. 1986 Culture of soybean mesophyll protoplasts in alginate beads. *Plant Cell Reports* 5: 334-337.

Xu X, Lu J, Dalling D, Jittayasothorn Y, Grosser JW. 2007. Isolation and culture of grape protoplasts from embryogenic cultures and leaves of *Vitis vinifera* and *Vitis rotundifolia*. Acta Horticulturae 738: 787-790.

FUNDING AGENCIES

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

BREEDING PIERCE'S DISEASE RESISTANT WINEGRAPES

Project Leader: Andrew Walker | Dept. of Viticulture and Enology | University of California | Davis, CA 95616 | awalker@ucdavis.edu
Cooperating Staff: Alan Tenscher | Dept. of Viticulture and Enology | University of California | Davis, CA 95616 | actenscher@ucdavis.edu

Reporting Period: The results reported here are from work conducted October 2018 to October 2019.

ABSTRACT

Breeding Pierce's disease (PD) resistant winegrapes continues to advance accelerated by aggressive vine training and selection for precocious flowering, resulting in a seed-to-seed cycle of two years. To further expedite breeding progress, we use marker-assisted selection (MAS) for PD resistance genes to select resistant progeny as soon as seeds germinate. These two practices have allowed us to produce four backcross generations with elite *Vitis vinifera* winegrape cultivars in 10 years. We have screened through about 2,000 progeny from the 2009, 2010, and 2011 crosses that are 97% *V. vinifera* with the *PdR1b* resistance gene from *V. arizonica* b43-17. 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 have been advanced to field testing with commercial-scale wine production, the first of which was planted in Napa in June 2013. To date, 20 scion and three PD resistant rootstocks based on *PdR1b* have been advanced to Foundation Plant Services (FPS) for certification. Five of these selections are now in pre-release to nurseries and are in the final stages of the patent process at UC Davis. Licenses allowing sales should be available to certified nurseries by December 2019. We have mapped an alternative form of PD resistance from a different form of *V. arizonica* collected in Baja California – *PdR2*, which resides on Chromosome 8. We are now combining (stacking) *PdR1b* with *PdR2* PD resistance and have advanced these lines to the 96% *V. vinifera* level using MAS to confirm the presence of the two forms of resistance. Other forms of *V. arizonica* are being studied and we will combine multiple resistance sources to ensure durable resistance. This spring, the first three scion selections that employ both *PdR1* and *PdR2* resistance were delivered to FPS. Pierce's disease resistance from *V. shuttleworthii* and BD5-117 is also being pursued but progress has been limited by their complex multigenic resistance and the absence of associated genetic markers. Very small-scale wines from 94% and 97% *V. vinifera PdR1b* selections have been very good and have been received well at public tastings in Sacramento (California Association of Winegrape Growers), Santa Rosa (Sonoma Winegrape Commission), Napa Valley (Napa Valley Grape Growers and Winemakers Associations), Temecula (Temecula Valley Winegrape Growers and Vintners), Healdsburg (Dry Creek Valley and Sonoma Grape Growers and Winemakers), the 2019 Unified Symposium, and UC Davis.

LAYPERSON SUMMARY

One of the most reliable and sustainable solutions to plant diseases is to create resistant plants. We use a classical plant breeding technique called backcrossing to combine Pierce's disease (PD) resistance from wild grape species with high quality winegrape varieties. To date we have identified two different PD resistance genes that exist on different chromosomes. These forms of PD resistance come from grape species native to Mexico and the southwestern United States.

Because we were able to locate these resistance genes - *PdR1* (Krivanek et al., 2006), and *PdR2* (Riaz, et al., 2018), we have been able to use marker-assisted selection to screen for the DNA markers linked with these PD resistance genes to greatly accelerate our breeding progress. We have evaluated many thousands of resistant seedlings for horticultural traits and fruit quality. The best of these are advanced to greenhouse testing, where only those with the strongest PD resistance after multiple greenhouse tests are advanced to wine quality testing at Davis and at PD hot spots around California. Twenty advanced *PdR1b* selections have been sent to Foundation Plant Services (FPS) over the past six winters to verify their virus-free status. Five of them have been sent in pre-release to nurseries and licenses allowing sales will be available to nurseries in December 2019. The first three winegrape selections that have both *PdR1* and *PdR2* resistance were delivered to FPS this past spring. Selections of other wild grape species are being studied and the best will be utilized in the PD resistance breeding program. Small-scale wines made from our advanced *PdR1* selections have been very good and well-received at professional tastings throughout California.

INTRODUCTION

We continue to make rapid progress breeding Pierce's disease (PD) resistant winegrapes. Aggressive vine training and selection for precocious flowering have allowed us to reduce the seed-to-seed cycle to two years. To further expedite breeding progress we are using marker-assisted selection (MAS) for the PD resistance loci *PdR1* and *PdR2* 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 select for fruit and vine quality and then move the best selections to greenhouse testing, where only those with the strongest resistance to *Xylella fastidiosa* (*Xf*), after multiple greenhouse tests, are advanced to multi-vine wine testing at Davis and other test sites. To date 20 scion and three PD resistant rootstocks based on *PdR1b* have been advanced to Foundation Plant Services (FPS) for certification. Five of these have been pre-released to grapevine nurseries to build up the amounts available for grafting and are now in the final stages of the campus patent process. Stacking of *PdR1b* with b42-26 PD resistance has advanced to the 96% *V. vinifera* level using MAS to confirm the presence of *PdR1* and the recently discovered (see companion report) PD resistance locus from another form of *V. arizonica* (b42-26) – *PdR2* on chromosome 8. This spring the first three scion selections that incorporate both *PdR1* and *PdR2* were delivered to FPS. Other forms of *V. arizonica* are being studied and the resistance of some will be genetically mapped for use in future efforts to combine multiple resistance sources and ensure durable resistance. Small-scale wines from 94% and 97% *V. vinifera PdR1b* selections have been very good and have been received well at public tastings, most recently at the 2019 Unified Symposium.

The Walker lab is uniquely positioned to undertake this important breeding effort, having developed rapid screening techniques for *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 *V. 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* 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 at the 94% *V. vinifera* level from the same resistance background for eleven years and from the 97% *V. vinifera* level for eight years. They have been very good and do not have typical hybrid flaws (blue purple color and herbaceous aromas and taste) that were prevalent in red wines from the 87% *V. vinifera* level. b43-17 is homozygous resistant to PD with the two forms/alleles of that locus named *PdR1a* and *PdR1b*. Screening results reported previously showed no significant difference in resistance levels in genotypes with either one or both alleles. We have primarily used *PdR1b* in our breeding, but we retain a number of selections at various backcross (BC) levels with *PdR1a* in the event that there is a yet unknown *Xf* strain-related resistance associated with the *PdR1* alleles. We also identified a PD resistance locus from *V. arizonica* b40-14 (*PdR1c*) that maps to the same region of Chromosome 14 as *PdR1* from b43-17. In the absence of a better understanding of how the PD resistance genes work and given the disparate origins of the b43-17 and b40-14 resistance sources, differences in preliminary DNA sequence data between them, and differences in their PD symptom expressions, we have continued to advance the b40-14 (*PdR1c*) resistance line as a future breeding resource. Our companion research project is pursuing the genetic basis of the differences between *PdR1b* and *PdR1c*. In 2005, we started a PD resistant breeding line from another Mexican accession, b42-26. Markers linked to this resistance proved elusive but strong resistance from this resistant accession was observed in our greenhouse screens as we advanced through the backcross levels. In 2011, we started stacking resistance from *PdR1b* with that of b42-26 using MAS to select for *PdR1b* and a higher than usual resistance in our greenhouse screen to move the b42-26 resistance forward. Late in 2016, our companion project identified the location of a significant PD resistance locus from b42-26 on chromosome (Chr) 8, which we have called *PdR2*. In 2014, we advanced our *PdR1* x *PdR2* line to the 92% *V. vinifera* level and in spring 2016 made crosses to advance it to the 96% *V. vinifera* level. MAS was used to advance only genotypes with both *PdR1b* and *PdR2* for the first time on these crosses. The resistance from southeastern United States species has been 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 PD 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 PD resistance.
3. Develop advanced lines of PD 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 PD resistant grapes with multiple sources of PD resistance and high-quality fruit and wine.

RESULTS AND DISCUSSION

Given the lack of information as to when I will be replaced after retirement in two years, no crosses were made in the spring of 2019. Instead, our focus has been on testing as many of the advanced crosses made in 2016 and 2017 as possible and assessing PD resistance in our wild species collection with our rapid greenhouse screen. Additionally, all the promising selections from these years have been evaluated in the field for fruit characteristics and horticultural quality. A special emphasis has been placed on the 96% *PdR1b* x *PdR2* line. Wines from three of these selections are scheduled to be made in fall 2020, and these three selections are already at FPS. We also intend to send at least a few advanced PD and powdery mildew (PM) resistant selections to FPS by June 2021.

Our rapid greenhouse screen is critical to our successful evaluation of PD resistance in wild accessions, new F1and BC1 mapping populations, and for the selection of advanced late generation backcrosses for release. **Table 1** provides a list of the PD greenhouse screens analyzed, initiated, and/or completed over the reporting period. In Group 1a, thirty 96% *PdR1b* x *PdR2* hermaphrodite genotypes were tested for resistance with the intent that, should they have sufficient resistance and have adequate fruit and wine quality, they would be candidates for release. An additional 55 genotypes homozygous at either *PdR1* or *PdR2* and having the other resistance source were tested to see if there is any pattern to high levels of resistance inheritance. Second or third screens were conducted on 54 genotypes with PD or PD x PM to validate previous results and confirming screens were run on five genotypes used as parents that didn't already have three completed screens. Regrettably, the conditions during this trial were such that we experienced only a low severity screen which doesn’t allow us to reproducibly differentiate our usual four categories (immune, promising-very resistant, resistant, and susceptible) of PD resistance. This trial will need to be repeated. As mentioned in previous reports, temperature has a large impact on our greenhouse screen. We continue to refine the relative importance of both the absolute levels and the average temperatures and their timing on observed severity of our greenhouse screen.

In Group 1b, two main groups were examined: 77 untested wild accessions to better characterize our collection and further elucidate PD resistance performance by geographical provenance and species (**Table 2**); and 148 97% *V. vinifera* PD x PM crosses from 2017. The latter is of interest as the lines involved have conferred, in the previous two generations, very high levels of resistance on a large percentage of their progeny. Resistance comes from *PdR1b* and b42-26 but with genotypes not having *PdR2*. Involved were four PD and PM susceptible 97% or pure *V. vinifera* seed parents by four PD and PM resistant 97% *V. vinifera* pollen parents. Although not a perfect matrix where all seed parents were crossed to all pollen parents, there were subgroups that could be rigorously compared. Of those, the pollen parent wasn’t significant and for only one seed parent was a significant difference found. This particular pure *V. vinifera* seed parent

was highly bred for certain fruit characteristics and must have left minor resistance factors behind that were present in the other three seed parents. A fit model analysis was performed on the four male pollen parents, observed PM resistance (clearly either R or S), and the interaction between the two. It is reassuring that resistance to PM wasn't related to PD status since previous results in other backgrounds indicated that PM resistance trended toward PD susceptibility. Recovery of resistant progeny, even for crosses to the one seed parent that conferred statistically lower resistance, was higher than average. Recovery ranged from a low of 25% to a high of 100%. With the one seed parent removed, the average recovery was approximately 73%. Especially noteworthy was that about 7% of the progeny were in the highest resistance category.

Table 1. Greenhouse PD screens analyzed, completed, and/or initiated during the reporting period. Projected dates in italics.

Group	Purpose	No. of Geno-types	Inoculation Date	ELISA Sample Date	PD Resistance Source(s)
1a	92 & 96% PD stack, retest of recent promising	170	08/23/2018	11/20/2018	*PdR1xPdR2*
1b	2017 PD x PM, PD Species, 2018 parents	241	10/16/2018	1/15/2019	Species, *PdR1b* x b42-26
1c	2017 PD Crosses, SWUS PD species	95	11/21/2018	2/21/2019	Species, *PdR1b* x b42-26
1d	2016 & 2017 PD Crosses	171	01/10/2019	4/11/2019	*PdR1b* x b42-26
1e	2017 PD crosses, SWUS PD species	255	03/28/2019	6/27/2019	Species, *PdR1b* x b42-26
1f	Species, 2017 *PdR1b* x b42-26 promising or untested	112	05/07/2019	8/6/2019	Species, *PdR1b* x b42-26
1g	SWUS Species, *PdR1* x *PdR2* Test 10, b41-13	168	07/02/2019	10/1/2019	Species, *PdR1b* x b42-26
1h	T 03-16 selfs and intercrosses, retests *PdR1* x *PdR2* promising, 2017 PD x PM	255	09/12/2019	*12/12/2019*	Species, *PdR1b* x b42-26

In February of this year, we completed the greenhouse screen for Group 1c. Fifty genotypes in this trial evaluated two 93% *V. vinifera* crosses from highly resistant *PdR1b* x b42-26 line parents (13329-09 and 13329-20) crossed back to elite *V. vinifera* to see if this high level of resistance carries forward another backcross generation. Eighteen *PdR1b* x *PdR2* genotypes at the 93% *V. vinifera* level that also carry PM resistance were tested for the first time as well as 15 southwestern United States (SWUS) *Vitis* accessions to continue characterization of our germplasm. Both parents in the 13-329 crosses have been tested four times and have the same minimum, total, and average R-rating scores of 5, 35, and 8.8, respectively. Although the *V. vinifera* parents are different, with the one exception discussed in 1a above, we haven't seen that matter in any past crosses in this line. Results show that by R-rating, 13329-20 produces

more resistant and less susceptible progeny than 13329-09. Importantly, we did see a higher than average rate of resistant progeny (76% compared to a more typical 30%) but none was as resistant as either of the parents. Similarly, a statistical comparison of the means found a lower progeny mean colony-forming unit (cfu)/ml for 13329-20. For the 94% *V. vinifera PdR1b* x *PdR2* PM resistant genotypes, results identified four promising candidates for consideration. Field evaluations this fall found none to be suitable for advancement. Of the 15 SWUS wild *Vitis* accessions, 10 were *V. arizonica* and, with the exception of one accession collected in mid-latitude Utah, as expected all were highly resistant to PD. The remaining accessions were either *V. californica* or *V. girdiana*. With the exception of one *V. californica* collected near Sacramento, all were highly susceptible. This is consistent with our previous findings in that occasional accessions of *V. californica* are highly PD resistant while the majority are highly susceptible. However, most Californian *V. girdiana* and *V. californica* appear to be hybrids with *V. vinifera* and are therefore expected to be less susceptible.

Table 2. Summary of species PD resistance results from Group 1b when more than seven accessions were tested.

Species	Average Mean (ln cfu/ml)	# Acces-sions	*Xf* titers Max (ln cfu/ml)	*Xf* titers Min (ln cfu/ml)	Comments
V. acerifolia	14.2	8	15.7	12.2	Kansas and Oklahoma, most highly susceptible.
V. arizonica	10.4	8	12.5	9.2	High are from Utah, low from Arizona and New Mexico, southern states.
V. candicans	12.4	12	15.4	9.8	Texas and Oklahoma, no clear geographical distribution of resistance.
V. girdiana	12.9	10	15.7	9.7	California, Nevada, and Utah. Most resistant accession had a highly susceptible accession collected nearby.
V. rupestris	15.5	7	15.7	14.6	Missouri and Oklahoma, all highly susceptible.

Group 1d consists of four main groups. Similar to Group 1c, we are testing twenty 93% *V. vinifera* genotypes from the highly resistant *PdR1b* x b42-26 line parent (13329-20) crossed to Dolcetto and Pedro Ximenez to validate results in Group 1c. As anticipated, there was no statistical difference between the observed PD resistance among the progeny families of the two *V. vinifera* parents. Fifty-three genotypes from the 96% *PdR1b x PdR2* 2017 crosses were also included. *Vinifera* parents included Arneis, Montepulciano, Morrastel, Pedro Ximenez, Pinot noir, and Sauvignon vert. Only six promising selections were identified based on their enzyme-linked immunosorbent assay (ELISA) and PD phenotype scores. Field evaluations this fall for fruit and horticultural traits found two that will be advanced. We also examined the role of the *V. vinifera* parent on observed cross means. **Table 3** summarizes the results for the three progeny

families where 10 or more genotypes were tested. Contrary to what we have observed in the many *PdR1* and *PdR1*xb42-26 (in the absence of *PdR2*) lines, we observed a clear effect of *V. vinifera* parent on the cross mean. This is consistent with a recent analysis of similar crosses made in 2016 to a different resistant female, 14309-111, where a difference was found in the cross means, with Cabernet Sauvignon having a lower mean (ln cfu/ml 11.9, n = 15) than Primitivo (ln cfu/ml 13.2, n = 22). Of the 54 PD x PM genotypes tested, 37 were susceptible, 11 resistant, four very resistant, and two appeared immune, our most resistant category, where no bacteria were detectable and phenotypic PD symptoms absent. However, this population contained lower levels of resistance than we expected. Filling out this group were 22 untested F1 progeny in the T 03-16 line to support our mapping project. Before putting these in testing, resistance in this line was identified as originating in the same region as *PdR1*. This line behaves very differently from the numerous other lines created from accessions with *PdR1* resistance – it appears to be heterozygous resistant and very few progeny are highly resistant and none immune: 77% were susceptible, 18% resistant, and 5% highly resistant.

Table 3. Subset of **Table 1d**, 96% *PdR1b x PdR2* with 10 or more progeny in a cross. All are crossed to the same resistant parent, 14388-029.

Vinifera Parent	PD R-rating			Progeny	Plants Tested	ln Mean cfu/ml
	S	R	Very R			
Pedro Ximenez	3	4	3	10	49	11.5
Morrastel	7	5	3	15	68	12.2
Sauvignon vert	14	6		20	96	13.1

Group 1e tests 149 untested species from our collection. Similar to Group 1d, an additional 80 96% *V. vinifera PdR1b* x *PdR2* genotypes from 2017 crosses are being tested. Elite *V. vinifera* parents, in addition to those mentioned above, include Alvarelhao, Mataro, and Refosco. The balance consists of bio-controls and the parents of the 2018 crosses. Samples are in the lab awaiting ELISA analysis. In Group 1f we are testing 42 untested wild *Vitis* accessions from our collection and, as in Group 1b, testing or retesting 61 genotypes from the 2017 PD x PM crosses.

Group 1g tests 70 additional wild vine selections from our collection, thirty-nine 96% *V. vinifera PdR1b* x *PdR2* genotypes from 2017 crosses, 25 *PdR1b* recombinants from 2018 crosses, and 17 b41-13 F1 genotypes to validate earlier findings, with the balance being bio-controls or untested selections with high field ratings. The trial has been sampled and ELISA results are pending.

Five main sets are being tested in Group 1h: 62 ANU67 resistance source F1 genotypes to confirm that their resistance also resides on LG14; fifty-seven 96% *V. vinifera PdR1b* x *PdR2* genotypes; fifty-two 97% *V. vinifera* PD x PM selections (both from 2017 crosses); thirty-five 97% *V. vinifera PdR1c* genotypes with resistance from wild accession b40-14; and 29 selections from crosses to the 13-329 line as in Groups 1c and 1d above. Also tested were small numbers of SWUS wild accessions, *PdR1b* recombinants from 2018 crosses, and bio-controls.
In 2017, we expanded the diversity of elite *V. vinifera* parents used in the 96% *V. vinifera PdR1* x *PdR2* breeding line. Parents included Alvarelhao, Arneis, Dolcetto, Mataro, Montepulciano,

Morrastel, Pedro Ximenez, two high yielding clones of Pinot noir, Refosco, Sauvignon vert and Touriga Nacional. These have given us progeny with a wide range of fruit and horticultural characteristics to choose from. Fruit evaluations were conducted this fall and three of the most promising also demonstrated satisfactory initial greenhouse PD resistance. Results of these are shown in **Tables 4a-4c**. These and other selections are currently being retested (Group 1g) in the greenhouse to verify the high level of PD resistance.

The 2018 PD x PM crosses are very exciting – some will contain progeny with strong resistance to PD and PM, and will be rapidly advanced to FPS for release. **Table 5** gives parentage, resistance sources, and percent *V. vinifera* for the first groups to be greenhouse tested this fall.

We continue to monitor our various field trials in PD hot spots around California. The first was planted at the Beringer vineyard in Yountville, Napa Valley in 2001. Decline from PD in the Riesling started in the fourth year after planting (most are now dead) while our vines continue to thrive after multiple years of needle inoculation in this area with high natural PD infection. The Riesling was only mechanically inoculated once.

Tables 6a and **6b** detail the vine, fruit and juice characteristics for the 12 PD resistant selections used to make wine lots in 2019. Selections relying on multigenic resistance from the Florida cultivar BD5-117 and *PdR1a* were not made this year after previous vintages found their wine quality to be poor. In addition, we made wines from a number of *V. vinifera* controls and Blanc du Bois and Lenoir as reference PD resistant cultivars. The wines were made from Davis grown fruit.

Table 4a. Three promising 96% *V. vinifera PdR1b* x *PdR2* PD resistant selections from the 2017 crosses: background and fruit characteristics.

Selection	Parentage	2019 Bloom Date	2019 Harvest Date	Berry Color	Berry Size (g)	Ave. Cluster Wt. (g)
17324-013	14309-111 x Dolcetto	05/15/2019	9/10/2019	B	1.4	214
17355-033	14309-111 x Mataro	05/19/2019	9/10/2019	B	1.5	356
17705-002	14388-029 x Morrastel	05/22/2019	9/10/2019	B	1.0	205

Table 4b. Juice analysis of three promising 96% *V. vinifera PdR1b* x *PdR2* PD resistant selections.

Selection	°Brix	TA (g/l)	pH	L-malic acid (g/l)	potassium (mg/l)	YAN (mg/l, as N)	catechin (mg/l)	tannin (mg/l)	Total antho-cyanins (mg/l)
17324-013	26.9	3.4	3.97	1.6	2,360	221	32	416	874
17355-033	24.2	4.9	3.66	2.0	2,200	189	24	325	935
17705-002	25.5	5.6	3.29	0.8	1,690	61	92	646	1,334

Table 4c. Three promising 96% *V. vinifera PdR1b* x *PdR2* resistant selections: Berry sensory analysis. Skin and seed tannin 1 (low) to 4 (high).

Genotype	Juice Hue	Juice Intensity	Juice Flavor	Skin Flavor	Skin Tannin Intensity	Seed Flavor	Seed Tannin Intensity
17324-013	Pink	Light	Jam, plum, fig, vs CS veg	Very fruity, soft, spice	2	Warm, bitter, spicy	4
17355-033	Pink-orange	Med-	Hay, wet straw, spice, hollow	Slight hay, spice, warm	3	Woody, hot	2
17705-002	Pink-orange	Med+	Prune, plum,	Neutral	2	Hot, slightly acrid, spicy, bitter	4

Table 5. 2018 PD x PM crosses currently entering our greenhouse testing. The PD resistance (PDR) type listed as A is a combination of b43-17 (*PdR1b*) enriched with resistance from b42-26, but lacking *PdR2*. *Ren1* and *Ren4* are PM resistance (PMR) loci from *V. vinifera* and *V. romanetii*, respectively. *Run1* and *Run2.1* are PMR loci derived from *Muscadinia rotundifolia*.

Cross ID	Parentage	PDR Type	PMR Type	% *V. vinifera*	# selections in field	# selections in testing
18-312	16376-008 x 16382-034	A	*Ren1* x *Run1*	93.6%	22	16
18-314	14305-078 x Fiano	A	*Ren4*	94.5%	17	5
18-315	14305-078 x Gouveio	A	*Ren4*	94.5%	34	14
18-316	14305-078 x Tinta Amarella	A	*Ren4*	94.5%	7	2
18-320	14305-078 x 09330-07	A	*Ren4*	93.0%	8	2
18-321	14305-078 x 09356-235	A	*Ren4*	93.0%	48	11
18-322	14305-078 x 10317-035	A	*Ren4*	93.0%	39	3
18-323	14305-078 x 09314-102	A	*Ren4*	93.0%	52	11
18-324	14305-078 x Alvarelhao	A	*Ren4*	94.5%	29	6
18-325	14305-078 x Pinot blanc	A	*Ren4*	94.5%	15	1
18-336	14305-078 x 14375-043	A	*Ren4* x *Run1*	93.0%	33	7
18-337	14305-078 x Bonarda	A	*Ren4*	94.5%	17	10
18-338	14305-078 x Teroldego	A	*Ren4*	94.5%	25	1
18-339	14305-078 x Tinta Cao	A	*Ren4*	94.5%	20	8
18-340	14305-078 x Cortese	A	*Ren4*	94.5%	22	17
18-342	14305-078 x 16382-034	A	*Ren4* x *Run1*	91.4%	23	8

Cross ID	Parentage	PDR Type	PMR Type	% *V. vinifera*	# selections in field	# selections in testing
18-370	16703-007 x 16344-003	A	*Ren1* x *Ren4 Run1*	93.6%	8	3
18-371	16703-007 x 16376-014	A	*Ren1* x *Ren4 Run1*	93.6%	39	21
18-377	16703-007 x 10317-035	*PdR1b*	*Ren1* x *Ren4 Run1*	95.3%	28	10
18-382	16703-007 x 16376-004	A	*Ren1* x *Ren4 Run1*	93.6%	26	12
18-384	15354-105 x 10317-035	*PdR1b*	*Ren1* x *Run2.1*	95.3%	22	10
18-390	14710-006 x 09314-102	*PdR1b*	*Run1*	96.9%	23	15

Table 6a. Parentage and fruit characteristics of the 12 PD resistant selections used in small scale winemaking in 2019. Those in bold are scheduled for release in spring 2020.

Selection	Parentage	2019 Bloom Date	2019 Harvest Date	Berry Color	Berry Size (g)	Ave Cluster Wt. (g)	Prod 9 = v high
07355-075	U0505-01 x Petite Sirah	5/10/2019	8/29/2019	B	1.5	341	7
09314-102	07370-028 x Cab Sauv	5/24/2019	8/27/2019	W	1.3	390	6
09330-07	07370-039 x Zinfandel	5/17/2019	9/3/2019	B	1.2	421	7
09331-047	07355-020 x Zinfandel	5/17/2019	8/29/2019	B	1.6	274	6
09333-370	07371-36 x Chard	5/15/2019	9/3/2019	B	1.6	615	7
09338-016	07371-20 x Cab Sauv	5/20/2019	8/27/2019	W	1.4	369	7
09356-235	07371-19 x Sylvaner	5/20/2019	8/27/2019	B	1.3	275	6
10302-178	07370-028 x Riesling	5/12/2019	8/20/2019	W	1	225	4
10302-238	07370-028 x Riesling	5/15/2019	8/20/2019	W	1.7	245	5
10302-293	07370-028 x Riesling	5/12/2019	8/13/2019	W	1.1	149	4
10302-309	07370-028 x Riesling	5/15/2019	8/22/2019	W	1.6	244	5
10317-035	07370-028 x Riesling	5/15/2019	8/20/2019	W	1.4	303	5

Table 6b. Juice analysis of PD resistant selections used in small scale winemaking in 2019.

Selection	°Brix	TA (g/l)	pH	L-malic acid (g/l)	potas-sium (mg/l)	YAN (mg/l, as N)	catechin (mg/l)	tannin (mg/l)	Total antho-cyanins (mg/l)
07355-075	27.6	3.63	5.4	2.14	2,180	169	9	570	1,231
09314-102	24.1	3.73	6.8	4.38	2,520	278			
09330-07	24.5	3.71	5.4	2.42	2,300	318	25	488	1,112
09331-047	27.5	3.75	5	1.96	2,240	289	12	561	1,606
09333-370	25.8	3.73	4.1	1.67	1,970	193	11	492	535
09338-016	23.1	3.62	5.2	1.65	1,820	260			
09356-235	25.6	3.69	6.4	2.93	2,290	311	88	586	2,395
10302-178	23.9	3.39	7.2	2.18	1,880	212			
10302-238	21.7	3.43	6.1	2.55	1,770	131			
10302-293	23.9	3.35	7.3	1.89	1,900	177			
10302-309	21.7	3.21	5.8	1.29	1,240	55			
10317-035	22.6	3.48	4.5	1.36	1,500	71			

CONCLUSIONS

We continue to make rapid progress breeding PD 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. 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 PD 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 20 advanced *PdR1b* scion selections to FPS over the past five winters to begin the certification and release process. Three PD resistant rootstocks were also sent to FPS for certification. This spring, the first three scion selections that employ both *PdR1* and *PdR2* resistance (*PdR2* is from b42-26 a more complex but very resistant form of *V. arizonica*) were delivered to FPS. Pierce's disease resistance from *V. shuttleworthii* and BD5-117 has been pursued, but progress and effort has been 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. Small-scale wines from 94% and 97% *V. vinifera PdR1b* selections have been very good and well-received at public tastings across California and in Texas, Georgia, and Virginia.

REFERENCES CITED

Baumgartel JE. 2009. Optimizing screening technology for breeding Pierce's disease resistant *Vitis*. M.S. Thesis. University of California, Davis.

Buzkan N, Krivanek AF, Eskalen A, Walker MA. 2003. Improvements in sample preparation and polymerase chain reaction detection techniques for *Xylella fastidiosa* in grapevine tissue. *American Journal of Enology and Viticulture* 54: 307-312.

Buzkan N, Kocsis L, Walker MA. 2005. Detection of *Xylella fastidiosa* from resistant and susceptible grapevine by tissue sectioning and membrane entrapment immunofluorescence. *Microbiological Research* 160: 225-231.

Krivanek AF, Stevenson JF, Walker MA. 2005a. Development and comparison of symptom indices for quantifying grapevine resistance to Pierce's disease. *Phytopathology* 95: 36-43.

Krivanek AF, Walker MA. 2005. *Vitis* resistance to Pierce's disease is characterized by differential *Xylella fastidiosa* populations in stems and leaves. *Phytopathology* 95: 44-52.

Krivanek AF, Famula TR, Tenscher A, Walker MA. 2005b. Inheritance of resistance to *Xylella fastidiosa* within a *Vitis rupestris* x *Vitis arizonica* hybrid population. *Theoretical and Applied Genetics* 111: 19-119.

Krivanek AF, Riaz S, Walker MA. 2006. The identification of *PdR1*, a primary resistance gene to Pierce's disease in *Vitis*. *Theoretical and Applied Genetics* 112: 1125-1131.

Riaz S, Huerta-Acosta K, Tenscher AC, Walker MA. 2018. Genetic characterization of *Vitis* germplasm from the southwestern U.S. and Mexico to expedite Pierce's disease-resistance breeding. *Theoretical and Applied Genetics* 131: 1589-1602.

FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board. Additional support from the Louise Rossi Endowed Chair in Viticulture, which helps fund our powdery mildew resistance breeding and collection trips across the southwestern United States, is also gratefully acknowledged.

ACKNOWLEDGEMENTS

We thank Gordon Burns of ETS Labs in St. Helena, CA for continued support with grape berry chemical analysis, and Ken Freeze of Brown-Miller Communications for help arranging and coordinating industry tastings.

MOLECULAR BREEDING SUPPORT FOR THE DEVELOPMENT OF PIERCE'S DISEASE RESISTANT WINEGRAPES

Project Leader: Andrew Walker | Department of Viticulture and Enology | University of California | Davis, CA 95616 | awalker@ucdavis.edu
Collaborator: Dario Cantu | Department of Viticulture and Enology | University of California | Davis, CA 95616 | dacantu@ucdavis.edu
Cooperating Staff: Summaira Riaz | Department of Viticulture and Enology | University of California | Davis, CA 95616 | snriaz@ucdavis.edu
Cooperating Staff: Cecilia Agüero | Department of Viticulture and Enology | University of California | Davis, CA 95616 | cbaguero@ucdavis.edu

Reporting Period: The results reported here are from work conducted July 2018 to October 2019.

ABSTRACT

This project provides support to our companion project "Breeding Pierce's Disease Resistant Winegrapes." Simple sequence repeat marker-based framework genetic maps were completed for two accessions (b40-14 and b41-13), and targeted limited mapping strategy was adopted for accession T03-16. The first two were collected from Mexico and the latter was collected from Texas. Quantitative trait loci analysis identified Pierce's disease resistance on chromosome 14, explaining greater than 50% of the genetic variation in three accessions. So far, 13 accessions have been identified with strong Pierce's disease resistance on chromosome 14. RNA sequencing was completed for a panel of six lines from accession b40-14. A pilot study to compare stem anatomy among grape species was initiated to gain better understanding of differences among species and Pierce's disease resistant and susceptible accessions. Physical maps were completed for b43-17 and b40-14 and sequence comparisons are being made to the susceptible PN40024 and Cabernet Sauvignon genome sequences. We are using *Agrobacterium* mediated transformation systems and embryogenic callus and meristematic bulks to regenerate plants. Two of the candidate genes [resistance gene analog (RGA)14 and 18] were sequence verified and plants were transformed. Promising results were obtained with one RGA14 line which, after inoculation, had better cane maturation and lower bacterial titers than untransformed plants. This finding agrees with results from sequencing of cDNA from b43-17, the original source of resistance, inoculated with *Xylella fastidiosa*, showing the amplification of fragments that comprise sequences identical to RGA14 but different from RGA18. Two St. George RGA18 lines did not have increased Pierce's disease tolerance. Co-transformations with both pCLB2301NK-14 and pCLB2301NK-18 have produced several lines of Thompson Seedless and Chardonnay that are growing in vitro and will be tested in 2020. We are also using a reverse genetic approach using clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 systems on b43-17 and U0505-01 to knock out candidate genes to expedite the process of identifying candidate genes.

LAYPERSON SUMMARY

This project provides molecular genetic support to the Pierce's disease resistant winegrape breeding program and conducts all of the DNA marker evaluations that dramatically accelerate the breeding program. It also identifies new sources of Pierce's disease resistance, studies the

genetic diversity of the southwestern United States and Mexican grape species and how they resist Pierce's disease, maps candidate resistance genes, and characterizes those genes so that they can be effectively included in the breeding program.

INTRODUCTION

This continuing project provides molecular support to the grape breeding project "Breeding Pierce's Disease Resistant Winegrapes." Previously, we identified a dominant form of Pierce's disease (PD) resistance termed *PdR1* in a *Vitis arizonica/candicans* accession (b43-17), which we mapped to chromosome (Chr) 14 (Riaz et al., 2006; Riaz et al., 2008). Markers linked to *PdR1* were used to breed PD resistant grapes (Riaz et al., 2009). We have surveyed over 250 accessions of *Vitis* species growing in the southern United States and Mexico to identify new sources of PD resistance. Analysis using population genetics tools has allowed us to better understand gene flow among resistant species and their taxonomic and evolutionary relationships. Fourteen promising resistant accessions were identified from this germplasm. Markers were used to determine their genetic diversity and relationships to each other so that the most different accessions could be used to broaden PD resistance. Small breeding populations were developed and more than 700 seedlings were marker tested. We used a limited mapping strategy by utilizing markers from chromosome 14 in conjunction with greenhouse screen data to determine if the resistance to PD in these 14 accessions is different from the previously identified locus *PdR1* (Riaz et al., 2018**)**. Three accessions (T03-16, ANU67, and b41-13) were identified to carry out further work with larger populations. We are also investigating potential differences in stem anatomy among the different grape species and their relationship with resistance / tolerance to *Xylella fastidiosa* (*Xf*; the bacterial causal agent of PD). Previous studies have shown that xylem anatomy could impact PD susceptibility (Pouzoulet et al., 2014; Deyett et al., 2019).

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). We are also utilizing a reverse genetic approach via the use of the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system on b43-17 and U0505-01 to identify the candidate genes by disruption of their function. Upstream and downstream sequences, as well as the gene sequences of two candidate genes, open reading frame (ORF)14 and ORF18, from *PdR1b* were verified, and constructs were developed to test their function. Transformation experiments with the *PdR1* resistance gene with a native grape promoter were completed with ORF18, and transgenic lines are being developed and maintained for later resistance verification. A multiple time point gene expression project was completed in the controlled environment and RNA extractions and libraries were completed for the RNA sequencing. Embryogenic callus cultures of *Vitis vinifera* cvs. Chardonnay and Thompson Seedless and *Vitis rupestris* St. George are being maintained to test the function of gene sequences. These efforts will help us identify candidate resistance genes by complementation and better understand how they function.

OBJECTIVES

The overall goal of this project is to provide molecular genetic support to the PD resistant winegrape breeding program. These efforts include discovering new sources of PD resistance; identifying unique resistant germplasm with the help of population genetics; creating genetic

maps to tag resistance regions; providing markers to assist the breeding program; and validating and characterizing the functions of candidate PD resistance genes and sequences. The candidate gene constructs will be developed with grape promoters and transformed into elite *V. vinifera* cultivars.

The specific objectives of this project are:
1. Provide genetic marker testing for mapping and breeding populations produced and maintained by the PD resistance breeding program, including the genetic mapping of three new highly resistant accessions (b41-13, T03-16, and ANU67) for use in stacking PD resistance genes.
2. Refine the genetic map of the *PdR2* region from the b42-26 background, and complete a physical map and carry out comparative sequence analysis with b43-17 (*PdR1a* and *b*) and b40-14 (*PdR1c*).
3. Employ RNA sequencing to understand genome-wide transcriptional changes of the pathways regulated by defense-related genes in b40-14.
4. Clone *PdR1c* and *PdR2* genes with native promoters.
5. Compare the PD resistance of plants transformed with native vs. heterologous promoters.

RESULTS AND DISCUSSION

Objective 1. Provide Genetic Marker Testing for Mapping and Breeding Populations Produced and Maintained by the PD Resistance Breeding Program, Including the Genetic Mapping of Three New Highly Resistant Accessions (b41-13, T03-16, and ANU67) for Use in Stacking PD Resistance Genes

This project provides molecular support to the companion PD resistant winegrape breeding project by conducting marker-assisted selection (MAS) on seedling populations. In spring 2019 we extracted DNA and marker-tested 2,400 seedling plants from 25 different crosses for the *PdR1* (b and c) and *PdR2* loci that were in all cases combined with powdery mildew (PM) resistance from one to three sources (**Table 1**). Marker screening is a time-intensive process, but it is extremely important and makes our breeding program extremely efficient and successful.

In 2018, we reported that a limited mapping strategy produced inconclusive results for three accessions chosen from the southwestern U.S./Mexico species screen (b41-13, T03-16, and ANU67) that focused on phenotypic data from small populations and markers from chromosome 14. These three accessions were chosen as candidates for further work and the development of framework maps with larger populations to detect new unique loci for PD resistance breeding. Accession T03-16 from the Big Bend region in Texas and b41-13 from Tamaulipas state in Mexico were strong candidates for large populations based on strong resistance and inheritance studies.

A total of 295 seedling plants from the b41-13 F1 population and 285 seedling plants from the T03-16 F1 population were established in the field. **Table 2** provides summary information on population sizes, number of markers tested for polymorphism for b41-13 and T03-16, and number of markers that were completed on larger populations to develop genetic maps. A manuscript titled “Genetic Mapping of Pierce’s Disease Resistance in Germplasm Collected from the Southwestern U.S. and Mexico” is ready for submission. Simple sequence repeat (SSR)

marker-based framework maps covering all 19 grape chromosomes were developed for b40-14 and b41-14, while the genetic map for T03-16 was only developed for chromosome 14. The quantitative trait locus (QTL) analyses determined that these three additional accessions also have PD resistance on chromosome 14 within the genetic window of the *PdR1* locus, bringing the total to 13 accessions with PD resistance on chromosome 14. The apparent lack of additional genomic regions conferring PD resistance, and the widespread distribution of the *PdR1* locus in wild germplasm collected from the southwestern U.S. and Northern Mexico, indicate that wild grapes developed resistance in response to the disease at or very near its center of origin, and that gene flow occurring over millennia has spread it to wider regions of the southwestern U.S. and Mexico. **Table 3** provides the summary statistics of the genetic maps of b41-13, T03-16, and b40-14.

Table 1. Number of seedlings marker tested for resistance to PD and PM. Numbers following PD or PM are the number of loci (genes) for resistance potentially stacked from each parent. The marker testing was completed in spring 2018 to support our PD resistance breeding program.

Cross ID	Resistance Stacking	MAS Test
18-340	PD2xPM1	125
18-324	PD2xPM1	175
18-320	PD2xPM1	35
18-314	PD2xPM1	70
18-323	PD2xPM1	140
18-315	PD2xPM1	125
18-339	PD2xPM1	125
18-316	PD2xPM1	25
18-342	PD2xPM2	75
18-321	PD2xPM1	100
18-336	PD2xPM2	100
18-338	PD2xPM1	100
18-337	PD2xPM1	100
18-322	PD2xPM1	75
18-703	PD1xPM1	100
18-377	PD1xPM3	100
18-375	PD1xPM2	100
18-325	PD2xPM1	75
18-391	PD1xPM1	150
18-384	PD1xPM2	75
18-376	PD1xPM2	100
18-390	PD1xPM1	100
18-373	PD1xPM3	70
18-374	PD1xPM3	70
18-371	PD2xPM3	90
Total		2,400

Table 2. List of accessions used to characterize additional sources of PD resistance with collection information, population identification, and number of seedlings tested in the study. Markers were tested on a set of eight samples including parents and progeny. Only polymorphic markers were added to the entire population for each genetic background.

Accession	Collection Location	Population Code and Size	No. of tested markers	No. of amplified markers	No. of polymorphic markers	No. of completed markers
b40-14	Near Chihuahua, Mexico	07744 / 120	607	449	323	225
b41-13	Near Ciudad Mante, Mexico	16337 / 250	596	543	295	244
T03-16	Near Lahitas, Texas	(13302, 13336, 16304, 17344) / 192	34	34	14	14

We are also making progress on the genetic mapping of b42-26, which was collected from Loreto, Baja California. A genetic map was completed with 189 SSR markers covering 825 centimorgans (cM) representing 18 grape chromosomes; no polymorphic markers were identified for chromosome 19. Results showed multiple small effect QTLs on chromosomes 8, 10, 14, and 17 on the paternal map that collectively explained up to 15% of the phenotypic variation for bacterial titers [colony-forming units (cfu)/ml]. Similar genomic regions were identified for the cane maturation index. **Figure 1** shows the distribution of genotypes for the cfu/ml values for the tested 323 genotypes of the F1 population (see more details in previous reports). Final results are being prepared for publication.

We also initiated a small pilot study to compare the stem anatomy between different grape species. Previous studies have shown that susceptible *V. vinifera* cultivars have xylem vessels with larger diameters and more connectivity due to extensive xylem relays in comparison to PD resistant accessions (Brodersen et al., 2013). We selected a subset of accessions from four grape species and also included accessions that are known to have PD resistance on chromosome 14 (**Table 4**) to study these anatomical traits.

Cuttings were made to produce plants for November/December inoculations, and resistance evaluations are expected in March 2020. We are planning to use high-resolution computed tomography, light microscopy, and scanning electron microscopy to measure vessel diameters and characterize vessel relays in stems, and compare their distributions and structure in four *Vitis* species as well as in accessions that carry the *PdR1* locus. This work will be carried out in collaboration with A. McElrone at UC Davis, with results expected in June 2020. We have carried out scanning electron microscopy on the stem sections from field-grown plants, however, field plants were grown in different environments under different water regimes that may have impacted the xylem anatomy. In this current experiment, all inoculated and control plants will be grown under similar greenhouse conditions to minimize environmental variation.

Table 3. Summary of parental framework maps of the three resistant backgrounds. R8918-05 was a resistant male seedling with b40-14 as the pollen parent.

	R8918-05 map		b41-13 map		T03-16 map	
Chromosome	Mapped Markers	Map length (cM)	Mapped Markers	Map length (cM)	Mapped Markers	Map length (cM)
Chr1	15	55.833	10	59.4		
Chr2	4	49.147	3	37.1		
Chr3	6	33.415	7	31.7		
Chr4	11	69.33	12	68.5		
Chr5	17	52.01	9	54.2		
Chr6	11	38.684	11	70.7		
Chr7	12	69.944	8	56.4		
Chr8	11	50.986	20	63.2		
Chr9	10	58.755	9	55.3		
Chr10	10	54.542	6	50.3		
Chr11	9	57.501	6	41.1		
Chr12	9	62.413	9	27.6		
Chr13	11	55.563	7	35.1		
Chr14	27	64.121	31	85.3	12	79.5
Chr15	8	29.554	3	7.8		
Chr16	9	59.326	2	2.5		
Chr17	12	43.963	6	45.1		
Chr18	12	58.062	14	88		
Chr19	13	40.443	5	27.5		
Total	217	1,003.592	178	906.8	12	79.5
Ave. marker distance (cM)	4.6		5.1		6.6	
Number of gaps > 20 cM	9		8		0	

Objective 2. Refine the Genetic Map of the *PdR2* Region from the b42-26 Background, and Complete a Physical Map and Carry Out Comparative Sequence Analysis with b43-17 (*PdR1a* and *b*) and B40-14 (*PdR1c*)

We completed the physical maps of the *PdR1*a, *PdR1*b, and *PdR1*c loci from the b40-14 and b43-17 backgrounds. In summary, the physical map of *PdR1b* spans 604 kilobase-pairs (Kb) that includes the flanking markers Chr14-77 and Chr14-81 used for marker-assisted screening. The physical map of b40-14 (*PdR1c*) covers 426 Kb and consisted of four overlapping bacterial artificial chromosome (BAC) clones. BAC clone H43I23 (206 Kb) that contains *PdR1a* was also sequenced, and it showed complete homology to the sequence of *PdR1b* haplotype, indicating that the parents of b43-17 must be closely related. **Figure 2** presents the physical maps of *PdR1b* and *PdR1c*.

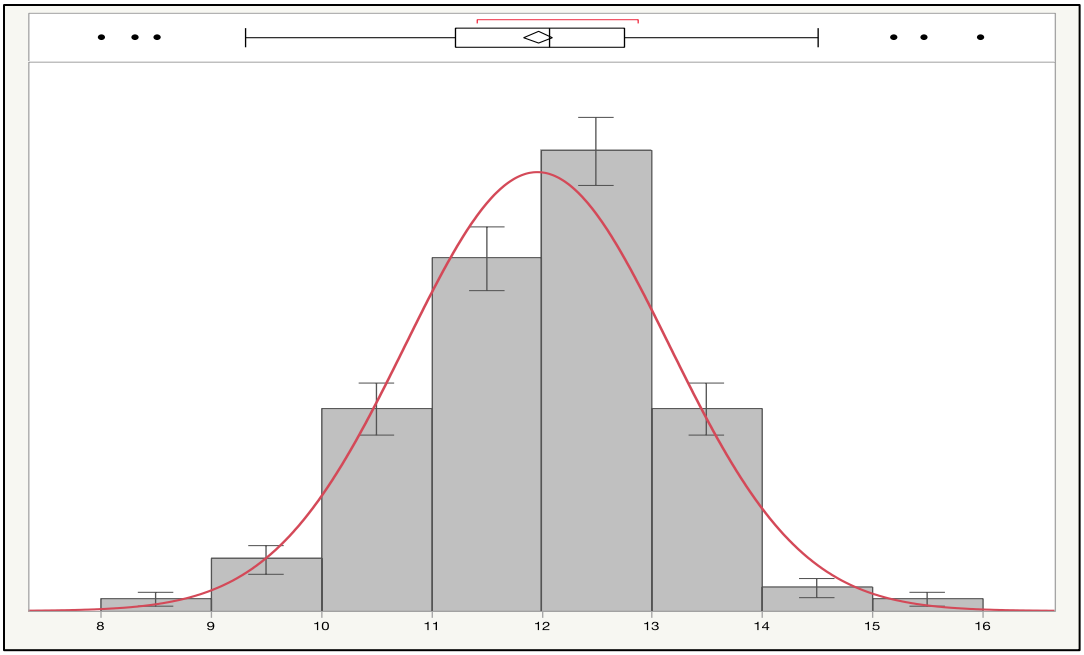


Figure 1. Distribution of enzyme-linked immunosorbent assay (ELISA) values for inoculated genotypes of the F1 05347 population with resistant accession b42-26 as the male parent.

Table 4. List of accessions that are part of pilot study to characterize the xylem anatomy in terms of vessel diameter and connectivity.

Accession	Species or Code	State / Country	PD Resistance Status
ANU56	*V. arizonica*	AZ	R
ANU09	*V. arizonica*	UT	S
NV11-118	*V. girdiana*	NV	R
NV12-041	*V. girdiana*	NV	S
b42-24	*V. cinerea*	MX	R
T65	*V. cinerea*	TX	S
DVIT1609	*V. aestivalis*	IL	R
DVIT1608	*V. aestivalis*	IL	S
T03-16	*V. arizonica*	TX	R-PdR1-chr14
b46-43	*V. arizonica*	TX	R-PdR1-chr14
b40-14	*V. arizonica*	MX	R-PdR1-chr14
b43-17	*V. arizonica hybrid*	MX	R-PdR1-chr14
b42-26	*V. arizonica hybrid*	MX	QTLs on chr8,10,14
U505-01	BC1, b43-17 background, PdR1-chr14		R-PdR1-chr14
U505-22	BC1, b43-17 background, PdR1-chr14		S-PdR1-chr14
U505-35	BC1, b43-17 background, PdR1-chr14		Intermediate-PdR1-chr14

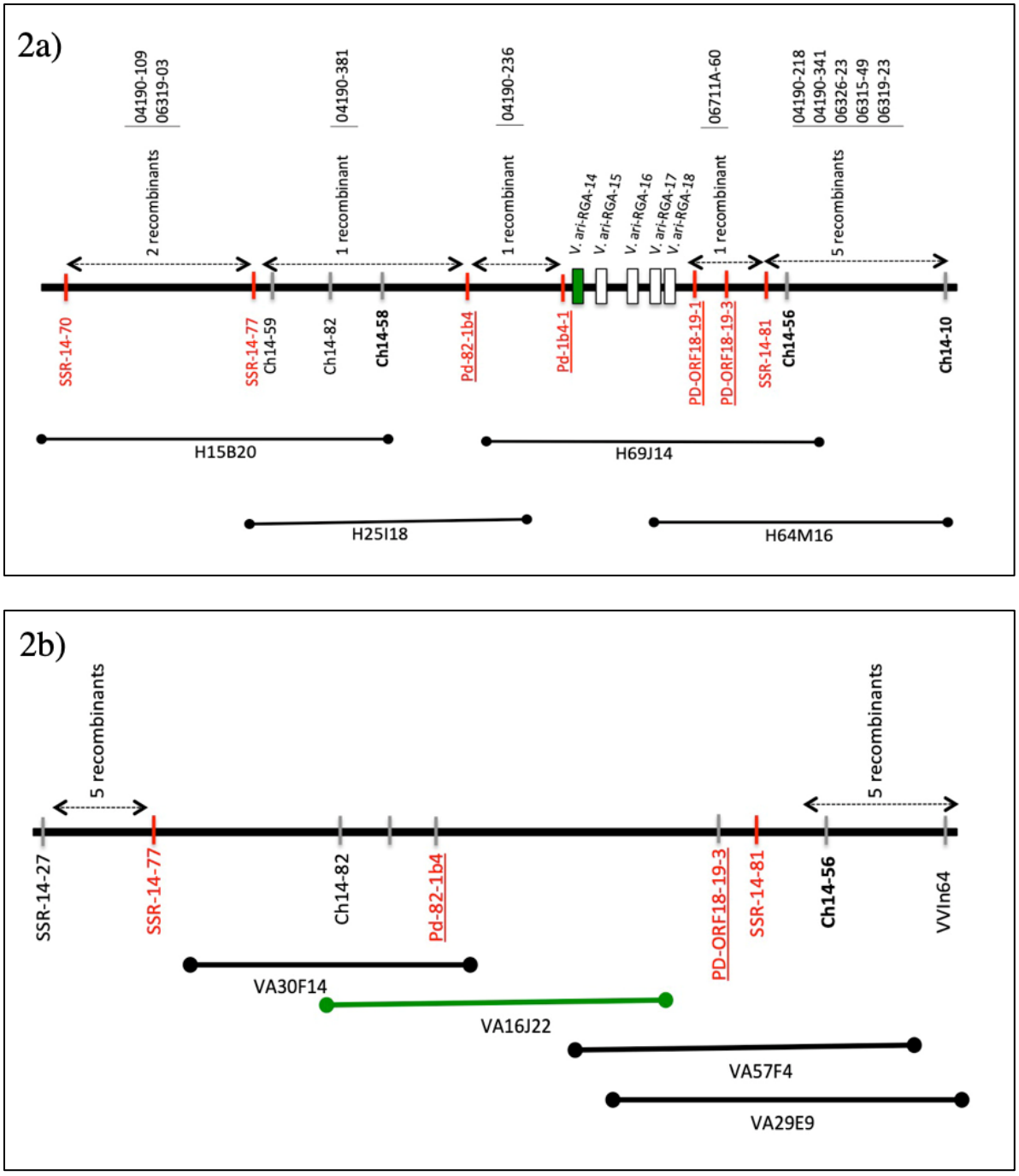


Figure 2. Physical map of PD resistant accessions. (2a) is the b43-17 map and (2b) is the b40-14 map. Markers in bold were used as probes to screen the library, markers in red are SSR markers. The four underlined markers were developed from the b43-17 sequence, the others were designed from the PN40024 sequence.

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. For the *PdR1b* locus, the genetic window is

limited to the 82 Kb between markers SSR-1b4-1 and PD-Orf18-19-1. Five ORFs in that region were associated with disease resistance (**Figure 3**). A total of 21 ORFs were identified in the 604 Kb sequence of *PdR1b* sequence in comparison to the 18 ORFs in the *PdR1c* sequence.

The PN40024 sequence was 230 Kb with many gaps, implying that some ORFs were not accounted for. The Cabernet Sauvignon sequence within the flanking markers was 527 Kb long. All three sequences had an abundance of transposable elements dispersed within the resistance gene analogs (RGAs). Genome sequence comparisons to the Cabernet Sauvignon sequence show sequence divergence in the region of the RGA, and the sequences had greater than 90% homology in the genomic region flanking the resistance. Comparison of the *PdR1* region in b43-17 and b40-14 also found sequence divergence for the resistance gene region, and for the number of transposable elements indicating significant differences between the two accessions. Currently, we are working to finalize the manuscript for publication documenting these physical maps.

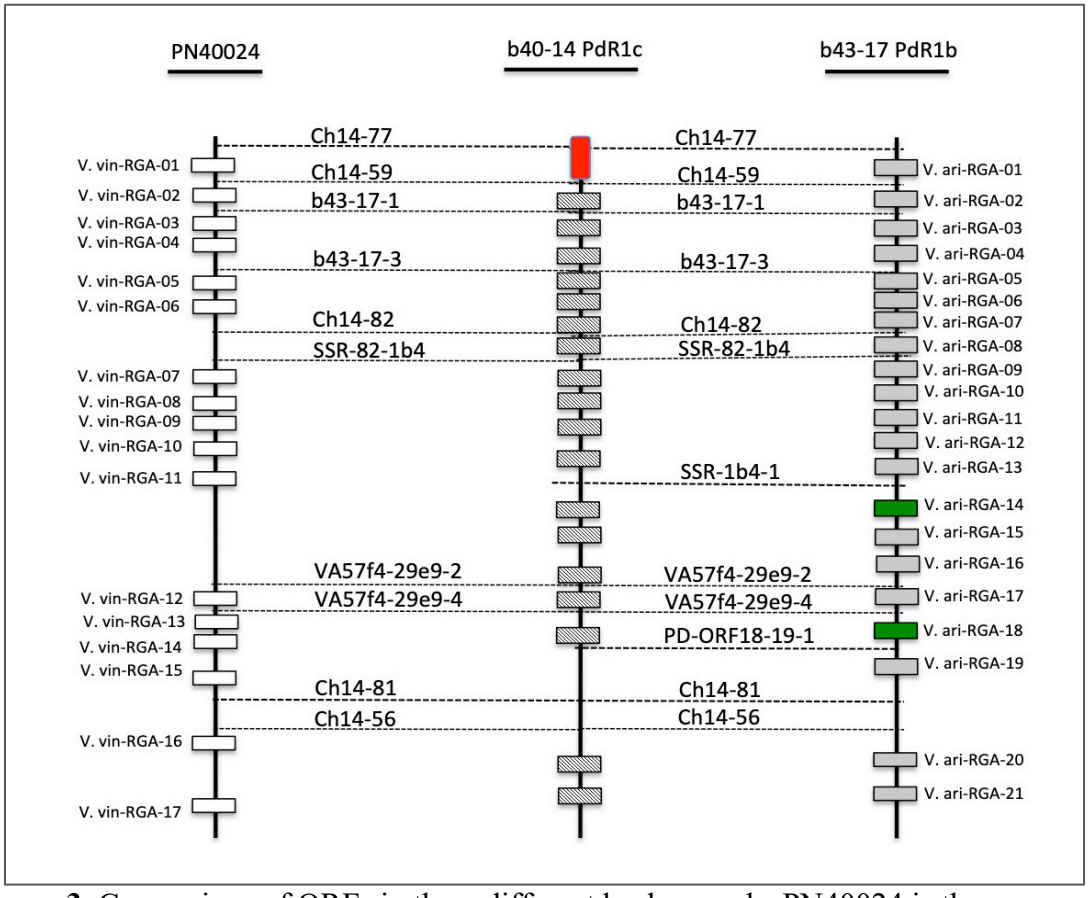


Figure 3. Comparison of ORFs in three different backgrounds. PN40024 is the susceptible Pinot Noir reference genome; dashed lines show the placement of markers to provide alignment for comparison among sequences. The red regions represent the gap between the Chr14-59 and Chr14-77 markers in the assembly. Green blocks in the *PdR1b* sequence are two candidate resistance genes for which constructs were developed for use in transformation experiments.

Objective 3. Employ RNA Sequencing to Understand Genome-Wide Transcriptional Changes of the Pathways Regulated by Defense-Related Genes in b40-14

RNA sequencing is a powerful approach for identifying transcripts and quantifying gene expression while combined with a single high-throughput sequencing assay. We completed a time-course experiment to monitor the bacteria levels in control and inoculated resistant and susceptible plants, to design an experiment capable of answering our biological questions with the maximum statistical power. For this purpose, three resistant and three susceptible plants from the 07744 population with resistance from b40-14 PD (*PdR1c*) were used. Plants were propagated and a time-course experiment was carried out in growth chambers with temperature and humidity control to reduce the variance. Stem samples were collected from positions 10 cm, 20 cm, 30 cm, and 40 cm above the point of inoculation, and weekly RNA extractions were performed. Samples were also collected from 30 cm above the point of inoculation for ELISA screening. RNA extractions were completed, and libraries were developed for sequencing. We have completed the sequencing and are currently analyzing the results.

Objective 4. Clone *PdR1c* and *PdR2* Genes with Native Promoters

With the help of molecular markers, we limited the genetic region that contains the *PdR1b* resistance locus to 82 Kb. Five ORFs of the leucine-rich repeat receptor kinase gene family, associated with disease resistance, were identified within the resistance region boundaries. Two ORFs, V.ari-RGA14 and V.ari-RGA18, 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. Fragments that contain the entire coding region of V.ari-RGA14 and V.ari-RGA18 plus ~3 Kb upstream and ~1 Kb downstream sequences were synthesized and cloned into pCLB2301NK (Feechan et al., 2013) at Genewiz Inc. to produce plasmids pCLB2301NK-14 and pCLB2301NK-18. See the final reports for CDFA agreements 14-0137-SA and 17-0427-000-SA for details.

Objective 5. Compare the PD Resistance of Plants Transformed with Native vs. Heterologous Promoters

We have established *Agrobacterium*-mediated transformation systems followed by regeneration of plants from embryogenic callus and meristematic bulks (Agüero et al., 2006; Xie et al., 2016). *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 the rootstock *V. rupestris* St. George. The evaluation of 44 transgenic lines of Chardonnay and Thompson Seedless (10-11 lines per genotype x two constructs) showed that all transgenic lines displayed disease symptoms, although with different degrees of intensity, with Thompson Seedless being considerably more susceptible than Chardonnay. Although some lines exhibited reduced symptoms or lower bacterial concentrations, none reached the levels of the resistant biocontrols. See final reports for CDFA agreements 14-0137-SA and 17-0427-000-SA for details.

Plant regeneration of transgenic St. George has been more challenging (**Table 5**); however, promising results were obtained with one RGA14 line that was inoculated in August 2018. The cane maturation index means of untransformed and transgenic plants were 4.9 and 1.7, respectively, while the leaf scorching index means were 4.5 and 3. **Figure 4** shows re-growth in transgenics after cutting back of plants for sampling 12 weeks after inoculation (right). None of

the untransformed plants resumed growth (left). Shoot lignification 30 cm above the point of inoculation is shown at the bottom of the figure. ELISA tests also produced significant differences between untransformed (410,000 cfu/ml) vs. transgenic (120,000 cfu/ml) plants. This finding agrees with results from sequencing of cDNA from b43-17, the original source of resistance, inoculated with *Xf*, showing the amplification of fragments that comprise sequences identical to RGA14, but different from RGA18. Two St. George RGA18 lines, inoculated in January and July 2019, did not show tolerance. The rest of the St. George lines were dwarf and grew very slowly; consequently more in vitro transformations have been initiated and are currently at the germination stage. Co-transformations with both pCLB2301NK-14 and pCLB2301NK-18 have produced several lines of Thompson Seedless and Chardonnay that are growing in vitro and will be transferred to the greenhouse this November.

Transformation of meristematic bulks of susceptible genotypes selected from the 04-191 population, which are 50% *V. vinifera*, 25% b43-17, and 25% *V. rupestris* A. de Serres, are also being pursued. One of these genotypes, designated 29-07, has produced one polymerase chain reaction (PCR)-positive line (**Table 5**) that has been acclimated to greenhouse conditions for further testing. Evaluating these lines plus additional transgenic St. George could help clarify the role of genetic background in *PdR1b* resistance.

Table 5. Data on meristematic bulks of susceptible progeny from the 04-191 population transformed with two constructs separately and together.

Genotype	No. Lines	No. Lines PCR Positive
pCLB2301NK-18		
St. George 29-42	4 3	4 0
pCLB2301NK-14		
St. George 29-07	2 6	1 2
Co-transformations		
T. Seedless	5	not tested
Chardonnay	2	not tested
St. George	0	

In addition, we have incorporated to this section of our project a reverse genetic approach using CRISPR-Cas9 systems on b43-17 and U0505-01. B43-17 is the *PdR1b* source of resistance and U0505-01, which is 87.5% *V. vinifera*, 6.25% *V. rupestris* and 6.25% b43-17, is heterozygous for the *PdR1b* molecular markers and highly resistant to PD. To this purpose, we have produced meristematic bulks (MB) of b43-17 and embryogenic cultures (EC) of U0505-01 (**Figure 5**) and completed the construction of a gRNA expression vector targeting RGA14 in collaboration with Mily Ron in Anne Britt's lab at UC Davis.

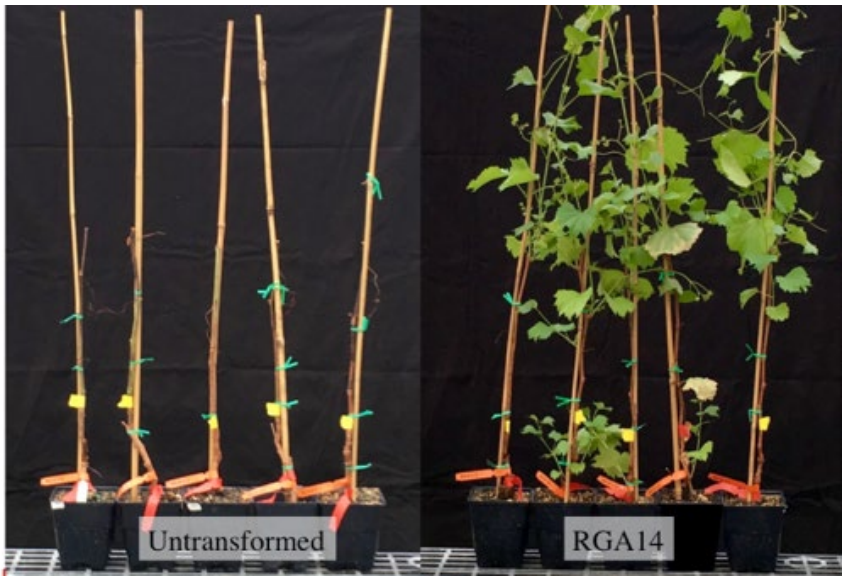
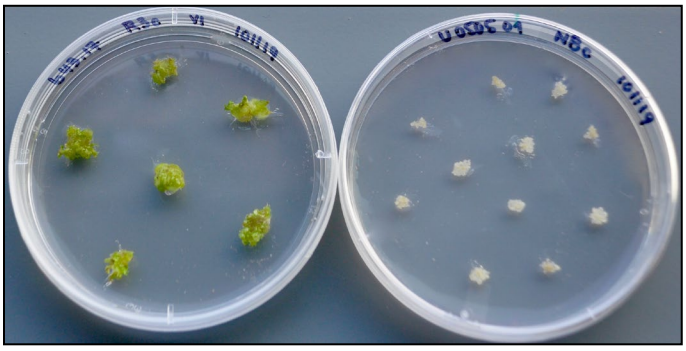


Figure 4. Regenerated St. George transformed with RGA14 or untransformed and inoculated with *Xf*. The line with RGA14 suppresses bacterial levels, regrows after being inoculated and sampled for analysis, and has reduced leaf and stem symptoms.



MB EC

Figure 5. Meristematic bulks (MB) and embryogenic callus (EC) of U505-01 in preparation for CRISPR-Cas9 resistance gene knockout to explore the *PdR1* resistance mechanism.

CONCLUSIONS

We completed greenhouse screening, marker testing, and QTL analysis of breeding populations from 13 new resistance sources. Genetic mapping and QTL analysis were completed for *V. arizonica* accessions b41-13 and T03-16. Results found that PD resistance in these two accessions also resides on chromosome 14 at the same genomic position as *PdR1*. This brings the total to 13 accessions with the *PdR1* locus. We have identified a new resistance locus, *PdR2*, from the b42-26 background, and closely-linked markers are being used in MAS to stack resistance loci from these different backgrounds. We have completed the genetic and physical mapping of PD resistance from b40-14 and b43-17. RNA sequencing is complete, and results are

being analyzed. A new pilot study is being initiated to characterize the xylem anatomy among different grape species and PD resistant and susceptible accessions. We completed greenhouse screening of Chardonnay and Thompson Seedless lines transformed with RGA18 and RGA14. Although some transgenic lines responded better than untransformed plants to *Xylella* infection, none reached the same level as our resistant biocontrols. Promising results have been obtained with one line of RGA14 transformed St. George. Testing of RGA14 and 18 in St. George and other genetic backgrounds, as well as more information about RGA15, 16, and 17 will help to clarify the meaning and importance of these results. To achieve a better understanding of how PD resistance from *PdR1* works, we have initiated CRISPR-Cas9 knockouts in resistant genotypes.

REFERENCES CITED

Agüero CB, Meredith CP, Dandekar AM. 2006. Genetic transformation of *Vitis vinifera* L. cvs. Thompson Seedless and Chardonnay with the pear PGIP and GFP encoding genes. *Vitis* 45: 1-8.

Brodersen GR, Choat B, Chatelet DS, Shackel KA, Matthews MA, McElrone AJ. 2013. Xylem vessel relays contribute to radial connectivity in grapevine stems (*Vitis vinifera* and *V. arizonica*; Vitaceae). American Journal of Botany 100: 314-321.

Deyett E, Pouzoulet J, Yang JI, Ashworth VE, Castro C, Roper MC, Rolshausen PE. 2019. Assessment of Pierce's disease susceptibility in *Vitis vinifera* cultivars with different pedigrees. *Plant Pathology* 68: 1079-1087. Doi: 10.1111/ppa.13027.

Feechan A, Anderson C, Torregrosa L, Jermakow A, Mestre P, Wiedemann-Merdinoglu S, Merdinoglu D, Walker AR, Cadle-Davidson L, Reisch B, Aubourg S, Bentahar N, Shrestha B, Bouquet A, Adam-Blondon, AF, Thomas MR, Dry IB. 2013. Genetic dissection of a TIR-NB-LRR locus from the wild North American grapevine species *Muscadinia rotundifolia* identifies paralogous genes conferring resistance to major fungal and oomycete pathogens in cultivated grapevine. *Plant Journal* 76: 661-674.

Holmes IB, Wendt T, Holm PB. 2013. Intragenesis and cisgenesis as alternatives to transgenic crop development. *Plant Biotechnology Journal* 11: 395-407.

Pouzoulet J, Pivovaroff AL, Santiago LS, Rolshausen PE. 2014. Can vessel dimension explain tolerance toward fungal vascular wilt diseases in woody plants? Lessons from Dutch elm disease and esca disease in grapevine. *Frontiers of Plant Science* 5: 253.

Riaz S, Krivanek AF, Xu K, Walker MA. 2006. Refined mapping of the Pierce's disease resistance locus, *PdR1*, and sex on an extended genetic map of *Vitis rupestris* x *Vitis arizonica*. *Theoretical and Applied Genetics* 113: 1317-1329.

Riaz S, Tenscher AC, Rubin J, Graziani R, Pao SS, Walker MA. 2008. Fine-scale genetic mapping of two Pierce's disease resistance loci and a major segregation distortion region on chromosome 14 of grape. *Theoretical and Applied Genetics* 117: 671-681.

Riaz S, Tenscher AC, Graziani R, Krivanek A, Ramming D, Walker MA. 2009. Using marker-assisted selection to breed Pierce's disease-resistant grapes. *American Journal of Enology and Viticulture* 60: 199-207.

Riaz S, Huerta-Acosta K, Tenscher AC, Walker MA. 2018. Genetic characterization of *Vitis* germplasm collected from the southwestern US and Mexico to expedite Pierce's disease resistance breeding. *Theoretical and Applied Genetics* 131: 1589-1602.

Xie X, Agüero CB, Wang Y, Walker MA. 2016. Genetic transformation of grape varieties and rootstocks via organogenesis. *Plant Cell, Tissue, and Organ Culture* 126: 541-552.

FUNDING AGENCIES
Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board. Additional support from the Louise Rossi Endowed Chair in Viticulture, which helps fund our powdery mildew resistance breeding and collection trips across the southwestern United States, is also gratefully acknowledged.

CULTIVAR SUSCEPTIBILITY AND TEMPERATURE-DEPENDENT RECOVERY OF *XYLELLA FASTIDIOSA* INFECTED GRAPEVINES

Project Leader: Lindsey Burbank | San Joaquin Valley Agricultural Sciences Center | USDA Agricultural Research Service | Parlier, CA 93648 | lindsey.burbank@usda.gov

ABSTRACT

Previous research in California demonstrated that *Xylella fastidiosa* (*Xf*) can be eliminated from infected grapevines and almond trees by exposure to cold winter temperatures. Duration and temperature of winter conditions, degree of host plant susceptibility, and time between inoculation and cold exposure are believed to play a role in rate of pathogen persistence and disease reoccurrence. Initial studies of overwintering survival of *Xf* in grapevine primarily focused on climate conditions experienced in northern California regions, and included a limited range of cultivars. To better understand the dynamics of vine recovery from *Xf* infection following cold stress, grapevines of three different cultivars were subjected to cold treatment at 4°C after either eight weeks or 16 weeks post-inoculation. Plants that underwent cold treatment at eight weeks post-inoculation were separated into two groups, one inoculated in June, and one inoculated in late August, to evaluate the effect of accelerated symptom development due to hot mid-summer temperatures. All vines were tested with quantitative polymerase chain reaction (qPCR) prior to cold treatment to determine infection status. Following eight weeks of cold treatment, all vines were grown back from dormancy for 20 weeks and evaluated for reoccurrence of disease by symptom observation and qPCR testing. In this study, cultivar susceptibility and higher temperatures during initial infection stages had a greater impact on disease reoccurrence than duration of infection prior to cold treatment. This suggests that use of tolerant or resistant plant material should be a priority for areas at risk of *Xf* infection, and that summer temperatures need to be considered in addition to winter temperatures and time of inoculation when gauging likelihood of vine recovery. Further information regarding the effect of climate factors such as temperature on pathogen persistence is important to inform region-specific management strategies and to evaluate risk of *Xf* spread in new areas.

SEARCHING FOR ENDOSYMBIONTS IN *KOLLA PAULULA*, A VECTOR OF *XYLELLA FASTIDIOSA* CAUSING PIERCE'S DISEASE OF GRAPEVINE IN TAIWAN

Co-Project Leader: Jianchi Chen | San Joaquin Valley Agricultural Sciences Center | USDA Agricultural Research Service | Parlier, CA 93648 | jianchi.chen@usda.gov
Co-Project Leader: Hsien-Tzung Shih | Applied Zoology Division | Taiwan Agricultural Research Institute | Council of Agriculture, Taiwan, ROC | htshih@tari.gov.tw
Collaborator: Chiou-Chu Su | Pesticide Application Division | Taiwan Agricultural Chemicals and Toxic Substances Research Institute | Council of Agriculture, Taiwan, ROC | auba@tactri.gov.tw
Collaborator: Chung-Jan Chang | Department of Plant Pathology | University of Georgia | Griffin, GA 30212 | cchang1@uga.edu

Reporting Period: The results reported here are from work conducted October 2018 to September 2019.

ABSTRACT

Kolla paulula (Hemiptera: Cicadellidae) is a plant sap-feeding insect and vector transmitting *Xylella fastidiosa* subsp. *fastidiosa* causing Pierce disease of grapevines in Taiwan. The insect was found in Taiwan and other regions in Asia but not in the Americas. Little is known about the biology of *K. paulula*. One important research area related to the recent next generation sequencing (NGS) technology is insect microbiota/microbiome. Manipulation of microbiota in insect vectors such as the glassy-winged sharpshooter (*Homalodisca vitripennis*) has been proposed as a potential strategy for Pierce's disease control in California. Members of the plant sap-feeding insect suborder Auchenorrhyncha (Hemiptera) have at least two obligate bacterial symbionts for essential amino acid synthesis: a highly conserved symbiont (*Candidatus* Sulcia muelleri) and a second symbiont species varying among different insect species. This research project explored the use of NGS technology to study endosymbionts of *K. paulula*. A colony of *K. paulula* was established and maintained on *Commelina diffusa* under laboratory conditions. Adult insects were collected and preserved in 70% alcohol solution. DNA was extracted from a single insect using a DNeasy blood and tissue kit (Qiagen). Whole-genomic DNA was amplified using an illustra GenomiPhi version 2 DNA amplification kit (GE Healthcare) and sequenced using Illumina MiSeq format after library preparation. A total of 28,327,432 reads (151 bp each) were generated. *De novo* assembling was performed with CLC Genomics Workbench software. The top 20 largest contigs were initially used for BLASTn search against GenBank nr (non-redundant) database. Sequences of "*Ca.* Sulcia muelleri" were detected and collected to assemble the draft genome sequence (designated as Strain KPTW1, GenBank accession QWZP00000000). The KPTW1 strain has a genome size of 253,942 bp, GC content of 22.7%, 237 predicted protein-coding genes, and 34 RNA genes. Interestingly, no taxonomy identity could be assigned to the largest *De novo* contig (251,844 bp) according to GenBank nr database. Sequence annotation was performed and a 1,530 bp 16S rRNA gene sequence was identified. According to GenBank 16S rRNA sequence database, the DNA sequence was similar (99% query coverage and 80% identity) to that of *Caedimonas varicaedens*, an endosymbiont bacterium of the Ciliate *Paramecium biaurelia*. It is suggested that the *Caedimonas*-like bacterium could be the second symbiont of *K. paulula*.

FUNDING AGENCIES
Funding for this project was provided by USDA Agricultural Research Service appropriated project 5302-22000-008-00D.

Additional Note: Mention of trade names or commercial products in this paper is solely for the purpose of providing specific information and does not constitute endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

INSIGHTS FOR UNDERSTANDING NEMAGUARD RESISTANCE MECHANISM AGAINST *XYLELLA FASTIDIOSA*

Project Leader: Rodrigo Krugner | San Joaquin Valley Agricultural Sciences Center | USDA Agricultural Research Service | Parlier, CA 93648 | rodrigo.krugner@usda.gov
Co-Project Leader: Craig Ledbetter | San Joaquin Valley Agricultural Sciences Center | USDA Agricultural Research Service | Parlier, CA 93648 | craig.ledbetter@usda.gov
Co-Project Leader: Elizabeth Rogers | San Joaquin Valley Agricultural Sciences Center | USDA Agricultural Research Service | Parlier, CA 93648 | elizabeth.rogers@usda.gov
Co-Project Leader: Lindsey Burbank | San Joaquin Valley Agricultural Sciences Center | USDA Agricultural Research Service | Parlier, CA 93648 | lindsey.burbank@usda.gov

Reporting Period: The results reported here are from work conducted March 2013 to October 2019.

ABSTRACT

The rootstock "Nemaguard" was selected by the USDA and released in 1959 as a rootstock for almond and stone fruits due to resistance to nematodes and enhanced scion vigor. Nemaguard happens to be resistant to *Xylella fastidiosa* (*Xf*), the causal agent of almond leaf scorch disease. Previous research showed that prior to June-budding, use of this rootstock prevents infection of almond nursery stock by *Xf*. Further, the rootstock also promotes an apparent complete pathogen elimination and remission of leaf scorching symptoms in infected susceptible scions. However, nothing is known about potential chemical and physical mechanisms of resistance to *Xf* infection. The goals of the current study were (1) to determine whether insect vector feeding periods on Nemaguard can reduce bacterial populations in vectors or its transmission efficiency to susceptible plants, and (2) to evaluate establishment and movement of *Xf* in Nemaguard compared to susceptible plants. After acquiring *Xf* from infected grapevines, vector access periods of up to 14 days on Nemaguard did not reduce pathogen population densities in vectors or transmission efficiency of *Xf* to susceptible plants when compared to controls. Mechanical inoculation of *Xf* to almond resulted in systemic infection and expression of typical almond leaf scorch symptoms, whereas ongoing analysis of Nemaguard samples have shown no survival or establishment of *Xf* at or beyond the point of inoculation. Results showed that Nemaguard xylem sap does not reduce *Xf* populations in infected vectors, indicating that natural chemical properties of Nemaguard xylem sap are not involved in resistance to *Xf*. Collectively, results suggest that future research should focus on identification of potential physical traits that prevent movement of bacterial cells within the plant.

FUNDING AGENCIES

Funding for this project was provided by the USDA Agricultural Research Service, appropriated project 2034-22000-012-00D.

MOLECULAR CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF *PILH* IN THE PATHOGENICITY OF *XYLELLA FASTIDIOSA*

Project Leader: Hong Lin | San Joaquin Valley Agricultural Sciences Center | USDA Agricultural Research Service | Parlier, CA 93648 | hong.lin@usda.gov
Cooperator: Xiangyang Shi | San Joaquin Valley Agricultural Sciences Center | USDA Agricultural Research Service | Parlier, CA 93648 | xiangyang.shi@usda.gov

Reporting Period: The results reported here are from work conducted October 2018 to September 2019.

ABSTRACT
Active movement mediated by bacterial twitching has been demonstrated to be an important component of pathogenic mechanisms. *In silico* analysis suggests that PilH of *Xylella fastidiosa* (*Xf*) regulates the type IV pilus system. To elucidate the roles of *pilH* in the twitching motility and virulence of *Xf*, a *pilH*-deletion mutant (*XfΔpilH*) and complemented strain (*XfΔpilH*-C) were generated. The *XfΔpilH* mutant showed a reduction in cell-matrix adherence, cell-to-cell aggregation, and biofilm production compared to *Xf* wild-type and *XfΔpilH*-C. The typical colony peripheral fringe was not observed for *XfΔpilH* but was observed in colonies of wild-type and *XfΔpilH*-C. Furthermore, the expression of type IV pilin *pilV*, *pilX*, fimbriae *fimA,* alginate synthesis *algH,* and virulence transcriptional regulator *csrA*, *lexA*, were down-regulated in *XfΔpilH* in comparison to wild-type and *XfΔpilH*-C. Disease indexes of grapevines inoculated with *XfΔpilH* were low compared to grapevines inoculated with wild-type and *XfΔpilH*-C. These results indicate that PilH contributes to the pathogenicity of *Xf* via regulation of type IV pilus and affects the twitching motility, cell aggregation, and biofilm formation required for development of Pierce's disease of grapevine.

LAYPERSON SUMMARY
Xylella fastidiosa (*Xf*) is the bacterium that causes Pierce's disease of grapevines. To reveal the role of pathogenicity associated with molecular mechanisms of *Xf*, genetic analyses were conducted to compare phenotypes of wild-type and a mutant strain of *Xf* with a defective pilH gene. Greenhouse experiments demonstrated that grapevines inoculated with the mutant strain showed significantly reduced Pierce's disease symptoms as compared to grapevines infected with *Xf* wild-type. Information derived from this study will improve our knowledge of the disease and facilitate development of Pierce's disease management.

FUNDING AGENCIES
Funding for this project was provided by the USDA Agricultural Research Service, appropriated project 2034-22000-010-00D.

Section 2:

Glassy-winged Sharpshooter

RIVERSIDE COUNTY GLASSY-WINGED SHARPSHOOTER PROGRAM: TEMECULA VALLEY AND COACHELLA VALLEY

Project Leader: Matt Daugherty | Department of Entomology | University of California | Riverside, CA 92521 | matt.daugherty@ucr.edu
Co-Project Leader: Carmen Gispert | Cooperative Extension | University of California | Indio, CA 92201 | cgispert@ucanr.edu
Researcher: Christina Hoddle | Department of Entomology | University of California | Riverside, CA 92521 | christina.hoddle@ucr.edu

Reporting Period: The results reported here are from work conducted November 2018 to October 2019.

ABSTRACT

For nearly 20 years portions of Riverside County have been part of an area-wide management program for an invasive vector, the glassy-winged sharpshooter (*Homalodisca vitripenni*s; 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. The area-wide management program originally consisted of insecticide applications to citrus groves along with monitoring of GWSS populations – both to evaluate the effectiveness of the treatments and to guide grape grower treatment decisions. The treatment element of the program was halted in 2013 for the Temecula Valley, and both monitoring and treatment were halted several years prior for the Coachella Valley. Monitoring of GWSS populations continues to occur in the Temecula Valley, with approximately 180 yellow sticky traps placed throughout citrus groves and select vineyards being inspected on a biweekly basis. This season approximately 150 traps were also deployed at the interface of vineyards and citrus groves throughout the Coachella Valley. In the Temecula Valley, GWSS peak activity was noticeably delayed compared to prior years and was, overall, of intermediate magnitude. In the Coachella Valley, GWSS appears to be rare if not absent from the area. Collectively these results show substantial differences in GWSS activity between the two regions, neither of which is indicative of the resurgence of GWSS populations that have been reported in other areas of California.

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 Riverside County, area-wide management programs played an important role in reducing the impact of this invasive vector and disease following severe disease outbreaks nearly 20 years ago. Monitoring continues to occur for GWSS to guide grape grower management decisions by identifying those areas and those times of year where the vector is most active. This season, results of monitoring in Temecula Valley citrus and winegrape vineyards showed a delay in GWSS activity compared to past years, with overall moderate numbers of insects. Monitoring near Coachella Valley table grape and raisin vineyards, which has not occurred for several years, indicates that GWSS is rare if not absent from the area.

INTRODUCTION

Recent economic analyses of the impact of Pierce's disease (PD) estimate its cost at more than $100 million per year in California (Tumber et al., 2014). In Southern California, the bulk of that impact is attributable to the activity of the invasive glassy-winged sharpshooter (Almeida et al., 2005). For example, in the PD epidemic of the early 2000s, at which time there were observations in the Temecula Valley of hundreds of glassy-winged sharpshooters (*Homalodisca vitripennis*; GWSS) per vine, there are anecdotal reports of 100% of vines in some vineyards becoming infected in a single season. Overall, it is estimated that 30% of vines in the Temecula Valley were lost to PD over the course of a few years.

In response to PD epidemics occurring in the Temecula Valley and other grape-growing areas in California, area-wide management programs were established to mitigate the effect of GWSS. These programs consisted of monitoring for GWSS populations and coordinated insecticide treatments of citrus to minimize the number of GWSS moving from citrus groves into vineyards in the summer. Insecticide applications typically include a spring application of a systemic neonicotinoid (e.g., imidacloprid) to citrus trees to target emerging nymphs, and perhaps spring or summer applications of foliar insecticides, especially in organic groves. Additionally, regular releases of biological control agents were made throughout the region to complement other GWSS control efforts, and growers were encouraged to reduce pathogen supply in the landscape by removing infected grapevines and other reservoir hosts. Collectively, these efforts were extremely successful at managing GWSS and PD. For example, surveys in the Temecula Valley approximately a decade after the initiation of the area-wide management program estimated that GWSS abundance had decreased 2,000-fold relative to its peak, and PD prevalence averaged less than 1% (Daugherty et al., 2015).

Despite the past success with GWSS and PD management, continued vigilance is needed to mitigate future impacts of this pest and disease. Indeed, in Kern County, GWSS populations have rebounded substantially over the last several years (Haviland and Stone-Smith, 2016). Moreover, in the Temecula Valley two seasons ago more than 50% more GWSS were caught than at any time since the area-wide management program was established (Daugherty and Soto, 2017).

In Riverside County there are two major grape production areas, both of which also have significant citrus production. In the Temecula Valley and surrounding areas there are approximately 4,500 acres of citrus (approximately 1/3 of which is grown in the Temecula Valley itself) and 2,500 acres of winegrapes. In the Coachella Valley, there are approximately 7,000 acres of table grapes and 8,500 acres of citrus. We conducted regular GWSS monitoring in both of these areas to help guide grower decision-making.

OBJECTIVES

1. Monitor regularly GWSS populations in citrus groves throughout the Temecula Valley.
2. Monitor GWSS in select Temecula Valley vineyards adjacent to identified GWSS hotspots in citrus.
3. Monitor GWSS populations in select citrus groves throughout the Coachella Valley.
4. Disseminate newsletters for stakeholders on sharpshooter seasonal abundance in the Temecula and Coachella valleys.

Double-sided yellow sticky cards (14 x 22 cm; Seabright Laboratories, Emeryville, CA) are being used to monitor for adult sharpshooters in citrus. Approximately 180 such sticky traps were deployed in citrus groves and select vineyards throughout the Temecula Valley. An additional 150 traps were deployed throughout the Coachella Valley at the interface between vineyards and citrus groves.

All traps were labeled, then geo-referenced with a hand-held global positioning system (GPS) monitor. Traps were attached with large binder clips to wooden stakes around the perimeter of the grove. For large groves traps were also placed in the interior. The total number of traps depended on the size of the block.

The traps were inspected and replaced approximately every two weeks during the summer and fall (May through October) and monthly the rest of the year. Trapping in the Coachella Valley began in July. At each inspection the number of adult GWSS and smoketree sharpshooters (*Homalodisca liturata*; STSS) were recorded, and the abundance of common generalist natural enemy taxa.

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

RESULTS AND DISCUSSION

Monitoring results from the Temecula Valley showed a distinct delay in peak trap catch compared to prior years (**Figure 1**). Specifically, peak GWSS catch occurred in early August, which is three to four weeks later than in most years, with a smaller secondary peak occurring in mid-September, as occurs in some but not all years. Overall GWSS catch was intermediate – far lower than in 2017, but also higher than especially low years, such as 2010 and 2011 (**Figure 1**).

In the Coachella Valley, no traps captured GWSS adults over the six censuses conducted to date (**Figure 2**). Over this same period, the native STSS was captured at variable but fairly low numbers (**Figure 2**).

CONCLUSIONS

GWSS in the Temecula Valley continues to exemplify an insect whose dynamics show substantial interannual variability, with overall trap activity this year that was intermediate relative to past years. In light of such variability, Temecula Valley grape growers are encouraged to remain vigilant with respect to the monitoring and management activities for GWSS and PD in their vineyards. Conversely, in the Coachella Valley, GWSS appears to be rare if not absent entirely from the area. Collectively these results indicate there are substantial differences in GWSS activity between the two growing regions, neither of which is obviously consistent with the resurgence of GWSS populations that has been reported in other areas of California.

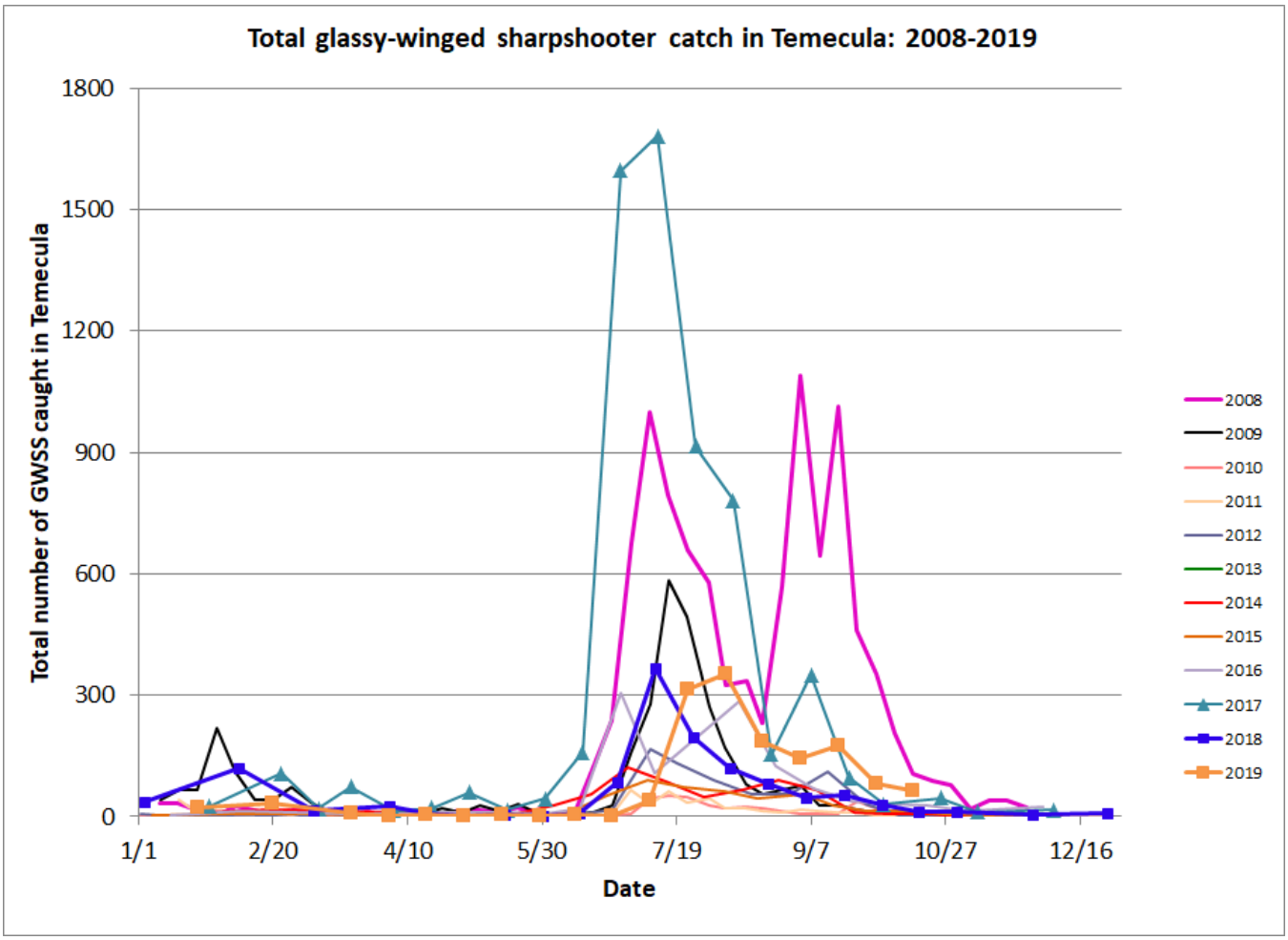


Figure 1. Seasonal total GWSS catch in 2019 compared to prior years.

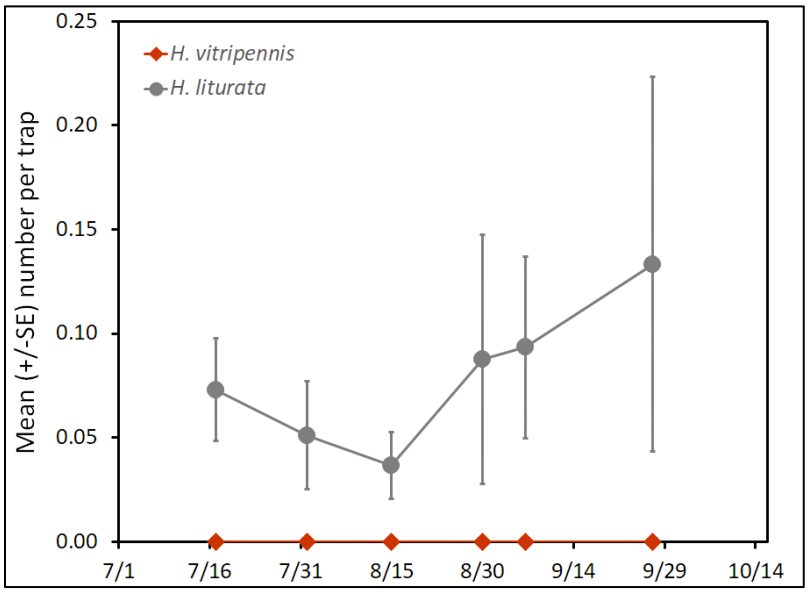


Figure 2. GWSS and STSS catch in Coachella Valley

REFERENCES CITED

Almeida RPP, Blua MJ, Lopes JRS, Purcell AH. 2005. Vector transmission of *Xylella fastidiosa*: Applying fundamental knowledge to generate disease management strategies. *Annals of the Entomological Society of America* 98: 775–786.

Daugherty MP, O'Neill S, Byrne F, Zeilinger A. 2015. Is vector control sufficient to limit pathogen spread in vineyards? *Environmental Entomology* 44: 789-797.

Daugherty MP, Soto D. 2017. The Riverside County glassy-winged sharpshooter program in the Temecula Valley. *Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes*. California Department of Food and Agriculture, Sacramento, CA, pp. 153-156.

Haviland D, Stone-Smith B. 2016. Monitoring glassy-winged sharpshooter and Pierce's disease in Kern County, California. *Proceedings of the 2016 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA, pp. 75-80.

Tumber K, Alston J, Fuller K. 2014. Pierce's disease costs California $104 million per year. *California Agriculture* 68: 20-29.

FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease Control Program.

ACKNOWLEDGEMENTS

We extend our thanks to the Temecula Valley and Coachella Valley citrus and grape growers for their continued cooperation in making this work possible.

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

Project Leader: Richard Redak | Department of Entomology | University of California | Riverside, CA 92521 | richard.redak@ucr.edu
Project Leader: Bradley White | Department of Entomology | University of California | Riverside, CA 92521 | bradley.white@ucr.edu
Project Leader: Frank Byrne | Department of Entomology | University of California | Riverside, CA 92521 | frank.byrne@ucr.edu
Cooperator: Matt Daugherty | Department of Entomology | University of California | Riverside, CA 92521 | matt.daugherty@ucr.edu
Cooperator: David Morgan | Pierce's Disease Control Program | California Department of Food and Agriculture | Riverside, CA 92501 | david.morgan@ucr.edu
Cooperator: David Haviland | Cooperative Extension | University of California | Bakersfield, CA 93307 | dhaviland@ucanr.edu
Cooperator: Judy Zaninovich | Exeter, CA 93221 | jsleslie@msn.com
Cooperator: Jason Stajich | Department of Microbiology and Plant Pathology | University of California | Riverside, CA 92521 | jason.stajich@ucr.edu

Reporting Period: The results reported here are from work conducted October 1, 2018 to October 1, 2019.

ABSTRACT

During 2018/2019, we have continued to assess the resistance status of glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) populations in the Central Valley area of California. Imidacloprid resistance is still widespread in areas east of Bakersfield, particularly in the General Beale Road area, where selection pressure has been historically high. However, GWSS populations collected from organic citrus are also showing resistance to imidacloprid, indicating movement of resistant insects from conventional groves. North of Bakersfield along Highway 65, the organic groves still show resistance to imidacloprid, albeit lower than that present at General Beale. Resistance in Tulare County is also more pervasive. In 2019, we revisited a Tulare organic site that we sampled during our 2016 monitoring campaign and found that the insects there were still susceptible to imidacloprid. GWSS collected from citrus in Temecula remain susceptible.

In 2019, we have been largely focusing on determining which compounds are effective at breaking the imidacloprid resistance. While the insects exhibited cross-resistance to the neonicotinoid acetamiprid (Assail), another neonicotinoid (thiamethoxam; Platinum and Actara) remains effective, with little separation in dose-response between the Highway 65 (organic), General Beale (conventional), Breckenridge (organic), and Tulare (conventional) populations.

Flupyradifurone (Sivanto) is a newer compound available to growers. There was a modest shift in susceptibility in the General Beale population, compared with Breckenridge and Highway 65. While we are not concerned at this time with the use of flupyradifurone, it is important to

continue monitoring GWSS populations in the valley over the next few years so that any shifts in tolerance can be documented and acted upon if necessary

The pyrethroid fenpropathrin remains highly effective against General Beale GWSS, with no change in tolerance compared with 2018. However, we have previously shown that imidacloprid-resistant populations do exhibit some cross-resistance to this compound, and we recommend monitoring for pyrethroid resistance as usage of fenpropathrin is likely to increase over the coming years as efforts to manage Asian citrus psyllid intensify in the Valley. We also compared the efficacies of pyrethrum extract and fenpropathrin in bioassays and found that the organic product (pyrethrum) is less effective than the synthetic option. This result further emphasizes the need to maintain vigilance in our use of pyrethroids, since resistance to the synthetic products could compromise their organic counterparts that are intrinsically less effective. With fewer products available for organic citrus pest management, the movement of resistant insects from conventionally managed groves into neighboring organic groves could have a serious impact on pest management within the organic system.

We are using biochemical and molecular techniques to investigate putative resistance mechanisms to imidacloprid. In 2019, we established a new RNAseq experiment using two resistant and two susceptible populations. In addition, we are conducting whole genome sequencing on susceptible and resistant populations, as this approach may provide greater insight into differences between the two genotypes. As part of a longer-term effort, we have also collected GWSS insects from nursery and urban locations, so that we can compare DNA sequence data for sodium channel (pyrethroid target site) and nicotinic acetylcholine receptor (neonicotinoid) genes, amongst others, in insects from broad geographical and host plant ranges 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 neonicotinoid, pyrethroid, and carbamate 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) between populations collected from citrus groves in Kern, Tulare, and Riverside Counties. Our data suggest that imidacloprid resistance confers cross resistance to acetamiprid (neonicotinoid) and mild cross resistance to fenpropathrin (pyrethroid). Historically, the imidacloprid resistance appears to be directly related to usage, with the highest levels of resistance occurring in populations receiving conventional insecticide treatments. However, we now routinely detect high levels of imidacloprid resistance in GWSS populations sampled from organic groves, presumably due to the movement of insects from conventional groves, where resistance was originally selected for, into adjacent organic groves. The neonicotinoid thiamethoxam still appears to be effective against the imidacloprid-resistant populations, despite belonging to the same insecticide class. Products not in the neonicotinoid class also remain effective against GWSS, including fenpropathrin. Despite

modest cross resistance, this compound is still effective under field conditions. In addition, the newer compound flupyradifurone (Sivanto) also shows good efficacy in 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 pyrethroid or carbamate treatments was 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 by USDA and CDFA 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 that area remains high, as similar management approaches are in use in the Temecula area 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 often required. These often include applications of fenpropathrin (pyrethroid insecticide class) or carbaryl (carbamate insecticide class). 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 have broadened the scope of our investigations to include populations from other geographical regions (Riverside County and Tulare County). This broader approach will result in a more comprehensive report on the overall resistance status of GWSS within southern California and will contribute to the establishment of 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 acetylcholinesterase (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

2019 Monitoring Data. In 2019, bioassays were conducted on insects collected from locations in Kern County and Tulare County. Resistance to imidacloprid was confirmed in populations in the General Beale Road area, and in organic citrus populations both east (Breckenridge) and north (along Highway 65) of Bakersfield (**Figure 1**). While the resistance in the General Beale Road region is not new, we continue to see resistance in organic groves, an indication of the movement of GWSS from the conventional groves where resistance was selected into the organic systems. Full details of the bioassays are provided in the caption to **Figure 1**.

Synergism of Imidacloprid Toxicity with Piperonyl Butoxide. Bioassays with synergists can assist with the elucidation of potential resistance mechanisms that occur in insects. In an attempt to identify the mechanism conferring insecticide resistance to imidacloprid in the Central Valley populations we conducted discriminating dose bioassays on the General Beale Road resistant strain. Pre-treatment of insects with a range of piperonyl butoxide concentrations, a known inhibitor of cytochrome P450 oxidase activity, followed by treatment with 50 ng imidacloprid, did not enhance the efficacy of imidacloprid. A number of dosing strategies were evaluated (pre-treatment with the synergist versus co-treatment), but none was able to improve the toxicity of imidacloprid applied at that dose. This result is in contrast to earlier findings that showed some synergism of imidacloprid toxicity by piperonyl butoxide in bioassays with Tulare conventional and Highway 65 insects, and suggests that either cytochrome P450s are not involved in the resistance in these insects, or that the synergist does not inhibit enzymes that are detoxifying the insecticide. We are hopeful that the RNAseq experiment (described below) will provide more insight into what the mechanism involved is.

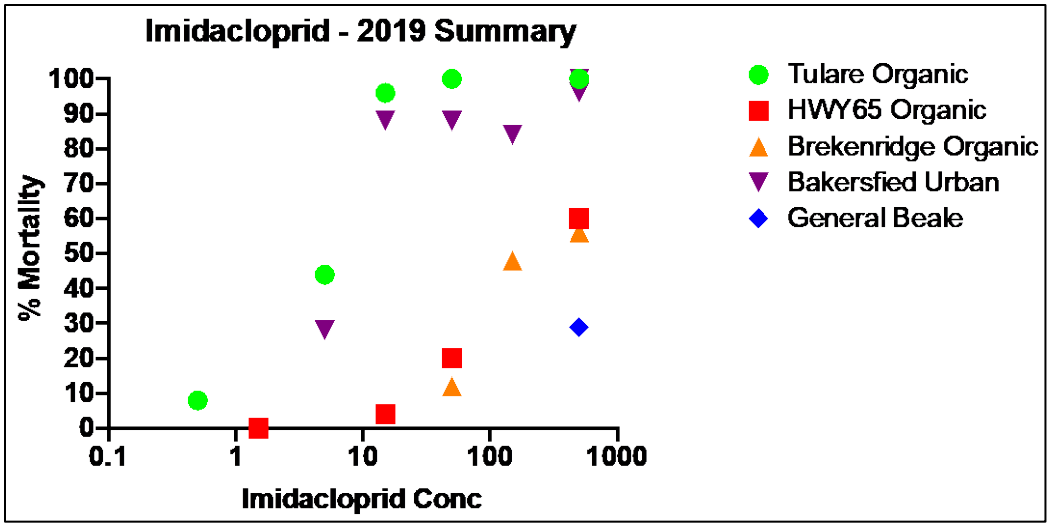


Figure 1. Toxicological response of GWSS adults to the neonicotinoid imidacloprid applied topically to the abdomen. Mortality was assessed at 48 hours post-treatment. Tulare Organic (green circles) was collected from an organic grove in Tulare County and showed similar levels of imidacloprid susceptibility to those measured in 2016 to 2018. Similar levels of susceptibility were also measured in an urban population from Bakersfield (purple triangle). A discriminating dose bioassay on insects from the General Beale Road (blue diamond) area confirmed a high degree of resistance to imidacloprid. GWSS sampled from organic citrus at two sites (Breckenridge and Highway 65) also expressed resistance to imidacloprid, although not at the same level as insects from General Beale.

Thiamethoxam Bioassays

Thiamethoxam is a neonicotinoid insecticide and belongs to the same insecticide class as imidacloprid. It is used as either a systemic soil treatment or as a foliar treatment. Thiamethoxam remains highly effective against insects expressing resistance to imidacloprid (**Figure 2**). While there is good separation between General Beale Road and Highway 65 populations with respect to imidacloprid resistance (**Figure 1**), no such separation occurs with thiamethoxam. In fact, there is good overlap of the dose response lines for insects from all sites tested to date, including the historical data for Agricultural Operations at UC Riverside that was measured in 2003 and the Tulare conventional site where high resistance to imidacloprid was recorded in 2017.

Flupyradifurone Bioassays

Flupyradifurone (Sivanto) is a member of the new class of butenolide insecticides and is used against a broad range of sucking insects, including those expressing resistance to neonicotinoids. Although still active against the nicotinic acetylcholine receptor (nAChR), it has a distinct toxicological profile that enables it to be used against resistant insects in which target site and metabolic resistance to neonicotinoids occur. In our bioassays, flupyradifurone toxicity levels were similar in Highway 65 and Breckenridge populations (**Figure 3**). While the insecticide was still highly toxic to General Beale insects, there was a slight separation of the dose-response lines that could indicate some level of tolerance (**Figure 3**).

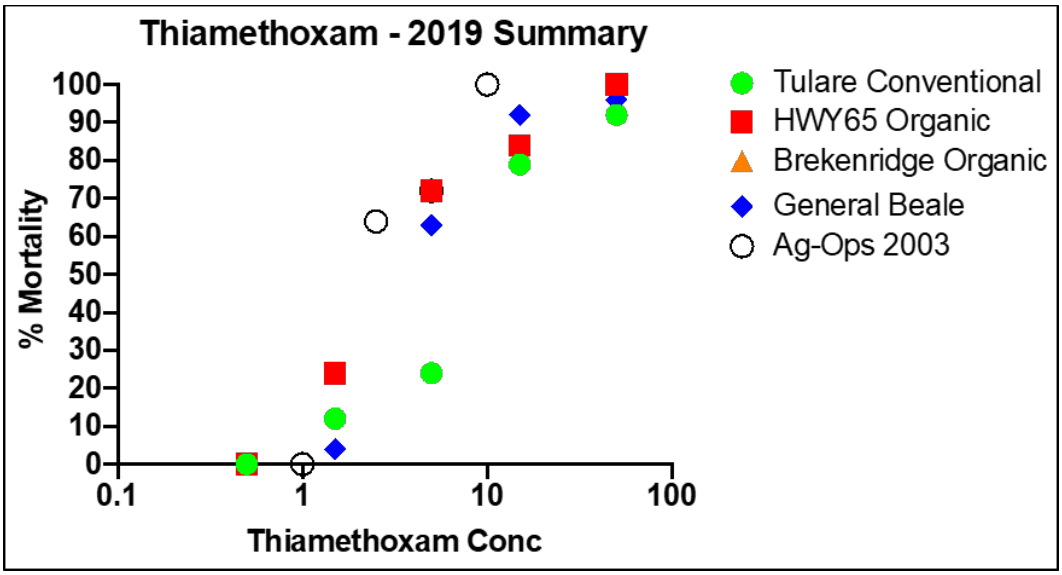


Figure 2. Toxicological response of GWSS adults to the neonicotinoid thiamethoxam applied topically to the abdomen. Mortality was assessed at 48 hours post-treatment. Data for Agricultural Operations at UC Riverside (open black circles) were generated in 2003 and are included for comparison. Despite varying degrees of resistance to imidacloprid, the insects from all these populations exhibit similar responses to thiamethoxam, indicating no apparent cross-resistance.

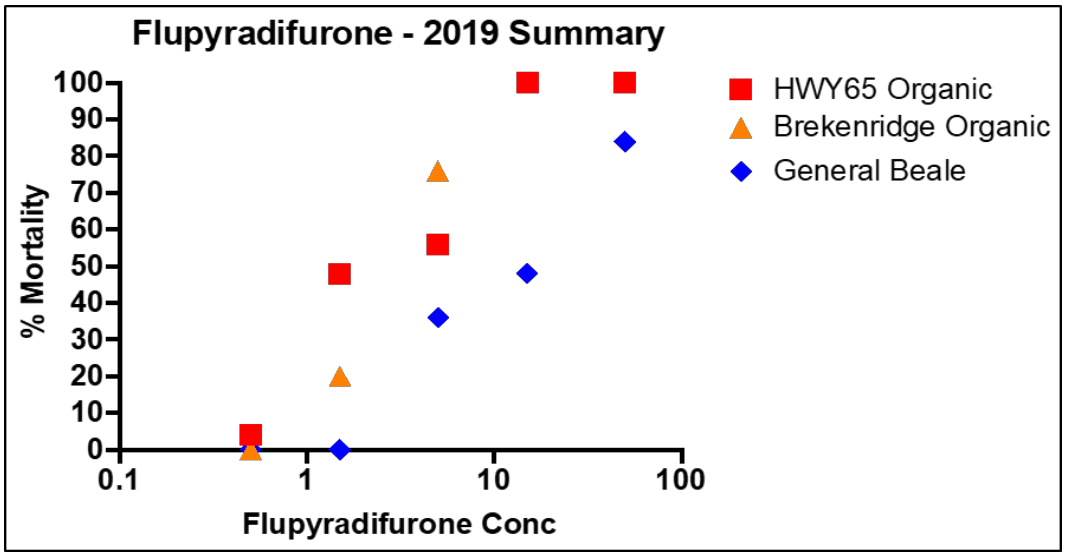


Figure 3. Toxicological response of GWSS adults to the butenolide insecticide flupyradifurone applied topically to the abdomen. Mortality was assessed at 48 hours post-treatment. Despite varying degrees of resistance to imidacloprid the insects from all these populations exhibited similar responses to flupyradifurone, although the slight separation of the General Beale population warrants scrutiny to ensure that the imidacloprid resistance is not conferring some level of cross-resistance.

Pyrethroid Bioassays

Fenpropathrin is an important pyrethroid insecticide used to control a range of pests within the citrus system. The General Beale GWSS populations that exhibit high levels of imidacloprid resistance show modest cross resistance to this insecticide. Despite distinct dose-response profiles for imidacloprid, the Breckenridge and General Beale populations showed similar responses to fenpropathrin in 2019 bioassays (**Figure 4**). We regard this result as an indication that the populations used for these bioassays are not expressing high levels of cross resistance to the pyrethroid, and that the insecticide is still largely effective against GWSS. Our first report of imidacloprid resistance in the General Beale Road area was in 2016, and was for a population of GWSS collected further south of the location where the population used in our most recent monitoring work was sampled (the decision is based on insect availability). The original population was practically immune to imidacloprid, whereas the more recent collections show some level of response, albeit still highly resistant.

In addition to fenpropathrin we also tested a pyrethrum extract, which contains the same active ingredients as the organic-approved insecticide Pyganic. Pyganic is one of the few products organic growers have available to them for pest control. For this series of bioassays we included insects from groves located in both the Riverside and Temecula areas (both susceptible to imidacloprid) and compared their responses to insects collected from the Breckenridge and General Beale areas. The response of the insects to the pyrethrum extract was similar for all populations (**Figure 5**). However, the pyrethrum extract was considerably less toxic than fenpropathrin for both the Breckenridge and General Beale insects (Riverside and Temecula insects were not included in this comparison).

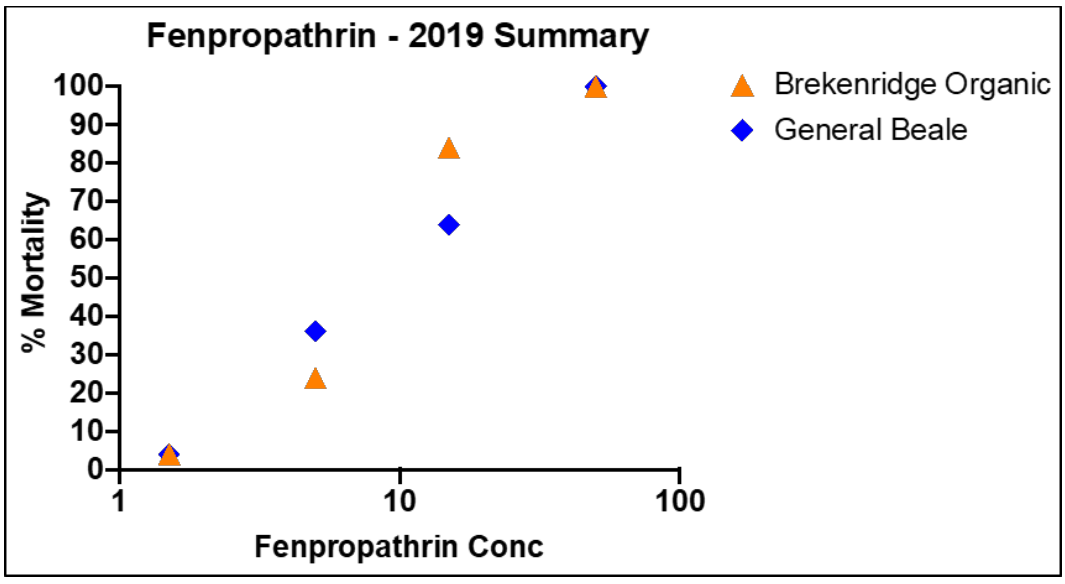


Figure 4. Toxicological response of GWSS adults to the pyrethroid fenpropathrin applied topically to the abdomen. Mortality was assessed at 48 hours post-treatment. Despite varying degrees of resistance to imidacloprid, the insects from all these populations exhibited similar responses to fenpropathrin, indicating a lack of significant cross-resistance.

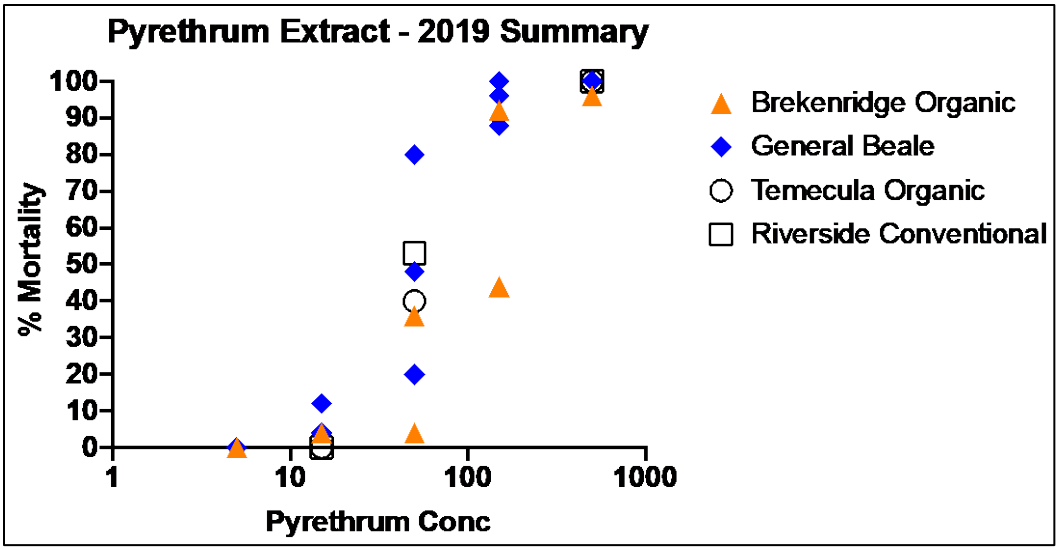


Figure 5. Toxicological response of GWSS adults to pyrethrum extract applied topically to the abdomen. Mortality was assessed at 48 hours post-treatment. Despite varying degrees of resistance to imidacloprid, the insects from all these populations exhibit similar responses to the extract, indicating a lack of significant cross-resistance. Note the different levels of toxicity between the natural pyrethrins and the synthetic pyrethroid (fenpropathrin data in **Figure 4**).

Genetic Analysis

The analysis of RNA-seq data generated for the Tulare, Highway 65 and General Beale Road populations is ongoing. In addition, we have initiated a new RNAseq study in which we have included two susceptible populations (Temecula and Tulare organic), and two resistant populations (General Beale and Tulare conventional). Bioassays were used to select out the most resistant insects from the General Beale and Tulare conventional strains, while bioassays were used to confirm the full susceptibility of a pool of insects from the Tulare organic and Temecula field sites from which insects used for the RNAseq study were chosen.

CONCLUSIONS

We have confirmed the variable levels of resistance to imidacloprid in Central Valley populations of GWSS and confirmed with the most recent monitoring data that the resistance not only extends into Tulare County, but is also pervasive within organic groves. 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 to 2017 bioassay data for a population collected from an organic grove in Tulare County. We have already reported on the presence of cross resistance to acetamiprid, which should preclude the use of this insecticide as an alternative management option for insects where imidacloprid resistance has been identified. In addition to imidacloprid resistance, we have also identified low levels of resistance to the pyrethroid fenpropathrin, although this resistance only seems to be expressed in the most highly imidacloprid-resistant populations. The pyrethroids continue to work effectively against imidacloprid-resistant GWSS. However, continued monitoring for pyrethroid resistance should be a high priority if this important insecticide class is to remain effective. In addition to the pyrethroids, we have shown that the butenolide insecticide

flupyradifurone is an effective insecticide against GWSS, providing similar levels of efficacy to insects that are susceptible and resistant to imidacloprid.

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, acetamiprid, and fenpropathrin will identify specific enzymes that are involved in conferring resistance.

REFERENCES CITED

Byrne FJ, Toscano NC. 2005. Characterization of neonicotinoids and their plant metabolites in citrus trees and grapevines, and evaluation of their efficacy against the glassy-winged sharpshooter and the egg parasitoid *Gonatocerus ashmeadi*. *Proceedings of the 2005 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA, pp. 287-289.

Byrne FJ, Toscano NC. 2006. Detection of *Gonatocerus ashmeadi* (Hymenoptera: Mymaridae) parasitism of *Homalodisca coagulata* (Homoptera: Cicadellidae) eggs by polyacrylamide gel electrophoresis of esterases. *Biological Control* 36: 197-202.

Nauen R, Hungenberg H, Tollo B, Tietjen K, Elbert A. 1998. Antifeedant-effect, biological efficacy and high affinity binding of imidacloprid to acetylcholine receptors in tobacco associated *Myzus persicae* (Sulzer) and *Myzus nicotianae* Blackman (Homoptera: Aphididae). *Pesticide Science* 53: 133-140.

Redak R, White B, Byrne FJ. 2015. Management of insecticide resistance in glassy-winged sharpshooter populations using toxicological, biochemical, and genomic tools. *Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes*. December 2015. California Department of Food and Agriculture, Sacramento, CA., pp. 152-158.

Redak R, White B, Byrne FJ. 2016. Management of insecticide resistance in glassy-winged sharpshooter populations using toxicological, biochemical, and genomic tools. *Proceedings of the 2016 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA, pp. 230-236.

Redak R, White B, Byrne FJ. 2017. Management of insecticide resistance in glassy-winged sharpshooter populations using toxicological, biochemical, and genomic tools. *Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes*. December 2017. California Department of Food and Agriculture, Sacramento, CA., pp. 163-169.

Redak R, White B, Byrne FJ. 2018. Management of insecticide resistance in glassy-winged sharpshooter populations using toxicological, biochemical, and genomic tools. *Proceedings of the 2018 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA, pp. 161-166.

FUNDING AGENCIES

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

Section 3:

Other Pests and Diseases of Winegrapes

GRAPEVINE VIRUS MANAGEMENT IN LODI: A COLLABORATIVE RESEARCH AND INTEGRATED OUTREACH EFFORT TO HELP SOLVE A STATEWIDE CHALLENGE

Project Leader: Stephanie L. Bolton | Lodi Winegrape Commission | Lodi, CA 95242 | stephanie@lodiwine.com
Cooperator: Kyle Brown | LangeTwins Family Winery and Vineyards | Acampo, CA 95220 | kbrown@langetwins.com
Cooperator: Matt Frank | Trinchero Family Estates | St. Helena, CA 94574 | mfrank@tfewines.com
Cooperator: Aaron Lange | LangeTwins Family Winery and Vineyards | Acampo, CA 95220 | aaron@langetwins.com
Cooperator: Neil McRoberts | Department of Plant Pathology | University of California | Davis, CA 95616 | nmcroberts@ucdavis.edu
Cooperator: Norm Peters | Trinchero Family Estates | St. Helena, CA 94574 | dpeters@tfewines.com
Cooperator: Nicholas Podsakoff | Wonderful Nurseries | Wasco, CA 93280 | nicholas.podsakoff@wonderful.com
Cooperator: Paul Precissi | Precissi Ag Services | Lodi, CA 95242 | paul.precissi@gmail.com
Cooperator: Tia Russell | Duarte Nurseries | Hughson, CA 95326 | tia@duartenursery.com
Cooperator: Charlie Starr, IV | Viticultural Services and Crush District 11 Grower | Acampo, CA 95220 | cstarriv@gmail.com
Cooperator: Chris Storm | Vino Farms and Crush District 11 Grower | Lodi, CA 95240 | cstorm@vinofarms.net
Cooperator: Keith Striegler | E. & J. Gallo | Acampo, CA 95220 | richard.striegler@ejgallo.com
Cooperator: Karen Suslow | Department of Natural Sciences and Mathematics | Dominican University of California | San Rafael, CA 94901 | karensuslow@gmail.com
Cooperator: Paul Verdegaal | Cooperative Extension (Emeritus) | Stockton, CA 95206 | psverdegaal@ucanr.edu

Reporting Period: The results reported here are from work conducted October 2017 to October 2019.

ABSTRACT

Three economically important viruses - leafroll, red blotch, and fanleaf - are devastating the winegrape industry by decreasing yields, lowering fruit quality, inhibiting cluster ripening, and decreasing the lifespan of vineyards. The Lodi Grapevine Virus Research Focus Group (Virus Focus Group), formed in October 2017, has begun to provide detailed, real-world advice on virus management topics such as how to rogue, how to economically test for viruses, how to replant after leafroll, and how to order clean grapevines. By taking into consideration a thorough review of virus management in the literature (previous studies), current virus research projects, regional perceptions of viruses, and management of viruses internationally (especially in South Africa and New Zealand), the Virus Focus Group is producing practical advice for growers while demonstrating why it is of utmost importance financially to manage viruses now. Additionally, the Virus Focus Group will serve as a communication network between growers, pest control advisors, nurseries, laboratories, extension personnel, County Agricultural Commissioners, the

California Department of Food and Agriculture, Foundation Plant Services, and researchers, ensuring a long-term sustainable strategy for virus management in California. The overall objective is to learn how to best manage and prevent grapevine virus diseases in the 110,000 acres of Crush District 11, providing outreach tools and strategies to be shared with other regions across California.

LAYPERSON SUMMARY

Grapevine viruses pose a severe threat to the sustainability of California viticulture. Unfortunately, there is little faith in virus prevention at any level. Growers are losing contract dollars as wineries reject grape loads due to virus-induced ripening problems. The good news is that there are virus management strategies which growers can implement right now in the short-term, which can be taught through real-world, hands-on integrated outreach from a team of growers, extension personnel, pest control advisors, and scientists. With the right communication, a long-term cooperative virus strategy can save the California industry from devastating future losses.

INTRODUCTION

Three main viruses - grapevine red blotch virus, grapevine leafroll-associated viruses, and grapevine fanleaf virus - are currently resulting in not only a great deal of confusion but also significant economic losses for winegrowers throughout California. Each of these viruses can cause general vine decline, decreased yields, difficulty ripening, poor fruit quality, shortened vineyard life spans, and decreased ability of a vine to handle other stresses (Martelli, 2014; Sudarshana, 2015). Virus infections have resulted in the loss of grape contracts, the need to rogue infected vines, and the need to remove entire vineyards (if the infection is greater than 26-30% of vines, depending on which economic model a grower chooses to follow) (Atallah, 2012; Ricketts, 2017). One recent study found that for grapevine red blotch disease alone, a high infection rate costs up to $27,741 per acre (Ricketts, 2017). For leafroll, a study in New York found the economic impact of ignoring the virus to be between $10,117 to $16,188 per acre (Atallah, 2012). Vine mealybugs (*Planococcus ficus*) complicate the virus challenge as they are an extremely efficient vector of at least five leafroll-associated viruses (Engelbrecht and Kasdorf, 1990; Tsai et al., 2010). It only takes one mealybug to infect a vine, and virus transmission can occur in as few as 1 to 24 hours (Golino et al., 2002; Tsai et al., 2008). Circumstantial evidence points towards a carryover effect with leafroll virus caused by mealybugs, where clean vines planted in the space where leafroll-infected vines existed previously can readily become infected (Pietersen, 2016). It is imperative to combine outreach on vine mealybugs with management of leafroll-associated viruses via collaboration between Lodi's Mealybug Biocontrol Research Focus Group (funded by the American Vineyard Foundation and the Lodi Winegrape Commission) and the Virus Focus Group.

In fact, it will take a joint effort by all sectors of the industry to find a sustainable solution which will allow growers to continue profitably farming winegrapes. Growers need more education to make responsible virus management decisions. Even when responsible growers plan ahead and pay extra for CDFA-certified material, viruses and/or mealybug vectors are too often slipping through registered nursery doors. Preliminary case study collections are uncovering a lack in formal reporting procedures for when this scenario occurs, making it difficult for the industry to know there is need for improvement in virus prevention protocols. When 300-acre vineyards

must be ripped out due to a virus infection after being in the ground for less than four years, there is a problem. The best way to learn is by doing, and Lodi growers are learning the hard way that ignoring grapevine viruses, either individually or as an industry, is one expensive mistake.

Despite many costly experiences with virus-infected grapevines, it has been surprising to discover that no one in Lodi has a working "virus best management protocol" in place. A true protocol would need to include nursery ordering, replanting following a leafroll infection, employee education, mealybug and ant control, scouting and roguing procedures, economic thresholds, sampling and testing procedures, mapping, and a great deal of organized record-keeping. For a grower or even a large vineyard operation to have the depth of knowledge and time required to create such a management protocol for viruses would be nearly impossible. Luckily, the Virus Focus Group is investing the time and skills of an entire team to learn everything they can about viruses and their management, and then distributing this knowledge in the form of easily understandable, integrated outreach.

Growers need answers on how to manage viruses now, and they need to hear economically relevant stories to decide for themselves why they should care about viruses. Even many well-educated growers are left thinking, "Is it worth it for me to worry about viruses if they are everywhere? Even if I knew how to manage for them, I couldn't afford it." Add in a general lack of knowledge about the different viruses - leafroll, red blotch, fanleaf, and vitiviruses - and it is easy to see that an integrated, extensive virus outreach program is needed immediately. On the flip side, the California winegrape industry needs stronger communication between growers, nurseries, laboratories, researchers, and government programs to find a long-term strategy for lowering the state's inoculum and reducing the spread of viruses.

OBJECTIVES

The overall objective is to learn how to best manage and prevent grapevine virus disease in the 110,000 acres of Crush District 11, providing outreach tools and strategies to be shared with other regions across California. This main objective will be accomplished by the following sub-objectives:

1. To investigate the current status of grapevine virus knowledge, both at the academic level and at the regional grower level. This ongoing investigation will include a grapevine virus literature search and the collection of case studies about grapevine viruses locally, statewide, and internationally.
2. To learn how to best test and rogue infected grapevines for virus management, developing and incorporating economic thresholds into outreach materials.
3. To learn best practices for replacement of an existing leafroll-infected vineyard.
4. To formulate a long-term management plan for economically feasible and impactful virus control strategies in Lodi and California.
5. To develop and deliver timely, relevant educational materials and approachable outreach for best virus management practices for growers.
6. To establish priorities for further grapevine virus research projects.

RESULTS AND DISCUSSION

Grapevine virus management has been established as a top outreach and research priority for Lodi, due to severe economic losses from region-wide virus infections and a general lack of

knowledge about viruses. Lodi's winegrowing community is fully committed to learning more about viruses in general and to discovering sustainable, economically viable management options to allow for profitable grape growing.

Objective 1. To Investigate the Current Status of Grapevine Virus Knowledge, Both at the Academic Level and at the Regional Grower Level. This Ongoing Investigation Will Include a Grapevine Virus Literature Search and the Collection of Case Studies About Grapevine Viruses Locally, Statewide, and Internationally

Monthly meetings of the Virus Focus Group, monthly pest management network breakfast meetings, tailgate talks, a large Mealybug and Virus Outreach meeting, as well as numerous personal conversations with local growers and other regional grower groups has revealed a great lack of knowledge about viruses in the California winegrape industry. Although the majority of growers are experiencing virus symptoms (red leaves or trouble ripening grapes), they have yet to understand the differences between the main economically important viruses or begin to manage for them. A significant amount of mis-information exists in all industry sectors from the nursery to the vineyard to the winery.

We've collected scientific articles, textbooks, online information, and a grower workbook on leafroll virus from sources in the USA and internationally. All information is shared within the Virus Focus Group and discussed at length during the monthly meeting, trying to understand how each piece of information applies locally in California. Information concerning leafroll virus from South Africa and New Zealand has been extremely useful and has allowed us to develop an overall virus strategy (**Figure 1**) at a faster pace.

Case studies regarding the economics of virus management and individual virus-related situations are being collected and used in research and outreach. The financial losses experienced due to viruses are much greater than our local winegrowing community had realized. For example, one 70-acre block planted in 2012 was infected with leafroll virus and had to be removed in 2018, at a total loss (including revenues) of at least $2.5 million. Through outreach and conversations, we've discovered that grower sentiments towards the California virus crisis are very similar to the stages of grief identified by the Modified Kubler-Ross Model - shock, denial, anger, bargaining, depression, testing, and finally acceptance. We've incorporated this grief model into our outreach presentations. The collection and sharing of local case studies is helping influence growers towards learning more about viruses and how to manage them, and will aid in bringing our industry towards the acceptance stage of thinking so that long-term solutions can be best realized. As we continue our outreach efforts, we are undoubtedly seeing increased understanding and acceptance about viruses and their vectors among our growers (based upon their conversational feedback).

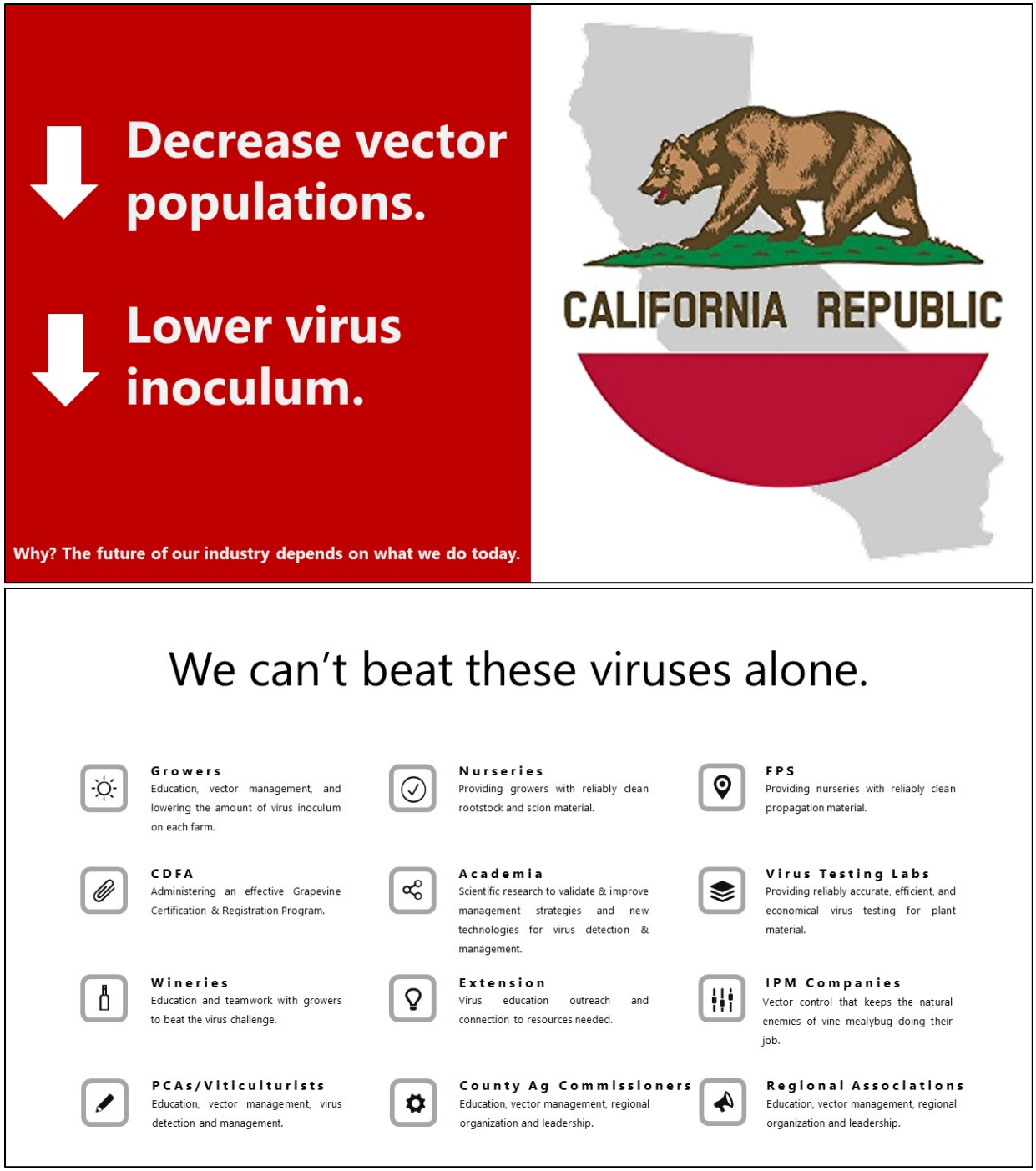


Figure 1. A visual representation of the overall virus strategy for California to guide the outreach initiatives of the Virus Focus Group. Created by Bolton for the 2018 Mealybug and Virus Outreach Meeting.

We've uncovered numerous myths about grapevine viruses that are believed by all types of people, from an average farmer to the leaders of industry and county organizations. Here are a few examples of these myths:

"Nurseries do not sell non-certified planting material."
– Grower Association Leader

"Certified vines are virus free."
– Extension publication

"Mealybugs won't develop resistance to Movento."
– A message told to growers by some pest control advisors

"Rootstocks don't get viruses."
– A message told to growers by some nurseries

"It is illegal to sell virus-infected vines."
– A County Agricultural Commissioner's office

"Someone or some group is in charge of orchestrating a solution to the virus crisis."
– Most people in the industry

We are continuously speaking with industry, extension leaders, and regional grower groups to discuss these myths and how we can use consistent messaging to overcome them.

Table 1. An overview of challenges uncovered through an in-depth look at the overall California grapevine virus situation.

Virus-Related Topic	Challenge(s)	Efforts to Help Solve the Issue
CDFA Grapevine Registration and Certification Program	The current CDFA Grapevine Registration and Certification Program is not robust enough to prevent viruses and their vectors from passing through the system.	We've spent over a year drafting a letter to the CDFA from industry seeking specific improvements in the Grapevine Registration and Certification Program (with input from the CDFA) and are currently gathering support from other industry groups for these improvements, which may ultimately turn into a voluntary program for nurseries.
	There was no list of nurseries registered under the CDFA Grapevine Registration and Certification Program.	We asked for a list of CDFA-registered nurseries, which is now posted online and is included in our outreach material so that growers know where to purchase CDFA-certified material.

Virus-Related Topic	Challenge(s)	Efforts to Help Solve the Issue
Ants	Ants play a key role in the protection and movement of vine mealybugs, but there is currently no effective and economically viable method available for ant control.	We worked with Dr. Kris Tollerup (UC Cooperative Extension) on ant trial research during the 2019 growing season, which was presented at our October 2019 Leafroll Virus Tailgate Talk.
Nursery Material	Vine mealybugs are being spread via nursery material, especially under the wax at the graft union.	We inform growers to inspect nursery material for mealybugs before planting and to ask for wax removal prior to shipment. We included nursery-specific outreach in our *Mealybug Management* Booklet and invite nurseries to all of our outreach events.
	There is a lot of mis-information from extension and others stating that nurseries sell certified virus-free material, when in reality it should be called "CDFA-certified virus-tested" material.	We wrote a peer-reviewed educational booklet explaining what the CDFA certification means and how to improve your chances of getting cleaner wood. We politely correct speakers at industry meetings when they use the term "virus-free," asking them to instead use "virus-tested."
	Many growers do not know how to order CDFA-certified rootstock and scion material (there are very specific questions which need to be asked at some nurseries).	Our *Nursery Ordering 101: Viruses* Booklet includes the questions a grower needs to ask to be able to order CDFA-certified rootstock and scion material.
Virus Testing Labs	There is no third-party oversight for virus testing labs, and no system of checks and balances in place. Each lab is operating separately using their own proprietary protocols. There is no industry "standard" established for virus testing. Growers do not trust lab results.	We held afternoon meetings during our Mealybug and Virus Outreach Meetings for the virus testing labs to speak with each other and our Virus Focus Group. At their suggestion, we organized a blind ring test in conjunction with Dr. Bob Martin of the USDA-ARS in Oregon, which was completed during winter 2018-2019. The results have been shared with individual laboratories. Laboratories are invited to all of our outreach events.

Virus-Related Topic	Challenge(s)	Efforts to Help Solve the Issue
Freedom Rootstock and Sudden/Mystery Vine Collapse	Growers in Lodi and other parts of California have been planting thousands of acres of vines with Freedom rootstock, which researchers have known to be very susceptible/sensitive to leafroll virus since the 1990s. This information was unknown by many growers, who use it in high vine mealybug/leafroll risk areas. Large patches of vineyards on Freedom rootstock (and others) have been collapsing across the state, succumbing to what we called the mystery vine collapse, now known as sudden vine collapse.	We worked with several scientists, including Dr. Akif Eskalen, to study collapsing vineyard patches and we organized case study collections where growers are interviewed to collect a large amount of data for each collapsing situation before testing these vines for viruses and other pathogens. High throughout sequencing provided by Dr. Maher Al Rwahnih revealed positive test results for a leafroll virus and a vitivirus in four collapsing sites on Freedom rootstock. We have a list of over 20 additional sites to study and test for viruses as we attempt to elucidate the cause of the collapse. Dr. Neil McRoberts will be studying the spread of the collapsing patches. We provide education on why growers may want to avoid Freedom and other possibly-sensitive rootstocks following the removal of a vineyard with mealybugs and leafroll, or for vineyards in high risk areas. We are now including vitiviruses in our outreach materials as an economically important virus group.
Vine Mealybug Control	Many growers falsely believe that they do not have vine mealybugs, an incredibly efficient leafroll virus vector, in their vineyards.	We tell all growers that they either have vine mealybugs or are at risk for them and include vine mealybug identification in all virus outreach meetings. We produced a *Vine Mealybug Management* booklet, which includes a laminated scouting card. We offer hands-on mealybug identification at our Leafroll Virus Tailgate Talks.
	Growers are heavily relying on only a few insecticide modes of action for mealybug control.	We include mealybug biocontrol in our outreach efforts and were awarded a Western Sustainable Agriculture Research and Education (SARE) grant to demonstrate effective mealybug biocontrol in Lodi. We strongly encourage the use of beneficial insects and pheromone mating disruption.

Virus-Related Topic	Challenge(s)	Efforts to Help Solve the Issue
USDA Financial Assistance for Losses due to Viruses - TAP	Leafroll virus is not currently an eligible disaster covered under the USDA Tree Assistance Program (TAP), but red blotch virus is covered.	Dr. Alan Wei pointed out to us that USDA TAP may decide to cover leafroll virus in addition to red blotch virus if enough growers applied for assistance. After hearing one of our outreach presentations, a grower filed for assistance under TAP due to leafroll virus, has appealed the rejection, and has had a hearing. We provided case studies and cost information to support this cause, in addition to spreading the word to other extension and grower groups. A formal request has been submitted to the Farm Service Agency California State Office to add leafroll to the approved list of diseases for the TAP.

Perhaps because there is no one organization "in charge" of the complex California grapevine virus crisis, there hasn't been adequate communication between all sectors of the industry (**Figure 1**). As **Table 1** shows, we are having great success in helping to facilitate these communication channels to discover industry-driven working solutions which can be implemented in both the short-term and long-term.

Objective 2. To Learn How to Best Test and Rogue Infected Grapevines for Virus Management, Developing and Incorporating Economic Thresholds into Outreach Materials

Research into virus testing procedures revealed that there is no standard protocol for virus testing in California, nor is there a virus-specific accreditation available for laboratories. Virus testing is expensive (in the range of $150 to $300 per sample or vine) and directions for sample collection need to be followed carefully for the most accurate results. On April 4, 2018, the Lodi Winegrape Commission hosted the first meeting where all seven grapevine testing laboratories came together with growers, nurseries, and pest control advisors (PCAs). At this meeting it was decided that a third-party ring test would help improve the accuracy and reliability of California virus testing. Dr. Bob Martin at the USDA-ARS in Oregon and Project Leader Stephanie Bolton orchestrated the blind ring test during winter 2018-2019 with positive samples donated by Dr. Maher Al Rwahnih from Foundation Plant Services (FPS). All seven commercial California laboratories, including FPS, and the new CDFA virus testing laboratory participated. The data was analyzed and used to help laboratories improve their methodology. We plan on repeating the blind ring test in winter 2019-2020, with improvements learned from the first ring test.

Efficient use of CDFA grant money awarded for regional virus testing will allow us to meet the following goals, all of which are currently in progress:

1. To experience virus testing with all seven laboratories as a grower would for improved, real-world educational materials on "how to test."
2. To determine the potential role of leafroll virus and vitiviruses in the mystery/sudden vine collapse disease complex, which may also elucidate rootstock and virus sensitivities in commercial vineyards.
3. To create and map virus spread in leafroll virus demonstration vineyards with scouting and training opportunities.
4. To gather virus case studies and photographs from across the Lodi American Viticultural Area (AVA) which are verified by testing and can be used in educational materials.
5. To teach growers and PCAs how to sample and test for viruses (each sampling is an opportunity to teach the grower and/or his or her PCA how to test).
6. To show growers, especially those in virus denial, how widespread grapevine viruses are across the Lodi AVA.
7. To determine which leafroll 3 virus strains exist in the Lodi AVA (Dr. Maher Al Rwahnih has tested selected samples to the strain level).

Thus far, every sample submitted for virus testing has come back positive for at least one virus, with leafroll 3 being the most common virus found. It appears that leafroll virus and vitiviruses play a key role in the mystery/sudden vine collapse disease, which also involves Freedom rootstock (and others), although further studies are needed. For the leafroll 3 positive samples tested to the strain level, we have observed Group I-V to dominate. Significant virus testing is planned to continue throughout the duration of this project and is crucial to understanding the nuances of virus management.

Objective 3. To Learn Best Practices for Replacement of an Existing Leafroll-Infected Vineyard

Grapevine root remnants remain alive for several years after the removal of a vineyard, and these root pieces can test positive for leafroll virus. The Cooperators on this grant are continuously experimenting with management methods at their own expense and generously sharing the results of these trials. There is a rootstock trial planted in a Cooperator vineyard (January 2019) to determine if there are commercially available rootstocks which may offer some tolerance to leafroll 3 virus and/or root mealybug vectoring. This trial includes nine replicates of fourteen rootstocks plus own-rooted vines (10 vines per replicate). The vineyard, which serves as Demonstration Vineyard #2, is a block which was removed due to leafroll virus and mealybugs.

Objective 4. To Formulate a Long-Term Management Plan for Economically Feasible and Impactful Virus Control Strategies in Lodi And California

As viruses are costing everyone a good deal of money, people have been more than willing to work together to find long-term strategies for virus control statewide. The first step was to get all the entities (nurseries, laboratories, extension personnel, County Agricultural Commissioners, scientists, the National Clean Plant Network, and the CDFA) talking to each other, with informed growers as part of these conversations. The key part in these conversations is having average growers be part of the conversation; it makes future decisions more applicable to the real world. Thus far, we have had discussions with every group listed. All entities are being invited

and encouraged to work with the Virus Focus Group, and as mentioned earlier, teamwork with other grower groups has begun as well.

On April 3, 2019, the evening before the last Mealybug and Virus Outreach Meeting, we hosted selected "virus influencers" from these entities for a dinner to discuss long-term strategy planning and how we can overcome some of our largest hurdles. Also present were Prof. Gerhard Pietersen, Dr. Marc Fuchs, Dr. Kent Daane, and Dr. James Stamp, each of whom bring a unique perspective to the long-term strategizing. Prof. Pietersen has possibly the world's best example in South Africa of a vineyard estate moving from a crisis-status to an "under control" status when it comes to leafroll virus. Everyone at the dinner expressed gratitude and encouragement surrounding the coming together of organizations to help solve the grapevine virus crisis.

Each guest discussed what he or she believes to be the one most significant action which could have the greatest impact on helping the winegrape industry combat the economically devastating viruses, and here is a summary of the resulting list (in no particular order):

1. Enhanced communication between all sectors of the industry and scientists.
2. Take an area-wide approach (biocontrol for vine mealybugs and virus management).
3. Ant control.
4. Teamwork (follow the example of other diseases such as boll weevil eradication).
5. Take a pre-emptive approach, considering vineyards to be infected.
6. Gene editing.
7. Pheromones for vine mealybug mating disruption.
8. Learn to live with the viruses.
9. A paradigm shift…long-term thinking is necessary because viruses have long-term effects.
10. Think BIG and cooperate with all stakeholders.
11. Higher grape prices would help growers be able to afford to reduce virus inoculum.
12. Think of viruses as a "pollutant."
13. Education for the average farmer - hands-on, in the field, focusing on the basics.
14. Stopping the blame game.
15. Create a model vineyard for vine mealybug and virus management in California.
16. Grapevine propagation method improvement.
17. Public education about viruses - red leaves in the fall are not pretty.
18. Improved virus detection methods.
19. Successful replanting strategies (following a virus infection).
20. Keeping the conversation going until it reaches the average farmer.
21. Taking responsibility for what is under your control - start virus management now, where you have influence.
22. Strengthened relationships for "cross-education" among stakeholders.

During the afternoon of April 4, 2019, after the Mealybug and Virus Outreach Meeting, we organized special technical leadership sessions for industry leaders in the areas of grapevine virus testing, nurseries, and extension personnel/regional grower groups. Each session included a roundtable discussion. In 2019, the nursery roundtable had the most successful meeting with an idea for improved efforts around virus management (above and beyond the CDFA Grapevine

Registration and Certification Program) and enhanced communication about CDFA-certified planting material with growers.

Objective 5. To Develop and Deliver Timely, Relevant Educational Materials and Approachable Outreach for Best Virus Management Practices for Growers

The Lodi Winegrape Commission has multiple established channels for communicating with growers and the industry. The 750 growers and 200 supporting members of the winegrowing community (as well as the additional LODI RULES community, reaching twelve other Crush Districts, and a network of Lodi wineries) receive information about virus educational workshops via mailings (postcards advertising events and biannual newsletters), email (a list-serve of over 800 people), twitter (@LodiGrower), a website (lodigrowers.com), and a blog (lodigrowers.com). Each method of communication listed provides an opportunity not only for educational outreach, but also for a conversation to begin between the recipient and the Virus Focus Group. We created a virus-specific email list-serve for anyone interested in virus outreach who does not want to be on our regular Lodi grower email list (as of October 23, 2019, this special virus email list has 183 members across the world). Due to the popularity of our virus outreach resources we also created a new page on our lodigrowers.com website under the Education tab called "Grapevine Virus Resources" where we post videos, articles, updates, and handouts (lodigrowers.com/growereducation/viruses/).

Our Integrated Outreach Strategy Progress

Open Communication Virus Meetings for Growers

(Impact: 1,000+ industry members invited to meetings; free and open to the public)

The Lodi Winegrape Commission continues to host monthly pest management network breakfast meetings where anyone in the Commission network (growers, PCAs, winemakers, etc.) can stop in and ask questions about grapevine pests and diseases. Beginning in April 2018 we devoted a portion of these roundtable meetings to viruses and their vectors so that the community has a consistent, approachable place to come with virus questions. At least three members of the Virus Focus Group are always in attendance. Prof. Gerhard Pietersen was the special guest at the April 2019 meeting; Dr. Akif Eskalen and Dr. Neil McRoberts were special guests at the well-attended June 2019 meeting, which focused on the topic of the mystery/sudden vine collapse; and Dr. Akif Eskalen, Dr. Neil McRoberts, and Dr. Maher Al Rwahnih were special guests at the October 2019 meeting (again on the topic of the mystery/sudden vine collapse) which astoundingly brought in 140 attendees from across California, representing all major groups in **Figure 1**.

Virus Management Demonstration Vineyards

(impact: 1,000+ industry members invited to annual tailgate talks; free and open to the public)

Two Virus Management Demonstration Vineyards have been established in Lodi, where growers can observe virus management in practice, learn symptom identification, and learn how to mark, test, and rogue vines during annual tailgate talks. The financials of the vineyards in terms of virus management, along with successes and failures, are discussed openly. Demonstration Vineyard #1 is an example of < 25% leafroll infection managed with roguing. Extensive mapping and testing by Cooperators Charlie Starr and Aaron Lange have provided a view of the virus infection over time. Prof. Gerhard Pietersen and Dr. Marc Fuchs spent a significant amount of time discussing how Demonstration Vineyard #1 can be used as a much-needed California

model for virus management, including scouting, mapping, testing, and roguing. Demonstration Vineyard #2, planted in January 2019, will hopefully provide an example of moving from > 60% leafroll infection to effective leafroll control.

Every fall we host a tailgate talk at one of the vineyards to discuss virus management. The October 2018 and 2019 Leafroll Virus Tailgate Talks were hosted at Demonstration Vineyard #1. Even during harvest, approximately 75 to 100 people showed up to learn about virus management. Hands-on learning stations were set up in the vines and the surrounding area and were led by members of the Virus Focus Group and invited educators on the following topics: mealybug biocontrol/beneficial insects, virus scouting, virus sampling and testing, virus roguing, pheromone mating disruption, the three-cornered alfalfa hopper, and the mystery/sudden vine collapse. Outreach materials created during this grant project were handed out to attendees, including the latest *Grapevine Virus Testing 101* booklet in 2019.

Annual Virus Workshop (in spring of every year)
(impact: 1,200+ industry members invited to annual workshops; free and open to the public)
Every year the Lodi Winegrape Commission hosts a virus workshop (called the Mealybug and Virus Outreach Meeting) with updated information and case studies from growers. This workshop provides timely, relevant information on nursery ordering, the CDFA Grapevine Registration and Certification Program, economically important viruses, virus management, mealybugs, ants, and replanting after a virus infection. The first workshop was held on April 4, 2018 and over 150 people from all over California attended the half-day meeting, with several people staying through the afternoon for more focused discussions.

In April 2019 the Mealybug and Virus Outreach Meeting included afternoon roundtable meetings for nurseries, laboratories, and regional grower associations along with extension personnel to discuss short-term and long-term strategies for reducing virus vectors and inoculum in California. Also in 2019, South African Prof. Gerhard Pietersen (leafroll expert) and Cornell University's Dr. Marc Fuchs (red blotch expert) were keynote speakers and, thanks to grant funding, they also were able to consult with growers throughout the week. Dr. James Stamp (nursery material expert) and Dr. Kent Daane (mealybug expert) were invited speakers, too. In attendance in 2019 were over 250 growers, pest control advisors, nursery representatives, beneficial insect company representatives (including a drone company which releases the beetles), pheromone mating disruption company representatives (Suterra provided free mealybug traps to attendees), staff from our County Agricultural Commissioner's office and the CDFA, virus testing laboratory representatives, Dr. Deborah Golino and Dr. Maher Al Rwahnih (FPS), Wine Institute representatives, UC Farm Advisors, a North Carolina viticulturist, and many other people with the power to help reduce vectors and inoculum.

The 2019 meeting, according to feedback, brought more "aha" moments about virus management and increased concern over the need to rogue and manage mealybugs. Attendees were impressed by the world-class speakers and the large audience, which added to the legitimacy of the meeting and the call to come together as an industry to help solve the grapevine virus challenge. The meeting received a lot of attention from press and resulted in several news and industry articles. Applicable management strategies were discussed in grower language by the speakers. The main take home message was that established California growers will get the

most bang for their buck by roguing virus-infected grapevines, which may seem like common sense, but the logic and reasoning behind the *need to rogue*, as well as clear examples where roguing was successful, were shared in a way that was easy to digest. Thanks to grant funds, we were able to capture the great advice given by the speakers in the form of videos. These videos were professionally produced and edited and are available on the Grapevine Virus Resources page at lodigrowers.com and on YouTube, and they will be included in the future electronic version of the Grapevine Virus Grower Workbook (lodigrowers.com/growereducation/viruses/).

The 2020 Mealybug and Virus Outreach Meeting is scheduled for April 9, 2020.

Grapevine Virus Grower Workbook
(impact: distributed through Lodi Winegrape Commission to 800+ community members; available to the public and to other winegrowing regions)
We're writing a *Grapevine Virus Grower Workbook* which teaches growers why they need to care about viruses (using financial examples and case studies), where to start if their vineyard is sparsely or completely infected, how to identify/sample/test vines, how to rogue, the differences between red blotch, leafroll, and fanleaf viruses, how to manage for vine mealybugs, and how to order certified virus-tested rootstocks and scions (protocol 2010) from a nursery, plus why that is financially and socially important. The educational material includes plentiful, recent photographs (verified by testing), case studies, myth-busters, question and answer sections, industry interviews, and very importantly, sections where the grower can record pertinent virus and vector management information for each vineyard.

Instead of waiting until the entire Workbook is ready for publishing (which will be too late), we are distributing small "draft" booklets on selected topics as the information is verified and available. Approximately four to five small booklets will each undergo a peer review process and a grower test run in Lodi before they are improved and published altogether as a robust *Virus Grower Workbook*, projected to be completed in spring 2020. Each booklet is being freely shared with any interested parties; we have received several requests for our *Nursery Ordering 101: Viruses* booklet especially. Additionally, booklets are shared with other winegrowing organizations with the hopes that they will customize the photos, information, and case study examples to their regional needs and grower audience. To date, outside of the California winegrape community, booklets and other virus resources have been shared with industry members in Washington, Texas, North Carolina, Portugal, Chile, and the California Table Grape industry.

Objective 6. To Establish Priorities for Further Grapevine Virus Research Projects
Thus far, it appears that research on the following topics is much needed:

1. An effective and efficient ant bait for use on large (50+) acre blocks to control ants which tend mealybugs.
2. A prevention strategy for leafroll replants (both individual vines and entire vineyard blocks).
3. The role of viruses in complexes with other biotic and abiotic stresses (including elucidating the mystery/sudden vine collapse).
4. Rootstock and scion combinations which are more or less prone to virus disease symptoms.

5. How to determine the percent of a vineyard which is infected with virus in a cost-effective manner.
6. Cost-effective methods of virus testing.

When new researchers are looking for grower-relevant virus-related projects, we have been able to provide them with this list of priorities, invite them to our monthly meetings and outreach events, and provide input or access to vineyards and information as needed.

CONCLUSIONS

Establishing the Virus Focus Group and developing an agreed-upon outreach strategy has brought new energy and momentum towards solving the virus challenge and has opened communication between all sectors of the industry to openly discuss successes and failures in virus management. The collaborative nature of this community, along with the immense experience of the Cooperators and openness of expert consultants Prof. Gerhard Pietersen and Dr. Marc Fuchs, sets the stage for quickly discovering and implementing both short-term and long-term virus management strategies first in Lodi and then statewide. There is a common recognition now that viruses are not just a nursery problem or one neighbor's bad luck. Grapevine viruses are everywhere and are thus everyone's problem, creating a unifying goal of finding real-world solutions so that everyone can stay in business.

These coordinated efforts directed by the Lodi Winegrape Commission, a trusted source for real-world grower education, are reaching over one thousand winegrape growers and PCAs to quickly and effectively implement virus management initiatives while establishing priorities for future research. Cooperators are investing their time and money into discovering virus management strategies for the greater good, and they are very capable of comparing management techniques due to the large number of acres they cover. Demonstration vineyards are managed by experienced growers in the LODI RULES sustainable winegrowing program, ensuring farming practices which are environmentally responsible and economically feasible. Outreach materials created, workshops and meetings hosted, and the communication channels which are opening between industry sectors are proving to be of utmost importance for the winegrape industry across the state of California as we collectively develop a long-term strategy for lowering the state's inoculum and reducing the spread of viruses.

REFERENCES CITED

Atallah SS, Gómez MI, Fuchs MF, Martinson TE. 2012. Economic impact of grapevine leafroll disease on *Vitis vinifera* cv. Cabernet franc in Finger Lakes vineyards of New York. *American Journal of Enology and Viticutlure* 63: 73-79.

Engelbrecht DJ, Kasdorf GGF. 1990. Transmission of grapevine leafroll disease and associated closteroviruses by the vine mealybug, *Planococcus ficus*. *Phytophylactica* 22 (3): 341-346.

Golino DA Sim ST, Gill R, Rowhani A. 2002. California mealybugs can spread grapevine leafroll disease. *California Agriculture* 56 (6): 196-201.

Martelli GP (ed.). 2014. Directory of virus and virus-like diseases of the grapevine and their agents. *Journal of Plant Pathology* 96 (1S): 1-4.

Pietersen G. 2016. Leafroll: Replacing whole, highly leafroll-infected vineyards with new healthy vineyards. *IGWS Factsheets*. University of Pretoria.

Ricketts KD, Gómez MI, Fuchs MF, Martinson TE, Smith RJ, Cooper ML, Moyer MM, Wise A. 2017. Mitigating the economic impact of grapevine red blotch: Optimizing disease management strategies in U.S. vineyards. *American Journal of Enology and Viticulture* 68: 127-135.

Sudarshana MR, Perry KL, Fuchs MF. 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Phytopathology* 105: 1026-1032.

Tsai CW, Chau J, Fernandez L, Bosco D, Daane KM, Almeida RPP. 2008. Transmission of grapevine leafroll-associated virus 3 by the vine mealybug (*Planococcus ficus*). *Phytopathology* 98 (10): 1093-8.

Tsai CW, Rowhani A, Golino DA, Daane KM, Almeida RPP. 2010. Mealybug transmission of grapevine leafroll viruses: an analysis of virus-vector specificity. *Phytopathology* 100 (8): 830-834.

FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board, the American Vineyard Foundation, Cooperators and their employers, and the Lodi Winegrape Commission.

ACKNOWLEDGEMENTS

We would like to thank the California Crush District 11 (Lodi) winegrape growers for their support and willingness to learn about yet another costly viticulture challenge. This effort would not be possible without support from the employers of our Cooperators: LangeTwins Family Winery and Vineyards, Trinchero Family Estates, Wonderful Nurseries, Precissi Ag Services, Duarte Nurseries, Viticultural Services, Vino Farms, and E. & J. Gallo. A big thanks to university researchers who answer our many questions and support us in research and outreach, including: Dr. Kamyar Aram, Dr. Deborah Golino, Dr. Maher Al Rwahnih, Dr. Akif Eskalen, and Dr. Kent Daane (University of California); Dr. Marc Fuchs (Cornell University); and Prof. Gerhard Pietersen (University of Pretoria). Stanton Lange (SC Lange Vineyards); John Duarte (Duarte Nurseries); Dustin Hooper and Brad Kroeker (Wonderful Nurseries); Vaughn Bell (New Zealand Institute for Plant and Food Research Limited); Ruby Andrew (Vino Vitis Communications); Dr. Bob Martin (USDA-ARS); and Joshua Kress (CDFA) have also greatly aided efforts. Thanks to the Velvet Grille in Lodi for providing us with a place to meet and for keeping our coffee cups full.

A big thanks to other regional organizations who support our efforts and learn along with us, especially the Vineyard Team, the California Association of Winegrape Growers, the Washington Winegrowers Association, and the Monterey County Vintners and Growers Association.

A STUDY ON THE IMPACT OF INDIVIDUAL AND MIXED LEAFROLL INFECTIONS ON THE METABOLISM OF RIPENING WINEGRAPE BERRIES

Project Leader: Dario Cantu | Department of Viticulture and Enology | University of California | Davis, CA 95616 | dacantu@ucdavis.edu
Co-Project Leader: Maher Al Rwahnih | Department of Plant Pathology | University of California | Davis, CA 95616 | malrwahnih@ucdavis.edu
Co-Project Leader: Susan Ebeler | Department of Viticulture and Enology | University of California | Davis, CA 95616 | seebeler@ucdavis.edu
Co-Project Leader: Deborah Golino | Department of Plant Pathology | University of California | Davis, CA 95616 | dagolino@ucdavis.edu
Cooperator: Amanda Vondras | Department of Viticulture and Enology | University of California | Davis, CA 95616 | amvondras@ucdavis.edu
Cooperator: Mélanie Massonnet | Department of Viticulture and Enology | University of California | Davis, CA 95616 | mmassonnet@ucdavis.edu

Reporting Period: The results reported here are from work conducted July 1, 2017 to October 23, 2019.

ABSTRACT

Grapevine leafroll-associated viruses (GLRaVs) are the most widespread and economically damaging viruses affecting viticulture (Goheen et al., 1959; Maree et al., 2013; Naidu et al., 2015; Atallah et al., 2012). Plant responses to viruses include a multitude of changes in metabolism, gene expression, and gene regulation (Alazem and Lin, 2014; Bester et al., 2016; Blanco-Ulate et al., 2017; Moon and Park, 2016). However, there is a gap in knowledge concerning the specific regulation of the response to GLRaVs and which pathways determine GLRaV symptoms and their severity. The effects of GLRaVs can include poor color development in red grapes, non-uniform or delayed ripening, reduced sugar content in berries, altered tannins, pigments, and acids, curling leaves, reddening or chlorotic interveinal areas, and high crop loss (Atallah et al., 2012; Guidoni et al., 2000; Vega et al., 2011; Alabi et al., 2016; Lee and Martin, 2009; Lee and Schreiner, 2010). The severity of GLRaV symptoms is influenced by host genotype (Guidoni et al., 2000), which virus or combination of viruses is present, scion-rootstock pairings (Fuchs et al., 2009; Prosser et al., 2007; Golino et al., 2003; Lee and Martin, 2009), and environmental factors (Cui et al., 2017). The experiments proposed will test our hypotheses that (1) GLRaVs disrupt berry development and the accumulation of flavor and aroma metabolites by altering hormone networks, and (2) the differences in symptoms associated with different GLRaVs are due to non-uniform impacts on some metabolite and gene regulatory pathways. We intend to integrate gene expression, hormone, and metabolite data to better understand how these viruses affect fruit metabolism during ripening given different rootstocks. This information will help inform future strategies to combat or resist leafroll viruses.

LAYPERSON SUMMARY

The purpose of this study is to understand the impact of individual and combinations of grapevine leafroll-associated viruses on ripening in Cabernet Franc grapevines grafted to two different rootstocks. Different virus combinations and different rootstocks were chosen because of their association with varying levels of symptoms given virus infection. This study has thus

far included data collection and analyses in two consecutive years. In each year, the first steps towards generating data include monitoring the infection status of the vines, sampling consistently each year, deseeding berries, and crushing tissue. Our previous reports stated that these steps were completed for 2017 and 2018 samples.

The next steps towards data generation include measuring total soluble solids (TSS; °Brix), choosing samples for sequencing, preparing RNA sequencing libraries, and sequencing those libraries. Our previous reports stated that these steps were completed for 2017 and 2018 samples.

In addition, hormone and ripening-related metabolite extractions were undertaken. For both hormone and ripening-related metabolite quantitation, this first involves optimizing extraction and detection methods for specific hormones and ripening-associated metabolites. Then, generating the data involves weighing crushed tissue for each sample in duplicate, performing the extractions, and subjecting the sample extracts to liquid chromatography and mass spectrometry (LC-MS). Then, data are normalized and statistical analyses are done. Our previous reports stated that the hormone analyses were completed for the 2017 samples, that ripening-related metabolite extractions for 2017 samples were ongoing, and that weighing 2018 tissue for both types of analyses were underway. As of now, the extractions of hormones and ripening-related metabolites have been completed for both years. In addition, the LC-MS analysis of hormones in both years was completed and we are proceeding with the data normalization and statistical analyses for that year. The LC-MS analyses of ripening-related secondary metabolites for both years are underway.

Finally, as described in the previous report, both years of RNA sequencing analyses are ongoing. We are comparing years to evaluate year-to-year effects of the viruses and rootstocks on berry response during ripening, looking closely at the affected genes under the various experimental conditions, and are examining condition-dependent changes in the relationships between genes. We are also working to integrate the hormone data that was generated and intend to integrate the forthcoming metabolite data into our analyses.

INTRODUCTION

Grapevine leafroll-associated viruses (GLRaVs) are the most widespread and economically damaging viruses affecting viticulture (Goheen et al.,1959; Maree et al., 2013; Naidu et al., 2015; Atallah et al., 2012). Plant responses to viruses include a multitude of changes in metabolism, gene expression, and gene regulation (Alazem and Lin, 2014; Bester et al., 2016; Blanco-Ulate et al., 2017; Moon and Park, 2016). However, there is a gap in knowledge concerning the specific regulation of the response to GLRaVs and which pathways determine GLRaV symptoms and their severity. The effects of GLRaVs can include poor color development in red grapes, non-uniform or delayed ripening, reduced sugar content in berries, altered tannins, pigments, and acids, curling leaves, reddening or chlorotic interveinal areas, and high crop loss (Atallah et al., 2012; Guidoni et al., 2000; Vega et al., 2011; Alabi et al., 2016; Lee and Martin, 2009; Lee and Schreiner, 2010). The severity of GLRaV symptoms is influenced by host genotype (Guidoni et al., 2000), which virus or combination of viruses is present, scion-rootstock pairings (Fuchs et al., 2009; Prosser et al., 2007; Golino et al., 2003; Lee and Martin, 2009), and environmental factors (Cui et al., 2017). The experiments proposed will test our hypotheses that (1) GLRaVs disrupt berry development and the accumulation of flavor

and aroma metabolites by altering hormone networks, and (2) the differences in symptoms associated with different GLRaVs are due to non-uniform impacts on some metabolite and gene regulatory pathways. We intend to integrate gene expression, hormone, and metabolite data to better understand how these viruses affect fruit metabolism during ripening given different rootstocks. This information will help inform future strategies to combat or resist leafroll viruses.

OBJECTIVES

1. Profile genome-wide transcriptional changes as a result of individual and combinations of GLRaV infections during grape berry development.
2. Identify secondary metabolic pathways that underlie the altered biochemical composition of GLRaV infected berries.
3. Determine changes in plant hormone biosynthesis, accumulation, and signaling that are associated with the abnormal ripening of GLRaV-infected berries.

RESULTS AND DISCUSSION

Pre-Objectives

Sampling and Sample Preparation in 2017 and 2018. GLRaV infection conditions were confirmed by molecular testing at Foundation Plant Services (FPS) prior to sampling. Photographs were taken and berries were collected at four distinct developmental stages (pre-véraison, véraison, post-véraison, and harvest) from Cabernet Franc grapevines grafted to MGT 101-14 and Kober 5BB rootstocks. Twenty berries were picked from each of six vines at each sampling date and from each viral treatment. Berries were sampled evenly throughout the plant. Following their sampling, berries were crushed. The D. Golino group oversaw re-testing of the experimental vines for viruses to ensure the same conditions in 2018 as in 2017. The grapevines were monitored throughout June in order to best estimate the beginning of samplings in 2018. Fruits were sampled at the same four developmental stages as in 2017. As in 2017, plants were photographed to monitor the onset of leafroll symptoms. Berries were deseeded and frozen at minus 80°C. These samples were crushed.

Measurement of Brix in 2017 and 2018. Differences in total soluble solids (TSS) were observed at each time point in the experiment that were dependent on the combination of infections and rootstock.

Objective 1. Profile Genome-Wide Transcriptional Changes as a Result of Individual and Combinations of GLRaV Infections During Grape Berry Development

Justification. The RNA-sequencing data to be generated will provide a quantitative, comprehensive view of the changes in gene expression due to GLRaVs; some may be associated secondary metabolism.

Selection of Samples for RNA-seq in 2017 and 2018. Following the collection, crushing, and measurement of TSS in six biological replicates, four of six were selected for the preparation of RNAseq libraries.

Library Preparation and Sequencing in 2017 and 2018. RNA extractions, library preparation, and sequencing have been completed.

Statistical Analysis and Differential Expression in 2017 and 2018. The library normalization and differential gene expression analyses have been completed and we are exploring the results. This includes determining whether certain types of genes involved in particular pathways or functions are overrepresented among the differentially expressed genes, and using the normalized expression data to look for similarities and differences between groups of samples. This also includes examining the reproducibility of the results between years. In addition, a gene co-expression network is being constructed to understand how possible relationships between genes change because of infection, whether they differ based on the rootstock present, and prioritizing which genes are central to changes in responses. We are approaching these questions using several tools. These analyses are ongoing, but a subset of the network analyses is presented herein. Infection by leafroll viruses is characterized by responses that are specific to particular infections, and responses that are common to all infections; some changes in co-expression also appear to be rootstock-specific (**Figure 1**).

Objective 2. Identify Secondary Metabolic Pathways That Underlie the Altered Biochemical Composition of GLRaV Infected Berries

Justification. Changes in the expression of secondary metabolism-associated genes can reveal mechanisms that underlie impaired berry metabolism and accumulation of commercially significant metabolites.

Overrepresented Gene Ontological Categories. To summarize the disparate impact of the viruses and rootstocks on gene expression during ripening, an overrepresentation test was used to identify overrepresented groups among differentially expressed genes, as well as disparately affected metabolite pathways.

Ripening-Related Metabolite Analysis in 2017 and 2018. After optimizing an extraction method and confirming our ability to detect ripening-related metabolites, tissue from the same samples used for RNAseq were weighed and ripening-related metabolites were extracted. This was completed for both years. Analysis by liquid chromatography and mass spectrometry (LC-MS) is ongoing and we intend to intersect the differential expression analyses with forthcoming ripening-related metabolite data.

Objective 3. Determine Changes in Plant Hormone Biosynthesis, Accumulation, and Signaling That Are Associated with the Abnormal Ripening of GLRaV-Infected Berries

Justification. Hormones play a major role in regulating ripening and disease responses, and the metabolic changes associated with both. Changes in the abundance of hormones will show which hormone pathways regulate GLRaV responses.

Hormone Identification and Quantitation by LC-MS). Pre-existing datasets were used by the Ebeler group to identify the correct signatures of several hormones of interest. The same samples used for RNA sequencing in both years were used for the measurement of hormones. We optimized our extraction method and completed the hormone analysis of 2017 and 2018 samples. The 2017 results indicate significant effects of GLRaVs on abscisic acid and salicylic acid. The data for the second year's samples are currently being normalized for subsequent statistical analyses.

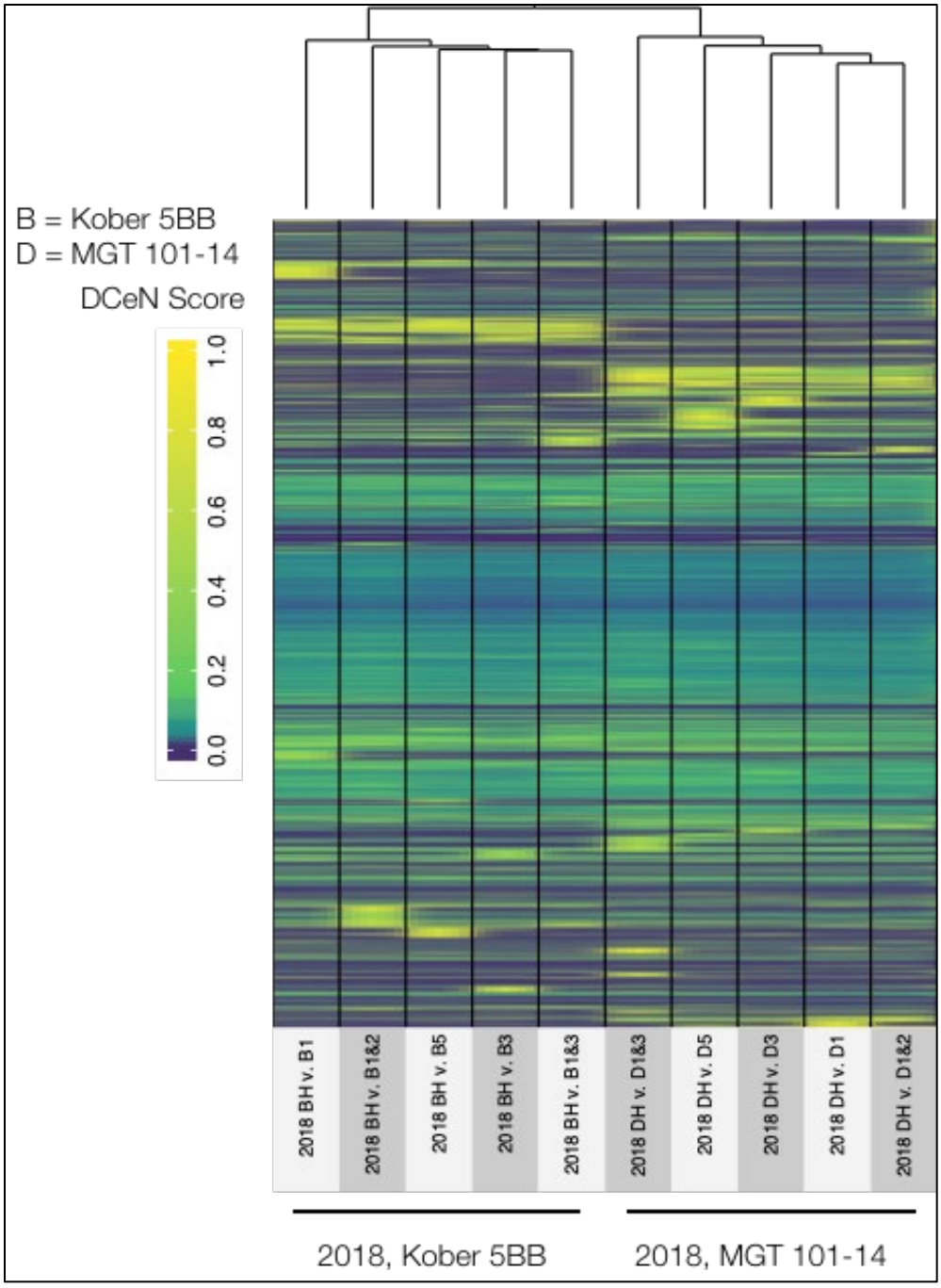


Figure 1. Dynamically co-expressed neighborhoods (DCeN) analyses, 2018 (second year of data only). DCeN scores indicate the proportion of the co-expression neighborhood of a single gene that differs between two conditions; high DCeN genes tend to have significant regulatory roles (Elo and Schwikowski, 2013). Key: B = Kober 5BB; D = MGT 101-14; H = healthy; GLRaV infections indicated by 1, 3, 5, 1 & 2, 1 & 3. Color scale indicates DCeN score. DCeN scores of 0 indicate no difference in neighborhood between the groups compared for a gene. DCeN scores of 1 indicate 100% difference in neighborhood between the groups compared for a gene. In this figure, each row is a single gene and each column is an independent comparison made.

CONCLUSIONS

This ongoing study is using RNA sequencing and metabolite profiling to explore the effects of individual and mixed infections of GLRaVs on ripening and to identify and better understand the pathways involved in responses and symptoms. The rootstocks, scions, and infections used in this study were selected to improve the likelihood of generating commercially transferable knowledge. The vineyard used consists of Cabernet Franc grapevines grafted to Kober 5BB or MGT 101-14 rootstocks and carrying consequential GLRaVs. Cabernet Franc was used because it produces clear symptoms to GLRaVs. Among the treatments established in the vineyard, vines carrying GLRaV-1, GLRaV-3, GLRaV-5, GLRaV-1 + GLRaV-2, and GLRaV-1 + GLRaV-3 were included because infections with one or more of these viruses are associated with a range of symptoms of varying severities. The data generated will improve our understanding of the basis of symptoms and to develop strategies to mitigate the detrimental effects of these viruses on ripening in the future. Thus far, significant differences in sugar accumulation, hormone abundance, gene expression, and gene co-expression have been found and associated with rootstock present and virus status. In addition, common co-expression effects have been observed across infection types.

REFERENCES CITED

Alabi OJ, Casassa LF, Gutha LR, Larsen RC, Henick-Kling T, Harbertson JF, Naidu RA. 2016. Impacts of grapevine leafroll disease on fruit yield and grape and wine chemistry in a wine grape (*Vitis vinifera* L.) cultivar. *Plos One* 11 (2), e0149666. http://doi.org/10.1371/journal.pone.0149666.

Alazem M, Lin N-S. 2014. Roles of plant hormones in the regulation of host-virus interactions. *Molecular Plant Pathology* 16 (5): 529-540. http://doi.org/10.1111/mpp.12204.

Atallah SS, Gomez MI, Fuchs MF, Martinson TE. 2012. Economic impact of grapevine leafroll disease on *Vitis vinifera* cv. Cabernet Franc in Finger Lakes vineyards of New York. *American Journal of Enology and Viticulture* 63 (1): 73–79. http://doi.org/10.5344/ajev.2011.11055.

Bester R, Burger JT, Maree HJ. 2016. Differential expression of miRNAs and associated gene targets in grapevine leafroll-associated virus 3-infected plants. *Archives of Virology* 162 (4): 987-996. http://doi.org/10.1007/s00705-016-3197-9.

Blanco-Ulate B, Hopfer H, Figueroa-Balderas R, Ye Z, Rivero RM, Albacete A, et al. 2017. Red blotch disease alters grape berry development and metabolism by interfering with the transcriptional and hormonal regulation of ripening. *Journal of Experimental Botany* 68 (5): 1225–1238. http://doi.org/10.1093/jxb/erw506.

Cui Z-H, Bi W-L, Hao X-Y, Li P-M, Duan Y, Walker MA, et al. 2017. Drought stress enhances up-regulation of anthocyanin biosynthesis in grapevine leafroll-associated virus 3-infected *in vitro* grapevine (*Vitis vinifera*) leaves. *Plant Disease* 101 (9): 1606–1615. http://doi.org/10.1094/PDIS-01-17-0104-RE.

Elo LL, Schwikowski B. 2013. Analysis of time-resolved gene expression measurements across individuals. *Plos One* 8.

Fuchs M, Martinson TE, Loeb GM, Hoch HC. 2009. Survey for the three major leafroll disease-associated viruses in Finger Lakes vineyards in New York. *Dx.Doi.org* 93 (4): 395-401. http://doi.org/10.1094/PDIS-93-4-0395.

Goheen AC, Hewitt WB, Alley CJ. 1959. Studies of grape leafroll in California. *American Journal of Enology and Viticulture* 66 (2): 112-119. http://doi.org/10.5344/ajev.2014.14055.

Golino D, Sim ST, Rowhani A. 2003. The role of rootstock genotype in the effects of single and mixed infection of grapevine viruses. *Proceedings of the 14th International Congress on Virus and Virus-Like Diseases of Grapevine*. Locorotondo, Italy, pp. 246-247.

Guidoni S, Mannini F, Ferrandino A, Argamante N, Di Stefano R. 2000. Effect of virus status on leaf and berry phenolic compounds in two wine grapevine *Vitis vinifera* cultivars. *Acta Horticulturae* (526): 445-452. http://doi.org/10.17660/ActaHortic.2000.526.49.

Lee J, Martin RR. 2009. Influence of grapevine leafroll associated viruses (GLRaV-2 and -3) on the fruit composition of Oregon *Vitis vinifera* L. cv. Pinot noir phenolics. *Food Chemistry* 112 (4): 889-896. http://doi.org/10.1016/j.foodchem.2008.06.065.

Lee J, Schreiner RP. 2010. Free amino acid profiles from Pinot noir grapes are influenced by vine N-status and sample preparation method. *Food Chemistry* 119 (2): 484-489. http://doi.org/10.1016/j.foodchem.2009.06.045.

Maree HJ. et al. 2013. Grapevine leafroll-associated virus 3. *Frontiers in Microbiology* 4 (82). http://doi.org/10.3389/fmicb.2013.00082.

Moon JY, Park JM. 2016. Cross-talk in viral defense signaling in plants. *Frontiers in Microbiology* 7 (307): 904. http://doi.org/10.3389/fmicb.2016.02068.

Naidu RA, Maree HJ, Burger JT. 2015. Grapevine leafroll disease and associated viruses: A unique pathosystem. *Annual Review of Phytopathology* 53 (1): 613-634. http://doi.org/10.1146/annurev-phyto-102313-045946.

Prosser SW, Goszczynski DE, Meng B. 2007. Molecular analysis of double-stranded RNAs reveals complex infection of grapevines with multiple viruses. *Virus Research* 124 (1-2): 151-159. http://doi.org/10.1016/j.virusres.2006.10.014.

Vega A, Gutiérrez RA, Peña-Neira A, Cramer GR, Arce-Johnson P. 2011. Compatible GLRaV-3 viral infections affect berry ripening decreasing sugar accumulation and anthocyanin biosynthesis in *Vitis vinifera*. *Plant Molecular Biology* 77 (3): 261-274.

FUNDING AGENCIES

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

ACKNOWLEDGEMENTS

We would like to acknowledge all Cantu lab members for participating in the sampling process and especially Eric Tran, Rosa Figueroa, Jadran Garcia Navarrete, Daniela Quiroz Larrain, Lucero Espinoza, and Diana Liang for preparing samples and extracts for downstream applications. We would also like to thank Larry Lerno for his assistance with LC-MS instrumentation.

IMPROVING EXTENSION OUTCOMES: IDENTIFYING DRIVERS AND BARRIERS TO ADOPTION OF MANAGEMENT PRACTICES USING LEAFROLL AND RED BLOTCH DISEASE AS MODEL SYSTEMS

Project Leader: Monica L. Cooper | Cooperative Extension | University of California | Napa, CA 94559 | mlycooper@ucanr.edu
Co-Project Leader: Malcolm B. Hobbs | Cooperative Extension | University of California | Napa, CA 94559 | mbhobbs@ucdavis.edu
Cooperator: Larry Bettiga | Cooperative Extension | University of California | Salinas, CA 93901 | lbettiga@ucanr.edu
Cooperator: Stephanie Bolton | Lodi Winegrape Commission | Lodi, CA 95242 | stephanie@lodiwine.com

Reporting Period: The results reported here are from work conducted July 2019 to October 2019.

ABSTRACT

Successful extension programs support the uptake of data-driven solutions to management challenges. This project seeks to improve extension outcomes by identifying drivers and barriers to adoption of best management practices, using two significant viral diseases of grapevine as model systems. Using quantitative surveys and qualitative interviews, the project team will explore the importance that decision-makers (grape growers and consultants) place on various educational resources, the ways in which they access these resources, how they use those resources and their professional networks to make informed decisions, and how they support adoption of best management practices among the greater community. The project aims to identify (1) individual or regional differences in how information is accessed, (2) where extension programs should focus resources to remove barriers to adoption, (3) how to improve resource allocation to optimize accessibility and efficiency, (4) critical successes and failures of leafroll and red blotch disease outreach efforts, and (5) ultimately to develop resources to improve current and future extension efforts.

LAYPERSON SUMMARY

Grapevine leafroll disease (GLD) is one of the most significant viral diseases of grapevines worldwide. Since 2012, grapevine red blotch disease (GRBD) has emerged as an important viral disease of grapevine in North America. Multiple studies of GLD epidemiology have coalesced into management guidelines that encourage the use of virus-tested plant material, the local eradication (through vine removal) of diseased vines, and management of vector populations. Since GRBD was more recently identified, management guidelines are still in development but will likely include similar practices. Uptake and implementation of disease management practices has varied among growers and across regions due to various factors such as resource limitations and communication challenges. Therefore, we will use GLD and GRBD as model systems to study potential drivers and barriers to the adoption of management recommendations in three California wine grape growing regions. Our goal is to identify critical successes and failures of outreach efforts. This will help us build effective extension programs that maximize accessibility and efficiency in the face of scare resources, while promoting the uptake of control

practices and positive outcomes for affected growers. This will improve current extension efforts and inform suitable responses to emerging pests and diseases.

INTRODUCTION

Grapevine leafroll disease (GLD) is one of the most significant viral diseases of grapevines worldwide, described for more than a century (Hoefert and Gifford 1967), occurring in every major grape-growing region, and infecting wine, juice and table grape cultivars, as well as rootstocks (Maree et al. 2013). The pathogens associated with GLD are known collectively as grapevine leafroll-associated viruses (GLRaV; Martelli et al. 2012). Of these, GLRaV-3 is the most widely reported, occurring in Europe, Africa, Asia, Oceania and the Americas (Maree et al. 2013). Decreased fruit quality and pigmentation (Guidoni et al. 2000), altered amino acid profiles (Lee et al. 2009), delayed maturity and yield reductions (Blaisdell et al. 2016; Woodrum et al. 1984) lead to significant economic losses (Atallah et al. 2012; Ricketts et al. 2015). Vine-to-vine transmission of GLRaV-3 occurs via mealybug and soft scale species (Herrbach et al. 2017; Almeida et al. 2013).

Grapevine red blotch disease (GRBD) has emerged in the last decade as an important viral disease of grapevine in North America. Grapevine red blotch virus (GRBV), the causal agent of red blotch disease (Yepes et al. 2018), is widespread in vineyards throughout the United States (Cieniewicz et al. 2017, Krenz et al. 2014, Sudarshana et al. 2015). GRBV affects the profitability of vineyards by reducing fruit quality and ripening (Blanco-Ulate et al. 2017; Martínez-Lüscher et al. 2019), resulting in losses up to $170,000 per acre over the lifespan of a vineyard, depending on the initial disease incidence, cultivar, region, and price penalty for low quality fruit (Ricketts et al. 2017).

Epidemiological studies have elucidated the impact of long and short distance spread on GLD outbreaks (Arnold et al. 2017; Charles et al. 2009; Poojari et al. 2017), resulting in a series of recommended management practices aimed at minimizing the introduction of the pathogen to new areas as well as local spread (Almeida et al. 2013; Pietersen et al. 2013; Sokolsky et al. 2013; Bell et al. 2018). Epidemiological studies for GRBD are ongoing (Bahder et al. 2016, Perry et al. 2016, Cieniewicz et al. 2018, Preto et al. 2018), and because of its more recent discovery have not coalesced into a clear set of actionable management guidelines, although these will likely also include the planting of virus-tested plant material and removal of diseased vines.

Anecdotally, we have observed differences in adoption rates for disease management practices across wine growing regions. It is imperative that the reasons for these differences be understood, not only to improve disease management outcomes for growers and grower communities, but also to inform research and outreach efforts for current and emerging pests and diseases. One barrier to adoption could be financial: removal of diseased vines and or blocks can be expensive (Ricketts et al. 2015, 2017; Cooper et al. 2012) and compounded by additional costs of training staff to make reliable observations and laboratory assays to confirm virus status of plant material. Faced with such costs, beneficial practices may only be partially employed, or growers may choose to tolerate the presence of GLD and absorb what they consider to be the lower cost option of reduced fruit yields or quality (Atallah et al. 2012; Ricketts et al. 2015; Andrew et al. 2015).

Other barriers to adoption may include misperceptions about scientific conclusions and personal capability to control the disease. Misunderstandings about the science of the disease may persist because growers were not exposed to resources, or because the information was not disseminated in an appropriate manner. An added challenge is the lag between the emergence of the pest or disease and the availability of science-based management recommendations. During the lag, growers often need to take management actions; in the absence of clear scientific direction, they may rely on their personal experience or anecdotal evidence. This could lead to the belief that control practices are ineffective, not worth implementing, or that the disease is not important. In addition, there are logistical and practical challenges to overcome, such as: (1) availability and reliability of monitoring tools for vectors and assays for pathogens; (2) training costs to prepare staff to identify vectors and diseased vines; (3) the role of external players (nurseries, neighbors) in disease epidemiology.

In order to improve uptake of best practices to realize positive disease management outcomes, we aim to understand what drives adoption and how barriers are overcome. The drivers of adoption are conceptualized as the effectiveness of outreach resources for promoting disease control. A range of outreach resources are available: seminars; trade & research articles; workshops & field days; videos; consultants; extension personnel; research trials; regional grower groups; and personal observations. Hoffman et al. (2011; 2015) reported on grower perceptions of the usefulness of many of these resources and the importance of knowledge networks for promoting vineyard management practices. We intend to extend Hoffman's work to the specific of issue of leafroll and red blotch disease in order to (1) quantify the usefulness of various resources; (2) assess differences in resource preference by position within the company (intern vs. vineyard director) to tailor outreach materials to different levels of responsibility and experience; (3) evaluate the effectiveness of well-connected individuals and regional grower groups to disseminate information. Understanding these factors is critical to identifying successes and failures of outreach efforts, to improve extension programs and build suitable responses to grapevine pests and diseases.

OBJECTIVES

The goal of this project is to improve the outreach response to current and future grapevine pests and diseases by identifying drivers and barriers to the adoption of management practices, using GLD and GRBD as model systems. The study has two phases (using quantitative and qualitative assessment tools) in three wine grape growing regions of California (Napa; Lodi; Monterey), and will produce recommendations for outreach strategies to promote adoption of disease management practices and guidelines for adapting these strategies to other grapevine pests and diseases.

1. Use quantitative and qualitative tools to assess potential drivers and barriers to the adoption of leafroll and red blotch disease management practices.
2. Develop recommendations for improving outreach programs that support greater adoption of best management practices for leafroll and red blotch disease, and guidelines for adapting these to improve current and future responses to grapevine pests and diseases.

RESULTS AND DISCUSSION

Objective 1. Use Quantitative and Qualitative Tools to Assess Potential Drivers and Barriers to the Adoption of Leafroll and Red Blotch Disease Management Practices

A quantitative tool was developed, consisting of 43 questions/statements aimed at measuring (1) demographic variables, (2) adoption rates of various disease management practices, (3) perceptions regarding usefulness of educational resources, (4) drivers and barriers to adoption of management strategies. Quantitative survey data were collected from a population (n = 93) of vineyard directors, managers and owners, viticulturists, consultants (pest control advisers) and field scouts working primarily in Napa County (70% of respondents) and growing *Vitis vinifera* cultivars Cabernet Sauvignon (87%) and Chardonnay (48%). Current efforts are underway to conduct similar surveys in the other study regions (Lodi and Monterey, California).

The surveyed population indicated most educational resources were useful (**Figure 1**), with observations of own vineyard, formal seminars/lectures, replicated research trials and public advisors ranked as most useful, and trade journals, webinars, online videos and social media as least useful. The surveyed population also ranked the importance of five identified virus disease management strategies: government/industry standards, commitment and cooperation from other growers, regional management programs, regional control of vectors and further research from scientists (**Figure 2**). This population ranked the implementation of government and industry standards as the most important factor for virus disease management and further research from scientists as least important, with regional programs and cooperation scoring intermediate categories. This indicates the important role regulatory agencies can play in supporting growers managing incurable viral diseases, and the value of regional and cooperative programs. Further analysis will explore demographic and regional differences to more fully assess the drivers and barriers to adoption of recommended practices.

A qualitative interview tool is under development and we plan to commence interviews in the second and third quarters of the project period.

Objective 2. Develop Recommendations for Improving Outreach Programs that Support Greater Adoption of Best Management Practices for Leafroll and Red Blotch Disease, and Guidelines for Adapting These to Improve Current and Future Responses to Grapevine Pests and Diseases

No results to report as we have not yet initiated work on this objective. We plan to work on this objective in project years 2 and 3, once we have collected data using the quantitative survey and qualitative interviews as described in objective 1.

CONCLUSIONS

Conclusions are preliminary given we are reporting on the first quarter's activities. We developed a quantitative survey and collected data in one study region. Qualitative interview questions are under development, with interviews scheduled to commence in the second and third quarters of the project period (Nov 2019 to Mar 2020). If we are able to recruit adequate participation in the survey and interviews, we expect to be able to meet the objectives as outlined.

REFERENCES CITED

Almeida RPP, Daane KM, Bell VA, Blaisdell GK, Cooper ML, Herrbach E, Pietersen G. 2013. Ecology and management of grapevine leafroll disease. *Frontiers of Microbiology* doi: 10.3389/fmicb.2013.00094.

Andrew R, Bell V, Hoskins N, Pietersen G, Thompson C. 2015. Leafroll 3 virus and how to manage it. *New Zealand Winegrowers*: Auckland, New Zealand. 108 pgs.

Arnold KL, Golino DA, McRoberts N. 2017. A synoptic analysis of the temporal and spatial aspects of grapevine leafroll disease in a historic Napa vineyard and experimental blocks. *Phytopathology* 107: 418-426.

Atallah SS, Gomez MI, Fuchs MF, Martinson TE. 2012. Economic impact of grapevine leafroll disease on *Vitis vinifera* cv. Cabernet Franc in Finger Lakes vineyards of New York. *American Journal of Enology and Viticulture* 63: 73-79.

Bahder BA, Zalom FG, Jayanth M, Sudarshana MR. 2016. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* as a vector of grapevine red blotch-associated virus. *Phytopathology* 106: 1223-1230.

Bell VA, Hedderley DI, Pietersen G, Lester PJ. 2018. Vineyard-wide control of grapevine leafroll-associated virus 3 requires an integrated response. *Journal of Plant Pathology* https://doi.org/ 10.1007/s42161-018-0085-z

Blaisdell GK, Cooper ML, Kuhn EJ, Taylor KA, Daane KM, Almeida RPP. 2016. Disease progression of vector-mediated grapevine leafroll-associated virus 3 infection of mature plants under commercial vineyard conditions. *European Journal of Plant Pathology* 146: 105-116.

Blanco-Ulate B Hopfer H, Figueroa-Balderas R, Ye Z, Rivero RM, Albacete A, Perez-Alfocea F, Koyama R, Anderson MM, Smith RJ, Ebeler SE, Cantu D. 2017. Red blotch disease alters grape berry development and metabolism by interfering with the transcriptional and hormonal regulation of ripening. *Journal of Experimental Botany* 68: 1225-1238.

Charles JC, Froud KJ, van den Brink R, Allan DJ, J.C. 2009. Mealybugs and the spread of grapevine leafroll-associated virus 3 (GLRaV-3) in a New Zealand vineyard. *Australasian Plant Pathology* 38: 576-583.

Cieniewicz EJ, Loeb G, Pethybridge S, Perry KL, Fuchs MF. 2018. Insights into the ecology of grapevine red blotch virus in a diseased vineyard. *Phytopathology* 108: 94-102.

Cieniewicz EJ, Perry KL, Fuchs MF. 2017. Grapevine red blotch: Molecular biology of the virus and management of the disease. Pages 303-314 in Meng B, Martelli FP, Golino DA, Fuchs M (eds.). *Grapevine Viruses: Molecular Biology, Diagnostics, and Management*. Springer.

Cooper ML, Klonsky KM, DeMoura RL. 2012. Sample costs to establish a vineyard and produce wine grapes in Napa County. *Cost and Return Studies*, UC Davis, Department of Agriculture and Resource Economics.

Guidoni S, Mannini F, Ferrandino A, Argamante N, di Stefano R. 2000. Effect of virus status on leaf and berry phenolic compounds in two winegrape *Vitis vinifera* cultivars. *Acta Horticulturae* 526: 445-452.

Herrbach E, Alliaume A, Prator CA, Daane KM, Cooper ML, Almeida RPP. 2017. Vector transmission of grapevine leafroll-associated viruses (Chapter 24). Pages 483-503 in Meng B, Martelli FP, Golino DA, Fuchs M (eds.). *Grapevine Viruses: Molecular Biology, Diagnostics, and Management*. Springer, doi: 10.1007/978-3-319-57706-7_24.

Hoefert LL, Gifford EM, Jr. 1967. Grapevine leafroll virus: History and anatomic effects. *Hilgardia* 38: 403-426.

Hoffman M, Lubell M, Hillis V. 2011. Learning pathways in viticulture management. *Research Brief*, Center for Environmental Policy and Behavior, UC Davis.

Hoffman M, Lubell M, Hillis V. 2015. Network-smart extension could catalyze social learning. *California Agriculture* 69: 113-122.

Krenz B, Thompson J, McLane H, Fuchs M, Perry KL. 2014. Grapevine red blotch-associated virus is widespread in the United States. *Phytopathology* 102: 232-240.

Lee J, Keller KE, Rennaker C, Martin RR. 2009. Influence of grapevine leafroll-associated viruses (GLRaV-2 and -3) on the fruit composition of Oregon *Vitis vinifera* L. cv. Pinot Noir: Free amino acids, sugars, and organic acids. *Food Chemistry* 117: 99-105.

Maree HJ, Almeida RPP, Bester R, Chooi K, Cohen D, Doljia VV, Fuchs MF, Golino DA, Jooste AEC, Martelli GP, Naidu RA, Rowhani A, Saldarelli P, Burger JT. 2013. Grapevine leafroll-associated virus 3. *Frontiers of Microbiology* doi: 10.3389/fmicb.2013.00082.

Martelli GP, Abou Ghanem-Sabanadzovic N, Agranowsky AA, Al Rawhanih M, Dolja VV, Dovas CI, Fuchs M, Gugerli P, Hu JS, Jelkmann W, Katis N, Maliogka VI, Melzer MJ, Menzel W, Minafra A, Rott ME, Rowhani A, Sabanadzovic S, Saldarelli P. 2012. Taxonomic revision of the family Closteroviridae with special reference to the grapevine leafroll-associated member of the genus Ampelovirus and the putative species unassigned to the family. *Journal of Plant Pathology* 94: 7-19.

Martínez-Lüscher J, Kurtural SK, Brillante L, Oberholster A, Cooper ML, Smith RJ, Al Rwahnih M. 2019. Grapevine red blotch virus may reduce carbon translocation leading to impaired berry ripening. *Journal of Agricultural and Food Chemistry* 67: 2437-2448.

Perry KL, McLane H, Hyder MZ, Dangel GS, Thompson JR, Fuchs MF. 2016. Grapevine red blotch-associated virus is present in free-living *Vitis* spp. proximal to cultivated grapevines. *Phytopathology* 106: 663-670.

Pietersen G, Spreeth N, Oosthuizen T, van Rensburg A, van Rensburg M, Lottering D, Russouw N, Tooth D. 2013. Control of grapevine leafroll disease spread at a commercial wine estate in South Africa: A case study. *American Journal of Enology and Viticulture* 64: 296-305.

Poojari S, Boule J, DeLury N, Lowery DT, Rott M, Schmidt AM, Urbez-Torres JR. 2017. Epidemiology and genetic diversity of grapevine leafroll-associated viruses in British Columbia. *Plant Disease* 101: 2088-2097.

Preto CR, Sudarshana MR, Zalom FG. 2018. Feeding and reproductive hosts of *Spissistilus festinus* (Say) (Hemiptera: Membracidae) found in Californian vineyards. *Journal of Economic Entomology* doi: 10.1093/jee/toy236

Ricketts KM, Gómez MI, Fuchs MF, Martinson T, Smith RJ, Cooper ML, Moyer MM, Wise A. 2017. Mitigating the economic impact of grapevine red blotch: Optimizing disease management strategies in U.S. vineyards. *American Journal of Enology and Viticulture* 68: 127-135.

Ricketts KD, Gomez MI, Atallah SS, Fuchs MF, Martinson TE, Battany MC, Bettiga LJ, Cooper ML, Verdegaal PS, Smith RJ. 2015. Reducing the economic impact of grapevine leafroll disease in California: Identifying optimal disease management strategies. *American. Journal of Enology and Viticultural* 66: 138-147.

Sokolsky T, Cohen Y, Zahavi T, Sapir G, Sharon R. 2013. Potential efficiency of grapevine leafroll disease management strategies using simulation and real spatio-temporal disease infection data. *Australian Journal of Grape and Wine Research* 19: 431-438.

Sudarshana MR, Perry KL, Fuchs MF. 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Phytopathology* 105: 1026-1032.

Woodrum RC, Antcliff AJ, Krake LR, Taylor RH. 1984. Yield differences between Sultana clones related to virus status and genetic factors. *Vitis* 23: 73-83.

Yepes LM, Cieniewicz E, Krenz B, McLane H, Thompson JR, Perry KL, Fuchs M. 2018. Causative role of grapevine red blotch virus in red blotch disease. *Phytopathology* 108: 902-909.

FUNDING AGENCIES

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

ACKNOWLEDGEMENTS

We gratefully acknowledge the participation of survey respondents and research support provided by S. Vengco.

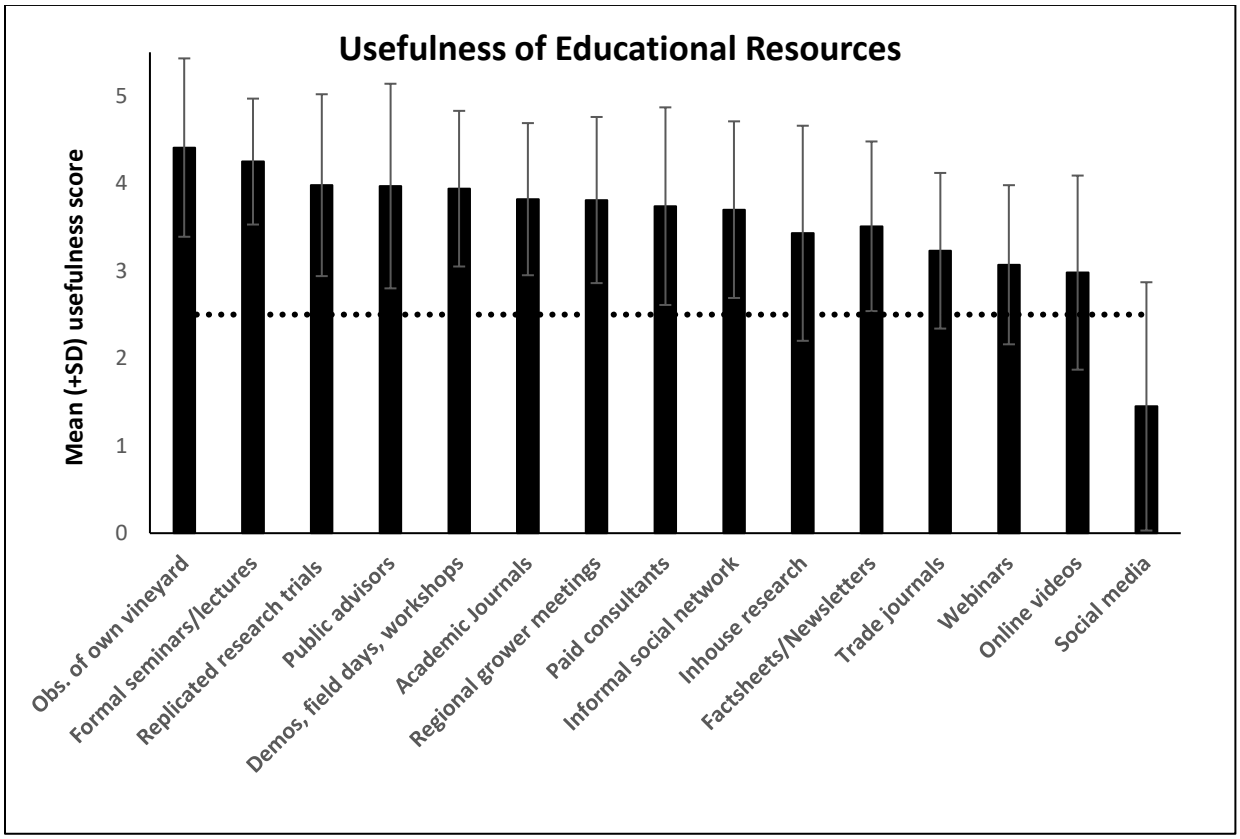


Figure 1. Perceived usefulness of educational resources to make management decisions among a surveyed population of 93 grape growers, managers and consultants. All educational resources ranked above average for usefulness, except social media. Most useful resources were vineyard observations, seminars/lectures, replicated research trials and public advisors, whereas trade journals, webinars, online videos and social media ranked least useful.

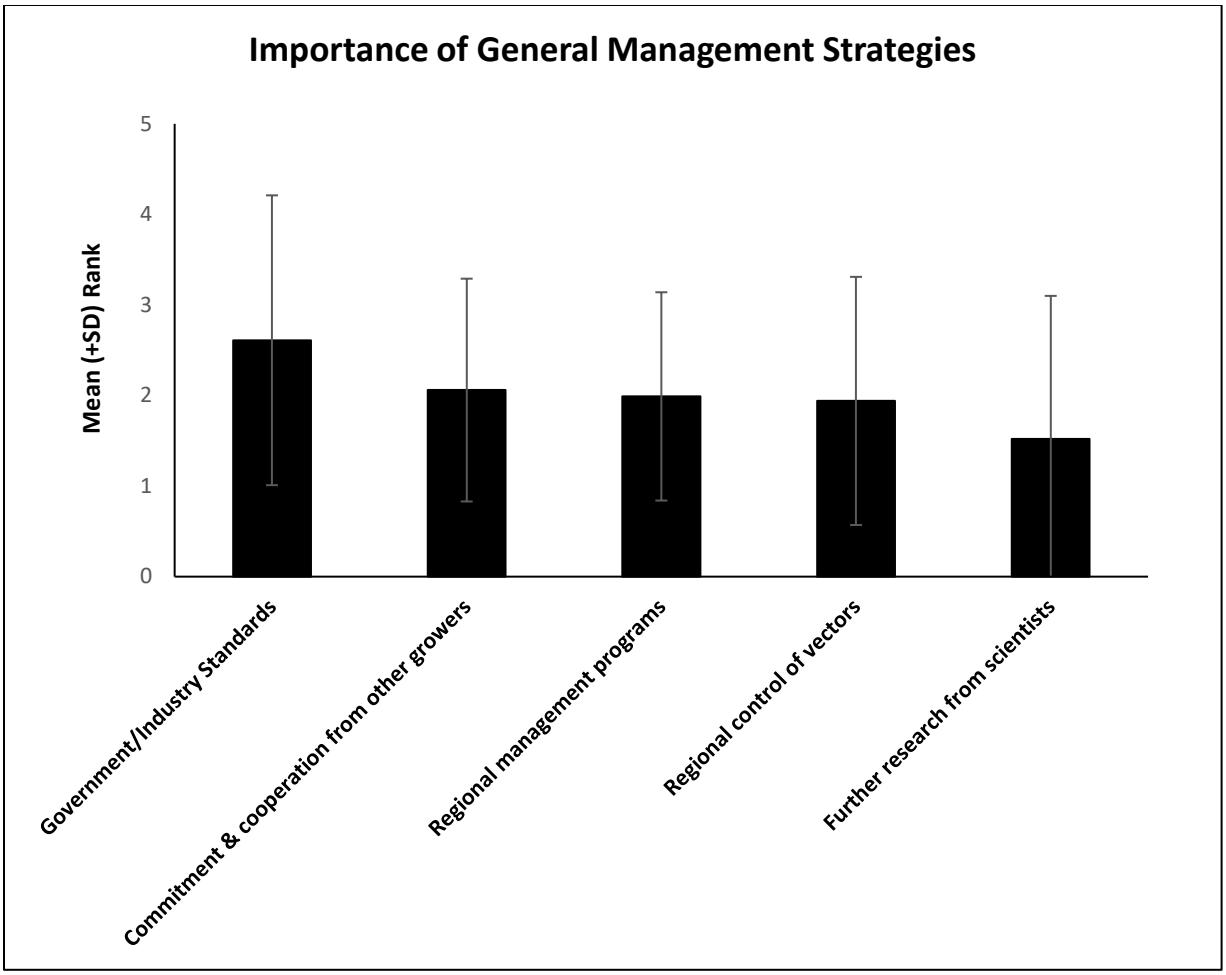


Figure 2. Perceived importance of general strategies for GLD and GRBD among a surveyed population of 93 grape growers, managers, and consultants. Government/industry standards ranked most important (score of 2.61); cooperation from other growers ranked second (score of 2.06); regional management program ranked third (score of 1.99); regional control of vectors ranked fourth (score of 1.94) and lastly was further research from scientists (score of 1.52).

SEASONAL ECOLOGY AND TRANSMISSION EFFICIENCY OF THREE-CORNERED ALFALFA HOPPER AND OTHER NOVEL INSECT VECTORS OF GRAPEVINE RED BLOTCH VIRUS

Project Leader: Kent M. Daane | Department of Environmental Science, Policy, and Management | University of California | Berkeley, CA 94720 | kdaane@ucanr.edu
Co-Project Leader: Houston Wilson | Department of Entomology | University of California | Riverside, CA 92521 | houston.wilson@ucr.edu
Cooperator: Monica Cooper | Cooperative Extension | University of California | Napa, CA 94559 | mlycooper@ucanr.edu
Post-Doctoral Researcher: Alexis Billings | Department of Environmental Science, Policy, and Management | University of California | Berkeley, CA 94720 | billinac@berkeley.edu
Laboratory Technician: Kristen Flores | Department of Environmental Science, Policy, and Management | University of California | Berkeley, CA 94720 | kmflores@berkeley.edu
Post-Doctoral Researcher: Kelsey Schall | Department of Environmental Science, Policy, and Management | University of California | Berkeley, CA 94720 | mccallak@berkeley.edu

Reporting Period: The results reported here are from work conducted September 2018 to October 2019.

ABSTRACT

Grapevine red blotch virus (GRBV) is a recently characterized DNA virus in the family *Geminiviridae*. This pathogen is the causal agent of grapevine red blotch disease, which affects cultivated grapevines and leads to negative effects on crop quality and yield. GRBV is present in vineyards and plant material repositories around the world, indicating spread that was largely human mediated. That said, recent surveys have demonstrated that there appears to be secondary transmission, most likely by an insect vector. Here, vineyard insects and plants were surveyed to identify potential candidate vectors and non-crop plants that may act as reservoirs for this pathogen. Results indicate that GRBV is limited to grapes (*Vitis* spp.), including both wild and cultivated grapes. Eight insect genera or species, field collected in vineyards, tested positive for GRBV; these were *Acinopterus angustatus*, *Colladonus coquilleti*, *C. montanus reductus*, *Colladonus* sp., *Lygus* sp., *Scaphytopius* spp., the three-cornered alfalfa hopper (*Spissistilus festinus*; TCAH), and an unknown Delphacid. Of these, TCAH is already known to be capable of transmitting GRBV while *Scaphytopius* spp. and the various species of *Colladonus* recovered merit closer evaluation as candidate vectors due to their affinity with grapes. Ongoing studies investigated vector transmission efficiency in both greenhouse studies and field-greenhouse studies, although we have yet to confirm transmission with any of the 10 species tested. Current studies are investigating the relationship between ground covers and neighboring vegetation to the number of TCAH and other potential vectors in the vineyard.

LAYPERSON SUMMARY

Grapevine red blotch virus (GRBV) is associated with grapevine red blotch disease in winegrapes (*Vitis vinifera*) and negatively impacts crop vigor, yield, and quality. Surveys have revealed that the virus only infects grapes (*Vitis* spp.). While multiple insects have tested positive for GRBV, in our studies we have yet to show that any of the common insects found in vineyards successfully transmit the pathogen. Other laboratories have shown that the three-cornered alfalfa

hopper (*Spissistilus festinus*; TCAH) transmits the virus between grapevines. We are now in the process of developing a better understanding of the seasonal ecology and transmission efficiency of TCAH in vineyards. Additionally, we plan to test the ability of any remaining candidate insect vectors to transmit GRBV. Our goal is to use this information to develop actionable management strategies for commercial grape growers to help reduce the incidence and spread of GRBV in vineyards.

INTRODUCTION

Grapevine red blotch virus (GRBV) is a circular, single-stranded DNA virus tentatively placed in the *Geminiviridae* and is associated with grapevine red blotch disease (GRBD), particularly important in winegrapes (*Vitis vinifera*) (Krenz et al., 2012; Al Rwahnih et al., 2013). Symptoms of GRBD include reddening of leaf veins and the appearance of blotchy red areas on the leaf surface and/or at the leaf margin, especially on basal leaves and towards the end of the growing season, although these symptoms are less apparent in white-berried cultivars, which exhibit irregular chlorotic and/or necrotic areas on leaves rather than any type of reddening of leaves (Sudarshana et al., 2015). GRBD negatively impacts crop vigor, yield, and quality. Diseased vines typically exhibit reduced photosynthesis and stomatal conductance, delayed fruit maturation, decreased accumulation of sugars and anthocyanins, and lower pruning and berry weights (Wallis and Sudarshana, 2016; Martínez-Lüscher et al., 2019; Blanco-Ulate et al., 2017; Girardello et al., 2019).

While this disease was first reported in 2008 in Napa County (California, USA), subsequent vineyard surveys have found GRBV to be present outside of North America (Krenz et al., 2014; Al Rwahnih et al., 2015b; Thompson et al., 2018; Luna et al., 2019; Gasperin-Bulbarela et al., 2019; Lim et al., 2016). Testing of archival plant material revealed the virus has been present in California since at least 1940 (Al Rwahnih et al., 2015a). The wide geographic distribution of GRBV implicates that this virus was likely distributed via infected nursery material, although many have also reported in-field spread of GRBD. While increased incidence of GRBD over time within vineyards and/or clustering of symptomatic vines gave reason to believe in the existence of one or more vectors, it could be argued that such trends were the result of environmental factors leading to latent expression of symptoms in some GRBV-positive vines. However, the argument for an insect vector was strengthened by surveys that revealed the presence of GRBV in wild grapes (*Vitis* spp.) naturally established outside of vineyards (Bahder et al., 2016a; Perry et al., 2016) and shortly thereafter it was shown that a treehopper, the three-cornered alfalfa hopper (*Spissistilus festinus*; TCAH) could successfully transmit GRBV between grapevines (Bahder et al., 2016b).

GRBV is or is closely related to *Geminiviridae* (Krenz et al., 2012; Al Rwahnih et al., 2013; Sudarshana et al., 2015; Varsani et al., 2017). The only known vectors of viruses in this family are hemipterans, in particular leafhoppers, treehoppers, and whiteflies (Briddon and Stanley, 2015; Bahder et al., 2016b). Key vineyard hemipterans that are known to regularly feed on grapevines include *Erythroneura* leafhoppers [Cicadellidae: western grape leafhopper (*E. elegantula*), variegated leafhopper (*E. variabilis*), and Virginia creeper leafhopper (*E. ziczac*)]; mealybugs [Pseudococcidae: vine mealybug (*Planococcus ficus*), *Pseudococcus maritimus*, obscure mealybug (*Ps. viburni*), and Gill's mealybug (*Ferrisia gilli*)]; blue-green sharpshooter (Cicadellidae: *Graphocephala atropunctata*), and, to a lesser extent, grape phylloxera

(Phylloxeridae: *Daktulosphaira vitifoliae*), grape whitefly (Aleyrodidae: *Trialeurodes vittatas*), and lecanium scale (Coccidae: *Parthenolecanium corni*). While many of these candidate vectors are frequently encountered and/or in high abundance in vineyards, so far experiments have shown that only TCAH can successfully transmit GRBV between grapevines (Daane et al., 2017).

While the ecology and management of TCAH has been well defined for multiple leguminous crops like alfalfa, soybeans, and peanuts (Meisch and Randolph, 1965; Mueller and Dumas, 1975; Moore and Mueller, 1976; Mitchell and Newsom, 1984; Wilson and Quisenberry, 1987; Johnson and Mueller, 1989; Wistrom et al., 2010; Beyer et al., 2017), very little is known about this insect in vineyards. Facing a lack of information, growers concerned about the spread of GRBV in their vineyards may be inclined to preemptively apply chemical controls for TCAH. As such, new information on TCAH population dynamics, transmission efficiency, and economic thresholds in vineyards will be critical to the development of sustainable integrated pest management programs.

In addition to TCAH, broad testing of numerous non-economic insects in vineyards has revealed a number of potentially novel candidate vectors, including *Melanoliarus* sp. (Cixiidae), *Osbornellus borealis* (Cicadellidae), and *Colladonus reductus* (Cicadellidae) (Cieniewicz et al., 2017; Fuchs et al., 2017) and *Scaphytopius* spp. (reported herein). Like TCAH, these organisms are typically found in low to moderate abundance in vineyards but are nonetheless present in and around these systems (Wilson et al., 2016; Daane et al., 2017). One issue of note with any of these vectors is their variation among vineyards and regions, annually and seasonally. For this reason, transmission of the pathogen two years ago may not be indicated by the presence of any particular insect in the current sampling season. Moreover, the role that different ground covers or nearby vegetation have on the presence of any of these species is not clearly understood. For example, we know that TCAH can reproduce on certain leguminous annual ground covers found in vineyards (Zalom et al., 2017); however, the role of perennial non-crop plants found outside of or adjacent to vineyards is less clear.

Recent work has demonstrated that TCAH densities in vineyards do not appear to be influenced by proximity to natural habitats such as oak woodland and riparian areas (Zalom et al., 2017). While many of the perennial plants found in such habitats can likely serve as suitable overwintering sites, or even reproduction sites (less likely), the TCAH does not appear to have an obligate relationship with any particular perennial species. That said, they do appear to make some use of these plants and more information on this will contribute to a better understanding of their seasonal ecology and movement between vineyards and natural habitats.

OBJECTIVES

1. Determine TCAH overwintering sites and reproduction on non-crop perennial plants to better understand its source populations.
2. Record the timing of TCAH colonization, movement into the vine canopy, and cane girdling to better determine the manager's optimal timing of control measures.
3. Evaluation of novel insect vector candidates.
4. Evaluation of TCAH transmission efficiency throughout the season, with different TCAH life stages and on different grape cultivars

RESULTS AND DISCUSSION

Objective 1. TCAH Overwintering Sites and Reproduction on Non-Crop Perennial Plants

TCAH populations have been sampled in natural vegetation surrounding vineyards with known TCAH populations. During the winter, it is assumed that TCAH is in the adult stage but breeding hosts other than legumes have been difficult to identify in the field. To date we have recovered TCAH adults on toyon (*Heteromeles arbutifolia*), wild grape, and various ground covers, primarily legumes during the summer, but TCAH has only been recovered from ground covers during the winter.

Objective 2. Timing of TCAH Colonization, Movement into the Vine Canopy, and Cane Girdling

TCAH Transect Study. We have sampled vineyards in Napa and Sonoma counties to evaluate the activity of TCAH populations along transects that extend out from large patches of natural habitat into vineyards. Field sites consist of vineyard blocks greater than two acres in size adjacent to riparian and/or oak woodland habitat. There are five total study sites. All vineyard blocks are red varietals that are at least five years old and located on level ground with similar trellis and irrigation systems. All plots are maintained insecticide free throughout the course of the study.

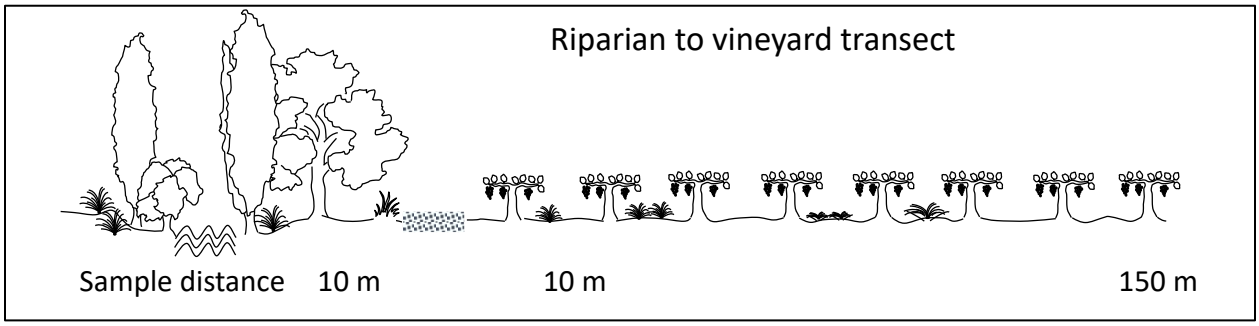


Figure 1. Diagram of vineyard study sites.

At each site insects are sampled along five parallel transects positioned 20 meters apart that extend out from the riparian or oak woodland habitat (i.e., "natural habitat") into the vineyard. Each transect is 160 meters long, going 10 meters into the natural habitat and 150 meters into the vineyard. Along each transect samples are taken at the interior of the natural habitat (10 meters into the habitat) as well as at the edge and interior of the vineyard (10 and 150 meters into the vineyard, respectively). The edge of the vineyard and natural habitat are typically separated by a roadway or path that is about 5 meters wide. Densities of TCAH, *Erythroneura* leafhoppers, and other hemipterans are being monitored along the transects approximately every two weeks using a combination of yellow sticky traps, sweep nets, and beat-sheet sampling. Two yellow sticky traps (16 x 10 cm, Seabright Laboratories, Emeryville, CA) are placed at each transect point. In the vineyard, one trap is placed in the vine canopy (approximately four feet above the ground surface) and another trap is hung from irrigation lines (approximately one foot above the ground surface). In the natural habitat, two sticky traps are hung from a pole at each transect point at a height equal to those in the vineyard (i.e., one trap four feet and the other one foot above the

ground surface). Traps are replaced approximately every two weeks between March 2017 and March 2019. Sweep-nets are used to sample ground covers. At each transect point, a set of 30 unidirectional sweeps are collected from the groundcovers using a 30.5 cm diameter sweep-net (BioQuip Products, Rancho Dominguez, CA). Proportion of ground cover to bare soil is recorded along with species composition and ground cover status (i.e., proportion of cover that was still green/healthy). A modified beat-sheet is used at each transect point to sample the canopy of grapevines (in the vineyard) and non-crop species (in the natural habitat). The beat-sheet consists of a one-square-meter nylon funnel that feeds into a detachable one-gallon plastic bag. For each sample, the funnel is held beneath the canopy while vigorously shaking the plant (or vine) for 30 seconds to dislodge insects into the funnel and plastic collection bag. Each month, vines along each vineyard transect point are evaluated for signs of TCAH feeding damage (i.e., girdling of leaf petioles). At each vineyard transect point, one cane from each of 10 randomly selected vines is visually inspected for leaf girdling. Total leaf nodes and leaf girdles per cane were recorded for each vine.

While survey and transect work has concluded in Napa/Sonoma and is currently being prepared for publication, work in San Luis Obispo was just initiated in June 2019. We report here on preliminary findings, with a full analysis at the end of the collection to be prepared for publication. At the Napa/Sonoma transect sites, TCAH activity showed a strong temporal trend, with densities generally increased between June and August along with some activity in March and October/November. Comparing the different sampling techniques, the highest TCAH densities were recorded on yellow sticky traps, followed by sweep nets and then beat-sheets. While there was no clear gradient of TCAH activity across the transect points, densities on the traps and in the sweep samples were slightly elevated in natural habitats in early June just prior to increases observed in the vine canopy at both the vineyard edge and interior in the following round of sampling. Changes in TCAH densities between the ground covers and vine canopy were not always clearly reflected in the data. While densities in the vine canopy did increase as the proportion of healthy/green ground covers diminished, some TCAH could still be found on the little bit of ground cover that remained later in the season. Surprisingly, these late season TCAH were most frequently encountered on ground covers in the vineyard interior. Finally, petiole girdling became apparent in August, with a higher proportion of girdles located at the vineyard interior. This increase in girdling in August follows increased TCAH densities observed in the vine canopy between June and August.

TCAH Groundcovers Study. Based on the findings of the above transect study, we initiated a new field study in March 2019 to evaluate the influence of two ground cover management strategies on TCAH populations and their movement into the vine canopy. This study includes five commercial vineyard sites with ground covers planted in every other row. Ground covers consist of either (a) an intentionally sown mix of grasses, legumes, and/or mustards, or (b) resident weedy vegetation. In both cases ground covers contain legumes, which are the preferred host of TCAH. At each site, four to five replicate sets of paired plots were assigned to either a “mow” or “mow/disc” treatment. Each plot is five rows x two treatments = 10 rows/replicate x five replicates/site = 50 rows experimental area at each site. Growers will typically mow and/or disc ground covers in the spring, depending on vine vigor and other management objectives. Previous data indicate that TCAH appear to complete one generation on vineyard ground covers (likely legumes) in the spring before moving into the vine canopy around

June/July. The natural dry-down of vineyard ground covers in the late spring roughly coincides with TCAH completion of development into adults, which are fairly mobile, and as the quality of ground covers declines these adults migrate into the vine canopy. TCAH nymphs, on the other hand, are fairly immobile and it may be that elimination of ground covers while they are still in the nymphal stage could reduce both populations and colonization of the vine canopy. Insects were sampled bi-weekly in the ground covers using sweep nets and in the vine canopy using yellow sticky traps. Additionally, we monitored petiole girdling every two weeks in these plots. Data collection ran for two years and was completed in October 2019; these data will be presented in the next report.

Objective 3. Evaluation of Novel Insect Vector Candidates

Field Surveys. In the survey work described above, we also collected and tested other potential candidate vectors. All insects from each sample type were transferred to a one-gallon plastic freezer bag, held in a cooler, and brought to the laboratory for further processing. Since GRBV is phloem-limited, testing efforts focused on all vineyard hemipterans that were known to be phloem rather than xylem feeders. In the laboratory, freezer bags were filled with carbon dioxid to immobilize the insects. While immobilized, all hemipterans were sorted out to genus or species, and then stored in 95% ethanol in a 1.5 ml microfuge tube and held at -80°C until virus testing occurred. Each unique group of insects from an individual sample was aggregated together for testing, so that in many cases it was not an individual insect but rather a group of five to ten specimens that were tested.

A DNA extraction of the complete insect was performed using DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). Homogenization of tissue was achieved in the same manner as the plant samples. Polymerase chain reaction (PCR) was used in the detection of GRBV in insects (protocol adapted from Krenz et al., 2014). A coat protein (CP) gene fragment of GRBaV was targeted for amplification using primer pair CP-Forward/CP-Reverse respectively (CP-for: 5'-AGCGGAAGCATGATTGAGACATTGACG-3', CP-rev: 5'-AAC GTA TGT CCA CTT GCA GAA GCC GC-3'). Reactions were conducted using a Taq 2x MasterMmix (New England Biolabs, Ipswich, MA; product #M0207) according to manufacturer's instructions. The thermocycler conditions were from Krenz et al. (2014) with three minutes at 95°C, 30 cycles of 15 seconds at 95°C, 15 seconds at 55°C, and 30 seconds at 72°C, finishing with a final extension of one minute at 72°C. Results were visualized on a 1.6% agarose gel stained with GelRed® Nucleic Acid Gel Stain (Biotium, Fremont, CA).

In total, 4,004 hemipterans across 13 families were tested (**Table 1**). Of these, eight genera or species were found to be positive; this included the cicadellids *Acinopterus angustatus*, *Colladonus coquilletti*, *Colladonus montanus reductus*, *Colladonus* sp., and *Scaphytopius* spp., an unknown delphacid, a mirid *Lygus* sp., and the membracid TCAH, a known potential vector of GRBV. Some of these species or genera frequently tested positive (e.g., *Colladonus* spp. and *Scaphytopius* spp.) while others did not (e.g., TCAH, *A. angulatus*, Delphacidae, and Miridae) (**Table 1**). Plant sampling included 36 non-crop plant genera or species across 21 families that included 15 annuals and 21 perennials. From this broad range of plants, only grape was found to be positive for GRBV, and this includes samples from both cultivated (*V. vinifera*) and wild grape (*V. californica*).

Insects that tested positive were not consistently associated with any specific host plant that also frequently tested positive. Furthermore, the likelihood that any given plant or insect species would test positive tended to vary over time. That is, certain insects that were tested throughout the year only returned positive for GRBV in the spring (Miridae), summer (Delphacidae and TCAH), or fall (*Acinopterus angulatus*, *Colladonus* spp., and *Scaphytopius* spp.). Insects that were found positive on grape included *Acinopterus angulatus*, *Colladonus coquilletti*, *Colladonus* sp., *Scaphytopius* spp., and TCAH. Notably, none of the insects that tested positive are considered economic pests of grapevines.

Table 1. Insect taxa tested for GRBV and mean aggregate sample size.

Family	Genus/Species	Mean ±SEM	n	%
Aphididae		9.8 ±1.2	0/122	0
Berytidae		2.7 ±1.1	0/7	0
Cicadellidae	*Acanalonia* sp.	1.0 ±0.0	0/3	0
	Aceratagallia spp.	4.0 ±1.1	0/55	0
	Acinopterus angulatus	1.3 ±0.1	1/26	4
	Alconeura sp.	2.0	0/2	0
	Colladonus coquilletti	1.3 ±0.2	4/6	67
	Colladonus montanus reductus	1.0	1/2	50
	Colladonus sp.	1.0	1/2	50
	Deltocephalus fuscinervosus	2.5 ±0.4	0/66	0
	Dikraneura rufula	1.0 ±0.0	0/3	0
	Dikrella californica	1.0 ±0.0	0/2	0
	Empoasca spp.	3.7 ±0.8	0/41	0
	Erythroneura elegantula	5.3 ±0.6	0/156	0
	Erythroneura variabilis	3.7 ±0.5	0/66	0
	Euscelidius schenkii	1.4 ±0.3	0/12	0
	Graphocephala atropunctata	1.0	0/2	0
	Macrosteles quadrilineatus	1.0 ±0.0	0/4	0
	Osbornellus sp.	2.0	0/2	0
	Scaphytopius spp.	1.1 ±0.1	9/19	47
	Thamnotettix zelleri	2.0 ±0.4	0/8	0
Cixiidae	*Melaniolarus* spp.	2.2 ±0.4	0/10	0
Delphacidae		1.2 ±0.1	1/21	5
Lygaeidae	*Nysius raphanus*	5.2 ±3.1	0/24	0
Membracidae	*Spissistilus festinus*	1.7 ±0.2	5/38	13
Miridae	*Lygus* spp.	3.8 ±0.5	1/99	1
Pentatomidae		1.5 ±0.5	0/2	0
Psyllidae		1.6 ±0.3	0/18	0
Rhopalidae	*Boisea rubrolineata*	4.0	0/2	0
Tingidae	*Corythuca* sp.	7.2 ±2.7	0/14	0

Acinopterus angulatus were frequently found in high abundance on ground covers in the summer and fall, but the only time this species tested positive for GRBV was the one time they were recovered from the vine canopy in the fall.

Colladonus coquilletti were generally in low abundance and recovered only on ground covers (toyon; *Heteromeles arbutifolia*) and on cultivated grapevine. The highest densities were observed in the fall, when positive specimens were recovered from both cultivated grapevine and on ground covers. *Colladonus montanus reductus* were in very low abundance and only sporadically recovered on *Prunus* spp. and ground covers, the latter of which yielded specimens in the fall that tested positive. A third species of *Colladonus* sp. was also in very low abundance, and only recovered on ground covers and on cultivated grapevine in the fall. The specimens that tested positive came from cultivated grapevine in the fall.

The unknown Delphacid was frequently recovered on ground covers and cultivated grapevine, as well as once from walnut (*Juglans regia*), but this species rarely ever tested positive. The few positive specimens recovered came from ground covers in the late summer. Miridae were collected from a very wide variety of plants over the course of the year, including ground covers, cultivated grapevine, *Alnus* spp., California laurel (*Umbellularia californica*), California buckeye (*Aesculus californica*), coyote brush (*Baccharis pilularis*), elderberry (*Sambucus* spp.), Pacific madrone (*Arbutus menziesii*), coast live oak (*Quercus agrifolia*), *Prunus* spp., toyon, walnut, willow (*Salix* spp.), and wild grape. High densities were most frequently recovered from ground covers, and the only specimens that tested positive came from ground covers in the spring. Specimens collected from wild grape in the spring did not test positive. *Scaphytopius* spp. were recovered from cultivated grapevine, ground covers, blackberry (*Rubus* spp.), toyon, and walnut. Most specimens were collected in the fall, and all of those that tested positive came at this time of year from both ground covers and cultivated grapes. TCAH was recovered in moderate abundance over the season from ground covers, cultivated grapevine, wild grape, *Quercus* spp., and toyon. While this species was most frequently found on ground covers, and some positive specimens were recovered from ground covers in the spring, most of the positive specimens came from cultivated and wild grape in the summer and early fall.

Transmission Studies. As of October 2019, we have completed multiple field transmission experiments with *Scaphytopius* sp. and are currently in the process of monitoring the status of healthy inoculation vines for symptoms of GRBD. In these trials mixed-sex cohorts of field-collected *Scaphytopius* were caged on GRBV positive vines in a commercial vineyard for 48 hours, and then moved over to healthy potted vines in a greenhouse where they were allowed to feed for 48 hours, after which they were removed from the potted vines. Potted vines are now being held in the greenhouse and will be tested for GRBV every three months over a two-year period.

Objective 4. TCAH Transmission Efficiency

Previous transmission experiments (2015 to 2017) were conducted under greenhouse conditions using potted grapevines. Candidate vectors evaluated included western grape leafhopper, Virginia creeper leafhopper, grape whitefly, vine mealybug, blue-green sharpshooter, and foliar-form grape phylloxera. To date, none of these candidates have been able to move GRBV between potted vines.

While Bahder et al. (2016b) demonstrated that TCAH can transmit GRBV between potted grapevines in a greenhouse, it remains unclear how well TCAH can move this virus under field conditions. As such, we are currently evaluating TCAH transmission using field vines for virus acquisition. That is, TCAH are caged on known GRBV-positive vines in commercial vineyards for a 48-hour period and then moved to clean potted vines in the greenhouse.

In 2018, we attempted to show transmission using a new experimental design that considered virus titer in the vine. In a commercial vineyard with known positive GRBV vines, we used organdy cages to cage TCAH adults on virus-free and virus-infected vine shoots for a 48-hour acquisition period. We then cut away the entire shoot (cane, leaves, cage, and all) and brought it to the UC Berkeley Laboratory. There, we transferred the caged TCAH to clean vines for a 48-hour transmission period. This study considered changes in virus titer in the vine during the season because we repeated this work four times during the season (we suspect that virus titer increases during the season). This also considers the possibility that earlier studies with potted vines supplied by Foundation Plant Services at UC Davis had GRBV but the titer in these potted vines was not comparable to field conditions, where some vines have had the virus for years if not decades.

To date, we have successfully shown TCAH on virus-infected vines have been positive for the GRBV (e.g., acquisition). However, we have not yet observed transmission to clean vines at UC Berkeley, although we note that symptoms are often slow to appear. These vines will be held for two years, being observed regularly and PCR-tested periodically for the GRBV.

CONCLUSIONS

Over the past five years we have drastically improved our understanding of GRBV epidemiology, host plants, and insect vectors. We have effectively defined a narrow list of non-crop reservoirs for this virus and whittled down the range of candidate insect vectors. While it has been demonstrated that TCAH can transmit GRBV between vines, many questions remain about transmission efficiency, especially under field conditions and, more generally, TCAH seasonal ecology in vineyards. Additional candidate vectors remain to be tested as well, including *Colladonus* spp. and *Scaphytopius* spp. As we enter this second phase of research, our goal is to better characterize TCAH activity in vineyards and adjacent natural habitats, quantify transmission efficiency, and test any remaining candidate vectors.

REFERENCES CITED

Al Rwahnih M, Rowhani A, Golino D. 2015. First report of grapevine red blotch-associated virus in archival grapevine material from Sonoma County, California. *Plant Disease* 99: 895.

Al Rwahnih M, Dave A, Anderson MM, Rowhani A, Uyemoto JK, Sudarshana MR. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology* 103: 1069-1076.

Bahder BW, Zalom FG, Sudarshana MR. 2016a. An evaluation of the flora adjacent to wine grape vineyards for the presence of alternative host plants of grapevine red blotch-associated virus. *Plant Disease* 100: 1571-1574.

Bahder BW, Zalom FG, Jayanth M, Sudarshana MR. 2016b. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* (Say) as a vector of grapevine red blotch-associated virus. *Phytopathology* 106(10): 1223-1230.

Beyer BA, Srinivasan R, Roberts PM, Abney MR. 2017. Biology and management of the three-cornered alfalfa hopper (Hemiptera: Membracidae) in alfalfa, soybean, and peanut. *Journal of Integrated Pest Management* 8(1):10; 1-10.

Blanco-Ulate B, Hopfer H, Figueroa-Balderas R, Ye Z, Rivero RM, Albacete A, Pérez-Alfocea F, Koyama R, Anderson MM, Smith RJ, Ebeler SE, Cantu D. 2017. Red blotch disease alters grape berry development and metabolism by interfering with the transcriptional and hormonal regulation of ripening. *Journal of Experimental Botany* 68: 1225-1238.

Briddon RW, Stanley J. 2015. Geminiviridae. *Encyclopedia of Life Sciences* (eLS). John Wiley & Sons, Chichester.

Cieniewicz EJ, Pethybridge SJ, Loeb G, Perry K, Fuchs M. 2017. Insights into the ecology of grapevine red blotch virus in a diseased vineyard. *Phytopathology* 108: 94-102.

Daane KM, Almeida R, Cooper M, Golino D, Wilson H, Anderson J. 2017. Searching for potential vectors of grapevine red blotch-associated virus. *Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes*. California Department of Food and Agriculture, Sacramento, CA, pp. 202-214.

Dalton DT, Hilton RJ, Kaiser C, Daane KM, Sudarshana MR, Vo J, Zalom FG, Buser JZ, Walton VM. 2019. Spatial associations of vines infected with grapevine red blotch virus in Oregon vineyards. *Plant Disease* 103(7): 1507-1514

Fuchs M, Perry K, Golino D. 2017. Ecology of grapevine red blotch virus. *Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes*. California Department of Food and Agriculture, Sacramento, CA, pp. 219-227.

Girardello RC, Cooper ML, Smith RJ, Lerno LA, Bruce RC, Eridon S, Oberholster A. 2019. Impact of grapevine red blotch disease on grape composition of *Vitis vinifera* Cabernet Sauvignon, Merlot, and Chardonnay. *Journal of Agricultural Food Chemistry* 67: 5496-5511.

Johnson M, Mueller A. 1989. Flight activity of the three-cornered alfalfa hopper (Homoptera: Membracidae) in soybean. *Journal of Economic Entomology* 82: 1101-1105.

Krenz B, Thompson JR, Fuchs M, Perry KL. 2012. Complete genome sequence of a new circular DNA virus from grapevine. *Journal of Virology* 86: 7715-7715.

Krenz B, Thompson J, McLane H, Fuchs M, Perry K. 2014. Grapevine red blotch-associated virus is widespread in the United States. *Phytopathology* 104: 1232-1240.

Lim S, Igori D, Zhao F, Moon JS, Cho IS, Choi GS. 2016. First report of Grapevine red blotch-associated virus on grapevine in Korea. *Plant Disease* 100: 1957-1957.

Luna F, Debat H, Moyano S, Zavallo D, Asurmendi S, Gomez-Talquenca S. 2019. First report of grapevine red blotch virus infecting grapevine in Argentina. *Journal of Plant Pathology* 101(4): 1239.

Martínez-Lüscher J, Plank CM, Brillante L, Cooper ML, Smith RJ, Al Rwahnih M, Yu R, Oberholster A, Girardello R, Kurtural SK. 2019. Grapevine red blotch virus may reduce carbon translocation leading to impaired grape berry ripening. *Journal of Agricultural Food Chemistry* 67: 2437-2448.

Meisch M, Randolph N. 1965. Life-history studies and rearing techniques for the three-cornered alfalfa hopper. *Journal of Economic Entomology* 58: 1057-1059.

Mitchell PL, Newsom L. 1984. Seasonal history of the three-cornered alfalfa hopper (Homoptera: Membracidae) in Louisiana. *Journal of Economic Entomology* 77: 906-914.

Moore G, Mueller A. 1976. Biological observations of the three-cornered alfalfa hopper on soybean and three weed species. *Journal of Economic Entomology* 69: 14-16.
Mueller A, Dumas B. 1975. Effects of stem girdling by the three-cornered alfalfa hopper on soybean yields. *Journal of Economic Entomology* 68: 511-512.
Perry KL, McLane H, Hyder MZ, Dangl GS, Thompson JR, Fuchs MF. 2016. Grapevine red blotch-associated virus is present in free-living *Vitis* spp. proximal to cultivated grapevines. *Phytopathology* 106: 663-670.
Sudarshana MR, Perry KL, Fuchs MF. 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Phytopathology* 105: 1026-1032.
Varsani A, Roumagnac P, Fuchs M, Navas-Castillo J, Moriones E, Idris A, Briddon RW, Rivera-Bustamante R, Zerbini FM, Martin DP. 2017. *Capulavirus* and *Grablovirus*: Two new genera in the family *Geminiviridae*. *Archives of Virology* 162: 1819-1831.
Wilson H, Quisenberry S. 1987. Impact of feeding by three-cornered alfalfa hopper (Homoptera: Membracidae): Greenhouse and field study. *Journal of Economic Entomology* 80: 185-189.
Wilson H, Miles AF, Daane KM, Altieri MA. 2016. Host plant associations of *Anagrus* spp. (Hymenoptera: Mymaridae) and *Erythroneura elegantula* (Hemiptera: Cicadellidae) in northern California. *Environmental Entomology* 45: 602–615.
Wistrom C, Sisterson MS, Pryor MP, Hashim-Buckey JM, Daane KM. 2010. Distribution of glassy-winged sharpshooter and three-cornered alfalfa hopper on plant hosts in the San Joaquin Valley, California. *Journal of Economic Entomology* 103: 1051-1059.
Zalom FG, Sudarshana MR, Daane KM, Wunderlich LR, Smith RJ, Cooper ML. 2017. Biology and role of treehoppers in grapevine red blotch disease. *Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes*. California Department of Food and Agriculture, Sacramento, CA, pp. 260-268.

FUNDING AGENCIES

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

QUANTIFYING VINE MEALYBUG SPATIO-TEMPORAL DYNAMICS: ASSESSING INVASION RISK TO REFINE MANAGEMENT STRATEGIES

Project Leader: Matt Daugherty | Department of Entomology | University of California | Riverside, CA 92521 | matt.daugherty@ucr.edu
Co-Project Leader: Monica Cooper | Cooperative Extension | University of California | Napa, CA 94559 | mlycooper@ucanr.edu
Researcher: Aubrey May | Cooperative Extension | University of California | Napa, CA 94559 | almay@ucanr.edu

Reporting Period: The results reported here are from work conducted November 2018 to October 2019.

ABSTRACT
Vine mealybug (*Planococcus ficus*; VMB) is a severe vineyard pest that contaminates fruit, debilitates vines, and transmits plant pathogens such as grapevine leafroll-associated virus-3. First reported in California from vines in the Coachella Valley, VMB soon spread throughout much of the state, likely on infested nursery stock. It is currently found in most California grape-growing regions and its range continues to expand, making this pest a serious threat to other grape-growing regions of the United States. The ongoing expansion of VMB in California and continued risk of its introduction into new areas necessitate better understanding of the factors driving its invasion. Here we use survey data on 2012-17 VMB occurrence to characterize the factors associated with VMB establishment and spread in Napa County, California. This work also identifies factors underlying hotspots in VMB activity, quantifies spatiotemporal patterns in VMB occurrence, and clarifies pathways that contribute to VMB spread. Ultimately, results of this investigation can improve understanding of the educational and regulatory steps needed to mitigate VMB impact in Napa vineyards.

LAYPERSON SUMMARY
The invasive vine mealybug (*Planococcus ficus*; VMB) is an aggressive pest in California vineyards, where it reduces vine health and contaminates fruit. VMB management is challenging and costly costing $300 to $500 per acre per year. Since VMB has proven difficult to eradicate once established, these costs are often incurred yearly for the life of the vineyard. VMB distribution is still expanding within California, and there is continued risk of introduction to other grape-growing regions of the United States. Although VMB biology and management have been intensively studied, the factors governing its invasion and spread are poorly characterized. Analyzing the patterns of VMB occurrence in surveys conducted in Napa County from 2012 to 2017 helps to explain why certain areas are heavily infested by this pest and identify which areas are most at risk of infestation in the near future. Such information is critical for developing a strategic response to this important pest.

INTRODUCTION
Geospatial analyses and niche-based/species distribution modeling have previously been used to characterize plant, aquatic invertebrate, amphibian, and insect invasions. Results of these and similar investigations have been applied, with varying degrees of success, to develop early detection strategies, identify and prioritize management in high risk areas, and minimize

monitoring expenditures (Thuiller et al., 2005; Bradley et al., 2010; Venette et al., 2010; Jiménez-Valverde et al., 2011; Vicente et al., 2016). An intriguing possibility is that information gained from geospatial analyses of invader spread and niche-based/species distribution modeling of suitable habitat for invaders may be used to simulate invader dispersal and predict invader distributions. Ensuing predictions of invader distributions could then guide detection and management efforts, as well as be evaluated and refined using field-collected data on invader occurrence. Here we use such tools to improve response to an important invasive insect in California, the vine mealybug (*Planococcus ficus*; VMB).

VMB is a severe vineyard pest that contaminates fruit, debilitates vines, and transmits plant pathogens such as grapevine leafroll-associated virus-3 (Daane et al., 2012; Almeida et al., 2013). Management of VMB has proven 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 winegrape-growing regions where climatic conditions are favorable and Argentine ants (*Linepithema humile*) disrupt biological control (Daane et al., 2007; Gutierrez et al., 2008). Management costs may range from $300 to $500 per acre, per year, and due to the aggressive nature of VMB populations, these practices cannot be neglected.

VMB was first reported in California from vines in the Coachella Valley (Gill, 1994) and soon spread throughout much of the state, 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). However, despite the continued expansion of VMB in California, its current distribution in Napa County and areas at risk of VMB introduction in this region are not well characterized.

OBJECTIVES

Given the ongoing expansion of VMB in California and continued risk of its introduction into new areas, a better understanding is needed of what is driving its invasion. The goal of this research is to characterize the factors associated with VMB establishment and spread in northern California vineyards, which will be addressed via the following objectives:

1. Quantify the spatiotemporal patterns in VMB occurrence to identify invasion hot spots and patterns of spread.
2. Characterize the landscape, climatic, and anthropogenic factors associated with current VMB occurrence to predict areas at risk of invasion.
3. Validate and update predictions of VMB risk via in-field monitoring.

RESULTS AND DISCUSSION

Objective 1. Quantify the Spatiotemporal Patterns in VMB Occurrence to Identify Invasion Hot Spots and Patterns of Spread

Prior analyses of trapping records provided by the Napa County Agricultural Commissioner's Office investigated spatiotemporal patterns of VMB throughout the county by quantifying the scale of spatial auto-correlation in trap detections, the scale and directionality of spread on an annual basis, and hotspot analysis. Results showed clustering of VMB detections, with persistent hotspots in the southern part of the county and increasingly up valley, but also highly

idiosyncratic (and method-dependent) patterns of spread in terms of magnitude and direction (Daugherty et al., 2018; **Table 1**).

Table 1. Mean estimates and standard errors (in meters) of yearly VMB spread generated via distance regression, square-root area regression, and boundary displacement methods.

	Distance		Square-Root Area		Boundary Displacement	
Threshold	Mean	Error	Mean	Error	Mean	Error
Presence-Only	365.9	31.0	779.8	261.2	832.6	41.0
10	310.1	39.9	572.8	230.1	848.1	41.0
100	299.5	87.8	94.9	257.3	890.3	62.6

Objective 2. Characterize the Landscape, Climatic, and Anthropogenic Factors Associated with Current VMB Occurrence to Predict areas at Risk of Invasion

Prior habitat suitability modeling used a suite of landscape, climatic, and anthropogenic variables to predict VMB occurrence throughout Napa County. Results from a grand ensemble model indictated that rainfall, elevation, and proximity to wineries contributed most strongly to the noted high heterogeneity in VMB suitability in the region, which was predicted to be greatest surrounding Napa and St. Helena, and the central-eastern portion of Napa County (**Figure 1**).

Objective 3. Validate and Update Predictions of VMB Risk Via In-Field Monitoring

Work on Objective 3 leveraged the results of Objectives 1 and 2 to evaluate the accuracy of predictions of habitat suitability and risk of VMB infestation via in-field monitoring. To do this we identified more than 300 unique vineyard properties throughout Napa Valley that collectively represent a large range in both estimated habitat suitability values (Objective 2) and distance to VMB trap detections in the prior year (Objective 1). Over the last season we confirmed the status of the vineyard with respect to VMB presence for approximately 250 of these, via one of three means. First, we interviewed growers and vineyard managers who oversee the properties to determine whether VMB was established at that site, and if so, what year the site was first invaded. For those sites for which the year of establishment was not known, we estimated it based on inspections of pesticide use reports to identify pronounced increases in VMB-effective insecticide use. Second, for more than a dozen independent sites, growers or vineyard managers reported suspected new VMB infestations during the current season, which we confirmed via field visits. Finally, at more than two dozen sites for which growers or managers believed VMB was not present or were not sure, we conducted detailed monitoring. The monitoring included timed visual inspection of 250 vines spread throughout up to approximately four acres, including inspection under the bark and on leaves. If any of the vines had evidence of VMB presence, that site was treated as infested (i.e., presence/absence).

Collectively, sites had estimated habitat suitability values ranging from 0.02 to 0.99, and distances to the nearest trap detection the prior year ranging from 0 to more than 4,500 meters. Overall, nearly 34% of sites had been invaded by VMB since 2014. Logistic regression analysis showed significant and positive effects of habitat suitability ($\chi^2 = 73.708$, df = 1, $P < 0.0001$;

Figure 2), whereas distance to the nearest trap detection was significantly negatively related to the proportion with VMB infestations ($\chi^2 = 30.68$, df = 1, $P < 0.0001$; **Figure 3**).

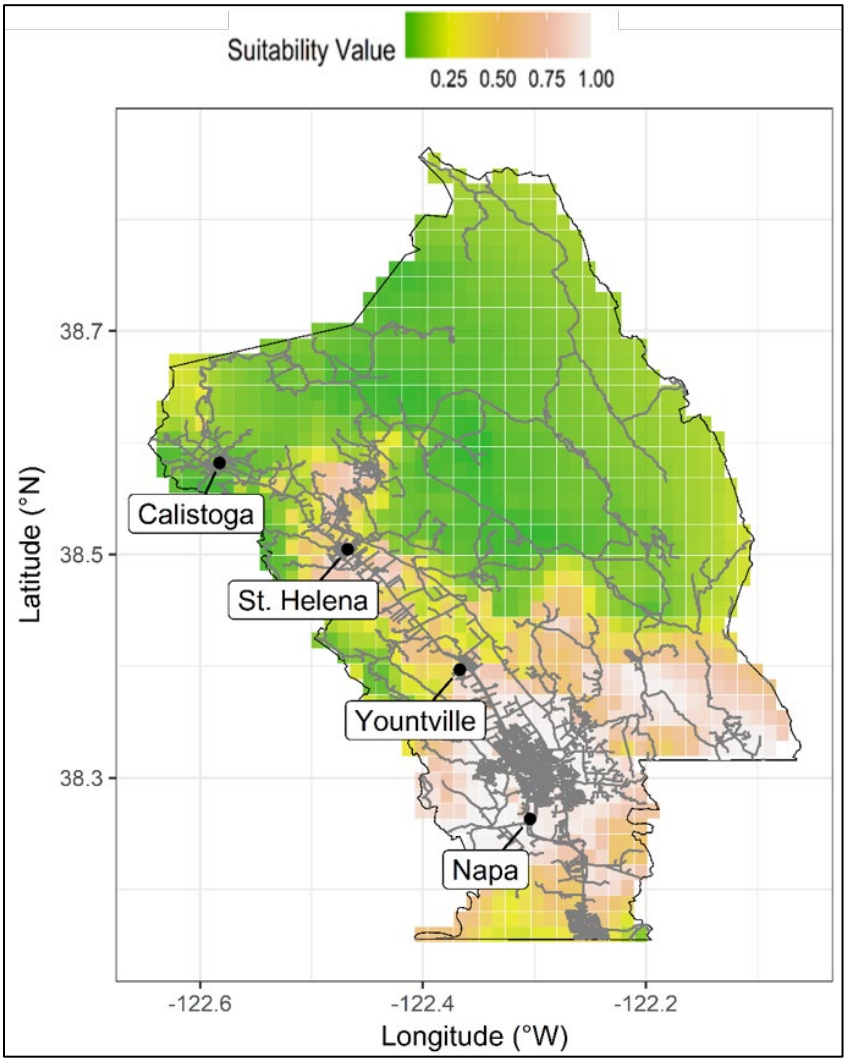


Figure 1. Grand ensemble prediction of habitat suitability for VMB in Napa County.

A few aspects of these results are noteworthy. First, the very strong positive association with habitat suitability indicates that the suitability modeling in Objective 2 is robust, and likely a useful indicator of the risk of VMB infestation in the near future at sites in the study region that are not already invaded. Second, the negative effect of distance from prior trap detections is biologically plausible (i.e., greater distances = lower probability of infestation), and may be useful in refining further estimates of VMB dispersal distances from known infestations. Results indicate that sites at distances up to approximately 250 meters from VMB trap detections are at elevated risk of invasion, which is congruent with the more conservative metrics of VMB spread based on analyses of trapping records alone (**Table 1**). Finally, given that almost all of the sites with distances to prior trap detections near 0 meters were invaded suggests that trap detections are a reliable indicator of a VMB infestation in the immediate area of a trap. In other words, the

network of traps in the study region, which forms the basis for the suitability modeling and grower management decisions, appears to capture accurately the true distribution of VMB in the region.

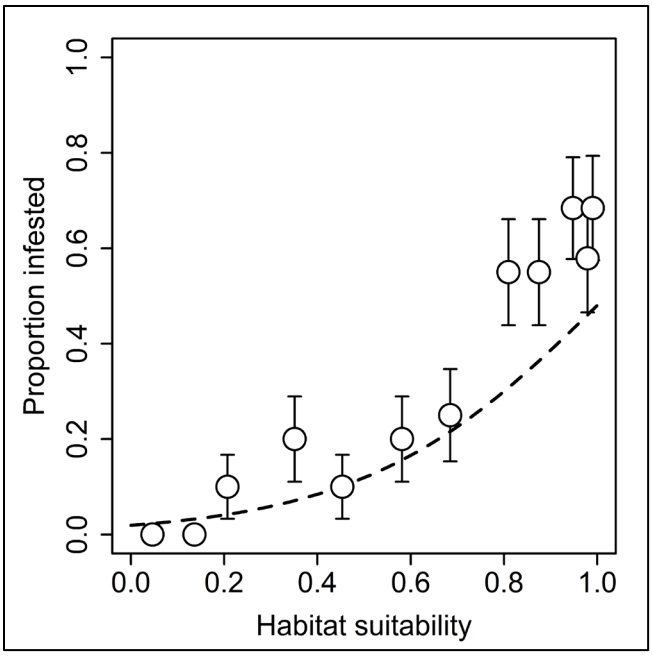


Figure 2. Proportion (±SE) of vineyards with VMB infestations as a function of estimated habitat suitability. The dotted line denotes model fit.

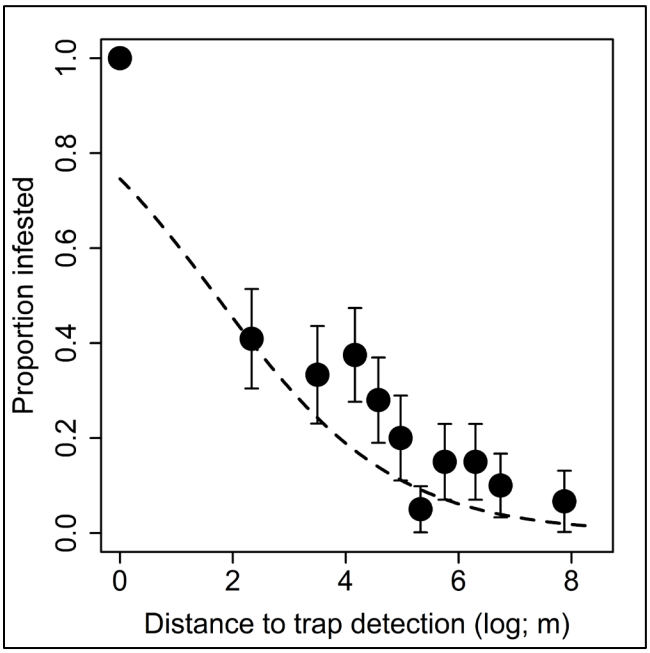


Figure 3. Proportion (±SE) of vineyards with VMB infestations as a function of distance to the nearest trap detection the prior year (natural log[x+1] transformed).

CONCLUSIONS

Our findings indicate that VMB invasion of Napa County is well beyond the initial stages and is actively spreading throughout this region. Future VMB spread may continue to occur via natural and/or human-assisted pathways at distances of hundreds of meters per year. We detected substantial heterogeneity in both the distribution of statistically significant hotspots of VMB detections and estimated habitat suitability for VMB over the study region. The amount of precipitation in the driest month, elevation, and trap distance to nearest winery were identified as the most important and strongly associated predictors of habitat suitability for VMB. Finally, results from the in-field monitoring both validate that the predictions of the habitat suitability modeling are robust and may help to refine further estimates of VMB dispersal rate. Collectively, these results suggest that geospatial analyses capture well the near term risk of VMB spread into areas of the study region that are not already infested. Such information may prove useful for early detection efforts, which are expected to improve the outcome of VMB management efforts by individual growers and vineyard managers.

REFERENCES CITED

Almeida RPP, Daane KM, Bell VA, Blaisdell GK, Cooper ML, Herrback E, Pietersen G. 2013. Ecology and management of grapevine leafroll disease. *Frontiers in Microbiology* 4: 94. doi:10.3389/fmicb.2013.00094

Bradley BA, Wilcove DS, Oppenheimer M. 2010. Climate change increases risk of plant invasion in the eastern United States. *Biological Invasions* 12: 1855-1872.

Daane KM, Weber EA, Bentley WJ. 2004a. Vine mealybug: Formidable pest spreading through California vineyards. *Practical Winery & Vineyard,* May/June: www.practicalwinery.com

Daane KM, Malakar-Kuenen R, Walton VM. 2004b. Temperature development of *Anagyrus pseudococci* (Hymenoptera: Encyrtidae) as a parasitoid of the vine mealybug, *Planococcus ficus* (Homoptera: Pseudococcidae). *Biological Control* 31: 123-132.

Daane KM, Sime KR, Fallon J, Cooper ML. 2007. Impacts of Argentine ants on mealybugs and their natural enemies in California's coastal vineyards. *Ecological Entomology* 32: 583-596.

Daane KM, Cooper ML, Triapitsyn SV, Walton VM, Yokota GY, Haviland DR, Bentley WJ, Godfrey K, Wunderlich LR. 2008. Vineyard managers and researchers seek sustainable solutions for mealybugs, a changing pest complex. *California Agriculture* 62: 167-176.

Daane KM, Almeida RPP, Bell VA, Walker JTS, Botton M, Fallahzadeh M, Mani M, Miano JL, Sforza R, Walton VM, Zaviezo T. 2012. Biology and management of mealybugs in vineyards. *Arthropod Management in Vineyards: Pests, Approaches and Future Directions*. Boustanian NJ, Vincent C, Isaacs R (eds.). Springer, New York.

Daugherty MP, Cooper MC, Schartel T. 2018. Quantifying vine mealybug spatiotemporal dynamics: Assessing invasion risk to refine management strategies. *Proceedings of the 2018 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA, pp. 215-218.

Gill R. 1994. Vine mealybug. *California Plant Pest and Disease Report*, January-June. California Department of Food and Agriculture, Sacramento, CA.

Godfrey KE, Daane KM, Bentley WJ, Gill RJ, Malakar-Kuenen R. 2002. Mealybugs in California vineyards. *UC ANR Publ. 21612*. Oakland, CA.

Gutierrez AP, Daane KM, Ponti L, Walton VM, Ellis CK. 2008. Prospective evaluation of the biological control of vine mealybug: Refuge effects and climate. *Journal of Applied Ecology* 45: 524-536.

Haviland DR, Bentley WJ, Daane KM. 2005. Hot water treatments to control *Planococcus ficus* (Hemiptera: Pseudococcidae) in grape nursery stock. *Journal of Economic Entomology* 98: 1109-15.

Jiménez-Valverde A, Peterson AT, Soberón J, Overton JM, Aragón P, Lobo JM. 2011. Use of niche models in invasive species risk assessments. *Biological Invasions* 13: 2785-2797.

Thuiller W, Richardson DM, Pyšek P, Midgley GF, Hughes GO, Rouget M. 2005. Niche-based modelling as a tool for predicting the risk of alien plant invasions at a global scale. *Global Change Biology* 11: 2234-2250.

Venette RC, Kriticos DJ, Magarey RD, Koch FH, Baker RH, Worner SP, Raboteaux NNG, McKenney DW, Dobesberger EJ, Yemshanov D, De Barro P.J. 2010. Pest risk maps for invasive alien species: A roadmap for improvement. *BioScience* 60: 349-362.

Vicente JR, Alagador D, Guerra C, Alonso JM, Kueffer C, Vaz AS, Fernandes RF, Cabral JA, Araujo MB, Honrado J.P. 2016. Cost-effective monitoring of biological invasions under global change: A model-based framework. *Journal of Applied Ecology* 53: 1317-132.

FUNDING AGENCIES

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

ACKNOWLEDGEMENTS

We thank A. Napolitano for help in acquiring the survey data on 2012-17 VMB occurrences. Thanks also to the Napa County grape growers and vineyard managers for their continued cooperation in making this work possible.

ECOLOGY OF GRAPEVINE RED BLOTCH VIRUS

Project Leader: Marc Fuchs | Section of Plant Pathology | Cornell University | Geneva, NY 14456 | mf13@cornell.edu
Co-Project Leader: Keith Perry | Section of Plant Pathology | Cornell University | Geneva, NY 14456 | klp3@cornell.edu
Collaborator: Deborah Golino | Foundation Plant Services | University of California | Davis, CA 95616 | dagolino@ucdavis.edu

Reporting Period: The results reported here are from work conducted July 1, 2019 to October 23, 2019.

ABSTRACT

Grapevine red blotch disease is one of the most important viral diseases of grapevine (*Vitis vinifera*) in the United States. Grapevine red blotch virus (GRBV), the causal agent of the disease, delays fruit ripening, reduces fruit quality, and causes economic losses ranging from $100 to $2,750 per hectare annually. There is no cure for GRBV in the vineyard. Management strategies rely on the use of clean plant material in newly established vineyards, and on roguing and parcel replacement in diseased vineyards. These strategies require an accurate detection of GRBV for the elimination of the virus from foundation stocks, and for the identification of infected vines to be removed from established vineyards. Ideally, testing for GRBV should be inexpensive, rapid, user-friendly, and performed on-site with limited training. Recently, we have developed a loop-mediated isothermal amplification (LAMP) assay to detect GRBV on-site. Efforts to validate this diagnostic assay with cooperating grower operations were undertaken. These were very satisfactory, although more work is needed for the growers to adopt the assay as a helpful diagnostic tool. In addition, cuttings of GRBV-infected wild grapes and Pinot Noir grapes were callused in the greenhouse. These vines will be used in transmission assays with the three-cornered alfalfa hopper (*Spissistilus festinus*) from a colony established on alfalfa or bean to determine the role of wild grapes as reservoirs of GRBV. Furthermore, the etiological role of wild *Vitis* virus 1 (WVV1), a virus identified in free-living grapes in northern California, is investigated by grafting and agroinoculation experiments. This will reveal whether WVV1 can infect cultivated grapes. Finally, information on the biology and ecology of GRBV was disseminated thus far to a group of growers in the Lodi, California district. Additional outreach efforts to share research findings on GRBV are planned in the coming months.

LAYPERSON SUMMARY

Grapevine red blotch virus (GRBV), the causal agent of grapevine red blotch disease, delays fruit ripening, reduces fruit quality, and causes substantial economic losses. There is no cure for GRBV in the vineyard. Management strategies, e.g., a careful selection of planting material, roguing, and parcel replacement, require an accurate detection of GRBV. Ideally, the diagnostic test should be inexpensive, rapid, and user-friendly for on-site performance. The recently developed loop-mediated isothermal amplification (LAMP) assay to detect GRBV on-site was validated with cooperating grower operations in Napa Valley, California. Also, cuttings of GRBV-infected wild grapes and Pinot Noir grapes were callused in the greenhouse. These vines will be used in transmission assays with the three-cornered alfalfa hopper (*Spissistilus festinus*) to determine the epidemiological role of wild grapes. Finally, the possible role of wild *Vitis*

virus 1 (WVV1), a virus identified in free-living grapes in northern California, in disease development is investigated by grafting and other inoculation methodologies. Information on the biology and ecology of GRBV was disseminated to growers in the Lodi, California district. Additional outreach efforts are planned in the near future.

INTRODUCTION

Grapevine red blotch disease (GRBD) is one of the most important viral diseases of grapevine (*Vitis vinifera*) in the United States (Cieniewicz et al., 2017a; Sudarshana et al., 2015). It was described for the first time on Cabernet Sauvignon at the University of California (UC) Oakville Research Field Station in 2008 (Calvi, 2011). Red or chlorotic blotches on leaves of red- and white-berried *V. vinifera* vines, respectively, delayed fruit ripening, and reduced fruit quality (Blanco-Ulate et al., 2017; Kurtural et al., 2019) are characteristic of GRBD. The estimated economic impact of GRBD ranges from $2,213 to $68,548 per hectare over a 25-year lifespan of a vineyard (Ricketts et al., 2017).

Grapevine red blotch virus (GRBV) is the type member of the genus *Grablovirus* in the plant virus family *Geminiviridae* (Varsani et al., 2017) and the causal agent of GRBD (Yepes et al., 2018). It has a single-stranded DNA genome that codes for seven open reading frames (Cieniewicz et al., 2017a; Vargas-Asencio et al., 2019). Analysis of the genetic diversity among GRBV isolates indicated two phylogenetic groups (Krenz et al., 2014). The two groups of isolates are involved in the etiology of the disease (Yepes et al., 2018).

GRBV is transmissible by grafting, which is likely the most significant mode of dispersal. Since its discovery in 2011, GRBV has been detected throughout the United States (Krenz et al., 2014) and Canada (Poojari et al., 2017; Xiao et al., 2015), with reports from Switzerland (Reynard et al., 2018), South Korea (Lim et al., 2016), Mexico (Gasperin-Bulbarela et al., 2018) and India (GenBank accession no. KU522121.1). GRBV was also isolated from numerous table grape accessions at the USDA germplasm repository in Davis, California (Al Rwahnih et al., 2015a), from an herbarium specimen at UC Davis (Al Rwahnih et al., 2015b), and from free-living grapes in northern California (Badher et al., 2016a; Cieniewicz et al., 2018; Perry et al., 2016). While long distance dispersal is attributed to dissemination of infected propagation material, short distance spread within vineyards has thus far only been observed in the western U.S. (Cieniewicz et al., 2017b, 2018, 2019; Dalton et al., 2019).

GRBV is transmitted by the three-cornered alfalfa hopper (*Spissistilus festinus;* TCAH) from infected to healthy vines under greenhouse conditions (Bahder et al., 2016b). It is also a vector of epidemiological relevance in vineyards (Cieniewicz et al., 2018). Populations of TCAH peaked from late June to early July in a Cabernet Franc vineyard in Napa Valley, but were low (20-30 individuals per sticky trap from March to November) (Cieniewicz et al., 2018). Differential dynamics of spread in the Cabernet Franc vineyard and an adjacent Cabernet Sauvignon vineyard were consistent with the abundance of TCAH populations; higher rates of spread occurred with higher TCAH populations (Cieniewicz et al., 2019). Legume species in vineyard cover crops in diseased vineyards tested negative for GRBV, suggesting no role in disease epidemiology. Similar epidemiological work in a Merlot vineyard in New York failed to document spread of GRBV and no TCAH or other vector candidates were caught on sticky traps (Cieniewicz et al., 2019). TCAH is known to prefer leguminous hosts over *Vitis* sp. for both

feeding and reproduction, and is an occasional pest of legumes in the southern U.S. (Beyer et al., 2017; Preto et al., 2018a). Although TCAH will feed on grapevine and ingest GRBV (Bahder et al., 2016a; Cieniewicz et al., 2018a), and will even oviposit in grapevine (Preto et al., 2018b), it does not seem to colonize grapevine, but rather stays near vineyard edges and overwinters on vineyard groundcover (Preto et al., 2019).

Management of GRBD relies upon the accurate detection of GRBV, eliminating the virus from foundational stocks and planting material, and in some cases removal of infected vines from established vineyards (Cieniewicz et al., 2017a). At present, detection of GRBV involves the use of a polymerase chain reaction (PCR) assay, requiring the sophisticated instrumentation and expertise of a testing laboratory. Ideally, testing for GRBV should be inexpensive, rapid, user-friendly, and performed in a vineyard facility (herein referred to as "on-site"), with limited training. A highly sensitive, on-site diagnostic assay is available, but there are limitations and it is relatively expensive (Li et al., 2017). Recently, we have developed a loop-mediated isothermal amplification (LAMP) assay to detect GRBV on-site that can be completed in approximately 45 minutes, without the need for significant training or instrumentation (Romero et al., 2019). LAMP assays are nucleic acid based, use DNA primers targeting the virus genome, and are isothermal, conducted at one temperature (65˚C). The assay is different from PCR, using a different enzyme, and the only required "major" equipment item is a simple heating block (relatively low tech). Additional needs are a clean work space, tubes, a pipetting device, and cold storage for the reagents. This means that samples of plant sap (a crude extract) can be taken in the vineyard.

In the workspace, a one µl drop is added to a tube with reagents, and after 35 minutes, a colorimetric change is observed for infected vines (yellow color) versus uninfected samples (pink color) (**Figures 1** and **2**).

The LAMP assay has tested reliably for all plants and GRBV isolates sampled, including those from both phylogenetic clades representing the genetic diversity of the virus (Romero et al., 2019). The assay has been shown to be robust (Romero et al., 2019); sampled materials (extracts) taken in a greenhouse remain stable and test reliably after 48 hours at ambient temperature (**Figure 1**).

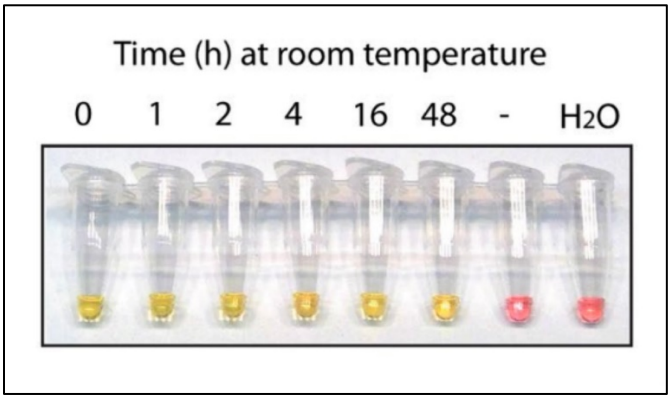


Figure 1. Colorimetric reactions showing the detection of GRBV in plant sap sampled and stored at ambient temperature for one to 48 hours.

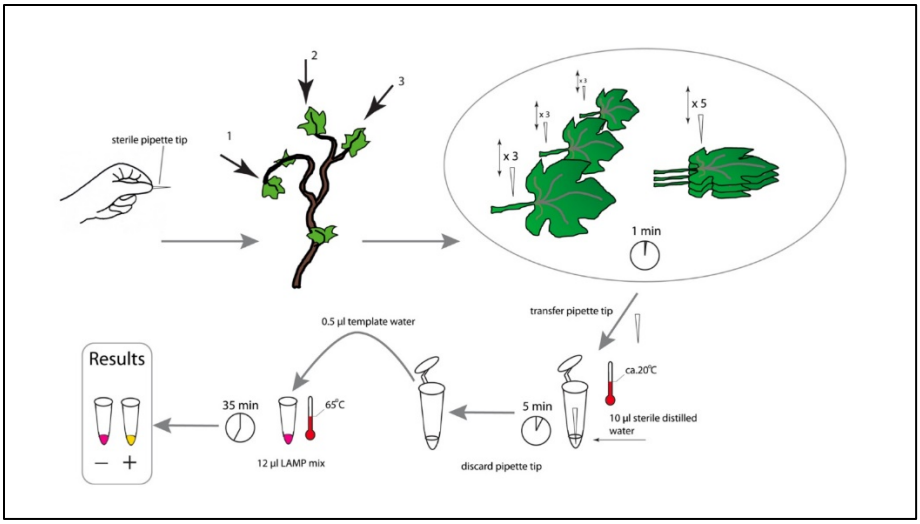


Figure 2. Flowchart of the steps involved in the pin-prick GRBV LAMP assay.

In spite of tremendous progress in recent years on the biology and spread of GRBV, research on ecological aspects of the disease is still needed. For example, refining the on-site diagnostic LAMP assay and validating it with cooperating vineyard managers would be of interest. This is because this assay could provide vineyard managers with a much-needed diagnostic tool to assess the incidence of GRBV in diseased vineyards. Assessing virus incidence in vineyards will facilitate the implementation of GRBD management strategies. Also, the role of wild grapes as virus reservoirs for secondary spread of GRBV via TCAH needs to be investigated to advance disease epidemiology. Furthermore, the etiological role of wild *Vitis* virus 1 (WVV1) is unknown. This virus was recently identified in free-living grape samples from Napa County and other areas in northern California. The genome of WVV1 most closely resembles that of GRBV in both sequence and organization, making it a new member of the genus *Grablovirus* in the family *Geminiviridae* (Perry et al., 2018). Surveys of wild grape populations in northern California showed a low incidence of WVV1 (7%; 15 of 203); nonetheless, it was significantly higher in counties with high grape production (Napa and Sonoma) compared to low grape production (Sacramento, Sutter, Butte, and Glenn) (Cieniewicz et al., 2019). Similar to GRBV, none of the WVV1-infected free-living vines exhibited disease symptoms. Analysis of the WVV1 genomic diversity was consistent with two distinct phylogenetic clades with 94-100% sequence identity with clade 1 isolates and 90-100% with clade 2 isolates (Cieniewicz et al., 2019). No information is available on the presence of WVV1 in production vineyards, planting material, or foundation stocks. Addressing the host range of WVV1 will help determine whether this GRBV-related virus can cause GRBD. In other words, knowing if this virus can infect cultivated grapes is critical. This is a prerequisite for the development of comprehensive disease management strategies. Finally, disseminating information on the biology and ecology of GRBV and WVV1 to the industry is essential, to communicate research accomplishments and share the latest knowledge on GRBD. These are significant components of this project.

OBJECTIVES

Our specific objectives are to:

1. Refine the efficacy of an on-site diagnostic LAMP assay for GRBV.
   a. Develop an additional LAMP assay for validation.
2. Validate the LAMP assay with cooperating vineyard managers.
   a. Identify cooperating vineyard managers.
   b. Assist cooperating vineyard managers with determining the incidence of GRBV.
3. Investigate the role of wild grapes as reservoirs of GRBV.
   a. Examine the transmissibility of GRBV by TCAH from infected wild grapes to healthy cultivated grapes.
   b. Determine the transmissibility of GRBV by TCAH from infected cultivated grapes to healthy wild grapes.
4. Determine the host range and transmissibility of WVV1.
   a. Develop a LAMP assay for WVV1.
   b. Inoculate *V. vinifera* cultivars and rootstock genotypes with WVV1.
5. Disseminate research results to the grape and wine industry, and to farm advisors.

RESULTS AND DISCUSSION

Objective 1. Refine the Efficacy of an On-Site Diagnostic LAMP Assay for GRBV

The full-length genome sequence of more than 120 GRBV variants was analyzed using bioinformatics tools to identify conserved short nucleotide sequence regions for the design of new primer sets. The efficacy of these primers will be assessed in LAMP to compare their efficacy with that of the three primer pairs designed in the predicted coat protein (V1) open reading frame of the GRBV genome previously used for the first version of the LAMP assay (Romero et al., 2019). The optimal assay will be validated using greenhouse and vineyard samples.

Objective 2. Validate the LAMP Assay with Cooperating Vineyard Managers

Several growers and vineyard managers interested in on-site testing for GRBV were identified in Napa County following extensive discussions at a 2019 winter growers meeting in Napa, California. These discussions led to the selection of two vineyard operations with distinct levels of GRBV-infected vines in several parcels in Napa County. These two vineyard operations were visited in June 2019. This visit was very fruitful. Growers were provided with all the equipment, tools, and reagents needed to carry out a LAMP assay for GRBV. They were also provided with protocols and hands-on training with the LAMP assay. The two vineyard operations expressed interest in using the LAMP assay independently sometime after grape picking. It will be interesting to see whether the two cooperating vineyard managers will validate the assay on their own and adopt it.

Objective 3. Investigate the Role of Wild Grapes as Reservoirs of GRBV

Cuttings of healthy and GRBV-infected free-living grapes were collected in Napa Valley in 2018 and early 2019 and shipped to Cornell. The cuttings were callused in the greenhouse in spring 2019. The corresponding vines are growing well (**Figure 3**). The virus status of these vines will be tested soon by multiplex PCR using specific primers and/or LAMP. Vines with detectable levels of GRBV will then be used in replicated transmission assays with TCAH using specimens from a colony maintained on alfalfa or bean in a growth chamber. These assays will determine if

TCAH can ingest GRBV from infected free-living vines and transmit it to healthy winegrapes. In parallel, cuttings of GRBV-infected *V. vinifera* cv. Pinot Noir were callused in the greenhouse (**Figure 4**). The presence of GRBV has been validated by multiplex PCR in these winegrapes. Subsequently, replicated transmission experiments will be carried out to determine if TCAH can transmit GRBV from winegrapes to free-living grapes.

Figure 3. Rooted vines derived from cuttings of healthy and GRBV-infected free-living vines from northern California.

Figure 4. Rooted vines of GRBV-infected Pinot Noir.

Objective 4. Determine the Host Range and Transmissibility of WVV1

Buds of healthy *V. vinifera* cv. Cabernet Franc were grafted onto rooted cuttings of WVV1-infected wild grapes that were collected in northern California (Cieniewicz et al., 2018). The

presence of WVV1 in Cabernet Franc tissue will be assessed over time by PCR with specific primers. In parallel, a full-length genome construct of WVV1 was engineered and cloned into a binary plasmid for mobilization in *Agrobacterium tumefaciens*. We will use this construct to agroinoculate *V. vinifera* and/or rootstocks, as previously done with infectious clones of GRBV (Yepes et al., 2018), and the presence of WVV1 will be verified over time in grafted and agroinoculated vines by PCR using specific primers. This work will indicate whether WVV1 has the potential to infect cultivated grapes. Additionally, we will develop a LAMP assay for WVV1 by using primers designed in conserved regions of the virus genome, paralleling the design approach of the assay for GRBV. The WVV1 LAMP assay will be validated using existing plant and nucleic acid samples.

Objective 5. Disseminate Research Results to the Grape and Wine Industry, and to Farm Advisors

Information on the biology and ecology of GRBV was communicated to growers (15 participants) in the Lodi, California district on October 16, 2019. Additional presentations will be made at grower meetings, conventions, and trade shows in the near future.

CONCLUSIONS

A cheap, rapid and user-friendly diagnostic assay was developed for GRBV based on LAMP. This assay was validated with two cooperating vineyard operations in Napa Valley. Equipment, tools, and supplies needed to run the LAMP assay were transferred to the cooperators and extensive hands-on training was provided during a visit in June 2019. The use of vineyard samples gave satisfactory test outputs. It will be interesting to see whether the two cooperators run the LAMP assay independently at a more optimal time of the growing season, e.g., before or after grape harvest, and adopt it for their operations. To determine the role of wild grapes as reservoirs of GRBV, cuttings of infected wild grapes were collected in Napa County and callused in the greenhouse. Similarly, cuttings of infected Pinot Noir were collected and callused in the greenhouse. Once the presence of GRBV is determined in some of these vines, they will be used in transmission assays with TCAH to assess whether wild grapes can serve as donors and recipients of GRBV. Outreach efforts to the grape industry have been initiated. These efforts targeted growers in the Lodi, California district. Exchanges on the biology and ecology of GRBV were fruitful and helpful in terms of disease management.

REFERENCES CITED

Al Rwahnih M, Rowhani A, Golino D, Islas C, Preece J, Sudarshana MR. 2015. Detection and genetic diversity of grapevine red blotch-associated virus isolates in table grape accessions in the National Clonal Germplasm Repository in California. *Canadian Journal of Plant Pathology* 37: 130-135.

Al Rwahnih M, Rowhani A, Golino D. 2015. First report of grapevine red blotch-associated virus in archival grapevine material from Sonoma County, California. *Plant Disease* 99: 895.

Bahder BW, Zalom FG, Sudarshana MR. 2016a. An evaluation of the flora adjacent to wine grape vineyards for the presence of alternative host plants of grapevine red blotch-associated virus. *Plant Disease* 100: 1571-1574.

Bahder B, Zalom F, Jayanth M, Sudarshana M. 2016b. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* (Say) as a vector of grapevine red blotch-associated virus. *Phytopathology* 106: 1223-1230.

Beyer BA, Srinivasan R, Roberts PM, Abney MR. 2017. Biology and management of the three-cornered alfalfa hopper (Hemiptera: Membracidae) in alfalfa, soybean, and peanut. *Journal of Integrated Pest Management* 8: 1-10.

Blanco-Ulate B, Hopfer H, Figueroa-Balderas R, Ye Z, Rivero RM, Albacete A, Pérez-Alfocea F, Koyama R, Anderson MM, Smith RJ, Ebeler SE, Cantu D. 2017. Red blotch disease alters grape berry development and metabolism by interfering with the transcriptional and hormonal regulation of ripening. *Journal of Experimental Botany* 68: 1225-1238.

Calvi BL. 2011. Effects of red-leaf disease on Cabernet Sauvignon at the Oakville experimental vineyard and mitigation by harvest delay and crop adjustment. M.S. thesis, University of California, Davis, CA.

Cieniewicz EJ, Perry KL, Fuchs M. 2017a. Grapevine red blotch virus: Molecular biology of the virus and management of the disease. Meng B, Martelli GP, Golino DA, Fuchs MF (eds.). *Grapevine Viruses: Molecular Biology, Diagnostics, and Management*. Springer Verlag, pp. 303-314.

Cieniewicz E, Pethybridge S, Gorny A, Madden L, Perry KL, McLane H, Fuchs M. 2017b. Spatiotemporal spread of grapevine red blotch-associated virus in a California vineyard. *Virus Research* 230: 59-62.

Cieniewicz E, Pethybridge SJ, Loeb GM, Perry KL, Fuchs, M. 2018. Insights into the ecology of grapevine red blotch virus in a diseased vineyard. *Phytopathology* 108: 94-102.

Cieniewicz E, Flasco M, Brunelli M, Onwumelu A, Wise A, Fuchs M. 2019. Differential spread of grapevine red blotch virus in California and New York vineyards. *Phytobiomes Journal* 3: 203-211.

Dalton DT, Hilton RJ, Kaiser C, Daane KM, Sudarshana MR, Vo J, Zalom FG, Buser JZ, Walton VM. 2019. Spatial associations of vines infected with grapevine red blotch virus in Oregon vineyards. *Plant Disease* 103: 1507-1514

Gasperin-Bulbarela J, Licea-Navarro AF, Pino-Villar C, Hernández-Martínez R, Carillo-Tripp J. 2018. First report of grapevine red blotch virus in Mexico. *Plant Disease* https://apsjournals.apsnet.org/doi/pdf/10.1094/PDIS-07-18-1227-PDN.

Krenz B, Thompson J, McLane H, Fuchs M, Perry KL. 2014. Grapevine red blotch-associated virus is widespread in the United States. *Phytopathology* 102: 1232-1240.

Kurtural K, Martínez-Lüscher J, Brillante L, Yu R, Plank C, Smith RJ, Cooper M, Oberholster A. 2019. Grapevine red blotch virus may reduce carbon translocation leading to impaired grape berry ripening. *Journal of Agriculture and Food Chemistry* 67: 2437-2448.

Li R, Fuchs M, Perry KL, Mekuriad T, Zhang S. 2017. Development of a fast amplifyrp acceler8 diagnostic assay for grapevine red blotch-associated virus. *Journal of Plant Pathology* 99: 657-662.

Lim S, Igori D, Zhao F, Moon JS. 2016. First report of grapevine red blotch-associated virus on grapevine in Korea. *Plant Disease* 100: 1957.

Perry KL, McLane H, Hyder MZ, Dangl GS, Thompson JR, Fuchs MF. 2016. Grapevine red blotch-associated virus is present in free-living *Vitis* sp. proximal to cultivated grapevines. *Phytopathology* 106: 663-670.

Perry KL, McLane H, Thompson JR, Fuchs M. 2018. A novel grablovirus from non-cultivated grapevine (*Vitis* sp.) in North America. *Archives of Virology* 163: 259-262.

Poojari S, Lowery DT, Rott M, Schmidt AM, Úrbez-Torres JR. 2017. Incidence, distribution, and genetic diversity of grapevine red blotch virus in British Columbia. *Canadian Journal of Plant Pathology* 39: 201-211.

Preto CR, Bahder BW, Bick EN, Sudarshana MR, Zalom F. 2019. Seasonal dynamics of *Spissistilus festinus* (Hemiptera: Membracidae) in a Californian vineyard. *Journal of Economic Entomology* 112: 1138-1144.

Preto CR, Sudarshana MR, Zalom FG. 2018a. Feeding and reproductive hosts of *Spissistilus festinus* (Say) (Hemiptera: Membracidae) found in Californian vineyards. *Journal of Economic Entomology* 111: 2531-2535.

Preto CR, Sudarshana MR, Bollinger ML, Zalom FG. 2018b. *Vitis vinifera* (Vitales: Vitaceae) as a reproductive host of *Spissistilus festinus* (Hemiptera: Membracidae). *Journal of Insect Science* 18: 1-7.

Reynard JS, Brodard J, Dubuis N, Zufferey V, Schumpp O, Schaerer S, Gugerli P. 2018. Grapevine red blotch virus: Absence in Swiss vineyards and analysis of potential detrimental effect on viticultural performance. *Plant Disease* 102: 651-655.

Ricketts KD, Gomez MI, Fuchs MF, Martinson TE, Smith RJ, Cooper ML, Wise A. 2017. Mitigating the economic impact of grapevine red blotch: Optimizing disease management strategies in U.S. vineyards. *American Journal of Enology and Viticulture* 68: 127-135.

Romero JLR, Carver GD, Johnson PA, Perry KL, Thompson JR. 2019. A rapid, sensitive, and inexpensive method for detection of grapevine red blotch virus without tissue extraction using loop-mediated isothermal amplification. *Archives of Virology* 164: 1453-1457.

Setiono FJ, Chatterjee D, Fuchs M, Perry KL, Thompson JR. 2018. The distribution and ability to detect grapevine red blotch virus in its host depends on time of sampling and tissue type. *Plant Disease* 102: 2187-2193.

Sudarshana MR, Perry KL, Fuchs MF. 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Phytopathology* 105: 1026-1032.

Vargas-Asencio J, Liou H, Perry KL, Thompson JR. 2019. Evidence for the splicing of grablovirus transcripts reveals a putative novel open reading frame. *Journal of General Virology* 100: 709-720.

Varsani A, Roumagnac P, Fuchs M, Navas-Castillo J, Moriones E, Idris I, Briddon RW, Rivera-Bustamante R, Murilo Zerbini F, Martin DP. 2017. Capulavirus and Grablovirus: Two new genera in the family Geminiviridae. *Archives of Virology* 162: 1819-1831.

Xiao H, Kim WS, Meng B. 2015. Comparison and improvement of methodologies for isolation of quality RNA from diverse woody plant species and utilization in detection of viral pathogens. *Virology Journal* 12: 171.

Yepes LM, Cieniewicz E, Krenz B, McLane H, Thompson JR, Perry KL, Fuchs M. 2018. Causative role of grapevine red blotch virus in red blotch disease. *Phytopathology* 108: 902-909.

FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board, and by the USDA National Institute of Food and Agriculture.

ACKNOWLEDGEMENTS

We are grateful to the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board for its support, to grower cooperators in California for their enthusiastic collaborative efforts, and to Maddie Flasco, Alex Clark, Tori Hoyle, Heather McLane, Fu-Wah Choi, and Yeng Mei Cheung for their valuable contributions.

I. RESISTANCE TO GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3 AND THE GRAPE MEALYBUG

Project Leader: Marc Fuchs | Section of Plant Pathology | Cornell University | Geneva, NY 14456 | mf13@cornell.edu
Co-Project Leader: Angela Douglas | Department of Entomology | Cornell University | Ithaca, NY 14853 | aes326@cornell.edu
Co-Project Leader: Greg Loeb | Department of Entomology | Cornell University | Geneva, NY 14456 | gmel@cornell.edu
Collaborator: Deborah Golino | Foundation Plant Services | University of California | Davis, CA 95616 | dagolino@ucdavis.edu

Reporting Period: The results reported here are from work conducted July 1, 2017 to October 23, 2019.

ABSTRACT

Grapevine leafroll disease is a devastating and widespread virus disease of grapevines. It causes economic losses by reducing yield, delaying fruit ripening, increasing titratable acidity, lowering sugar content in fruit juices, modifying aromatic profiles of wines, and shortening the productive lifespan of vineyards. There are six distinct viruses associated with leafroll disease, but grapevine leafroll-associated virus 3 (GLRaV-3) is dominant in vineyards. This virus is transmitted by several species of mealybugs including the grape mealybug (*Pseudococcus maritimus*), its most abundant and widely distributed vector and a pest of grapes. The vine mealybug (*Planococcus ficus*) is another important pest of grapes and a vector of GLRaV-3. Management of leafroll viruses and their mealybug vectors remains challenging due to a lack of recognized host resistance. We are exploring RNA interference (RNAi), a technology that has been successfully applied against viruses of fruit crops and phloem-feeding insects, to achieve resistance against GLRaV-3 and the grape mealybug. To apply RNAi against the grape mealybug, the osmoregulation genes *AQP1* and *SUC1*, as well as a nonspecific nuclease (*NUC*), were characterized by reverse transcription polymerase chain reaction (RT-PCR) using total RNA from specimens from a colony maintained on Pixie grapes in the greenhouse with overlapping degenerate primer pairs designed in conserved regions of the genes of interest based on alignments of similar sequences of other hemipterans. The cloned *AQP1, SUC1,* and *NUC* fragments are 490, 394, and 877 base pairs in size, respectively. Sequence analysis of the cloned PCR amplicons validated the nature of the *AQP1*, *SUC1*, and *NUC* products obtained. Efforts to characterize the orthologues of these osmoregulatory genes in the vine mealybug using specimens generously offered by Kent Daane (University of California, Berkeley) are underway. To evaluate the performance of double-stranded RNA (dsRNA) constructs against the grape mealybug, a transient assay based on artificial diet was developed. This bioassay was selected because a detached grape leaf assay proved suboptimal to test the effect of dsRNA constructs on the survival of grape mealybugs. Preliminary bioassay results revealed a 20% reduction in the survival of grape mealybug nymphs on an artificial diet containing dsRNA constructs to *AQP1*, *SUC1*, and *NUC* relative to a control diet. This effect was significant ($p = 0.0436$). Work is underway to verify these very encouraging results and identify a potentially more effective version of *SUC*. In parallel, RNAi against GLRaV-3 focused on conserved nucleotide regions within the open reading frame coding for protein p19.7 (*p19.7*), a viral RNA silencing

suppressor, the coat protein (*CP*), the RNA-dependent RNA polymerase (*RdRp*), and the heat shock 70 homolog (*HSP70h*) of GLRaV-3. Sets of overlapping primer pairs covering conserved regions of *p19.7*, *CP*, *RdRp*, and *HSP70* were designed and used in RT-PCR. Amplicons of the expected size were obtained, cloned, and validated by sequencing. One inverted-repeat *p19.7* construct was engineered and transferred into embryogenic calli of rootstock 110R via *Agrobacterium tumefaciens*-mediated transformation for the production of transgenic grapevines. Efforts to develop embryogenic cultures of grapevine (*Vitis vinifera*) cultivars Pinot Noir and Cabernet Franc are underway. Additionally, pyramided GLRaV-3 dsRNA constructs are being stacked with grape mealybug dsRNA constructs for expression by the phloem-specific promoter s*ucrose-H+ symporter* (*SUC2*). It is anticipated that a pyramided approach for the simultaneous engineering of resistance against GLRaV-3 and the grape mealybug will protect grapevines against the major virus of leafroll disease and its widely distributed insect vector.

LAYPERSON SUMMARY

Grapevine leafroll disease affects yield, fruit ripening, and aromatic profiles of wines. Grapevine leafroll-associated virus 3 (GLRaV-3) is the predominant virus associated with leafroll disease in vineyards. This virus is transmitted by several species of mealybugs including the grape mealybug (*Pseudococcus maritimus*), its most abundant and widely distributed vector and a pest of grapes (*Vitis vinifera*). The vine mealybug (*Planococcus ficus*) is another pest of grapes and vector of GLRaV-3. Management of leafroll viruses and their mealybug vectors is challenging due to a lack of recognized host resistance. We explore RNA interference (RNAi) technologies to achieve resistance against GLRaV-3 and the grape mealybug by simultaneously interfering with the expression of key genes of the virus and its major vector. For RNAi against the grape mealybug, our targets are osmoregulatory genes that are expressed in the gut and required for water balance and survival. Two osmoregulation genes from the grape mealybug, as well as another gene that is essential for RNAi efficacy, were isolated and characterized. The same osmoregulation genes are being characterized for the vine mealybug. In parallel, a transient assay based on artificial diet was developed, as a detached grape leaf assay proved suboptimal. Preliminary results revealed a 20% reduction in the survival of grape mealybug nymphs on an artificial diet containing double-stranded RNA (dsRNA) constructs to *AQP1*, *SUC1*, and *NUC* relative to a control diet. This effect was significant ($p = 0.0436$). Efforts to characterize the same osmoregulation genes in the vine mealybug using specimens kindly provided by Kent Daane (UC Berkeley) are ongoing. Options to improve the efficacy of our approach are being explored. For RNAi against the virus, conserved nucleotide sequence regions within four coding viral regions were identified and characterized. Among these four regions, an inverted-repeat p19.7 construct was engineered and used for the production of transgenic grapevines via *Agrobacterium tumefaciens*-mediated transformation. GLRaV-3 and grape mealybug dsRNA constructs are currently being stacked for expression by a phloem-specific promoter for maximal efficacy. We anticipate that combining resistance against GLRaV-3 and the grape mealybug will protect grapevines against the major virus of leafroll disease and its widely distributed insect vector.

INTRODUCTION

Grapevine leafroll disease is one of the most devastating and widespread viral diseases of grapevines (*Vitis vinifera*). It reduces yield, delays fruit ripening, increases titratable acidity, lowers sugar content in fruit juices, modifies aromatic profiles of wines, and shortens the productive lifespan of vineyards (Almeida et al., 2013; Naidu et al., 2014). The economic cost of leafroll is estimated to range from $12,000 to $92,000 per acre in California (Ricketts et al., 2015) and from $10,000 to $16,000 in New York (Atallah et al., 2012).

Six major viruses named grapevine leafroll-associated viruses (GLRaVs), i.e., GLRaV-1, -2, -3, -4, -7, and -13, have been identified in diseased vines (Ito and Nakaune, 2016; Naidu et al., 2014; Naidu et al., 2015). Among these viruses GLRaV-3 is the dominant leafroll virus in vineyards, including in California (Maree et al., 2013; Naidu et al., 2014; Naidu et al., 2015). This virus is phloem-limited and semi-persistently transmitted by several species of mealybugs, with acquisition and inoculation occurring within one-hour access periods of feeding by immature mealybug stages (Almeida et al., 2013). There is no significant effect of host plant tissue on transmission efficiency, nor is there specificity of transmission (Almeida et al., 2013; Naidu et al., 2014), indicating that many mealybug species may disseminate all transmissible strains of GLRaV-3.

Mealybugs are sap-sucking insects in the family Pseudococcidae. They are pests of grapes and many other important crops. At high densities mealybugs can cause complete crop loss, rejection of fruit loads at wineries, and death of spurs, although small infestations may not inflict significant direct damage. Feeding on plant sap, mealybugs excrete honeydew that often becomes covered with a black sooty mold, additionally damaging fruit clusters. Several mealybug species feed on grapevines, but the grape mealybug (*Pseudococcus maritimus*) is the most abundant and widespread in U.S. vineyards (Almeida et al., 2013). Another pest of grapes of importance is the vine mealybug (*Planococcus ficus*). Unassisted, mealybugs have limited mobility, but first instar immatures (crawlers) can be dispersed over long distances by wind and other means (Almeida et al., 2013).

In diseased vineyards, management strategies rely on the elimination of virus-infected vines and the reduction of mealybug populations through the application of systemic insecticides, primarily spirotetramat (Pietersen et al., 2013). However, managing leafroll viruses and their mealybug vectors remains challenging due to several factors, including a lack of recognized host resistance (Oliver and Fuchs, 2011). Resistance can be achieved by applying RNA interference (RNAi) technologies. This approach relies on the development of double stranded (ds) RNA constructs targeting specific pathogen or insect genes and their use to specifically downregulate their expression upon infection or feeding. The RNAi approach is highly specific and anticipated to reduce hazards of chemical pesticide applications. The fact that mealybugs transmit leafroll viruses offers an opportunity to explore a two-pronged approach to simultaneously target virus and vector (Fuchs, 2017).

The goal of our research is to develop a robust RNAi-based strategy against GLRaV-3 and the grape mealybug, and eventually the vine mealybug. The basis for our approach is three-fold. First, mealybug survival depends on two gene functions localized to the gut that prevent osmotic collapse and dehydration of the insect as it feeds on its sugar-rich diet of plant phloem sap. These

genes are the water channel aquaporin *AQP1* and the sucrase-transglucosidase *SUC1* (Jing et al., 2016), with evidence that insect mortality is enhanced by co-targeting these two genes with different molecular functions but related physiological roles (Tzin et al., 2015). Perturbing the expression of osmoregulatory genes required for water balance, specifically *AQP1* and *SUC1*, in the gut of phloem-feeding insects causes the insects to lose water from the body fluids and dehydrate, dying within two to three days (Karley et al., 2005; Shakesby et al., 2009; Tzin et al., 2015). Second, the functions of *AQP1* and *SUC1* can be targeted by in planta RNAi with evidence from related phloem-feeding insects that RNAi efficacy is enhanced by stacking these RNAi constructs with RNAi against the gut nuclease *NUC1* (Luo et al., 2017). Third, RNAi has been successfully applied against viruses of fruit crops such as papaya (Gonsalves et al., 2008) and plum (Hily et al., 2004). The goal of this research is to develop grapevines resistant to GLRaV-3, grape mealybug, and vine mealybug using RNAi by pyramiding double-stranded (dsRNA) constructs against several targets of the virus and the insect vectors, providing for greater efficacy in disease management and greater opportunities in impeding the development of virus and insect vector populations capable of overcoming the resistance.

OBJECTIVES

Our specific objectives are to:

1. Optimize RNAi constructs against the grape mealybug.
2. Develop a high throughput transient expression system to test the efficacy of RNAi constructs against the grape mealybug.
3. Characterize stably transformed RNAi grapevines.
4. Disseminate information to stakeholders through presentations at conventions and workshops.

RESULTS AND DISCUSSION

Objective 1. Optimize RNAi Constructs Against the Grape Mealybug

AQP1 and *SUC1* have been characterized by reverse transcription polymerase chain reaction (RT-PCR) using total RNAs isolated from crawlers of a grape mealybug colony maintained on Pixie grapes in the greenhouse and overlapping primers. The cloned *AQP1* fragment is 490 base pairs in size and the cloned *SUC1* fragment is 394 base pairs in size. The sequences of *AQP1* and *SUC1* were used to design dsRNA constructs which were cloned in a binary plasmid for expression in planta.

To enhance the efficacy of RNAi against the grape mealybug, dsRNA constructs against the osmoregulation genes *AQP1* and *SUC1* were stacked. Additionally, we identified *NUC1*, a non-specific nuclease that is expressed in the gut and functions to degrade ingested dsRNA (Christiaens et al., 2014; Luo et al., 2013) by RT-PCR using overlapping primers and total RNA from crawlers. A dsRNA *NUC1* construct should protect dsRNA against degradation and dramatically increase insect mortality by stacking dsRNA against the osmoregulation genes with dsRNA against the nuclease, as recently documented (Luo et al., 2017). The *NUC1* dsRNA construct was stacked with dsRNA constructs to *AQP1* and *SUC1*. The feasibility of a gene stacking approach is assured by our previous research, in which up to five dsRNA constructs for in planta delivery were used with no effect on plant growth or development but with high mortality of psyllid and whitefly pests (Luo et al., 2017; Tzin et al., 2015). Efforts to develop other versions of *SUC1* are considered for increased efficacy of RNAi against mealybugs. Also,

orthologues of *AQP1*, *SUC1*, and *NUC1* in the vine mealybug are characterized from specimens generously provided by Kent Daane (UC Berkeley). The sequence of the vine mealybug orthologue will be determined and compared with the sequence from the grape mealybug. These efforts will provide clues on the potential of RNAi against the grape mealybug at protecting against the vine mealybug.

For GLRaV-3, dsRNA constructs to the suppressor of RNA silencing *p19.7* and the coat protein (*CP*) open reading frame were engineered. Additional dsRNA constructs from conserved regions of the viral genome were developed by analysis of aligned virus nucleotide sequences available in GenBank and identification of short stretches of conserved regions. Emphasis was placed on the RNA-dependent RNA polymerase (*RdRp*) and the heat shock protein 70 homolog (*HSP70h*) open reading frames of GLRaV-3. Conserved regions were identified for *RdRp* and *HSP70h*. We retrieved full-length GRLaV-3 genome sequences available in GenBank and analyzed them to identify highly conserved nucleotide sequence regions. Search outputs revealed conserved nucleotide stretches of 100-300 nucleotides in size for *CP*, *RdRp*, and *HSP70h*. Individual conserved regions were amplified by RT-PCR using specific primers and total RNA from GLRaV 3-infected grapevines as template. The integrity of these constructs was verified by restriction digestions and sequencing. Each of these fragments was cloned into the plasmid pEPT8, a plasmid derived from pUC19 that contains the cauliflower mosaic virus 35S promoter sequence and nopaline synthase terminator sequence, and subsequently into binary plasmid pGA482G for mobilization into *Agrobacterium tumefaciens* strain C58 for plant transformation. DsRNA constructs to GLRaV-3 *RdRp* and *HSP70h* complete the *CP* and *p19.7* dsRNA constructs previously engineered.

Anticipating the engineering of stacked dsRNA constructs to the grape mealybug and GLRaV-3 for combined resistance to the virus and its most abundant vector, targeting the viral silencing suppressor *p19.7* (Gouveia et al., 2012) is not optimal. This is because RNAi should be fully effective and no silencing suppressor should be used for maximal efficacy. Therefore, dsRNA constructs of GLRaV-3 *CP*, *RdRp*, and *HSP70h* will be stacked first, and these constructs will then be stacked with dsRNA constructs of *AQP1*, *SUC1*, and *NUC1* from the grape mealybug. The GLRaV-3 dsRNA construct pGA482G-LR3p19.7-4 (against the viral silencing suppressor *p19.7*) will continue to be used, but only for resistance against GLRaV-3. Expression of pyramided dsRNA constructs to *AQP1*, *SUC1*, NUC, *CP*, *RdRp*, and *HSP70* will be driven by the phloem-specific promoter s*ucrose-H+ symporter* (*SUC2)* to target RNAi expression to the grape phloem, the preferred feeding sites of the grape mealybug and preferred localization tissue of GLRaV-3 in grape.

Objective 2. Develop a High Throughput Transient Expression System to Test the Efficacy of RNAi Constructs Against the Grape Mealybug

Optimizing the delivery of dsRNA constructs to grape tissue was an initial priority. This work is critical for the future development of RNAi transient bioassays to identify the most promising dsRNA constructs against the grape mealybug. Efforts included the monitoring of the behavior of the grape mealybug on tissue-culture-grown grape plantlets, anticipating that transient assays will be carried out on this type of plant material, perhaps via vacuum-assisted infiltration (Yepes et al., 2018). Crawlers were deposited on leaves and stems of tissue-culture-grown *V. vinifera* or rootstock plantlets and observed over time (**Figure 1**). Unfortunately, this new habitat was not

optimal for crawlers, as the majority of specimens did not survive the transfer from Pixie grapes onto stems or leaves of tissue-culture-grown grapevines, regardless of the nature of the plant material, i.e., *V. vinifera* cultivars or rootstock genotypes, as shown by repeated counts within two to three weeks.

Figure 1. Grape mealybug crawler on a stem of a tissue-culture-grown *V. vinifera* cultivar Syrah grape plantlet.

Since tissue culture grape material was shown to be suboptimal for transient assays with dsRNA constructs based on the behavior of the grape mealybug, the use of detached leaves of Pixie grapes was investigated. Pixie is a natural dwarf grapevine derived from the periclinal chimera of *V. vinifera* cultivar Pinot Meunier. It has short internodes and is a preferred host of the grape mealybug. To test the feasibility of a detached leaf assay, we excised young Pixie leaves and placed them in microfuge tubes containing distilled water or a red food dye (10%). Red pigmentation was visible in the veins of Pixie leaves within one hour and more pigment continued to disperse is subsequent hours (**Figure 2**). This initial work revealed that a food dye spreads from the stem of a detached Pixie grape petiole throughout the leaf, particularly to its very small veins. This result was very encouraging for the delivery of dsRNA constructs against the grape mealybug in transient assays.

Next, grape mealybugs from a colony maintained on potted Pixie vines in the greenhouse were deposited on detached Pixie leaves to evaluate their behavior on this new habitat. A high survival rate (more than 80%) of grape mealybugs was consistently obtained in replicated assays even after two weeks of exposure (**Figure 3**).

Such conditions are anticipated to be well adapted to evaluate the effect of dsRNA constructs against the grape mealybug in a transient assay based on excised Pixie leaves. The next step was to determine if a dsRNA construct can be administered to an excised petiole of a Pixie leaf. We used a dsRNA construct to the green fluorescent protein (GFP) as a proxy for dsRNA constructs to the grape mealybug. First, we tested the stability of the GFP dsRNA construct in water over time. No degradation was observed for the GFP dsRNA construct over the course of the experiment (0 to 24 hours), as shown by electrophoresis on an agarose gel (**Figure 4**).

Figure 2. Absorption of red food coloring by detached leaves of Pixie grape. Left: a leaves exposed to red food coloring (top) vs. distilled water (bottom). Middle: primary, secondary, and tertiary veins of leaves exposed to water. Right: primary, secondary, and tertiary veins of leaves exposed to red food coloring. Pictures were taken 18 hours after exposure.

Figure 3. Close-up the excised a Pixie leaf with its petiole immersed in water and mealybug adults feeding on secondary veins.

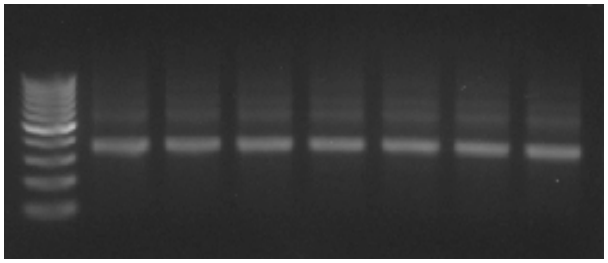


Figure 4. Analysis of the stability of a GFP dsRNA construct kept in water after 0 (lane 2), 0.5 (lane 3), 1 (lane 4), 2 (lane 5), 6 (lane 6), 12 (lane 7) and 24 (lane 8) hours by electrophoresis on an agarose gel.

Then the GFP dsRNA construct (0.05 µg/µl in 200 µl solution) was added to the microfuge tubes containing excised Pixie leaves and its presence was tested by Northern blot hybridization in tissue collected from Pixie leaves at 24 hours post-soaking using a specific ^{32}P-labeled probe (**Figure 5**). Analysis of the Northern blot image showed an uptake of the GFP dsRNA construct by excised Pixie leaves. Results were also consistent with the integrity of the GFP dsRNA detected in leaf tissue and some degradation possibly due to the plant RNAi machinery, since several DNA products of lower molecular mass than the 0.4 kb full-length GFP dsRNA construct were detected.

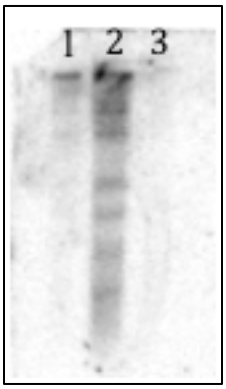


Figure 5. Northern blot hybridization of total RNA extracted from excised Pixie leaves for which the petiole was immersed into a GFP dsRNA solution for 24 hours (lane 2) or water (lane 3). Lane 1 is the GFP dsRNA construct in water as positive control.

Based on these encouraging preliminary results, we initiated Northern blot hybridization experiments to determine whether the GFP dsRNA construct can be detected in grape mealybugs exposed to excised Pixie leaves soaked in a GFP dsRNA construct for 24 to 48 hours. This analysis is critical for determining whether the intact dsRNA construct is delivered to the insect and diced by the RNAi machinery of the insect to 21-nucleotide small interfering RNA (siRNA), with minimal nonspecific degradation. Optimizing such conditions is vital prior to running separate experiments with dsRNA constructs and testing their effect on the survival of grape mealybugs. Unfortunately, the detached grape leaf assay provided inconsistent data in terms of the efficiency of the dsRNA construct uptake in Pixie leaves, and subsequently in mealybugs. Therefore, this assay was abandoned and the use of a diet to be supplemented with dsRNA constructs of interest was investigated. First, we determined if adults and nymphs that were reared on Pixie grapes could survive on an artificial diet. The artificial diet used in this study was similar to the one that is routinely used for work with other hemipterans. The objective was to see if this type of food source could sustain grape mealybugs for a few days. Results showed that at least 70% of the specimens survived for three to five days when exposed to the artificial diet. These conditions were deemed appropriate for the testing of the effect of dsRNA constructs on the survival of grape mealybugs.

Experiments with dsRNA constructs focused on nymphs, as they are the most efficient stage for GLRaV-3 transmission (Almeida et al., 2013). Approximately 20 one-to-ten-day-old grape mealybug nymphs were exposed to the artificial diet, and their survival was measured at three days post-exposure. Mealybugs were fed first an artificial diet (without any dsRNA construct) or the same diet supplemented with the dsRNA construct against *NUC* at 0.2 µg/µl. Then, mealybugs were allowed to feed for 72 hours on the control diet or the same diet supplemented with various dsRNA constructs. Experiments were triplicated. Results showed a 20% reduction in the survival of grape mealybugs exposed to a diet supplemented with dsRNA constructs against *AQP1* (0.1 µg/µl), *SUC1* (0.1 µg/µl), and *NUC* (0.3 µg/µl) relative to control diets, i.e., a diet without any supplements and a diet supplemented with a dsRNA construct against *GFP* (0.4 µg/µl) (**Figure 6**). This reduction was significant ($p = 0.0436$). The survival of nymphs on a diet supplemented with dsRNA constructs against *AQP1* (0.2 µg/µl) and *SUC1* (0.2 µg/µl) was also significantly reduced relative to the two control diets ($p = 0.0824$) (**Figure 6**). As expected, the effect of dsRNA constructs against *NUC*, *AQP1*, and *SUC1* on mealybug mortality was more pronounced than the dsRNA constructs against *AQP1* and *SUC1*. Similarly, as expected, the survival of nymphs exposed to a diet supplemented with *NUC* (0.2 µg/µl) and GFP (0.2 µg/µl) was identical to that of nymphs on the control diets ($p = 0.3184$) (**Figure 6**). These results are very encouraging. New experiments to verify these trends are underway.

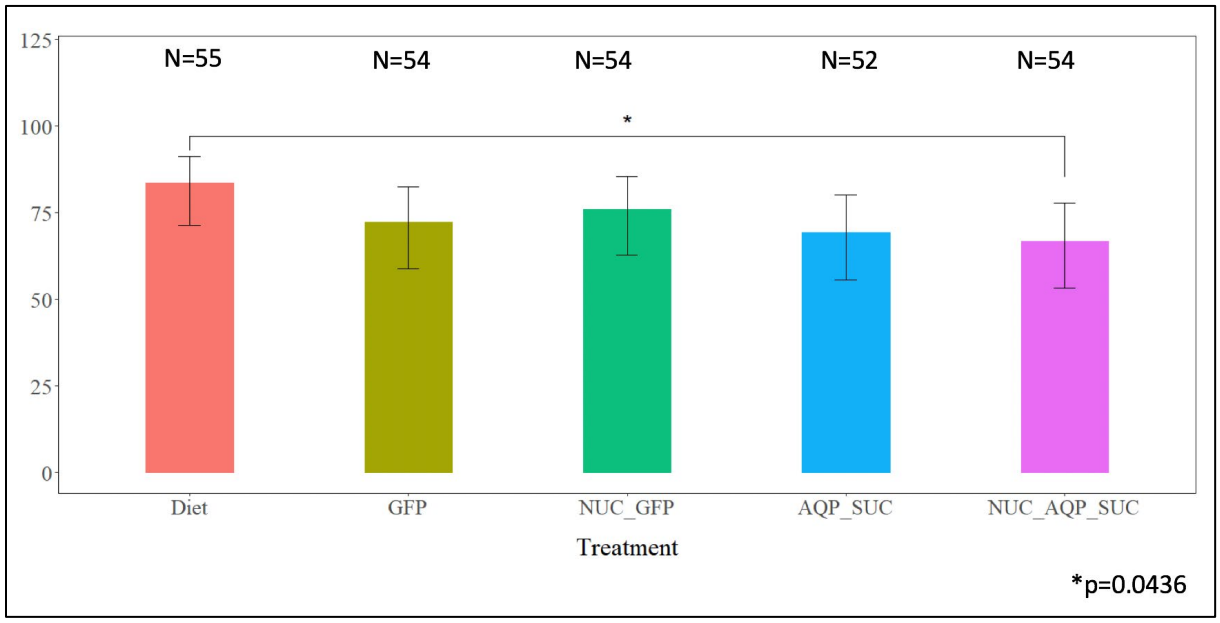


Figure 6. Survival of grape mealybug nymphs at three days post-exposure to an artificial diet supplemented with various dsRNA constructs.

Efforts to optimize the efficacy of RNAi are underway. They focus on different versions of *SUC1* that potentially could augment the potency of RNAi against the grape mealybug. In addition, *AQP1*, *SUC1*, and *NUC* are characterized in the vine mealybug from a colony maintained by K. Daane.

Objective 3. Characterize Stably Transformed RNAi Grapevines

An inverted-repeat p19.7 construct was engineered and used for the production of transgenic grapevines via *A. tumefaciens*-mediated transformation. Embryogenic cultures of rootstock genotypes 110R and 101-14 were used for stable transformation experiments. Following transformation with *A. tumefaciens* elongation of embryogenic cultures was observed, with the highest efficacy obtained with 110R followed by 101-14. A few plants of the rootstock genotypes 110R that were subjected to transformation experiments were regenerated and micropropagated in tissue culture. Some putative transgenic plantlets were transferred to soil in the greenhouse by removing them from test tubes or polyethylene tissue culture bags using forceps, rinsing roots in water, and trimming roots to about one third in length to stimulate growth prior to transfer to Cornell mix in individual plastic pots. Plants were covered with plastic bags to avoid dehydration. Plastic bags were gradually opened following active growth in the greenhouse. Transgene insertion will be characterized by PCR and Southern blot hybridization using total plant DNA isolated from leaves of actively-growing putative transgenic plants. In the near future, RT-PCR and Northern blot hybridization will be carried out to confirm transgene expression and the accumulation of siRNA, respectively. Additional putative transgenic 110R and 101-14 rootstock plants were transferred from tissue culture to the greenhouse for characterization of transgene insertion and expression.

Efforts to engineer stable grapevine transformants with other GLRaV-3 dsRNA constructs have focused on stacked dsRNA constructs of *CP*, *RdRp*, and *HSP70h*. These dsRNA constructs are vital for combining resistance to the virus and the grape mealybug, as the dsRNA *p19.7* construct, which is coding for a silencing suppressor, would not be optimal for inclusion as one of the stacked constructs. Next, stacked GLRaV-3, *AQP1*, *SUC1*, and *NUC* dsRNA constructs will be engineered and used in stable transformation experiments. Recently, we initiated efforts to develop embryogenic cultures of *V. vinifera* cultivars Cabernet Franc and Pinot Noir from immature inflorescences (**Figure 7**). These cultures will be used in stable transformation experiments with RNAi constructs against the grape mealybug and GLRaV-3.

Figure 7. Anther callus of *V. vinifera* cultivar Pinot Noir.

Objective 4. Disseminate Information to Stakeholders Through Presentations at Conventions and Workshops

Research results were communicated to 380 growers, vineyard managers, vintners, farm advisors, extension educators, crop consultants, researchers, and regulators in California, North Carolina, and Ontario, Canada at the following meetings:

- Fuchs M. 2019. Leafroll disease management: Current recommendations and future prospects, October 16, Acampo, CA (participants = 15).
- Fuchs M. 2019. Biology of grapevine viruses. Mealybug and Virus Outreach Meeting, April 4, Stockton, CA (participants = 250).
- Fuchs M. 2019. Impact of leafroll and red blotch diseases. Vinedresser Meeting, March 28, Dobson, NC (participants = 20).
- Cieniewicz E, Fuchs M. 2018. Virus diseases: Why should I care and what can I do? California State University - Fresno, October 3, Jordan College of Agriculture Sciences and Technology, Department of Viticulture and Enology, Fresno, CA (participants = 30).
- Fuchs M. 2018. Grape virus research updates. Biennial Grape Research Tailgate Tour, August 30, Niagara-on-the-Lake, Ontario, Canada (participants = 80).

These presentations provided opportunities to communicate on research progress and discuss the future of RNAi technology for leafroll and mealybug management.

CONCLUSIONS

Grapevine leafroll disease is one of the most devastating and widespread viral diseases of grapevines. GLRaV-3 is the dominant virus in leafroll-diseased vineyards. This virus is transmitted by several species of mealybugs including the grape mealybug, which is its most abundant and widely distributed vector in vineyards and a pest of grapes. We are exploring RNAi to protect grapevines against GLRaV-3 and the grape mealybug. For RNAi to GLRaV-3, conserved nucleotide sequence regions of *p19.7*, *CP*, *RdRp*, and *HSP70* were used to engineer dsRNA constructs. Putative transgenic plants of the rootstock genotype 110R were obtained following *A. tumefaciens*-mediated transformation with a dsRNA *p19.7* construct and established in the greenhouse. Transgene insertion was confirmed in transgenic 110R plants. The development of embryogenic cultures from immature inflorescences of *V. vinifera* cultivars Pinot Noir and Cabernet Franc is underway. For the grape mealybug, key osmoregulatory genes *AQP1* and *SUC1* and the nonspecific nuclease *NUC* were obtained from crawlers of a grape mealybug colony established on Pixie grapes in the greenhouse. A bioassay based on artificial diet was developed. This assay revealed a 20% reduction in the survival of grape mealybug nymphs that fed on an artificial diet containing dsRNA constructs to *AQP1*, *SUC1*, and *NUC* relative to a control diet. This effect was significant ($p = 0.0436$). These results are very encouraging. Orthologues of *AQP1*, *SUC1*, and *NUC* from the vine mealybug are being characterized from specimens generously provided by K. Daane. We will pyramid dsRNA constructs against several targets of the virus and the insect vector, anticipating a greater efficacy in disease management and greater opportunities in impeding the development of virus and insect vector populations capable of overcoming the resistance.

REFERENCES CITED

Almeida RPP, Daane KM, Bell VA, Blaisdell GK, Cooper ML, Herrbach E, Pietersen G. 2013. Ecology and management of grapevine leafroll disease. *Frontiers in Microbiology.* doi: 10.3389/fmicb.2013.00094.

Atallah S, Gomez M, Fuchs M, Martinson T. 2012. Economic impact of grapevine leafroll disease on *Vitis vinifera* cv. Cabernet Franc in Finger Lakes vineyards of New York. *American Journal of Enology and Viticulture* 63: 73-79.

Christiaens O, Swevers L, Smagghe G. 2014. DsRNA degradation in the pea aphid (*Acyrthosiphon pisum*) associated with lack of response in RNAi feeding and injection assay. *Peptides* 53: 307-314.

Fuchs M. 2017. Pyramiding resistance-conferring gene sequences in crops. *Current Opinion in Virology* 26: 36-42.

Gonsalves D, Ferreira S, Suzuki J, Tripathi S. 2008. Papaya. Kole C, Hall TC (eds.). *Compendium of Transgenic Crop Plants*. Oxford UK, Blackwell Publishing, pp. 131-162.

Gouveia P, Dandlen S, Costa S, Marques N, Nolasco G. 2012. Identification of an RNA silencing suppressor encoded by grapevine leafroll-associated virus 3. *European Journal of Plant Pathology* 133: 237-245.

Hily JM, Scorza R, Malinowski T, Zawadzka B, Ravelonandro M. 2004. Stability of gene silencing-based resistance to plum pox virus in transgenic plum (*Prunus domestica* L.) under field conditions. *Transgenic Research* 13: 427-436.

Ito T, Nakaune R. 2016. Molecular characterization of a novel putative ampelovirus tentatively named grapevine leafroll-associated virus 13. *Archives of Virology* 161: 2555-2559.

Jing XF, White TA, Luan J, Jiao C, Fei Z, Douglas AE. 2016. Evolutionary conservation of candidate osmoregulation genes in plant phloem-sap feeding insects. *Insect Molecular Biology* 25: 251-258.

Karley AJ, Ashford DA, Minto LM, Pritchard J, Douglas AE. 2005. The significance of gut sucrase activity for osmoregulation in the pea aphid, *Acyrthosiphon pisum*. *Journal of Insect Physiology* 51: 1313-1319.

Luo Y, Wang X, Wang X, Yu D, Chen B, Kang L. 2013. Differential responses of migratory locusts to systemic RNA interference via double-stranded RNA injection and feeding. *Insect Molecular Biology* 22: 574-583.

Luo Y, Chen Q, Luan J, Chung SH, Van Eck J, Turgeon ER, Douglas AE. 2017. Towards an understanding of the molecular basis of effective RNAi against a global insect pest, the whitefly *Bemisia tabaci. Insect Biochemistry and Molecular Biology* 88: 21-29.

Maree HJ, Almeida RPP, Bester R, Chooi KM, Cohen D, Dolja VV, Fuchs MF, Golino DA, Jooste AEC, Martelli GP, Naidu RA, Rowhani A, Saldarelli P, Burger JT. 2013. Grapevine leafroll-associated virus 3. *Frontiers in Microbiology* 4: 94.

Naidu RA, Rowhani A, Fuchs M, Golino DA, Martelli, GP. 2014. Grapevine leafroll: A complex viral disease affecting a high-value fruit crop. *Plant Disease* 98: 1172-1185.

Naidu RA, Maree HJ, Burger JT. 2015. Grapevine leafroll disease and associated viruses: A unique pathosystem. *Annual Review of Phytopathology* 53: 613-634.

Oliver JE, Fuchs M. 2011. Tolerance and resistance to viruses and their vectors in *Vitis* sp.: A virologist's perspective of the literature. *American Journal of Enology and Viticulture* 62: 438-451.

Pietersen G, Spreeth N, Oosthuizen T, van Rensburg A, van Rensburg M, Lottering D, Rossouw N, Tooth D. 2013. Control of grapevine leafroll disease spread at a commercial wine estate in South Africa: A case study. *American Journal of Enology and Viticulture* 64: 296-305.

Ricketts KD, Gomez MI, Atallah SS, Fuchs MF, Martinson T, Smith RJ, Verdegaal PS, Cooper ML, Bettiga LJ, Battany MC. 2015. Reducing the economic impact of grapevine leafroll disease in California: Identifying optimal management practices. *American Journal of Enology and Viticulture* 66: 138-147.

Shakesby AJ, Wallace IS, Isaacs HV, Pritchard J, Roberts DM, Douglas AE. 2009. A water-specific aquaporin involved in aphid osmoregulation. *Insect Biochemistry and Molecular Biology* 39: 1-10.

Tzin V, Yang X, Jing X, Zhang K, Jander G, Douglas AE. 2015. RNA interference against gut osmoregulatory genes in phloem-feeding insects. *Journal of Insect Physiology* 79: 105-112.

Yepes LM, Cieniewicz E, Krenz B, McLane H, Thompson JR, Perry KL, Fuchs M. 2018. Causative role of grapevine red blotch virus in red blotch disease. *Phytopathology* 108: 902-909.

FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board, and by the USDA National Institute of Food and Agriculture.

ACKNOWLEDGEMENTS

We are grateful to the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board for its support, to Arinder Arora and Luz Marcela Yepes for spearheading research efforts on resistance to the grape mealybug and GLRaV-3, respectively, and to Noah Clark, Stephen Hesler, Karen Wentworth, and Yeng Mei Cheung for their valuable contributions.

II. RESISTANCE TO GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3 AND THE GRAPE MEALYBUG

Project Leader: Marc Fuchs | Section of Plant Pathology | Cornell University | Geneva, NY 14456 | mf13@cornell.edu
Co-Project Leader: Angela Douglas | Department of Entomology | Cornell University | Ithaca, NY 14853 | aes326@cornell.edu
Co-Project Leader: Greg Loeb | Department of Entomology | Cornell University | Geneva, NY 14456 | gmel@cornell.edu
Collaborator: Deborah Golino | Foundation Plant Services | University of California | Davis, CA 95616 | dagolino@ucdavis.edu

Reporting Period: The results reported here are from work conducted July 1, 2019 to October 23, 2019.

ABSTRACT

Grapevine leafroll disease is one of the most devastating and widespread viral diseases of grapevines (*Vitis vinifera*). There is no cure for grapevine leafroll disease in the vineyard. In addition, grapevines resistant to the viruses associated with the disease and their mealybug vectors are not available. Therefore, developing grapevines resistant to grapevine leafroll-associated virus 3 (GLRaV-3), the dominant leafroll viruses in diseased vineyards, and to the grape mealybug (*Pseudococcus maritimus*; GMB) and vine mealybug (*Planococcus ficus*; VMB), the two most important mealybug vectors of GLRaV-3, using RNA interference (RNAi) is a prime objective of our research. Our strategy is to combine RNAi against targets of the virus and of the two insect vectors, providing for greater efficacy in disease management, as well as greater opportunities in impeding the development of virus and insect vector populations capable of overcoming the resistance. We have identified the candidate GMB genes coding for the water channel aquaporin AQP1 and the sucrase-transglucosidase SUC4, as well as a gut nonspecific nuclease NUC1, and developed a system to assess the impact of RNAi trigger molecules against these genes on GMB survival. Our findings documented a significantly increased mortality (20%) of GMB feeding on a diet supplemented with RNAi against *AQP1*, *SUC4*, and *NUC1*. Efforts to apply these findings to the VMB are underway using specimens kindly provided by Kent Daane at the University of California, Berkeley. For resistance to GLRaV-3, RNAi constructs against the coat protein (CP), RNA-dependent RNA polymerase (RdRp), and heat shock 70 homologue (HSP70h) genes were developed and used in grape transformation experiments for the recovery of stable transformants. To augment the efficacy of our RNAi approach, constructs against the GMB (*AQP1*, *SUC4*, and NUC1) and GLRaV-3 (*CP, RdRp,* and *HSP70h*) are being stacked in various combinations. Their expression in planta will be driven by the phloem-specific promoter *AtSUC2*. This promoter was a gift from Robert Turgeon. The production of grape plants stably transformed with RNAi constructs against GLRaV-3 and the mealybug pests is underway.

LAYPERSON SUMMARY

Grapevine leafroll disease is one of the most devastating and widespread viral diseases of grapevines (*Vitis vinifera*). It reduces fruit production and quality. There is no cure for grapevine leafroll disease in the vineyard and resistant grapevines are not available. The objective of our

research is to develop grapevines resistant to grapevine leafroll-associated virus 3 (GLRaV-3), the dominant leafroll viruses in diseased vineyards, and to the grape mealybug (*Pseudococcus maritimus*; GMB) and the vine mealybug (*Planococcus ficus*; VMB), the two most important mealybug vectors of GLRaV-3, using RNA interference (RNAi). Our strategy is to combine RNAi against targets of the virus and of the two insect vectors. Target GMB genes were identified and characterized. RNAi against these genes resulted in a significantly increased mortality of GMB following feeding on a diet supplemented with the anti-GMB constructs. Efforts to apply these findings to VMB are underway. For resistance to GLRaV-3, target RNAi constructs were developed and used in grape transformation experiments for the recovery of stable transformants. For increased efficacy, RNAi against GMB and GLRaV-3 are stacked and their expression in planta will be directed to the phloem tissue by a specific promoter. The production of grape plants stably transformed with RNAi constructs against GLRaV-3 and the mealybug pests are underway.

INTRODUCTION

Grapevine leafroll disease is one of the most devastating and widespread viral diseases of grapevines (*Vitis vinifera*). It reduces yield, delays fruit ripening, increases titratable acidity, lowers sugar content in fruit juices, modifies aromatic profiles of wines, and shortens the productive lifespan of vineyards (Almeida et al., 2013; Naidu et al., 2014). The economic cost of leafroll is estimated to range from $12,000 to $92,000 per acre in California (Ricketts et al., 2015) and from $10,000 to $16,000 in New York during a productive 25-year lifespan of a vineyard (Atallah et al., 2012).

Six major viruses named grapevine leafroll-associated viruses (GLRaVs; GLRaV-1, -2, -3, -4, -7, and -13) have been identified in diseased vines (Naidu et al., 2014, 2015). Among these viruses GLRaV-3 is the dominant leafroll virus in vineyards (Maree et al., 2013; Naidu et al., 2014, 2015). This virus is phloem-limited and semi-persistently transmitted by several species of mealybugs, with acquisition and inoculation occurring within a one-hour access period of feeding by immature mealybug stages (Almeida et al., 2013). There is no significant effect of host plant tissue on transmission efficiency, nor is there specificity of transmission (Almeida et al., 2013; Naidu et al., 2014), indicating that many mealybug species may disseminate all transmissible strains of GLRaV-3.

Mealybugs are sap-sucking insects in the family Pseudococcidae. They are pests of grapes and many other important crops. At high densities, mealybugs can cause complete crop loss, rejection of fruit loads at wineries, and death of spurs, although small infestations may not inflict significant direct damage. In the feeding process on plant sap, mealybugs excrete honeydew (a sugary egesta) that often becomes covered with a black sooty mold, which additionally damages fruit clusters under high infestation levels. Several mealybug species feed on grapevines, but the grape mealybug (*Pseudococcus maritimus*; GMB) and the vine mealybug (*Planococcus ficus*; VMB) are the most abundant and widespread species in California vineyards (Almeida et al., 2013). Unassisted, mealybugs have limited mobility, but first instar immatures (crawlers) can be dispersed over long distances by wind and other means (Almeida et al., 2013).

In diseased vineyards, management strategies rely on the elimination of virus-infected vines and the reduction of mealybug populations through the application of insecticides. Managing leafroll

viruses and their mealybug vectors remains challenging due to several factors, including a lack of recognized host resistance (Oliver and Fuchs, 2011). Resistance can be achieved by applying RNA interference (RNAi) technologies. The approach relies on the development of RNAi constructs targeting specific pathogen or insect genes to specifically downregulate their expression upon infection or feeding. The RNAi approach is highly specific and anticipated to reduce hazards of chemical pesticide applications. The fact that mealybugs transmit leafroll viruses offers an opportunity to explore a two-pronged approach to simultaneously target virus and vector.

The goal of our research is to develop a robust RNAi-based strategy against GLRaV-3, GMB, and VMB. The basis for our approach is three-fold. First, mealybug survival depends on two gene functions localized to the gut that prevent osmotic collapse and dehydration of the insect, as it feeds on its sugar-rich diet of plant phloem sap. These genes are the water channel aquaporin *AQP1* and the sucrase-transglucosidase *SUC4* (Jing et al., 2016; Arora et al., in prep), with evidence that insect mortality is enhanced by co-targeting these two genes with different molecular functions but related physiological role (Tzin et al., 2015). Second, these gene functions can be pyramided for RNAi application with evidence from related phloem-feeding insects that RNAi efficacy is enhanced by stacking these RNAi constructs with RNAi against the gut nonspecific nuclease (*NUC1*) (Luo et al., 2017). Third, RNAi is being successfully applied against viruses of fruit crops such as papaya and plum (Ibrahim and Aragão, 2015).

The RNAi strategy has two key strengths: (i) the localization of expression of the RNAi trigger molecule can be controlled to minimize contact with non-target organisms, including beneficial arthropods, and (ii) the sequence of the RNAi trigger molecules, i.e., hairpin (hp) constructs in plant transformations, can be designed to the desired specificity, enabling us to target the pest species (GMB, VMB, and GLRaV-3) with minimal impact on non-target organisms. We will design the transformed plants to maximize efficacy against the pests with minimal bulk concentration of the RNAi trigger molecule in the plant and minimal release to the environment. Our strategy is to place the hairpin constructs under a plant phloem-specific promoter, so that the RNAi trigger molecules are expressed in the phloem sap, which is the feeding site of the mealybugs and the tissue where GLRaV-3 is preferentially located, with minimal leakage to the rest of the plant.

OBJECTIVES

Our specific objectives are to:

1. Complete the first generation of double-stranded RNA (dsRNA) constructs under a phloem-specific promoter (first generation of grape transformations), and initiate new grape transformations with dsRNA constructs (second generation of grape transformations).
2. Quantify the impact of RNAi on the survival of GMB and expression of the target GMB genes.
3. Characterize homologous genes in VMB and design RNAi constructs with predicted efficacy against both GMB and VMB, for the second generation of grape transformations.
4. Test whether the RNAi molecules against GMB and GLRaV-3 in the transformed grape plants are targeted correctly to the phloem sap.
5. Determine whether RNAi molecules can be detected in honeydew after GMB feeding on dsRNA constructs.

6. Disseminate information to stakeholders through presentations at conventions and workshops.

RESULTS AND DISCUSSION

Objective 1. Complete the First Generation of Double-Stranded RNA (dsRNA) Constructs Under a Phloem-Specific Promoter, and Initiate New Grape Transformations with dsRNA Constructs

dsRNA constructs against the coat protein (*CP*), the heat shock protein 70 homolog (*HSP70h*), and the RNA-dependent RNA polymerase (*RdRp*) open reading frame fragments of GLRaV-3 will be stacked with dsRNA constructs against *AQP1*, *SUC4,* and *NUC* of GMB. Expression of the stacked genes will be driven by the *SUC2* promoter of *Arabidopsis thaliana*, *AtSUC2*. Using *AtSUC2* will target RNAi expression to the phloem where the virus is preferentially restricted and GMB is preferentially feeding. First GLRaV-3 dsRNA constructs will be concatenated using sets of specific primers, then these constructs will be stacked with the GMB dsRNA constructs using other sets of specific primers in a tiered polymerase chain reaction (PCR) approach. Specific primer pairs have been designed to stack both GLRaV-3 and GMB dsRNA constructs.

Perturbing the expression of osmoregulatory genes required for water balance, specifically *AQP1* and *SUC4*, in the gut of phloem-feeding insects causes the insects to lose water from the body fluids and dehydrate, dying within two to three days (Karley et al., 2005; Shakesby et al., 2009; Tzin et al., 2015; Luo et al., 2017). In addition, non-specific nuclease(s) (*NUC)* genes expressed in the gut of phloem-feeding insects act to degrade ingested dsRNA, thereby preventing effective RNAi (Christiaens et al., 2014; Luo et al., 2017; Chung et al., 2018). The use of a dsRNA construct against *NUC* will enhance RNAi efficacy.

Transformation efforts will focus on *V. vinifera* cvs. Cabernet Franc and Pinot Noir. Cabernet Franc and Pinot Noir were selected for stable transformation because they are good indicators of GLRaV-3 symptoms and support large GMB populations. In addition, transformation of rootstock genotypes 101-14 and 110R will continue as previously.

Objective 2. Quantify the Impact of RNAi on the Survival of GMB and Expression of the Target GMB Genes

We will quantify the impact of stably transformed Cabernet Franc and Pinot Noir with different combinations of RNAi constructs on survival of GMB. For these assays we will use a lab colony of GMB that is successfully maintained on Pixie grapevines in a greenhouse.

Objective 3. Characterize Homologous Genes in VMB and Design RNAi Constructs with Predicted Efficacy Against Both GMB and VMB, for the Second Generation of Grape Transformations

We are determining the nucleotide sequence of the homolog GMB dehydration genes *AQP1*, *SUC4,* and *NUC1* in VMB. Specimens of VMB from a colony maintained on butternut squash (*Cucurbita moschata*) were kindly provided by Kent Daane at the University of California, Berkeley. Total RNA was isolated from VMB and used in reverse transcription (RT)-PCR with degenerate primers designed to characterize *AQP1*, *SUC4,* and *NUC1* in this mealybug species, as previously done to obtain the corresponding sequences for GMB (Arora, Clark, and Douglas, unpublished). DNA products obtained by RT-PCR will be sequenced and used for in silico

prediction of the efficacy of RNAi against VMB by dsRNA sequences against GMB. This is important to design constructs with predicted maximal efficacy against both mealybug pests. The sequences of the VMB and GMB genes will also be analyzed for predicted activity against non-target taxa, particularly beneficial insects.

Objective 4. Test Whether the RNAi Molecules Against GMB and GLRaV-3 in the Transformed Grape Plants Are Targeted Correctly to the Phloem Sap
Experiments will be conducted after transgenic Cabernet Franc and Pinot Noir plants are obtained.

Objective 5. Determine Whether RNAi Molecules Can Be Detected in Honeydew After GMB Feeding on dsRNA Constructs
We will check whether RNAi molecules are released to the honeydew of mealybugs using first a transient delivery system and then stably transformed plants. This research will determine if RNAi has the potential of deleterious effects on non-target organisms feeding on the honeydew.

Objective 6. Disseminate Information to Stakeholders Through Presentations at Conventions and Workshops
Research findings will be communicated to the grape and wine industry during grower conventions and meetings.

CONCLUSIONS

Progress is being made toward the development of grapevines resistant to GLRaV-3, the dominant leafroll virus in diseased vineyards, and to GMB and VMB, the two most important mealybug vectors of GLRaV-3, using RNAi. Target genes, i.e., *AQP1*, *SUC4*, and *NUC1*, for RNAi against GMB were identified and characterized. A biological assay showed a significantly increased mortality (20%) of GMB feeding on a diet supplemented with these RNAi constructs. Efforts to apply these findings to the VMB have been undertaken, with the goal of designing RNAi that would be efficient against both mealybug species. For GLRaV-3, RNAi constructs were designed in target genes, i.e., *CP*, *RdRp*, and *HSP70*, and used in grape transformation experiments. RNAi constructs against GMB and GLRaV-3 are being stacked for expression in planta and directed to the phloem by a specific promoter, *AtSUC2*. The production of grape plants stably transformed with RNAi constructs against GLRaV-3 and the mealybug pests is underway. Future efforts will focus on assessing RNAi constructs on the survival of GMB in stably transformed plants, determining expression of RNAi against mealybugs and GLRaV-3 in the phloem sap, analyzing detectability of RNAi molecules in the mealybug honeydew, and disseminating information on research findings to the wine and grape industry.

REFERENCES CITED

Akman Gunduz E, Douglas AE. 2009. Symbiotic bacteria enable insect to use a nutritionally inadequate diet. *Proceedings of Biological Sciences* 276: 987-991.

Almeida RPP, Daane KM, Bell VA, Blaisdell GK, Cooper ML, Herrbach E, Pietersen G. 2013. Ecology and management of grapevine leafroll disease. *Frontiers in Microbiology* 4: 94. doi: 10.3389/fmicb.2013.00094.

Atallah S, Gomez M, Fuchs M, Martinson T. 2012. Economic impact of grapevine leafroll disease on *Vitis vinifera* cv. Cabernet franc in Finger Lakes vineyards of New York. *American Journal of Enology and Viticulture* 63: 73-79.

Christiaens O, Swevers L, Smagghe G. 2014. DsRNA degradation in the pea aphid (*Acyrthosiphon pisum*) associated with lack of response in RNAi feeding and injection assay. *Peptides* 53: 307-314.

Chung SH, Jing X, Luo Y, Douglas AE. 2018. Targeting symbiosis-related insect genes by RNAi in the pea aphid-*Buchnera* symbiosis. Insect Biochemistry and Molecular Biology 95: 55-63.

Cousins P. 2007. Tiny grape could do big things. *Agricultural Research* 55: 23.

Daane KM, Bell VA, Walker JTS, Botton M, Fallahzadeh M, Mani M, Miano JL, Sforza R, Walton VM, Zaviezo T. 2012. Biology and management of mealybugs in vineyards. Bostanian NJ (ed.). *Arthropod Management in Vineyards: Pests, Approaches, and Future Directions*. Springer, pp. 271-307.

Dias-Lara A, Klaassen V, Stevens K, Sudarshana MR, Rowhani A, Maree H, Chooi KM, Bluin AG, Habili N, Song Y, Aram K, Arnold K, Cooper ML, Wunderlich L, Battany MC, Bettiga LJ, Smith RJ, Bester R, Xiao H, Meng B, Preece JE, Golino D, Al Rwahnih A. 2018. Characterization of grapevine leafroll-associated virus 3 genetic variants and application towards RT-qPCR assay design. PLOS One, https://doi.org/10.1371/journal.pone.0208862.

Dunoyer P, Himber C, Ruiz-Ferrer V, Alioua A, Voinnet O. 2007. Intra- and intercellular RNA interference in *Arabidopsis thaliana* requires components of the microRNA and heterochromatic silencing pathways. *Nature Genetics* 39: 848-856.

Fuchs M, Agranoswsky AA, Bar-Joseph M, Candresse T, Dolja V, Livieratos I, Martelli GP, Maree H, Melzer MJ, Menzel W, Minafra A, Sabanadzovic S. 2017. Three new species in the family *Closteroviridae*. A taxonomic proposal submitted to the executive committee of the international committee on taxonomy of viruses. https://Talk.Ictvonline.Org/Files/Proposals/Taxonomy_Proposals_Plant1/M/Plant04/6359

Gouveia P, Dandlen S, Costa S, Marques N, Nolasco G. 2012. Identification of an RNA silencing suppressor encoded by grapevine leafroll-associated virus 3. *European Journal of Plant Pathology* 133: 237-245.

Ibrahim AB, Aragão FJ. 2015. RNAi-mediated resistance to viruses in genetically engineered plants. *Methods in Molecular Biology* 1287: 81-92.

Jing XF, White TA, Luan J, Jiao C, Fei Z, Douglas AE. 2016. Evolutionary conservation of candidate osmoregulation genes in plant phloem-sap feeding insects. *Insect Molecular Biology* 25: 251-258.

Karley AJ, Ashford DA, Minto LM, Pritchard J, Douglas AE. 2005. The significance of gut sucrase activity for osmoregulation in the pea aphid, *Acyrthosiphon pisum*. *Journal of Insect Physiology* 51: 1313-1319.

Luan JB, Ghanim M, Liu SS, Czosnek H. 2013. Silencing the ecdysone synthesis and signaling pathway genes disrupts nymphal development in the whitefly. *Insect Biochemistry and Molecular Biology* 43: 740-746.

Luo Y, Wang X, Wang X, Yu D, Chen B, Kang L. 2013. Differential responses of migratory locusts to systemic RNA interference via double-stranded RNA injection and feeding. *Insect Molecular Biology* 22: 574-583.

Luo Y, Chen Q, Luan J, Chung SH, Van Eck J, Turgeon ER, Douglas AE. 2017. Towards an understanding of the molecular basis of effective RNAi against a global insect pest, the whitefly *Bemisia tabaci*. *Insect Biochemistry and Molecular Biology* 88: 21-29.

Maree HJ, Almeida RPP, Bester R, Chooi KM, Cohen D, Dolja VV, Fuchs MF, Golino DA, Jooste AEC, Martelli GP, Naidu RA, Rowhani A, Saldarelli P, Burger JT. 2013. Grapevine leafroll virus-associated 3. *Frontiers in Microbiology* 4: 94.

Naidu RA, Rowhani A, Fuchs M, Golino DA, Martelli GP. 2014. Grapevine leafroll: A complex viral disease affecting a high-value fruit crop. *Plant Disease* 98: 1172-1185.

Naidu RA, Maree HJ, Burger JT. 2015. Grapevine leafroll disease and associated viruses: A unique pathosystem. *Annual Review of Phytopathology* 53: 613-634.

Oliver JE, Fuchs M. 2011. Tolerance and resistance to viruses and their vectors in *Vitis* sp.: A virologist's perspective of the literature. *American Journal of Enology and Viticulture* 62: 438-451.

Ricketts KD, Gomez MI, Atallah SS, Fuchs MF, Martinson T, Smith RJ, Verdegaal PS, Cooper ML, Bettiga LJ, Battany M.C. 2015. Reducing the economic impact of grapevine leafroll disease in California: Identifying optimal management practices. *American Journal of Enology and Viticulture* 66: 138-147.

Scott JG, Michel K, Bartholomay LC, Siegfried BD, Hunter WB, Smagghe G, Zhu KY, Douglas AE. 2013. Towards the elements of successful insect RNAi. *Journal of Insect Physiology* 59: 1212-1221.

Shakesby AJ, Wallace IS, Isaacs HV, Pritchard J, Roberts DM, Douglas AE. 2009. A water-specific aquaporin involved in aphid osmoregulation. *Insect Biochemistry and Molecular Biology* 39: 1-10.

Truernit E, Sauer N. 1995. The promoter of the *Arabidopsis thaliana* SUC2 sucrose-H+ symporter gene directs expression of beta-glucuronidase to the phloem: Evidence for phloem loading and unloading by SUC2. *Planta* 196: 564-570.

Tzin V, Yang X, Jing X, Zhang K, Jander G, Douglas AE. 2015. RNA interference against gut osmoregulatory genes in phloem-feeding insects. *Journal of Insect Physiology* 79: 105-112.

Wilson H, Wong JS, Thorp RW, Miles AF, Daane KM, Altieri MA. 2018. Summer flowering cover crops support wild bees in vineyards. *Journal of Economic Entomology* 47: 63-69.

Zhang C, Turgeon R. 2018. Mechanisms of phloem loading. *Current Opinion in Plant Biology* 43: 71-75.

FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board, and by the USDA National Institute of Food and Agriculture.

ACKNOWLEDGEMENTS

We are grateful to the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board for its support, to Arinder Arora and Luz Marcela Yepes for spearheading research efforts on resistance to GMB and GLRaV-3, respectively, and to Noah Clark, Stephen Hesler, Karen Wentworth, and Yeng Mei Cheung for their valuable contributions.

VIRUS-BASED DELIVERY OF INTERFERING RNAS TARGETING GRAPEVINE LEAFROLL-ASSOCIATED VIRUS(ES) AND ASSOCIATED MEALYBUGS

Project Leader: Yen-Wen Kuo | Department of Plant Pathology | University of California | Davis, CA 95616 | ywkuo@ucdavis.edu
Co-Project Leader: Bryce W. Falk | Department of Plant Pathology | University of California | Davis, CA 95616 | bwfalk@ucdavis.edu
Cooperator: Maher Al Rwahnih | Foundation Plant Services | University of California | Davis, CA 95616 | malrwahnih@ucdavis.edu
Cooperator: Kristine E. Godfrey | Contained Research Facility | University of California | Davis, CA 95616 | kegodfrey@ucdavis.edu

Reporting Period: The results reported here are from work conducted July 2019 to October 2019.

ABSTRACT

Grapevine leafroll disease is the most complex viral disease of grapevines worldwide and is economically important in all grape-growing regions. At least eleven viruses and several species of mealybugs and scale insects were reported to be associated with the disease complex. Given the lack of natural resistance in *Vitis vinifera* grapevines and challenges in developing disease resistance by conventional breeding, more control strategies are needed for this disease and its associated insect vectors. For the funded project, we aim to develop grapevine virus-based approaches for RNA interference (RNAi) targeting grapevine leafroll-associated viruses and their insect vector mealybugs. The objectives for the first phase of this project are to acquire the full-length viral genomes of the two proposed viruses, grapevine virus A (GVA) and grapevine geminivirus A (GGVA), and make them into infectious clones that will be used to deliver RNAi in grapevine plants. During the first three and a half months of this project we have successfully acquired full length viral sequences of GGVA and constructed two different clones based on the two GVAs with slightly different sequences from different California grapevine samples. The GGVA clones contain ~1.2-mer of the full-length viral genome, which are yet to be tested for infectivity. We also have acquired 95% of GVA full-length sequences of several variants in California grapevine samples. We are starting to test the 1.2-mer GGVA clones for their replication capability in grapevine plants. This project will provide new important information and contemporary strategies to incorporate into the existing management approaches for the grapevine leafroll disease and associated mealybugs.

LAYPERSON SUMMARY

For a disease such as grapevine leafroll, which is the most complex and economically important viral disease in all grape-growing regions, different control and management strategies are needed. Given the lack of natural resistance in *Vitis vinifera* grapevines and challenges in developing disease resistance by conventional breeding, novel disease control strategies could provide sufficient protection for grapevines from the devastating disease, and with even better efficacy when combined with proper management. For the funded project, we aim to utilize a virus-based RNA interference (RNAi) strategy and develop grapevine virus-based approaches to target grapevine leafroll-associated viruses and their insect vector mealybugs. We will modify two grapevine viruses to be non-pathogenic viral vectors and attempt to use them to deliver and

enhance the RNAi efficacy in grapevine rootstocks and scions. The objectives for the first phase of this project are to acquire the full-length viral genomes of the two proposed viruses, grapevine virus A (GVA) and grapevine geminivirus A (GGVA), and make them into infectious clones that will be used to deliver RNAi in grapevine plants. During the first three and a half months of this project we have successfully acquired full-length viral sequences of GGVA and constructed two different multimer (~1.2-mer) clones based on two GVAs from different California grapevine samples. Because of the nature of the virus, the multimer of the GGVA genome is needed for the clones to be infectious in plants. The ~1.2-mer clones are yet to be tested for infectivity. We also have acquired 95% of GVA full-length sequences of California grapevine samples. We are starting to test the ~1.2-mer GGVA clones for their replication capability in grapevine plants. This project will provide new important information and contemporary strategies to incorporate into the existing management approaches for grapevine leafroll disease and its associated mealybugs.

INTRODUCTION

Grapevine leafroll disease (GLD) is one of the economically important diseases of winegrape (*Vitis vinifera*) cultivars across many grapevine-growing regions (Atallah et al., 2012). After the discovery of several serologically and genetically distinct closteroviruses (family *Closteroviridae*) in grapevines, designated as grapevine leafroll-associated viruses (GLRaVs) (Karasev, 2000; Martelli et al., 2012), and their transmission by different species of mealybugs (Hemiptera: Pseudococcidae) and scale insects (Hemiptera: Coccidae), GLD was recognized as the most complex viral disease for winegrape production (Almeida et al., 2013; Naidu et al., 2014). In this funded research we apply our expertise on virology and RNA interference (RNAi) to assess new, effective approach(es) to target GLD and associated mealybugs.

Plant viruses have been used to enhance the RNAi effects targeting a variety of plant pathogens (especially viruses) and insects (Tang et al., 2010; Khan et al., 2013; Wuriyanghan and Falk, 2013; Rosa et al., 2018). Viruses can increase the expression level of the cloned sequences compared to that achieved by transgenic plant approaches. They can be engineered to yield specific interfering sequences, induce stronger RNAi effects, and sometimes give better mobility of interfering RNAs in plants. For our funded research, we aim to develop two recombinant viral vectors or plant virus replicons [grapevine geminivirus A (GGVA)-based and grapevine virus A (GVA)-based] to deliver RNAi in grapevine rootstocks and scions to target grapevine leafroll-associated virus(es) and one of the insect vectors, mealybugs.

OBJECTIVES

The objectives for the first period of this project are:

1. Obtain full-length sequences of the California isolates of GGVA and GVA sequence.
2. Construct infectious clones of GGVA and GVA.

RESULTS AND DISCUSSION

Objective 1. Obtain Full-Length Sequences of the California Isolates of GGVA and GVA Sequence

GGVA. We have obtained full length sequences of two California isolates of GGVA that were kindly provided by Foundation Plant Services (FPS) at UC Davis. One sample, with PID number 81693, cultivar Super Hamburg, was collected from the Davis Virus Collection (DVC) at the

Armstrong Plant Pathology Research Farm (DVC: B:24:8). The other sample, with PID number 81676, cultivar Longyan, was also collected from the DVC (DVC: B:24:9).

Both GGVA-76 and GGVA-93 genome clones were sequenced with Sanger sequencing. The full length of GGVA-76 sequence contains 2,904 nucleotides, while GGVA-93 contains 2,905 nucleotides. The two isolates share 98.0% identity of their nucleotide sequences with three gaps in the non-coding region between the replication associated protein (Rep) and V2 open reading frames (**Figure 1**). The identity and similarity of the amino acid sequences are shown in **Table 1**. Although both GGVA isolates share over 95% identity of the nucleotide and amino acid sequences, we will evaluate the efficiency of infectivity and the possible titer differences of both viruses. The differences may affect RNAi delivery and efficacy.

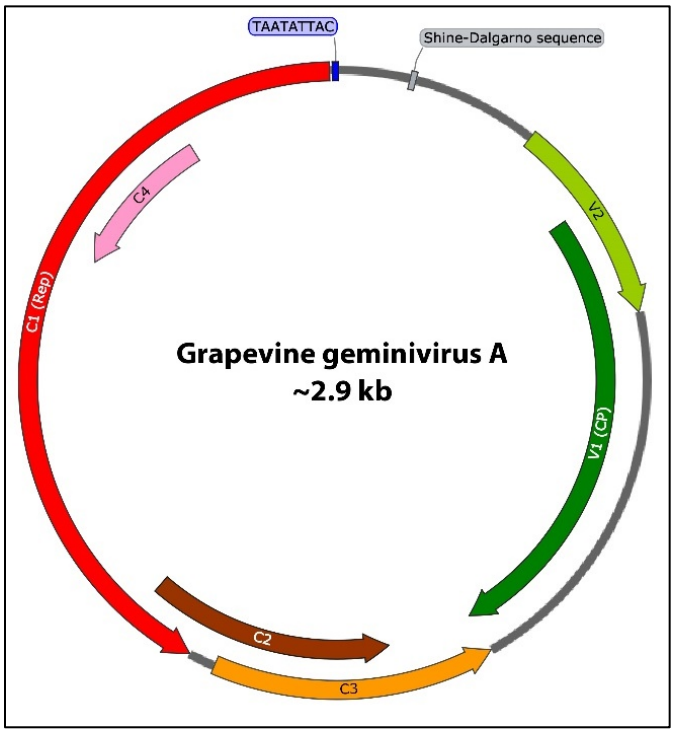


Figure 1. Genome organization of GGVA. The virus contains six open reading frames (ORFs): V1 (coat protein, CP), V2, C1 (Rep), C2, C3, and C4.

Table 1. The identity and similarity of the amino acid sequences of GGVA-76 and GGVA-93.

	V1 (CP)	V2	C1 (Rep)	C2	C3	C4
Identity	257/257 (100%)	100/104 (96.2%)	402/404 (99.5%)	137/140 (97.9%)	141/143 (98.6%)	83/86 (96.5%)
Similarity	257/257 (100%)	102/104 (98.1%)	404/404 (100.0%)	139/140 (99.3)	143/143 (100.0%)	83/86 (96.5%)

GVA. We have obtained 95% of the full-length sequence of several GVA variants from two samples that were kindly provided by FPS at UC Davis. One sample, with PID number 83846, cultivar Aledo, was collected from DVC: B:2:11. The other sample, with PID number 83847, cultivar Aledo, was collected from DVC: B:2:12.

We have cloned ~4.4 kb from the 3'-end of the viral genome of eight variant sequences and ~2.9 kb from the 5'-end of the viral genome of six variant sequences from two California isolates: 211 2R 1F-1, 211 2R 1F-3, 2113R 1F-2, 211 3R 1F-2, 211 3R 1F-3, 212 2R 2F-1, 212 2R 2F-2, 212 2R 2F-3, 211 4K-2, 211 4K-9, 211 4K-15, 212 4K-4, 212 4K-6, and 212 4K-8 (**Figure 2**). All the fragments were cloned into pCRTM-XL-2-TOPOTM vector (ThermoFisher Scientific) and sequenced by Sanger sequencing.

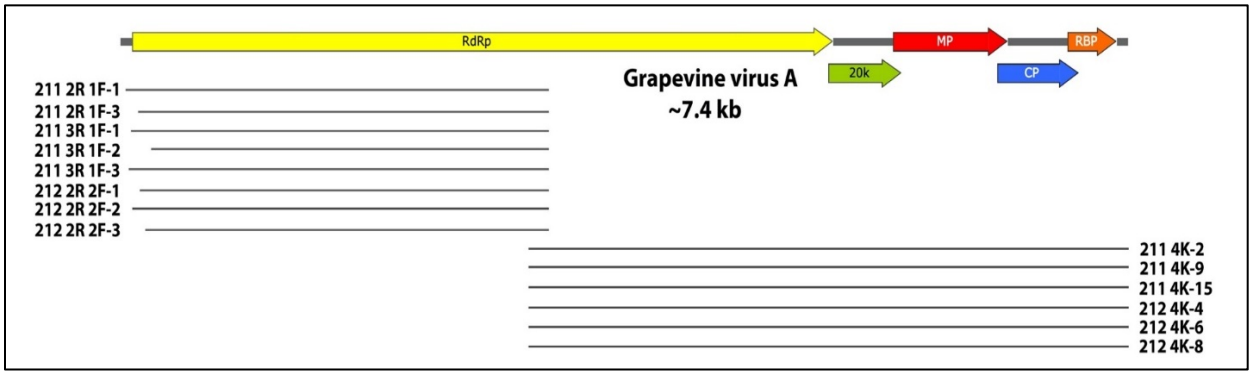


Figure 2. Genome organization of GVA and current constructs.

We are currently working on 3'- and 5'-rapid amplification of cDNA ends (RACE) to confirm the end sequences of the viral genome. We will also amplify the full genome sequences of the variants in single polymerase chain reaction from the infected grapevine samples.

Objective 2. Construct Infectious Clones of GGVA and GVA

GGVA. We have cloned two clones of GGVA from two different California isolates, GGVA-76 and GGVA-93. Both clones are constructed to have ~1.2-mer of the GGVA genome that contains double nona-nucleotide (TAATATTAC) sequences (the cleavage site for the Rep protein) to release the natural mono-full genome sequence (**Figure 3**). The ~1.2-mer clones are constructed in the pCB301 mini-binary vector and transformed into the disarmed *Agrobacterium tumefaciens* GV3101 strain. These will be agroinfiltrated into grapevine plants by a vacuum approach following the methods described by Ben-Amar et al. (Ben-Amar et al., 2013).

GVA. The variants of GVA in grapevine plants increased the amount of work to obtain the full genome sequences, therefore the progress for GVA is not as fast as the progress for GGVA. We will select two to three more different variants to construct them into infectious clones. We will start to construct GVA infectious clones after we obtain the full viral genome sequences. The viral genome sequence will be cloned into pJL89 binary vector (Lindbo, 2007).

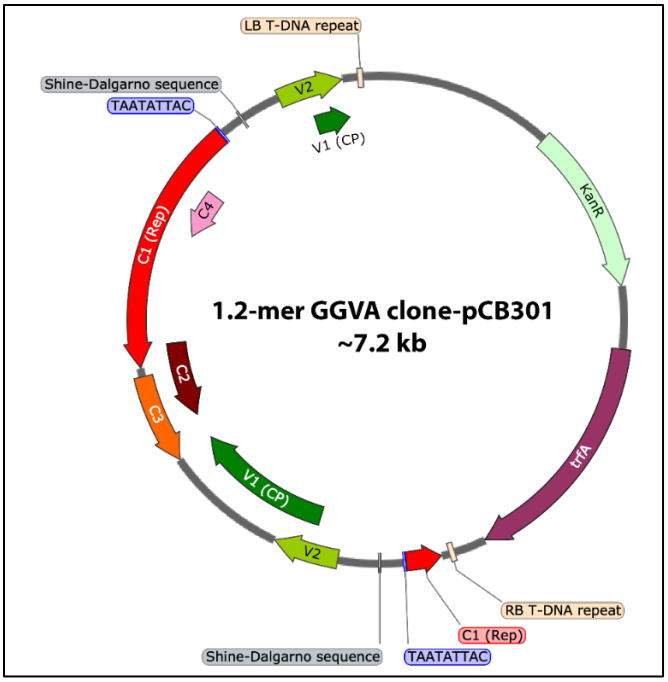


Figure 3. 1.2-mer GGVA clone structure.

CONCLUSIONS

During the first three and a half months of the project we have obtained the full-length viral sequence of GGVA and 95% genome sequence of GVA. We also have constructed two 1.2-mer GGVA clones and transformed them into *A. tumefaciens* GV3101. We are preparing and will start testing agroinfiltration for grapevine plants. We will first test the infiltration technique with a green fluorescent protein expression vector to evaluate the efficiency of the vacuum agroinfiltration approach (Ben-Amar et al., 2013) and make adequate adjustments to better fit our conditions. Meanwhile, we will also start to test positions on the viral genome in the infectious clones for inserting desired sequence(s) to have high expression levels of the inserted sequence(s). We will test different lengths of the insertion sequences and evaluate their stability and viability.

We are making good progress to achieving our goals to use viral vectors to deliver RNAi targeting grapevine leafroll-associated virus(es) and one of the insect vectors, mealybugs. This project will provide new important information and contemporary strategies to incorporate into the existing management approaches for grapevine leafroll disease and its associated mealybugs.

REFERENCES CITED

Almeida RPP, Daane KM, Bell VA, Blaisdell GK, Cooper ML, Herrbach E, Pietersen G. 2013. Ecology and management of grapevine leafroll disease. *Frontiers in Microbiology* 4.

Atallah SS, Gomez MI, Fuchs MF, Martinson TE. 2012. Economic impact of grapevine leafroll disease on *Vitis vinifera* cv. Cabernet franc in Finger Lakes vineyards of New York. *American Journal of Enology and Viticulture* 63: 73-79.

Ben-Amar A, Cobanov P, Buchholz G, Mliki A, Reustle G. 2013. In planta agro-infiltration system for transient gene expression in grapevine (*Vitis* spp.). *Acta Physiologiae Plantarum* 35: 3147-3156.

Karasev AV. 2000. Genetic diversity and evolution of Closteroviruses. *Annual Review of Phytopathology* 38: 293-324.

Khan AM, Ashfaq M, Kiss Z, Khan AA, Mansoor S, Falk BW. 2013. Use of recombinant tobacco mosaic virus to achieve RNA interference in plants against the citrus mealybug, *Planococcus citri* (Hemiptera: Pseudococcidae). *PLOS ONE* 8: e73657.

Lindbo JA. 2007. TRBO: A high-efficiency tobacco mosaic virus RNA-based overexpression vector. *Plant Physiology* 145: 1232-1240.

Martelli GP, Abou Ghanem-Sabanadzovic N, Agranovsky AA, Al Rwahnih M, Dolja VV, Dovas CI, Fuchs M, Gugerli P, Hu JS, Jelkmann W, et al. 2012. Taxonomic revision of the family Closteroviridae with special reference to the grapevine leafroll-associated members of the genus Ampelovirus and the putative species unassigned to the family. *Journal of Plant Pathology* 94: 7-19.

Naidu R, Rowhani A, Fuchs M, Golino D, Martelli GP. 2014. Grapevine leafroll: A complex viral disease affecting a high-value fruit crop. *Plant Disease* 98: 1172-1185.

Rosa C, Kuo YW, Wuriyanghan H, Falk BW. 2018. RNA interference mechanisms and applications in plant pathology. *Annual Review of Phytopathology* 56: 581-610.

Tang Y, Wang F, Zhao J, Xie K, Hong Y, Liu Y. 2010. Virus-based microRNA expression for gene functional analysis in plants. *Plant Physiology* 153: 632-641.

Wuriyanghan H, Falk BW. 2013. RNA interference towards the potato psyllid, *Bactericera cockerelli*, is induced in plants infected with recombinant tobacco mosaic virus (TMV). *PLOS ONE* 8: e66050.

FUNDING AGENCIES

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

ACKNOWLEDGEMENTS

We would like to thank Dr. Maher Al Rwahnih, Dr. Minsook Hwang, and Isela Guerra from FPS for providing the GGVA and GVA samples, and grapevine plants for this project.

TIMING OF FIELD TRANSMISSION OF GRAPEVINE RED BLOTCH VIRUS

Project Leader: Robert R. Martin | Horticultural Crops Research Unit | USDA Agricultural Research Service | Corvallis, OR 97330 | bob.martin@usda.gov
Cooperator: Michael Moore | Quail Run Vineyards | Talent, OR 97540 | michael.qrv@gmail.com
Cooperator: Daniel Sweeney | Quail Run Vineyards | Talent, OR 97540 | daniel.qrv@gmail.com

Reporting Period: The results reported here are from work conducted July 2016 to October 2019.

ABSTRACT

The goal of this project is to determine when grapevine red blotch virus (GRBV) is spreading in the vineyard. Knowing when the virus is spreading will provide important information on effective management of GRBV 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 GRBV has been spreading were used in 2016 and four vineyards were used in 2017. One vineyard was adjacent to a riparian zone, with most virus spread occurring near that edge of the vineyard nearest the riparian zone. In this case the trap plants were placed in a grassy area between the riparian zone and the vineyard. The second vineyard was adjacent to an alfalfa field, 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. This vineyard was removed after the 2016 season, and another nearby vineyard with GRBV was substituted for the 2017 field trials. The third vineyard had most spread adjacent to a recently disturbed wooded area. In each vineyard, every plant was given a unique number and the location of each plant was mapped so that where virus spread occurs in each vineyard can be determined. Fifteen plants were placed in each vineyard each month starting April 15 and going through September 15. After one month in the field the plants were returned to Corvallis, treated with a systemic insecticide, and maintained in a screenhouse. In 2017, plants were placed in four vineyards, two in southern Oregon and two in the Willamette Valley. All 300 plants from the 2016 field trials were tested for GRBV in late October 2016, November 2017, and October 2018 by polymerase chain reaction (PCR) and in September 2019 by GRBV loop mediated isothermal amplification (LAMP) assay. Given the lack of positive results in the 2016 trials in the fall testing in 2017, only 25% of the 400 plants from the 2017 field trials were tested in early November 2017. All 700 plants were tested by PCR in the fall of 2018 and with the GRBV LAMP assay in September 2019.

LAYPERSON SUMMARY

The goal of this project is to determine when grapevine red blotch virus (GRBV) is spreading in the vineyard. Knowing when the virus is spreading will provide important information on effective management of GRBV 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 GRBV has been spreading were used in 2016 and four vineyards were used in 2017. One vineyard was adjacent to a riparian zone, with most virus spread occurring near that edge of the vineyard nearest the riparian zone. In this case the trap plants were placed in a grassy area between the riparian zone and the vineyard. The second

vineyard was adjacent to an alfalfa field, 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. This vineyard was removed after the 2016 season, and another nearby vineyard with GRBV was substituted for the 2017 field trials. The third vineyard had most spread adjacent to a recently disturbed wooded area. In 2017 a fourth vineyard was added to the study, adjacent to a grassy/wooded area, where GRBV movement had been observed. In each vineyard, every plant was given a unique number and the location of each plant was mapped so that where virus spread occurs in each vineyard can be determined. Fifteen plants were placed in each vineyard each month starting April 15 and going through September 15 in 2016, and starting May 2 and continuing until September 23 in 2017. After one month in the field the plants were returned to Corvallis, treated with a systemic insecticide, and maintained in a screenhouse. All 300 plants from the 2016 trials were tested for GRBV in November 2016 and were negative for GRBV in polymerase chain reaction (PCR) testing. After overwintering, a set of 90 plants that represented trap plants for the 2016 growing season were tested by PCR in May 2017. Again, all plants were negative for GRBV. The entire set of 300 plants was tested in November 2017 and October 2018. Twenty-five percent of the plants from the 2017 trial were tested in November 2017 and were negative for GRBV. All 400 of the test plants from the 2017 field trial were tested in the fall of 2018 by PCR and in 2019 using the GRBV loop mediated isothermal amplification (LAMP) assay.

INTRODUCTION

In 2012, a new virus was identified in Cabernet Franc in New York's Finger Lakes region and also in Cabernet sauvignon plants 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 of 2012, the name grapevine red blotch-associated virus (GRBaV) was agreed upon for this new virus. After the virus was shown to cause grapevine red blotch disease in single infections, the name was changed to grapevine red blotch virus (GRBV).

The goal of this research is to determine when GRBV spread in the field. The three-cornered alfalfa hopper (*Spissistilus festinus*) has been shown to transmit GRBV, but this vector is not common in many vineyards where the virus is spreading. Also, the Virginia creeper leafhopper has been reported as a vector, but this insect had not been reported in Oregon. Movement of GRBV 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 GRBV 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 GRBV. 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 time frame 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 vineyards that are adjacent to riparian areas, this will provide clues as to where one should look to identify potential vectors.

This project was started in March 2016 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 was used for the remainder of the project. Three hundred grapevines (Merlot on 3309 rootstock) were obtained from (donated by Duarte Nursery, repotted into three-gallon pots, and held in a screenhouse until being used in the field, or held in a canyard near Corvallis isolated from any vineyards. Plants were tested for GRBV prior to use in the field experiment and all plants tested negative for GRBV in polymerase chain reaction (PCR) assays using two sets of primers. Beginning in 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 was returned to the greenhouse in Corvallis in mid-October. There were six sets of plants in each vineyard for a total of 270 trap plants, with an additional 30 plants that were not taken to a vineyard and remained in the screenhouse or canyard during the summer. In 2017 four vineyards were used in the study, two in southern Oregon and two in the Willamette Valley. Again, 15 plants per vineyard per month were placed in the vineyards, for a total of 360 plants in the field over the season, with 40 control plants maintained as in 2016. After the last set of plants was collected in 2016 all 300 plants were tested for GRBV in November 2016. A subset of the plants was tested in May 2017, and all were tested in November 2017 and again in October 2018. A subset (25%) of the trap plants from the 2017 study was tested in November 2017 and all 400 were tested in the fall of 2018 by PCR, and in September 2019 using the GRBV loop mediated isothermal amplification (LAMP) assay.

OBJECTIVES

1. Determine the timing of field transmission of grapevine red blotch virus.

RESULTS AND DISCUSSION

Three hundred plants were provided for this work in 2016 and 450 plants were provided in 2017, all donated by Duarte Nursery. All plants were tested for GRBV prior to the start of the experiment in 2016 and a subset of the plants was tested for the trial prior to potting in 2017. Plants were potted in three-gallon pots and maintained in a canyard 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 GRBV 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. In 2017, plants in this vineyard were placed in the vineyard in an area that had low incidence of grapevine red blotch disease in 2015 and very high (> 60%) incidence in 2016, suggesting rapid spread in 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 and there was a greater than 90% GRBV incidence in this vineyard. This vineyard was removed after the 2016 season, and the second vineyard used in southern Oregon in 2017 was also near Medford, Oregon, with documented spread of GRBV, with the plants placed in GRBV hot spots in the vineyard that exhibited year-over-year increase of grapevine red blotch disease symptoms. The third vineyard was in the Willamette Valley near Yamhill, Oregon. In this vineyard the spread was occurring throughout the vineyard, with high rates of spread along the east edge of the vineyard where there had 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. A fourth vineyard was added in 2017, another vineyard in the Willamette Valley, with spread of GRBV based on discussions with the grower.

Each plant was numbered (1-300 in 2016, and 1-400 in 2017) and the location of each plant and the month it was in the vineyard was recorded. Thus, if GRBV spread was observed, we knew the location of the plants in the vineyards as well as which month the plants were in the field and exposed to potential GRBV transmission.

All plants were tested for GRBV in November 2016 by PCR and all were negative for GRBV. A subset of 90 plants representing one vineyard in southern Oregon was tested in May 2017 and all were negative for GRBV. All plants from 2016 were tested in October 2017 and all were negative for GRBV. The last set of plants from the 2017 field experiments were brought back from the fields in mid-October. A subset of the 2017 plants (25% of the plants from the field) were tested the first week of November 2017, and all were negative for GRBV. In all cases, the nucleic acid extracts were tested for the amplification of a plant gene to ensure the quality of the nucleic acid was such that it did not inhibit the enzymatic reactions of the PCR testing. All samples tested positive for the plant gene. Based on work from Marc Fuchs' lab at Cornell, showing the unreliability of testing for GRBV until two years after infection, the plan was changed to keep these plants for two full years after coming back from the field. The plants from 2016 and 2017 were tested in the fall of 2018 and 2019.

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. Recent work by entomologists Frank Zalom (University of California, Davis) and Vaughn Walton (Oregon State University, Corvallis) suggests that sticky cards are not effective for monitoring membracid insects. The entomologists have been working on insect monitoring in vineyards in Oregon in 2016 and 2017. The entomologists working on membracids in Oregon (V. Walton and Rick Hilton) did catch several species of membracids in Oregon vineyards in 2016 and 2017 and the feeding damage was observed in the fields where we had our trap plants in 2017. Work on transmission by the membracid species identified from Oregon vineyards is ongoing by V. Walton's group at Oregon State University and as of meetings we had in January of 2019, they had not obtained any positive transmissions in the greenhouse using these two membracids.

We had transmission in one of the trap plants in July 2016. This plant was in vineyard #2 in southern Oregon adjacent to the alfalfa field. All other trap plants from 2016 and 2017 tested

negative for GRBV in the fall of 2018 and 2019. This extremely low level of transmission suggests that the vector is uncommon in vineyards in Oregon and/or very inefficient at transmitting GRBV. As of writing this report, there has not been any successful transmission of GRBV reported in Oregon by the entomologists working with the three membracids identified in vineyards (*Spissistilus festinus*, *Tortistilus wickhami,* and *Tortisstilus albidosparsus*). Given the rate of apparent spread observed in some vineyards in Oregon and California and the difficulty in documenting or obtaining transmission by these insects under controlled conditions, it appears that there may well be other vectors of importance in the spread of this virus in the field. The work with the Virginia creeper leafhopper has been discounted by most researchers working with GRBV since others have not been able to repeat transmissions with this vector. It may be that there are biotypes of one of these insects that are more efficient vectors of this virus than those that have been used in controlled transmission studies. Biotype specific transmission has been observed in other virus/vector interactions, most notably aphid transmissions of viruses in the *Luteoviridae* family of plant viruses.

REFERENCES CITED

Al Rwahnih M, Dave A, Anderson MM, Rowhani A, Uyemoto JK, Sudarshana MR. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology* 103: 1069-76. doi:10.1094/PHYTO-10-12-0253-R.

Bahder BW, Zalom FG, Sudarshana MR. 2016. An evaluation of the flora adjacent to wine grape vineyards for the presence of alternative host plants of grapevine red blotch-associated virus. *Plant Disease* 100: 1571-1574. http://dx.doi.org/10.1094/PDIS-02-16-0153-RE.

Bahder BW, Zalom FG, Jayanth M, Sudarshana MR. 2016. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* as a vector of grapevine red blotch-associated virus. *Phytopathology* 106: 1223-1230.

Krenz B, Thompson JR, Fuchs M, Perry KL. 2012. Complete genome sequence of a new circular DNA virus from grapevine. *Journal of Virology* 86: 7715. doi:10.1128/JVI.00943-12.

Krenz B, Thompson JR, McLane HL, Fuchs M, Perry KL. 2014. Grapevine red blotch-associated virus is widespread in the United States. *Phytopathology* 104: 1232-40. doi: 10.1094/PHYTO-02-14-0053-R. PubMed PMID: 24805072.

Perry KL, McLane H, Hyder MZ, Dangl GS, Thompson JR, Fuchs MF. 2016. Grapevine red blotch-associated virus is present in free-living *Vitis* spp. proximal to cultivated grapevines. *Phytopathology* 106: 663-70. doi:10.1094/PHYTO-01-16-0035-R.

Poojari S, Alabi OJ, Fofanov VY, Naidu RA. 2013. Evolutionary lineage in the Family *Geminiviridae* implicated in the grapevine redleaf disease by next-generation sequencing. *PLOS One* 8: 364194.

Romero JLR, Carver GD, Johnson PA, Perry KL, Thompson JR. 2019. A rapid, sensitive and inexpensive method for detection of grapevine red blotch virus without tissue extraction using loop-mediated isothermal amplification. *Archives of Virology* 164: 1453-1457.

Sudarshana MR, Perry KL, Fuchs MF. 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Phytopathology* 105: 1026-32. doi: 10.1094/PHYTO-12-14-0369-FI.

FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-Winged Sharpshooter Board, the Erath Family Foundation, and the USDA Agricultural Research Service.

ACKNOWLEDGEMENTS
We would like to acknowledge Quail Run Vineyards, RoxyAnn Vineyards, Marsh Vineyards, and Shea Vineyards for allowing us to work in their vineyards; Duarte Nursery for donating plants for the project; staff in the Martin Lab for their work in getting the plants potted and ready before we had funding in place, and also for plant maintenance and virus testing; Daniel Sweeney for watering the plants in the vineyards in southern Oregon; and Karl Mohr for watering the plants at Marsh vineyard.

IDENTIFICATION OF GRAPE CULTIVARS AND ROOTSTOCKS WITH RESISTANCE TO VINE MEALYBUG

Project Leader: Rachel Naegele | San Joaquin Valley Agricultural Sciences Center | USDA Agricultural Research Service | Parlier, CA 93648 | rachel.naegele@usda.gov
Cooperator: Kent Daane | Department of Environmental Science, Policy, and Management | University of California | Berkeley, CA 94720 | kdaane@ucanr.edu

Reporting Period: The results reported here are from work conducted April 2019 to October 2019.

ABSTRACT
Mealybugs cause economic loss to vineyards through physical damage, fouling fruit and leaves with honeydew, and the transmission of viruses. Vine mealybug (*Planococcus ficus*) is one of several mealybug species in vineyards, and one that causes economic damage over a relatively large global range. To develop novel management tools, host resistance to vine mealybug, which has not previously been identified for any grape cultivars, was studied. Previously, ten grape lines (species, cultivars, and rootstocks) were evaluated for vine mealybug resistance across two separate potted plant assays. Significant differences were detected among cultivars and rootstocks in the recorded number of vine mealybug juveniles, adults, and egg sacs. Cabernet Sauvignon and Chardonnay were two of the most favorable grape cultivars for mealybug population growth, whereas rootstocks IAC 572, 10-17A, and RS-3 all demonstrated some level of resistance. Southern fire ant (*Solenopsis xyloni*) was positively associated with mealybug populations but did not have a negative effect on the observed presence of other arthropod species, including potential predators. This work is being repeated in the greenhouse, without the pressure of ants, to confirm host resistance and identify new sources.

LAYPERSON SUMMARY
Vine mealybug (*Planococcus ficus*) is a major pest to the California grape industry. Growers spend an estimated $123 to $500/acre annually to manage mealybugs. Insecticide sprays provide inconsistent control due to problems associated with timing and poor contact with the insect. As concerns about the development of insecticide resistance increase, alternate systems for controlling mealybugs are essential. Resistant grape cultivars are not currently available and could take more than a decade to breed. In the interim, resistant rootstocks could provide sufficient control either alone or in combination with insecticides. Two potential sources of resistance to at least one species of mealybugs have been identified in lab and outdoor pot tests. This project evaluates previously identified and new materials in a greenhouse to remove outside pressures of ants and beneficials to confirm host resistance.

INTRODUCTION
Mealybugs are soft-bodied, sap-sucking insect pests of grapevines and other plants. Besides the direct losses attributed to damaged leaves and fruit in grapes, mealybugs can transmit the economically important grapevine leafroll-associated virus. It is estimated that grapevine leafroll disease control costs growers $12,106 to $91,623 per acre annually in California (Ricketts et al., 2015). Of that expenditure, mealybug control costs are estimated at $50 per acre in vineyards with small mealybug populations and many natural predators, to $500 per acre for vineyards

with moderate populations and few parasitoids (Ricketts et al., 2015). Vine mealybug (*Planococcus ficus*) is one of six mealybug species that threaten the California grape industry. This introduced (ca. 1994) pest can rapidly reproduce and spread, outcompeting other mealybug species and making it the most important mealybug pest of grape in California (Daane et al., 2012).

Insecticides are the main form of mealybug control. Mating disruption and parasitoids have been implemented with success in vineyards, however, these forms of control are more expensive or can be impeded by Argentine ant populations which "tend" the mealybugs (Daane et al., 2007; Mansour et al., 2011; Varela et al., 2019). An effective complement to insecticides is the use of resistant grapes. Resistant grapes, and specifically resistant rootstocks, could directly reduce mealybug populations developing or overwintering under the bark and on roots in the vineyard.

Few sources of natural resistance to mealybug have been identified in grape. In Brazil, one study identified a single rootstock with lab-based resistance to mealybug (Filho et al., 2008). This resistance was described as a reduction in the number of viable offspring produced per female compared to susceptible cultivars, Cabernet Sauvignon and Isabel (Filho et al., 2008). This was later confirmed in a similar lab experiment performed by a different lab group (Bertin et al., 2013). These results, while promising, are based on mealybug species [pineapple mealybug (*Dysmicoccus brevipes*) and citrus mealybug (*Planococcus citri*)] of minor importance to California. The only other report of mealybug resistance in grape comes from observations by Michael McKenry and David Ramming (unpublished), suggesting that rootstock RS-3 has resistance to an unknown species of mealybug in addition to nematode resistance. Work on our previously funded CDFA project has shown that IAC 572 and RS-3 appear to be resistant to vine mealybugs and differ in their level of resistance. Other species with potential resistance based on our previous research include rootstock 10-17A, which needs to be confirmed.

Our proposed work will continue to identify sources of resistance to vine mealybug and evaluate these materials for their effect on mealybug overwintering and survival. This project will have long-term impact by facilitating the breeding of mealybug resistance into commercial wine, table, and raisin grape cultivars. In the short term, these materials may be used as rootstocks to reduce mealybug populations on susceptible commercial cultivar scions. The information from these studies will be distributed to growers, nurseries, breeders, pest control advisors, and extension personnel to accelerate the implementation of these materials into breeding programs, rootstock evaluations, and nurseries.

OBJECTIVES

This proposal seeks to develop novel control strategies for vine mealybug using host resistance as part of an integrated management program. This will be accomplished by identifying grape material with resistance to vine mealybug that can be used for rootstocks and traditional breeding.

1. Evaluate grape materials with identified resistance to vine mealybug.

RESULTS AND DISCUSSION

Objective 1. Evaluate Grape Materials with Identified Resistance to Vine Mealybug

Twenty replicate plants for each of the eight cultivars were evaluated for resistance to vine mealybug in a greenhouse at the Kearney Agricultural Research and Extension Center (**Table 1**). Each vine was inoculated with 200 first or second stage mealybug juveniles and evaluated bi-weekly for eight weeks for the number of visible third stage juveniles/adult females and ovisacs. Plant health was evaluated at the end of the study based on a 0 to 5 scale, with 0 representing a dead plant and 5 representing a completely healthy plant. Area under the insect growth curve (AIGC) was calculated modified from the Area Under the Disease Progress Curve (AUDPC) described by Shaner and Finney (1977), and the average AIGC was calculated per line using SAS statistical analysis software. Data was normalized using a log transformation prior to analysis of variance (ANOVA) and statistical differences were determined based on Tukey's honestly significant difference test.

Table 1. Grape material tested for resistance.

Cultivar	Species	Type	Resistance
Cabernet Sauvignon	*V. vinifera*	wine grape	none
Chardonnay	*V. vinifera*	wine grape	none
Autumn King	*V. vinifera*	table grape	none
Thompson Seedless	*V. vinifera*	table grape	none
Flame Seedless	*V. vinifera*	table grape	none
Freedom	interspecific hybrid	rootstock	nematode
IAC 572	interspecific hybrid	rootstock	citrus mealybug

The experiment is currently being repeated with the same genotypes. Statistical analyses are ongoing for round 1 of the experiment. In brief, mealybugs were observed on each genotype, however, the number of mealybugs was reduced on rootstocks 10-17A, Freedom, and IAC 572 compared to the cultivated varieties Autumn King, Cabernet Sauvignon, Chardonnay, Flame Seedless, and Thompson Seedless. Plant health was low for highly susceptible cultivars at the end of the experiment. 10-17A and Freedom had the highest plant health scores at the end of the first round of the experiment.

Table 2. Round 1 results for mealybug resistance testing in greenhouse.

Cultivar	AIGC Adults	AIGC Ovisac	Plant Health
10-17a	465.15	246.925	4.4
Autumn King	475.475	205.975	1.5
Cabernet	636.125	309.4	2.05
Chardonnay	641.025	385.35	3.05
Flame Seedless	623.525	316.925	3.05
Freedom	256.55	105.875	3.9
IAC 572	481.95	180.25	3
Thompson Seedless	575.4	279.475	2.75

CONCLUSIONS

Vine mealybug is one of several mealybug species found in grape vineyards globally. Resistant grape cultivars, which are an important component of integrated pest management, are not available to manage this insect pests. Previous work funded by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board identified differences in mealybug population growth among cultivars and rootstocks. Both juvenile and adult female mealybugs and Southern fire ant populations were lower on rootstocks than on cultivated varieties. Because of the variability in mealybug growth even on the rootstocks, it is likely that there are cultivar-specific mechanisms contributing to mealybug resistance. In this current project we evaluated one additional rootstock (Freedom), and confirmed the susceptibility/resistance of select rootstocks and cultivars in a greenhouse setting. However, the experiment will need to be repeated an additional time.

REFERENCES CITED

Berning D, Dawson G, Foggia M. 2014. *Planococcus ficus* (On-line), *Animal Diversity Web*. Accessed December 28, 2016 at http://animaldiversity.org/accounts/Planococcus_ficus/.

Bertin A, Bortoli LC, Botton M, Parra JRP. 2013. Host plant effects on the development, survival, and reproduction of *Dysmicoccus brevipes* (Hemiptera: Pseudococcidae) on grapevines. *Annals Entomol. Soc. Amer*. 106: 604-609.

Daane KM, Almeida RPP, Bell VA, Walker JTS, Botton M, Fallahzadeh M, Mani M. 2012. Chapter 12: Biology and Management of Mealybugs in Vineyards in Arthropod Management. Pages 271-307 in: Bostanian NJ, Vincent C, Isaacs R. (*Eds*) *Vineyards: Pests, Approaches, and Future Directions*. Springer, Dordrecht.

Daane KM, Sime KR, Fallon J, Cooper ML. 2007. Impacts of Argentine ants on mealybugs and their natural enemies in California's coastal vineyards. *Ecol. Entomol*. 32: 583-596.

Filho M, Grutzmacher AD, Botton M, Bertin A. 2008. Biology and fertility life table of *Plannococcus citri* in different vegetative structures of grape cultivars. *Pesq. Agropec. Bras*. 43: 941-947.

Geiger CA, Daane KM. 2001. Seasonal movement and distribution of the grape mealybug (*Homoptera: Pseudococcidae*): Developing a sampling program for San Joaquin Valley vineyards. *J. Econ. Entomol*. 94: 291-301.

Gutierrez AP, Daane KM, Ponti L, Walton VM, Ellis CK. 2008. Prospective evaluation of the niological control of vine mealybug: Refuge effects and climate. *J. Appl. Ecol*. 45: 524-536.

Johnson-Cicalese J, Baxendale F, Riordan T, Heng-Moss T. 1998. Identification of mealybug (Homoptera: Pseudococcidae) resistant turf-type buffalo grass germplasm. *J. Econ. Entomol.* 91: 340-346.

Mansour R, Suma P, Mazzeo G, Lebedi KG, Russo A. 2011. Evaluating side effects of newer insecticides on the vine mealybug parasitoid *Anagyrus* sp. near *Pseudococci*, with implications for integrated pest management in vineyards. *Phytoparasitica* 39: 369 doi:10.1007/s12600-011-0170-8.

Ricketts KD, Gomez MI, Atallah SS, Fuchs MF, Martinson TE, Battany MC, Bettiga LJ, Cooper ML, Verdegaal PS, Smith RJ. 2015. Reducing the economic impact of grapevine leafroll disease in California: Identifying optimal disease management strategies. *Amer. J. Enol. Vitic*. 66: 138-147.

Santa-Cecilia LVC, Prado E, Borges CM, Correa LRB, Souza B. 2008. Methodology for biological studies of mealybugs (Hemiptera: Pseudococcidae). *Coffee Science* 3: 152-155.

Shaner G, Finney RE. 1977. The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. *Phytopathology* 67: 1051-1056.

Varela LG, Haviland DR, Bentley WJ, Zalom FG, Bettiga LJ, Smith RJ, Daane KM. 2019. Insects and mites. *UC IPM Pest Management Guideline: Grape*. *UC ANR Publication 3448*. http://ipm.ucanr.edu/PMG/r302302111.html.

Waterworth RA, Wright IM, Millar JG. 2011. Reproductive biology of three cosmopolitan mealybug (Hemiptera: Pseudococcidae) species, *Pseudococcus longispinus*, *Pseudococcus viburni*, and *Planococcus ficus*. *Annals Ent. Soc. Amer*. 104: 249-260.

FUNDING AGENCIES

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

ACKNOWLEDGEMENTS

The work described in this report was performed by Alanna Burhans, Noor Abdelsamad, Abelardo Rodriguez, Evelin Munoz, Molly Irwin, and Nick Banda.

INVESTIGATION OF THE IMPACT OF GRAPEVINE RED BLOTCH VIRUS ON GRAPE RIPENING AND METABOLISM

Project Leader: Anita Oberholster | Department of Viticulture and Enology | University of California | Davis, CA 95616 | aoberholster@ucdavis.edu
Cooperator: Mysore Sudarshana | USDA-ARS and Department of Plant Pathology | University of California | Davis, CA 95616 | mrsudarshana@ucdavis.edu
Cooperator: Larry Lerno | Department of Viticulture and Enology | University of California | Davis, CA 95616 | lalerno@ucdavis.edu

Reporting Period: The results reported here are from work conducted July 1, 2018 to October 23, 2019.

ABSTRACT

Grapevine red blotch disease (GRBD) is a recently identified disease caused by grapevine red blotch virus (GRBV). Since its discovery in 2011, its widespread presence has been confirmed in 14 states in the United States as well as in Canada, and it has been found in white and red winegrape varieties, table and raisin grapes, interspecific hybrids, and rootstocks. Prior to our research little was known about the impact of GRBD on grape and wine composition. After four years of study across multiple varieties and sites we have good baseline data about the range of impacts. Results indicate mostly a substantial impact on berry ripening in all varieties studied, along with variable impacts on primary and secondary metabolites depending on site and season, which had a larger impact than variety. However, the impact of GRBD on metabolic pathways remains to be explored in depth. Limited previous research indicated transcriptional suppression of primary and secondary metabolic pathways by GRBV when studied in Zinfandel for one season. The current project aims to expand this research to other varieties and sites over multiple seasons to determine any potential varietal, as well as environmental, impact on GRBD expression. Only after viral impact is well understood can tools be developed to mitigate the impact thereof. Currently, the differential expression of genes between healthy and diseased grapes has been determined. Gene expression data was overlaid with different Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways to understand the impact of GRBV on gene regulation and thus grape metabolism. These results highlighted the impact of GRBV on the phenylpropanoid pathway, which is responsible for flavonoid synthesis. These compounds are crucial to the color, flavor, and mouthfeel of a final wine. It was also observed that the impact of GRBV is dependent on variety, rootstock, season, and ripening stage.

LAYPERSON SUMMARY

Prior to our research over the past four years, little was known about the impacts of grapevine red blotch virus (GRBV) on grape composition and the resulting wine quality. Through our research, it was found that there are variable impacts on levels of primary and secondary metabolites, depending on the variety, season, and rootstock. In addition, in research performed by Blanco-Ulate et al. in 2017, there were observed changes in transcriptional factors and regulatory networks relating to an inhibition of berry ripening in infected fruit. The current project aims to further this research across varieties, seasons, sites, and rootstocks to understand the potential variable impacts the disease has on berry ripening. By doing so, a deeper

knowledge of the viral impact will be gained, and possible mitigation strategies can be suggested.

The first portion of this project was method validation. We have finalized and implemented the methodology needed to obtain pure total RNA extracts of high concentrations to enable successful analysis by the Expression Analysis Core at UC Davis. The isolated RNA samples have been analyzed by the Expression Analysis Core for RNA sequencing and the data processed by the Bioinformatics Core. The differential expression of genes between healthy and diseased grapes have been determined. In addition, gene expression data was overlaid with different Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways to understand the impact of GRBV on gene regulation and thus grape metabolism. These results highlighted the impact of GRBV on the phenylpropanoid pathway, which is responsible for flavonoid synthesis. These compounds are crucial to the color, flavor, and mouthfeel of a final wine. It was observed that the impact of GRBV depended on variety, rootstock, season, and ripening stage.

The data collection of volatile compounds for all samples is completed, and data analysis is underway. Non-volatile data collection will begin in the end of October and will be completed by the end of 2019. Quantifying volatile and non-volatile grape metabolites will aid our understanding in how gene regulation impacts biosynthesis. Finally, the methodology for phytohormones will undergo validation in January, and data collection will follow shortly after.

INTRODUCTION

Grapevine red blotch virus (GRBV), a causative agent for grapevine red blotch disease (GRBD), is a recently discovered virus that has been identified in vineyards in 14 states across the United States as well as in Canada. Symptoms of GRBV include red blotches on leaves as well as reddening of primary and secondary veins for red varieties and chlorotic regions within leaf blades and marginal burning similar to potassium deficiency on white varieties (Sudarshana, Perry, et al., 2015). Over the past four years the Oberholster group has researched the impacts of GRBV on grape development and composition and the resulting impact on wine quality across varieties, sites, seasons, and rootstocks. Results indicate mostly a substantial impact on berry ripening in all varieties studied (Oberholster, 2015; Oberholster, 2016), along with variable impacts on primary and secondary metabolites, depending on site and season (Oberholster, 2015; Eridon, 2016; Oberholster, 2016). Through transcriptomics and metabolomics the present study aims to investigate the impact the virus has on transcriptional factors and regulatory networks. Previous research investigated the impact of GRBV on Zinfandel infected fruit and found that there was an inhibition of the phenylpropanoid metabolic pathway, along with other regulatory networks responsible for berry ripening (Blanco-Ulate, Hopfer, et al., 2017). This research needs to be expanded across varieties, sites, seasons, and rootstocks to determine any potential varietal, as well as environmental, impact on GRBV and GRBD expression. Only once virus functioning is understood can tools be developed to mitigate the impact of GRBV other than the removal of infected vines.

OBJECTIVES

The main objectives of this project are the following:

1. To determine the impact of GRBV on grape metabolism during ripening.
2. To determine the potential impact of variety, rootstock, and season on GRBV functioning.

RESULTS AND DISCUSSION

The first step is to understand GRBV and grapevine interaction. How does GRBV infection influence grape metabolism and thus ripening? What potential synergy exists between environmental stresses and GRBD expression? Answers to these questions are the first step in developing a GRBD management strategy. Outcomes from this study will add much needed information for understanding the influence of GRBV on grape metabolism and development. This can be used to develop a measurement tool to determine disease impact as well as vineyard management recommendations to mitigate potential impacts on grape quality and guide judicious removal of grapevines.

To answer these questions, grape berries sampled during ripening from two different sites (*Vitis vinifera* cultivars Cabernet Sauvignon and Merlot) over two seasons will be analyzed as described in Objective 1. Grapes collected from the 2015-2016 and 2016-2017 seasons as part of a previous proposal funded by the American Vineyard Foundation (2016-1953 and 2017-1675) were stored at -80°C. Studies have shown that tissue samples stored at -80°C are stable for several years (Andreasson, Kiss, et al., 2013). The Cabernet Sauvignon grapevines are grafted onto two different rootstocks, 420A and 110R, allowing us to investigate the potential impact of rootstock selection.

Objective 1. To Determine the Impact of GRBV on Grape Metabolism During Ripening

Grapes sampled from two vineyards planted with Cabernet Sauvignon (Oakville Experimental Station, Napa County) and Merlot (Paso Robles) were utilized for this investigation. Treatment vines designated red blotch positive (RB+) and red blotch negative (RB-) were identified and marked according to detailed visual mapping of the last few years, and confirmed with GRBV and leafroll virus (grapevine leafroll-associated virus types 1 to 4, and strains of 4) quantitative polymerase chain reaction (qPCR) testing of a subset of vines until 20 data vines for each treatment have been identified. These sites have been utilized previously for red blotch investigations and there is a consistent association with the virus in symptomatic vines in red varieties (Sudarshana, Perry, et al., 2015). Only healthy vines (i.e., vines that tested negative for viruses and did not show symptoms of viral disease, RB-) and vines which only tested positive for GRBV and which are symptomatic (RB+) were used as data vines. Data vines were randomly subdivided into five biological replicates of four vines each using a random sequence generator (http://www.random.org.sequences). Five berries were collected from each data vine randomly (top, middle, and bottom of grape bunches on the outer and inner side of the canopy) for a total of 20 berries per biological replicate. Grapes were sampled three times during ripening at pre-veraison, 50% veraison (berry softening and color change), and harvest for 2017, and four times during ripening for 2016 at all the previous points with the addition of post-veraison. The post-veraison sampling was missed in 2017 due to a heat spike and unexpected fast increases in sugar content. Previous research utilizing untargeted metabolomics found indications that both primary (organic acids, amino acids, sugars) and secondary (volatile aroma compounds and phenolics) metabolites are affected by GRBD (Oberholster, 2015; Blanco-Ulate, Hopfer, et al., 2017). Thus, sampled grape berries will be analyzed by targeted metabolomic analysis focusing on the primary and secondary metabolites (organic acids, amino acids, sugars, varietal aroma compounds, and phenolics). Solid phase micro-extraction gas chromatography mass spectrometry (SPME-GC-MS, Agilent Technologies) will be used for the analysis of volatile aroma compounds (Hjemeland, King, et al., 2013; Hendrickson, Lerno, et al., 2016). Whereas,

ultra-high-performance liquid chromatography high resolution time-of-flight mass spectrometry (UHPLC/TOF/MS, Agilent Technologies) analysis will be utilized for non-volatile metabolites (Toffali, Zamboni, et al., 2011; Theodoridis, Gika, et al., 2012; Blanco-Ulate, Hopfer, et al., 2017). Finally, proton nuclear magnetic resonance (1H NMR) will be used for analysis of primary metabolites (sugars, amino acids, and organic acids) (Fortes et al., 2011). Metabolic profiling data will be combined with transcriptomic approaches using next generation RNA sequencing (RNA-seq) which will allow changes in gene expression to be monitored and the impact of GRBV on the metabolic pathways during ripening to be elucidated.

Grapes sampled at pre-veraison, veraison, post-veraison, and harvest were immediately processed upon arrival at the laboratory and berries were deseeded, frozen in liquid nitrogen, and stored at -80°C until further analysis. Data processing and normalization will be performed using Agilent's MassHunter Qualitative Analysis software with Molecular Feature Extraction. Compounds will be identified by authentic standards and/or cross-referenced with metabolite databases (e.g., METLIN Metabolite Database, Tandem Mass Spectrum Database, Human Metabolite Database). Transcriptomic approaches using next generation 3'-Tag RNA-seq will allow the monitoring of gene expression changes.

The proposed transcriptomics experiments will require the isolation of total RNA from all GRBV-infected and control samples (five biological replicates for each treatment and site) using the Qiagen RNeasy Plant MiniKit. The purity of the extracted and purified RNA will be analyzed by measuring the absorbance at 260 and 280 with a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific) and integrity 2100 Bioanalyzer (Agilent Technologies). Sequencing will be performed at the Expression Analysis Core Facility (UC Davis) using the Illumina HiSeq 3000 platform. These analyses will provide a potential list of genes that have been down or up-regulated and alterations that took place in molecular pathways as a result of GRBD. Blanco-Ulate et al. (Blanco-Ulate, Hopfer, et al., 2017) found that GRBV infection restricted the biosynthesis and accumulation of phenylpropanoids and derivatives in Zinfandel grape berries. Therefore, further information should be gained to understand the impacts on other varieties. Integration of the transcriptomics and metabolomics data will be carried out using multivariate analyses.

In order to fully understand the impacts of GRBV on grape ripening, investigating the effects of the virus on hormone abundances and enzymatic activity is crucial. Similar approaches to those detailed in Blanco-Ulate et al. (2017) will be followed for both enzymatic activity and phytohormone analyses to verify the impacts found on the phenylpropanoid metabolism. Lower abundances of the hormone abscisic acid, known to be linked to anthocyanin biosynthesis, and increased levels of auxin, known to suppress berry ripening (Blanco-Ulate, Hopfer, et al., 2017) have previously been documented. Further investigation into the interactions of transcriptional regulators and hormone networks needs to be performed. Confirming hormonal response to red blotch infection can lead to the development of hormone treatments that could potentially decrease the negative impacts of the disease. More information regarding grape responses to GRBV infection in other grape varieties and environmental conditions is necessary to ascertain how both grapevine genotype and environment may impact disease outcomes, especially as previous research by the Oberholster group found clear influences of variety, site, and season on GRBV expression.

After several months of researching and testing methods to determine the most reliable method with the highest throughput, a protocol for RNA extraction and quality assurance was established. The RNA from each sample was isolated using a guanidine thiocyanate lysate buffer made in-house and the Qiagen RNeasy Plant Mini Kit in conjunction with the Qiagen PowerClean Pro Cleanup kit. DNA was removed prior to the library preparation using the DNaseI RNase free kit from New England Biolabs. RNA integrity and purity were analyzed using a 2100 Bioanalyzer and NanoDrop 2000c spectrophotometer, respectively. Afterwards, the Expression Analysis Core at the Genomic Center at UC Davis prepared libraries for and sequenced each sample using 3' Tag RNA-seq method. In December 2018 a subset of samples was sent to be sequenced by the Expression Analysis Core. Two samples from each rootstock (1103P, 110R, and 420A) and from each year were analyzed for a total of 12 samples. Overall, 93% of the reads aligned to the grape genome and 65% of the reads aligned uniquely to the genes, indicating that the methodology was successful and could be applied to the complete sample set.

In April 2019 the RNA extracts from the complete set of 210 samples were submitted to the Expression Analysis Core and sequencing began June 25, 2019. Once the samples were sequenced the differential expression data was analyzed by the Bioinformatics Core at UC Davis. We have been able to analyze the impact of ripening stage, site, season, variety, and rootstock on disease expression. To ensure there was no batch effect (variance caused by day-to-day sample preparation) samples were randomized across all variables in this study. Through multi-dimensional scaling (MDS) it was also observed that the gene variance between the samples was mainly derived from ripening stage (**Figure 1)**. This data agrees with observations found in the work by Blanco-Ulate et al. (2017). This indicates that the differences in gene expression are primarily driven by ripening stage, irrespective of variety, season, rootstock, and disease status. However, evaluating disease expression through ripening remains crucial for our understanding of disease functioning.

Differential expression (DE) data was collected by taking the log fold change between healthy and infected fruit for a single variety/rootstock at one time point (i.e., the healthy and diseased grapes of Cabernet Sauvignon 110R at veraison in 2016 were compared). This was done for each genotype for each ripening stage for both years. Data was filtered to determine the genes that were significantly differentially expressed for each combination of variables. The DE data was analyzed by overlaying the results with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. We aimed to look first at pathways known to be impacted by the virus, such as the phenylpropanoid pathway. First, the two varieties were compared at each time point for both years to understand not only the difference in disease expression between genotypes, but also through ripening and across seasons. It should be noted that disease expression was either low or not present in pre-veraison samples, and therefore is not shown in the following figures. **Figures 2** and **3** depict veraison and harvest in 2016. Each box indicates a gene, and the color indicates level of expression. As the legend in the top right indicates, purple indicates that gene is downregulated, and yellow indicates that gene is upregulated, due to the presence of the virus. The intensity of the color indicates the respective level of up or down regulation. Each box is separated into three parts by rootstock/variety. From left to right, results for 420A/Cabernet Sauvignon, 110R/Cabernet Sauvignon, and 1003P/Merlot are shown. **Figures 2** and **3** indicate that several genes in the phenylpropanoid pathway were affected by GRBV, including several

genes that are responsible for the biosynthesis of hydroxycinnamic acids. The effect on these genes differed depending on genotype. In the cases where these genes were downregulated, downregulation increased with ripening as the season progressed.

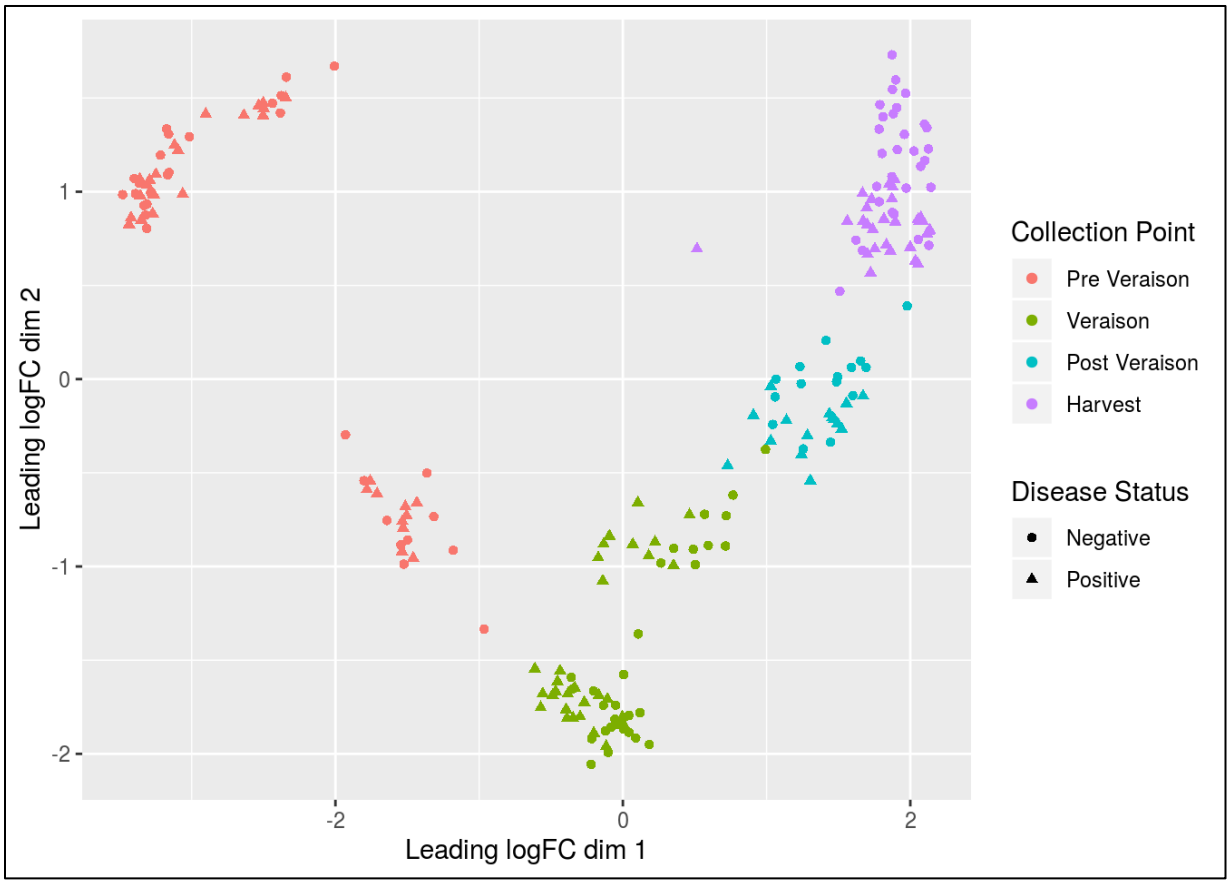


Figure 1. MDS plot of gene counts based on ripening stage across all sites for two seasons.

Similar observations were made in 2017. **Figure 4** shows the DE of the phenylpropanoid pathway at veraison in 2017, whereas **Figure 5** is at harvest in 2017. Again, downregulation of genes in this pathway increases as the season progresses, indicating that between veraison and harvest, the effects of the virus increase. This agrees with findings in work done by Blanco-Ulate et al. (2017).

In addition, there are several genes that are upregulated through ripening. Some of these genes are responsible for the synthesis of compounds such as eugenol, which potentially could be due to a stress response. However, to better understand the role of these genes in grape physiology, levels of metabolites should be compared with these KEGG pathways. Therefore, the completion of the metabolomic work will aid in our understanding of GRBV impacts on gene regulation.

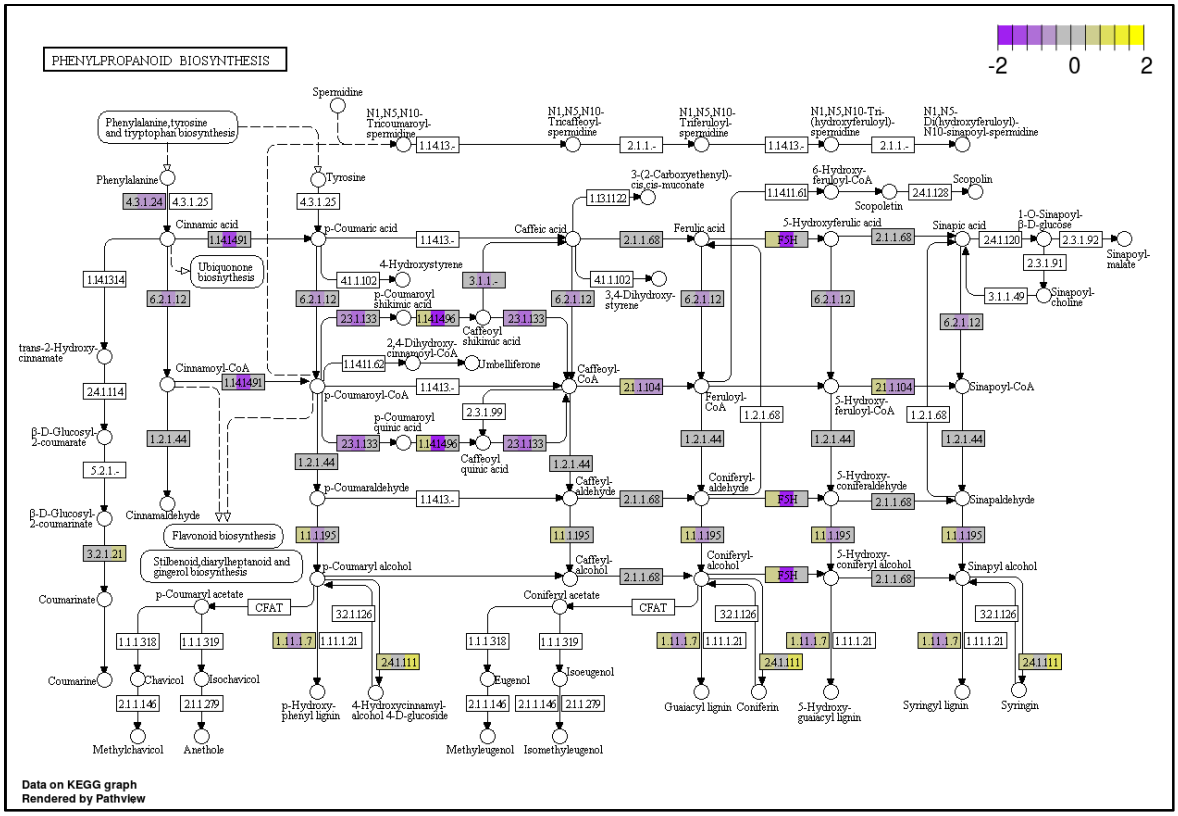


Figure 2. GRBV impact on the phenylpropanoid pathway at veraison in 2016 using DE and KEGG pathways. Boxes indicate genes, circles indicate metabolites, yellow indicates upregulated, and purple indicates downregulated. The intensity of the color is indicative of the extent of gene up or down regulation. The boxes are separated into three parts indicating, from left to right, 420A/Cabernet Sauvignon, 110R/Cabernet Sauvignon, and 1003P/Merlot.

Previous research (Girardello et al., 2019; Martínez-Lüscher et al., 2019; Blanco-Ulate et al., 2017) has indicated that GRBV can severely impact the synthesis of anthocyanins (color pigments). These compounds are synthesized through the phenylpropanoid pathway. However, **Figures 2** to **5** do not show the genes involved in flavonoid biosynthesis, such as anthocyanins. **Figures 6a** and **6b** show the DE results overlaid with anthocyanin biosynthesis for grapes at veraison and harvest in 2016, respectively. **Figures 7a** and **7b** show the DE results overlaid with anthocyanin biosynthesis for grapes at veraison and harvest in 2017, respectively. In 2016, similar results were observed as seen in **Figures 2** and **3**, where the extent of downregulation increased as the season progressed from veraison to harvest. The opposite was observed for 2017, indicating the impact of season. Previous work on geminiviruses like GRBV have indicated that temperature can impact the accumulation of viral DNA in a plant due to plant responses to the pathogen (Chellappan et al., 2015). Therefore, it is possible that average temperatures of seasons can impact the levels of disease expression observed.

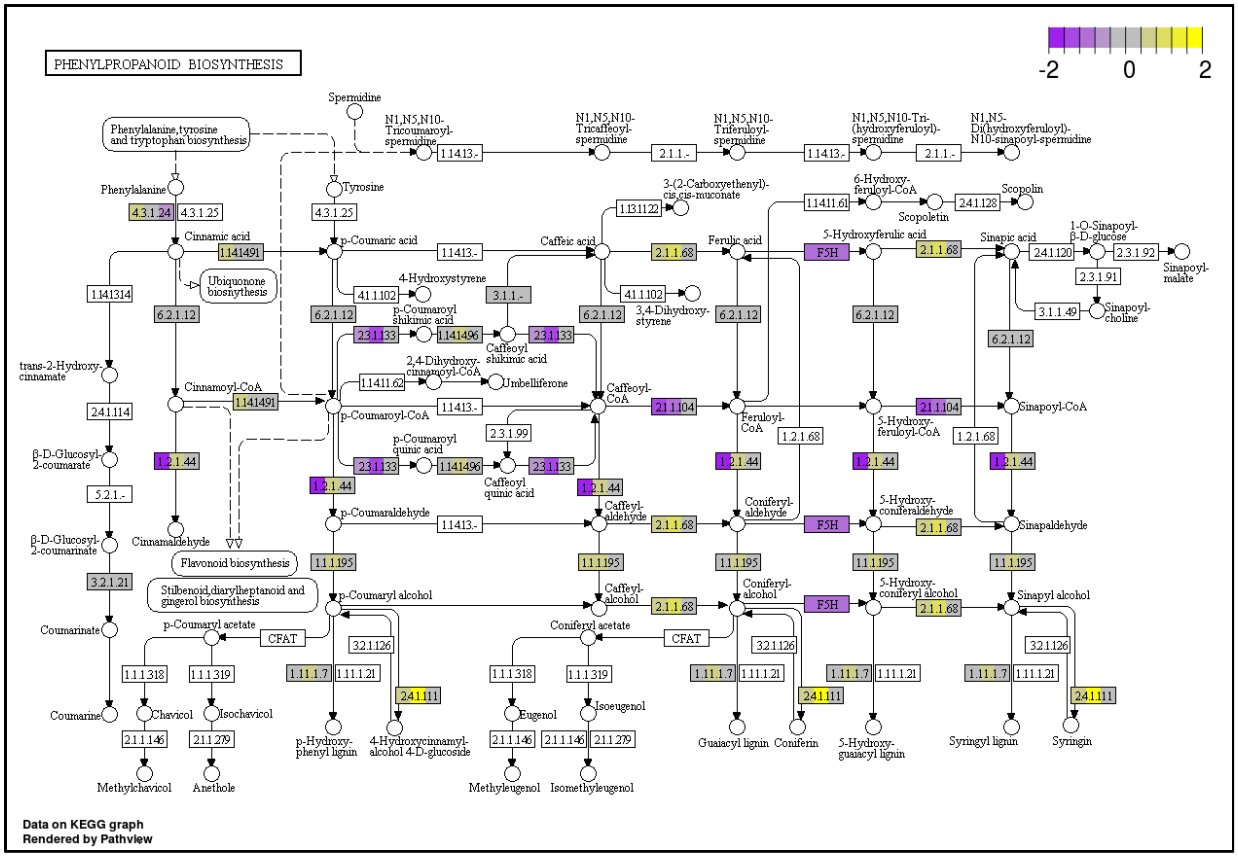


Figure 3. GRBV impact on the phenylpropanoid pathway at harvest in 2016 using DE and KEGG pathways. Boxes indicate genes, circles indicate metabolites, yellow indicates upregulated, and purple indicates downregulated. The intensity of the color is indicative of the extent of gene up or down regulation. The boxes are separated into three parts indicating, from left to right, 420A/Cabernet Sauvignon, 110R/Cabernet Sauvignon, and 1003P/Merlot.

The three main genes responsible for the synthesis of grape anthocyanins were impacted. However, in 2016 they were downregulated for Merlot at harvest, and upregulated for 420A Cabernet Sauvignon. This indicates differences in disease expression due to genotypic differences, and metabolomic data can support if gene regulation was translated into metabolite concentrations. Differences could also be indicative of differences in ripening due to climatic conditions at each site irrespective of disease status. Similar variability in disease expression due to genotype was observed in 2017 (**Figures 7a** and **7b**).

In addition, sample preparation techniques for metabolomics analysis have been finalized. Non-volatile metabolites will be analyzed using 1H NMR and ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC/QTOF/MS). Extractions will be performed using one gram of homogenized grape tissue and four ml of 60:20:20 methanol:water:cholorform that is acidified with 1% formic acid. Decyl β-D-glucopyranoside will be used as an internal standard. The methodologies for both UPLC/QTOF/MS and NMR have been optimized for this study. Volatile metabolites were analyzed using HS-SPME-GC-

MS. In a 10 ml amber vial, one gram of NaCl, 500 mg homogenized grape tissue, one ml of one molar sodium citrate buffer, 25 µl of 0.2 g/l of ascorbic acid solution, and 25 µl of 0.5 mg/l 2-undecanone were added. The data collection for volatile secondary metabolites has been completed and data analysis is underway and set for completion by the end of October. Data collection for UPLC/QTOF/MS and NMR will be completed by the end of 2019, with data analysis being performed through January and February 2020.

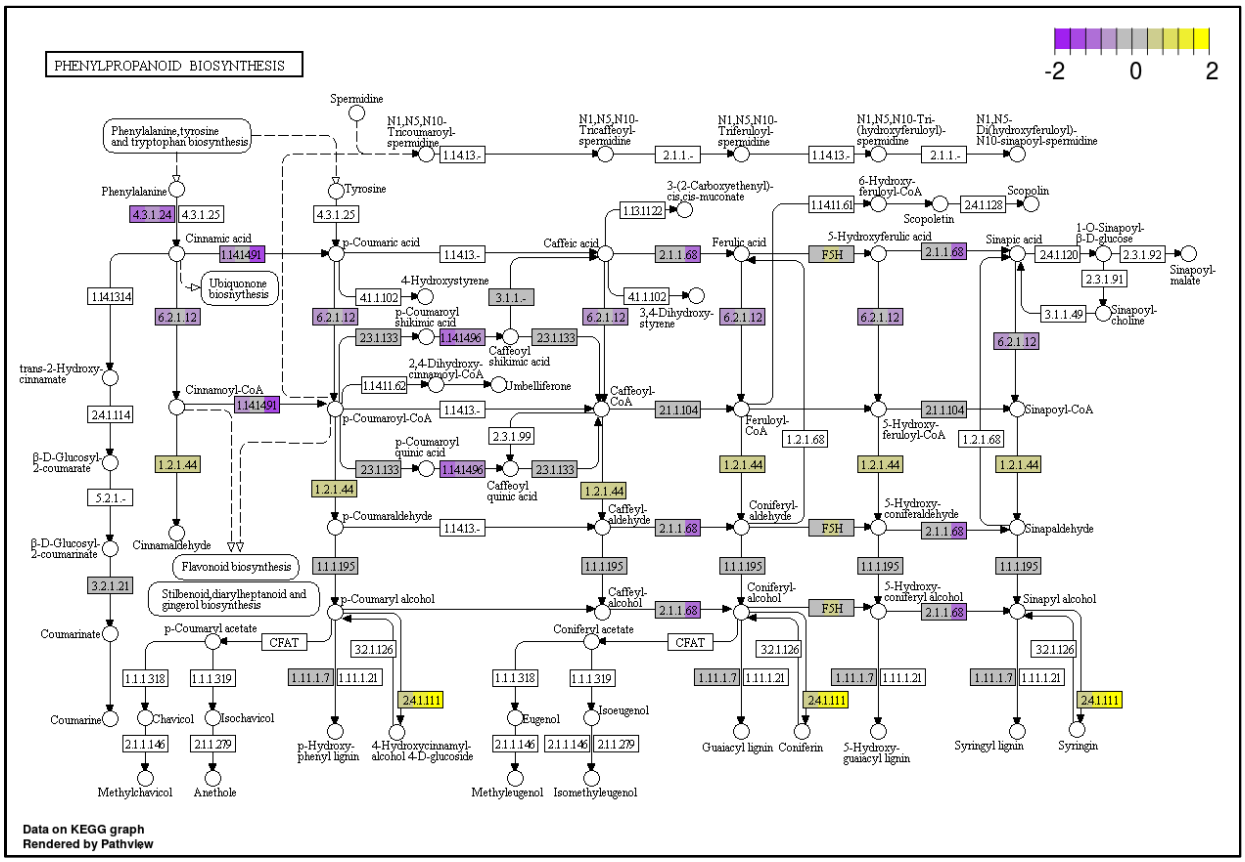


Figure 4. GRBV impact on the phenylpropanoid pathway at veraison in 2017 using DE and KEGG pathways. Boxes indicate genes, circles indicate metabolites, yellow indicates upregulated, and purple indicates downregulated. The intensity of the color is indicative of the extent of gene up or down regulation. The boxes are separated into three parts indicating, from left to right, 420A/Cabernet Sauvignon, 110R/Cabernet Sauvignon, and 1003P/Merlot.

Currently, method development has begun on the extraction and analysis of phytohormones in grape samples. Methods outlined in Pan et al. (2010) and Muller and Bosch (2011) will be used for the extraction of major hormones in grapes. The hormones of interest due to their importance during grape ripening are abscisic acid, salicylic acid, indole-3-acetic acid, trans-zeatin, and gibberellic acid (GA3). For each sample, 100 mg of grape tissue powder (from liquid nitrogen grinding) will be extracted with one ml of 20:79:1 (v/v/v) water:isopropanol:glacial acetic acid. An internal standard mix will be added (50 µl to each sample) which contains all hormones of

interest as well as their deuterated forms in a concentration of 50 µg/ml. This solution is sonicated at 4°C to 7°C for 30 minutes and then briefly vortexed. To each sample, one ml of dichloromethane is added and sonicated for 30 minutes at 4°C to 7°C. The samples are vortexed and then centrifuged at 13,000 x g for 15 minutes at 4°C. The solvent is removed from the plant tissue and kept separate. To the remainder of the plant tissue, another 200 µl of extraction solvent and 200 µl of dichloromethane is added, sonicated similarly, vortexed, and centrifuged for 15 minutes at 4°C. The supernatant is again collected and combined with the previous fraction. This process is repeated twice more for a total of four collection fractions. To the supernatant collected, two layers would have formed. The bottom layer is collected (one ml) and transferred to a pre-weighed vial. The solvent is removed using a constant flow of nitrogen at 4°C to 7°C. Once dried, the vial is weighed again to obtain sample mass. Then the sample is re-dissolved in 0.1 ml of methanol and then analyzed using ultra-performance liquid chromatography-electrospray tandem mass spectrometry (UPLC/ESI-MS/MS) with multiple reaction monitoring (MRM).

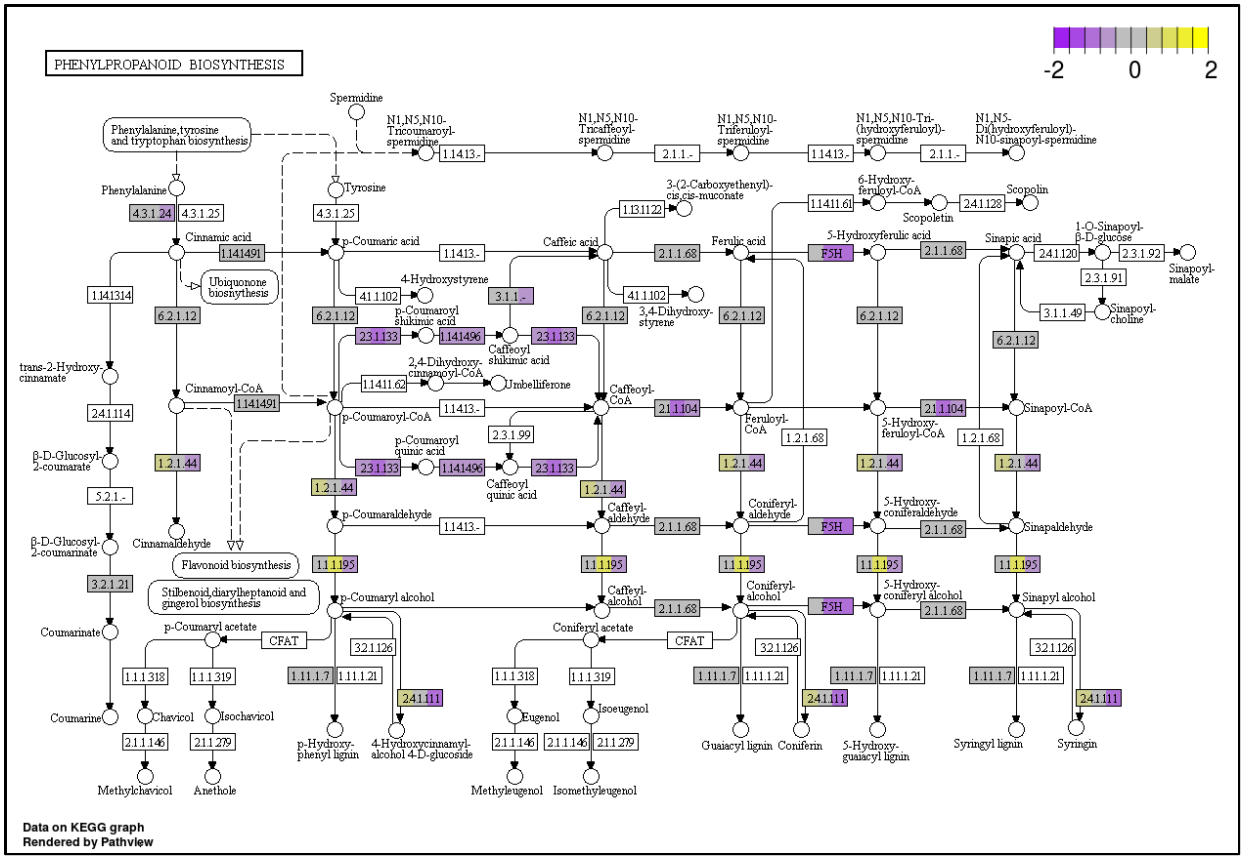


Figure 5. GRBV impact on the phenylpropanoid pathway at harvest in 2017 using DE and KEGG pathways. Boxes indicate genes, circles indicate metabolites, yellow indicates upregulated, and purple indicates downregulated. The intensity of the color is indicative of the extent of gene up or down regulation. The boxes are separated into three parts indicating, from left to right, 420A/Cabernet Sauvignon, 110R/Cabernet Sauvignon, and 1003P/Merlot.

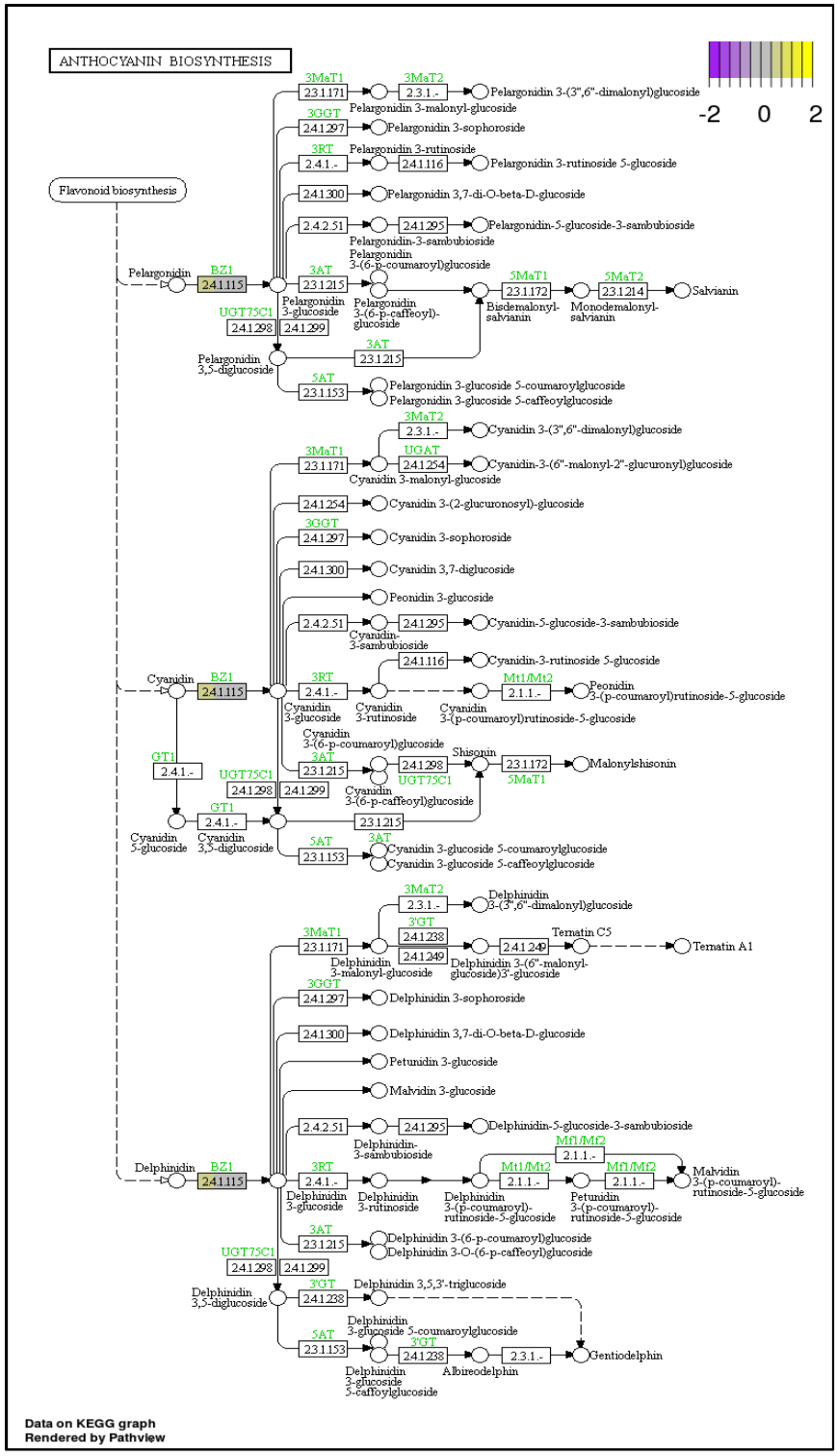


Figure 6a. GRBV impacts on the anthocyanin pathway at veraison in 2016 using DE and KEGG pathways. Boxes indicates genes, circles indicate metabolites, yellow indicates upregulated, and purple indicates downregulated. The intensity of the color is indicative of the extent of gene up or down regulation. The boxes are separated into three parts

indicating, from left to right, 420A/Cabernet Sauvignon, 110R/Cabernet Sauvignon, and 1003P/Merlot.

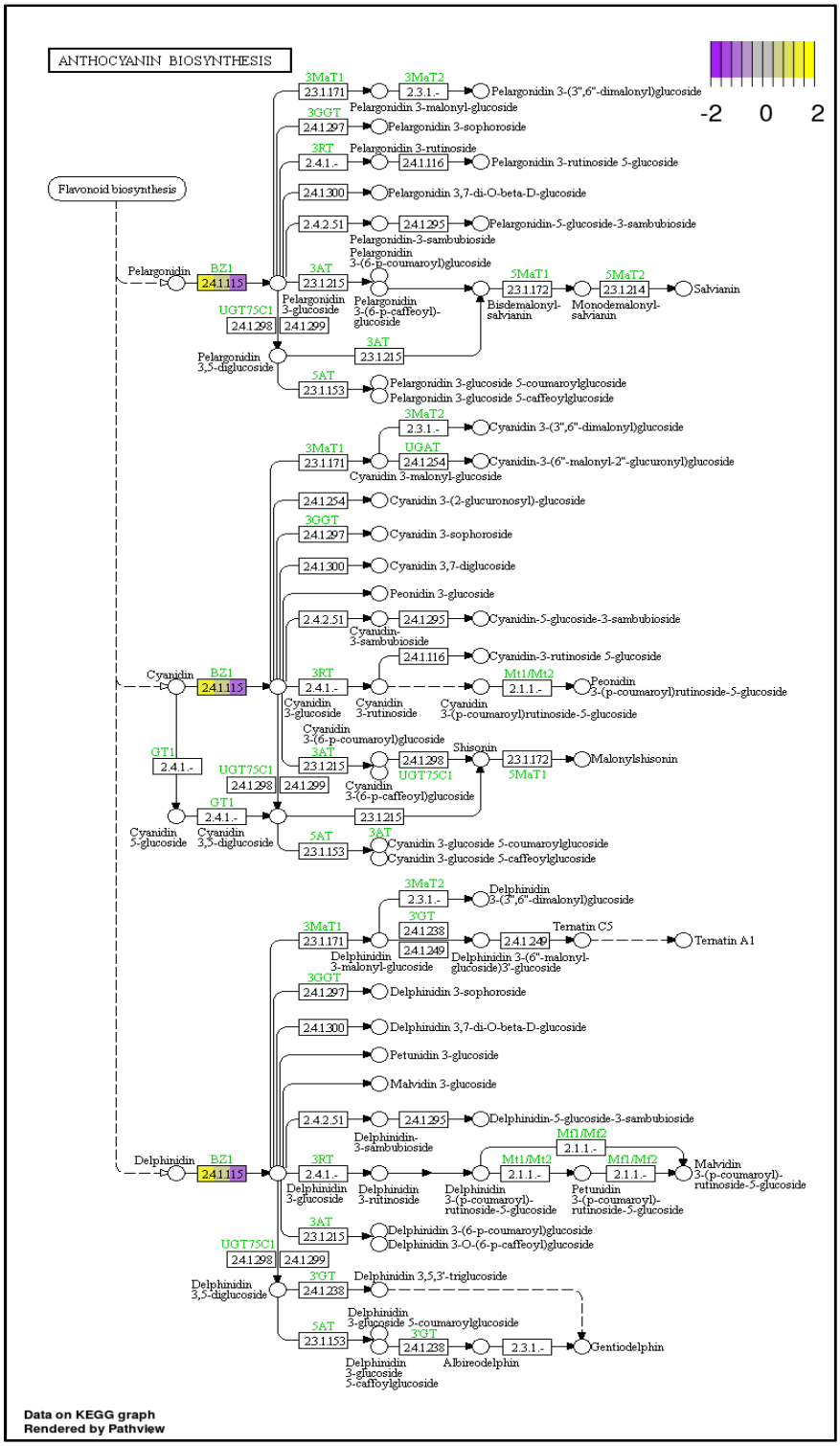


Figure 6b. GRBV impacts on the anthocyanin pathway at harvest in 2016 using DE and KEGG pathways. Boxes indicates genes, circles indicate metabolites, yellow indicates upregulated, and purple indicates downregulated. The intensity of the color is indicative of the extent of gene up or down regulation. The boxes are separated into three parts

indicating, from left to right, 420A/Cabernet Sauvignon, 110R/Cabernet Sauvignon, and 1003P/Merlot.

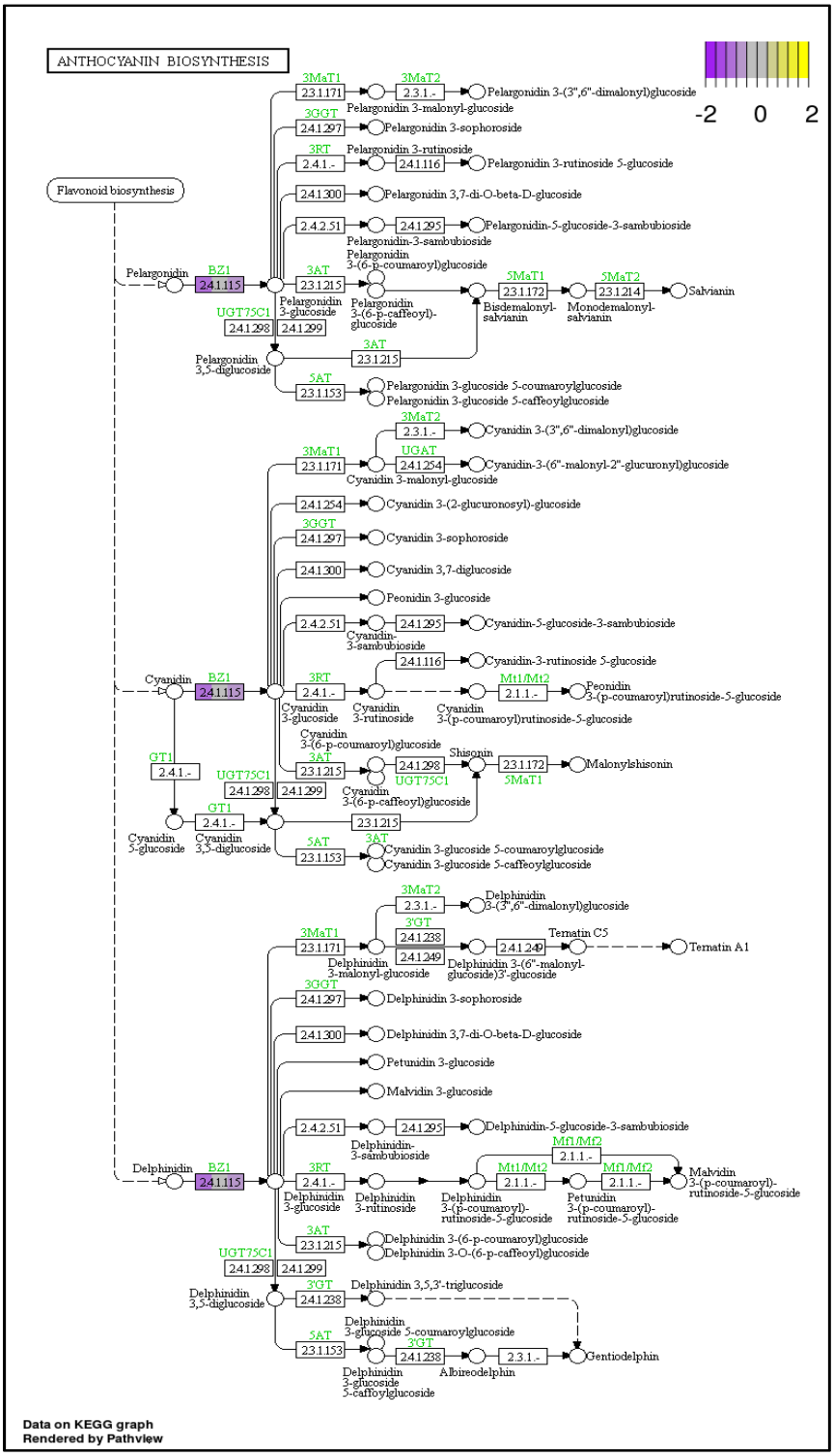


Figure 7a. GRBV impacts on the anthocyanin pathway at veraison in 2017 using DE and KEGG pathways. Boxes indicates genes, circles indicate metabolites, yellow indicates upregulated, and purple indicates downregulates. The intensity of the color is indicative of the extent of gene up or down regulation. The boxes are separated into three parts

indicating, from left to right, 420A/Cabernet Sauvignon, 110R/Cabernet Sauvignon, and 1003P/Merlot.

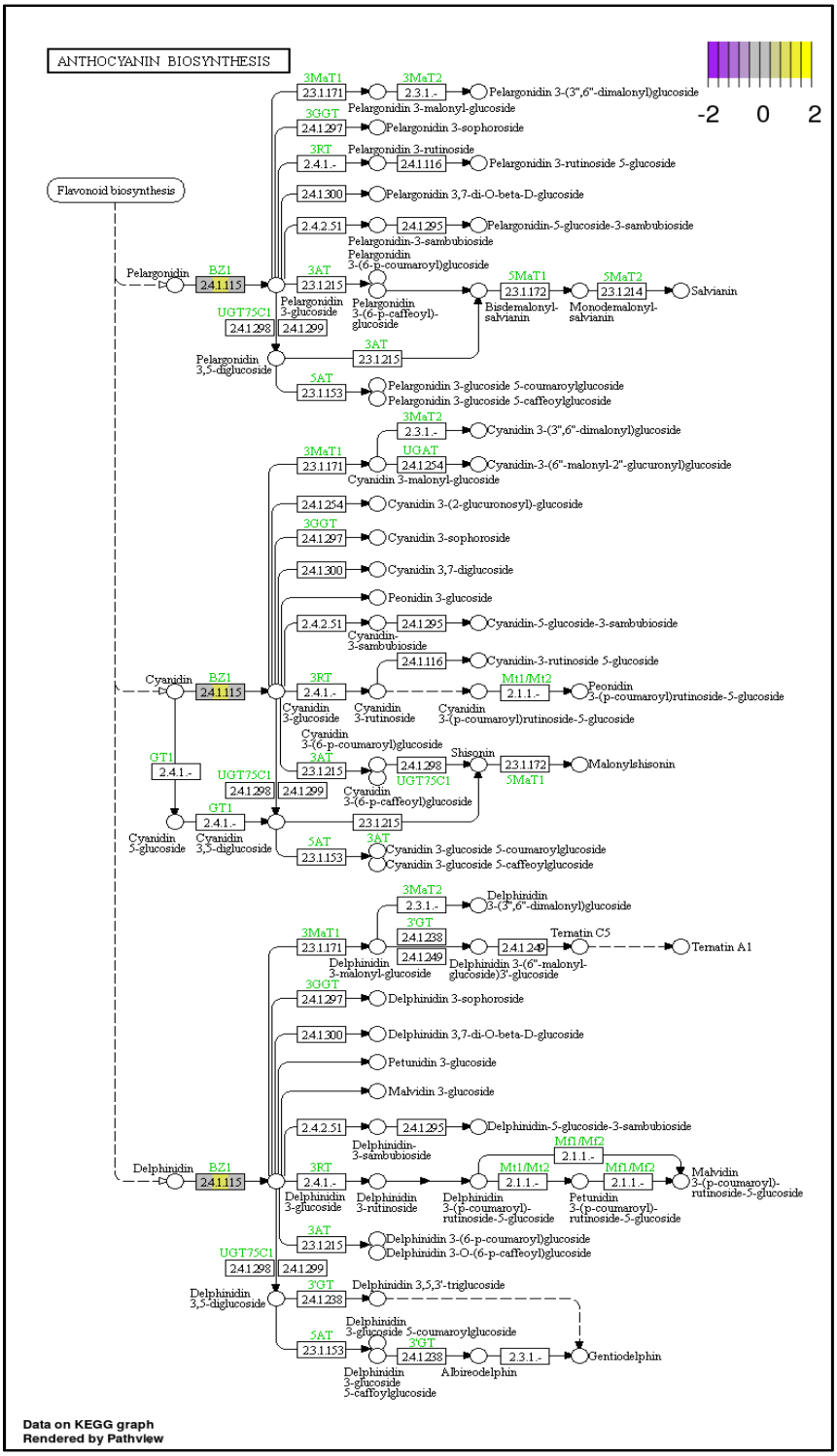


Figure 7b. GRBV impacts on the anthocyanin pathway at harvest in 2017 using DE and KEGG pathways. Boxes indicates genes, circles indicate metabolites, yellow indicates upregulated, and purple indicates downregulates. The intensity of the color is indicative of the extent of gene up or down regulation. The boxes are separated into three parts

indicating, from left to right, 420A/Cabernet Sauvignon, 110R/Cabernet Sauvignon, and 1003P/Merlot.

Objective 2. To Determine the Potential Impact of Variety, Rootstock, Site, and Season on GRBV Functioning

Grape samples were collected from two different varieties (*V. vinifera* cultivars Cabernet Sauvignon and Merlot) over two seasons as described above. Targeted metabolomic and transcriptomic analysis (Objective 1) of each variety will enable us to determine whether variety has any influence on the functioning of the disease on a molecular level. Additionally, the Cabernet Sauvignon vineyard block contains two rootstocks, 110R and 420A. Initial investigations suggest that grape berries from GRBV symptomatic vines on 110R rootstocks are more impacted by GRBD than those on 420A rootstocks. Additionally, previous research indicated a large environmental (seasonal) impact on both visual expression of GRBD as well as its impact on grape composition. No correlation has been found between GRBD visual expression and grape and wine compositional impact.

A potential pitfall and limitation is the spread of GRBD to data vines after qPCR testing. Vines were monitored for any visual signs of infections during the season and re-tested if needed. Further limitation is the capabilities of the analytical instrumentation that will be utilized.

This objective is partly completed with the completion of RNA-seq. However, metabolomic data is needed to determine the impact of genotypic and environmental factors on disease expression.

REFERENCES CITED

Andreasson A, Kiss NB, Juhlin CC, Höög A, 2013. Long-term storage of endocrine tissues at -80°C does not adversely affect RNA quality or overall histomorphology. *Biopreservation and Biobanking* 11(6): 366- 370.

Blanco-Ulate B, Hopfer H, Figueroa-Balderas R, Ye Z, Rivero RM, Albacete A, Perez-Alocea F, Koyama R, Anderson MM, Smith RJ, Ebeler SE, Cantu D. 2017. Red blotch disease alters grape berry development and metabolism by interfering with the transcriptional and hormonal regulation of ripening. *Journal of Experimental Botany* 10.1093/jxb/erw506.

Chellappan P, Vanitharani R, Ogbe F, Fauquet CM. 2005. Effect of temperature on geminivirus-induced RNA silencing in plants. *Plant Physiology* 138(4): 1828-1841. https://doi.org/10.1104/pp.105.066563.

Eridon SS. 2016. Assessing the effect of different percentages of red blotch affected fruit on wine composition for Cabernet Sauvignon. MSc, University of California, Davis.

Fortes AM, Agudelo-Romero P, Silva MS, Ali K, Sousa L, Maltese F, Choi Y, Grimplet J, Martinez-Zapater JM, Verpoorte R, Pais MS. 2011. Transcript and metabolite analysis in Trincadeira cultivar reveals novel information regarding the dynamics of grape ripening. *BMC Plant Biology* 11: 149.

Girardello RC, Cooper ML, Smith RJ, Lerno LA, Bruce RC, Eridon S, Oberholster A. 2019. Impact of grapevine red blotch disease on grape composition of *Vitis vinifera* Cabernet Sauvignon, Merlot, and Chardonnay. *Journal of Agricultural Food Chemistriy* 67(19): 5496-5511. https://doi.org/10.1021/acs.jafc.9b01125.

Hendrickson DA, Lerno LA, Hjelmeland AK, Ebeler SE, Heymann H, Hopfer H, Block KL, Brenneman CA, Oberholster A. 2016. Impact of mechanical harvesting and optical berry

sorting on grape and wine composition. *American Journal of Enology and Viticulture* 67(4): 385-397.
Hjemeland AK, King ES, Ebeler SE, Heymann H. 2013. Characterizing the chemical and sensory profiles of United States Cabernet Sauvignon wines and blends. *American Journal of Enology and Viticulture* 64(2): 169-179.
Martínez-Lüscher J, Plank CM, Brillante L, Cooper ML, Smith RJ, Al-Rwahnih M, Yu R, Oberholster A, Girardello R, Kurtural SK. 2019. Grapevine red blotch virus may reduce carbon translocation leading to impaired grape berry ripening. *Journal of Agricultural Food Chemistry* 67(9): 2437-2448. https://doi.org/10.1021/acs.jafc.8b05555.
Oberholster A. 2015. Investigation of the impact of grapevine red blotch-associated virus on grape and wine composition and quality, American Vineyard Foundation.
Oberholster A. 2016. Investigation of the impact of grapevine red blotch-associated virus on grapevine health and subsequent grapes and wine composition and style., American Vineyard Foundation.
Sudarshana M, Perry K, Fuchs M. 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Plant Disease* 105: 1026-1032.
Theodoridis G, Gika H, Franceschi P, Caputi L, Arapitsas P, Scholz M, Masuero D, Wehrens R, Vrhovsek U, Mattivi F. 2012. LC-MS based global metabolite profiling of grapes: solvent extraction protocol optimisation. *Metabolomics* 8: 175-185.
Toffali K, Zamboni A, Anesi A, Stocchero M, Pezzotti M, Levi M, Gusso F. 2011. Novel aspects of grape berry ripening and post-harvest withering revealed by untargeted LC-ESI-MS metabolomics analysis. *Metabolomics* 7: 424-436.

FUNDING AGENCIES

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

EFFECTS OF GRAPEVINE RED BLOTCH DISEASE ON FLAVOR AND FLAVOR PRECURSOR FORMATION IN THE GRAPE AND ON WINE QUALITY

Project Leader: Michael Qian | Food Science and Technology | Oregon State University | Corvallis, OR 97331 | michael.qian@oregonstate.edu
Co-Project Leader: Alexander D. Levin | Department of Horticulture | Oregon State University | Central Point, OR 97502 | alexander.levin@oregonstate.edu

Reporting Period: The results reported here are from work conducted July 2017 to October 2019.

ABSTRACT
A field experiment was established with two irrigation treatments (wet and dry) and two disease conditions, red blotch affected and non-affected grapevines. A wet treatment was irrigated at 100% estimated crop evapotranspiration (ETc) and dry treatment was irrigated at 66% ETc. Wines were made in triplicate using standard protocols in a pilot winery. Highly volatile compounds were quantified by headspace gas chromatography (GC) flame ionization detection, and other volatiles were analyzed using stable isotope dilution approach with solid-phase micro-extraction-GC-mass spectrometry (MS) and stir bar sorptive extraction GC-MS techniques. Preliminary results showed red blotch disease decreased the total soluble solids of grape berries and total phenolic content of wines. Wines with wet treatment had higher concentrations of isoamyl acetate, and red blotch affected wines with wet treatment revealed lower levels of ethyl hexanoate, phenylethyl alcohol, and hexanoic acid. The impacts of red blotch and irrigation on other volatile compounds were not obvious. Continued studies of red blotch affected grapes will further investigate effects on volatile aroma compounds.

LAYPERSON SUMMARY
Two irrigation treatment main plots are randomized in two blocks of fields and characterized by varying water application rates on both red blotch infected and non-infected grapevines. The impact of red blotch disease on grape and wine quality was studied. Berry maturity parameters, wine anthocyanins, phenolics, and flavor profiles were investigated. The results indicated that red blotch infected grapes had a lower level of total soluble solids. Wines made from red blotch infected grapes showed lower total phenolic content compared to wine from non-infected grapes. Most volatile compounds did not show statistical differences between red blotch positive and red blotch negative wines.

INTRODUCTION
Grapevine red blotch virus (GRBV) is a single-stranded circular DNA virus correlated with red blotch disease (Krenz et al., 2014). It was first found in Cabernet Sauvignon in California in 2008 and is widespread in North America, especially the U.S. In recent years, red blotch was found in grapevines in Canada (Xiao et al., 2018) and Korea (Lim et al., 2016). The grape species that can be infected by GRBV include Cabernet franc, Cabernet Sauvignon, Chardonnay, Malbec, Merlot, Mourvèdre, Petite Syrah, Petit Verdot, Pinot Noir, Riesling, and Zinfandel (Al Rwahnih et al., 2013). GRBV inhibits grape ripening pathways involved in the generation of color, flavor, and aroma compounds by altering transcription factors and hormone networks which then disrupt normal grape berry development (Blanco-Ulate et al., 2017). The symptoms

of red blotch are similar to leafroll (Cieniewicz et al., 2017; Cieniewicz et al., 2018), but the leaves infected by GRBV turn red and fruit maturity delays (Krenz et al., 2014). The disease causes the decrease of grape production (Eridon, 2017) and increases costs in the wine industry (Ricketts et al., 2017).

It has been reported that the virus is transmitted by grafting, so it is likely that spread primarily occurs through propagation of material (Cieniewicz et al., 2017). A leafhopper has been reported to also transmit the virus (Poojari et al., 2013). Bahder et al. (2016) found that the three-cornered alfalfa hopper (*Spissistilus festinus*) can be a vector of grapevine red blotch by using the phylogeny of geminivirus coat protein sequences and digital polymerase chain reaction. It is suggested that roguing symptomatic vines and replanting with clean vines derived from virus-tested stocks will minimize losses if disease incidence is below 30%, while a full vineyard replacement should be pursued if disease incidence is higher (Ricketts et al., 2017).

GRBV infections can affect berry physiology, causing uneven ripening, higher titratable acidity, and lower sugar and anthocyanin contents (Blanco-Ulate et al., 2017). It has been reported in the literature that red blotch affected fruit at harvest had a major decrease in Brix and significantly higher concentrations of tannins and non-tannin phenolics. Anthocyanins were found to be significantly higher in all wines made with fruit from healthy vines, and tannin concentrations were significantly higher in wines made with fruit from red blotch affected vines. Many of the differences in volatiles found may be attributable to fermentation and harvest Brix levels. Four mouthfeel and taste sensory attributes were found to be significantly different among the wines (Eridon, 2017).

OBJECTIVES

1. To identify the impact of grapevine red blotch disease on grape berry development.
2. To identify the impact of grapevine red blotch disease on Pinot Noir wine monomeric anthocyanin and total phenolic content.
3. To identify the impact of grapevine red blotch disease on Pinot Noir wine volatile profiles.

RESULTS AND DISCUSSION

Vine Water Status. Despite the change in vineyard sites between the 2018 and 2019 seasons (due to vineyard removal by the previous collaborator), there were consistent and statistically significant effects of red blotch disease status on vine water status (midday stem water potential; Ψstem) (**Table 1**). However, irrigation treatments only significantly affected Ψstem in 2018. Finally, there were no significant interaction effects between irrigation and disease status in either year.

In general, infected vines had a significantly higher water status compared to healthy vines in both years (**Figure 1**). Yet, while this difference was observed immediately following veraison in 2018, it was not observed until just prior to harvest in 2019. Nevertheless, the fact that the effects of disease status on Ψstem were only observed post-veraison was consistent between years.

Table 1. Analysis of variance (ANOVA) results for the response of midday Ψstem to irrigation treatment, disease status, and sample date. *P*-values were considered statistically significant at $P < 0.05$.

Source of Variation	*P*-values	
	Year 2018	Year 2019
irrigation	0.004	0.334
status	0.203	< 0.001
date	< 0.001	< 0.001
Irrigation * status	0.572	0.631
Irrigation * date	< 0.001	0.636
Status * date	0.008	0.132
Irrigation * status * date	0.537	0.804

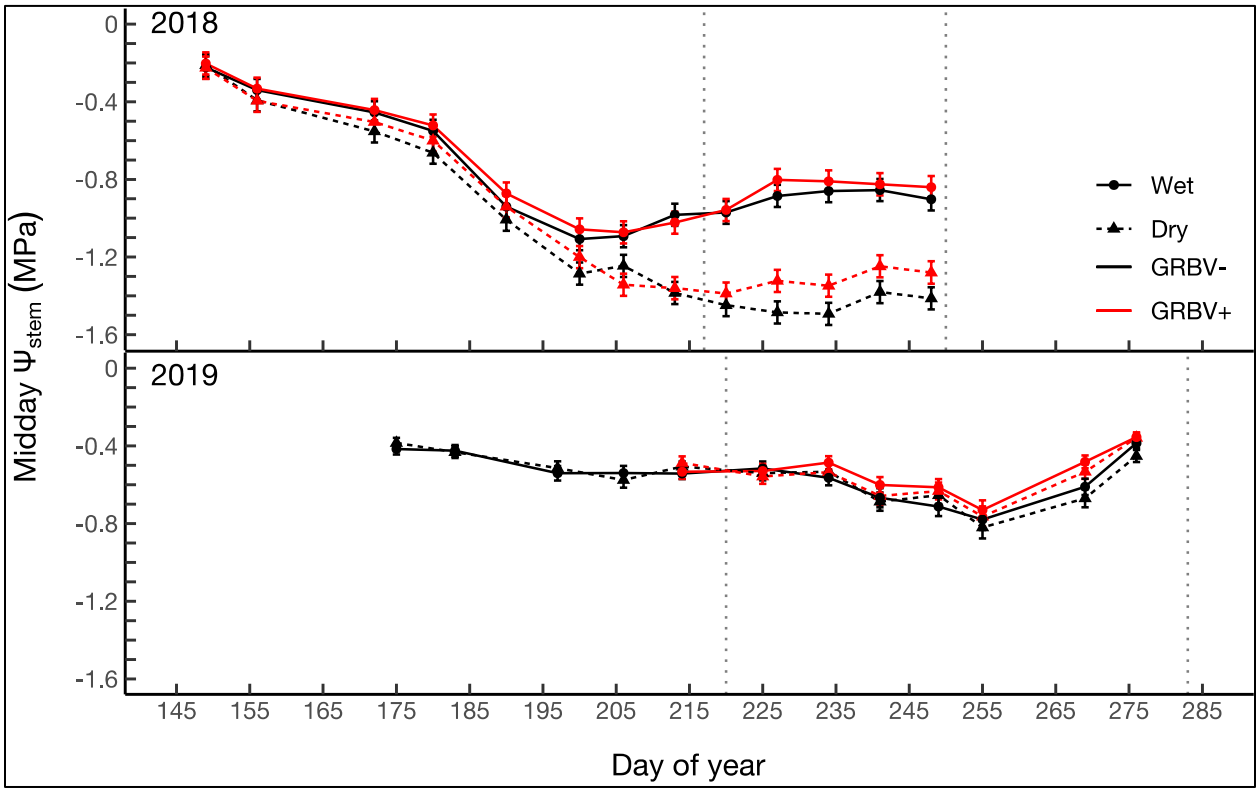


Figure 1. Response of midday Ψstem to experimental treatments in 2018 and 2019. Data are means ± 1 standard error (n = 5 and 4 in 2018 and 2019, respectively). Left-most and right-most vertical dotted lines signify approximate date of veraison and date of harvest, respectively.

Objective 1. Grape Berry Organic Acids and Total Soluble Solids (TSS)

TSS increased in both red blotch positive (RB+) and red blotch negative (RB-) grapes with maturity (**Figure 2**). In 2019, RB- grapes showed higher levels of TSS than RB+. No consistent trend is observed across the two years between RB+/-. All major organic acids decreased during

berry development (**Figures 3a and 3b**). No difference was observed in organic acid concentration of RB+ and RB- grape berries across both years.

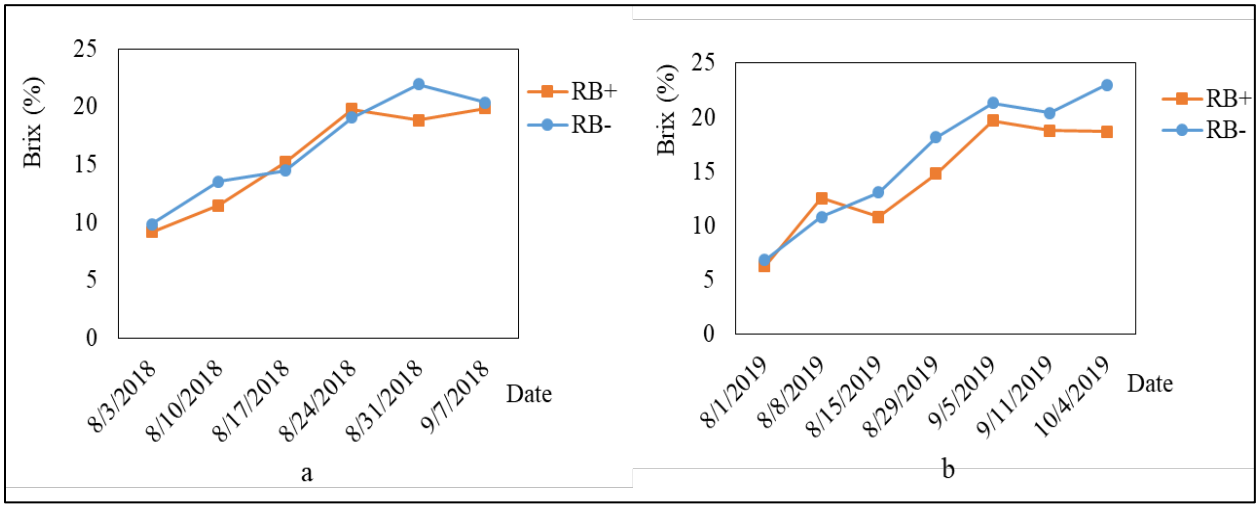


Figure 2. TSS of grape juice. Different harvest years are distinguished by letter, with a = grapes harvested in 2018 (n = 4), and b = grapes harvested in 2019 (n = 4).

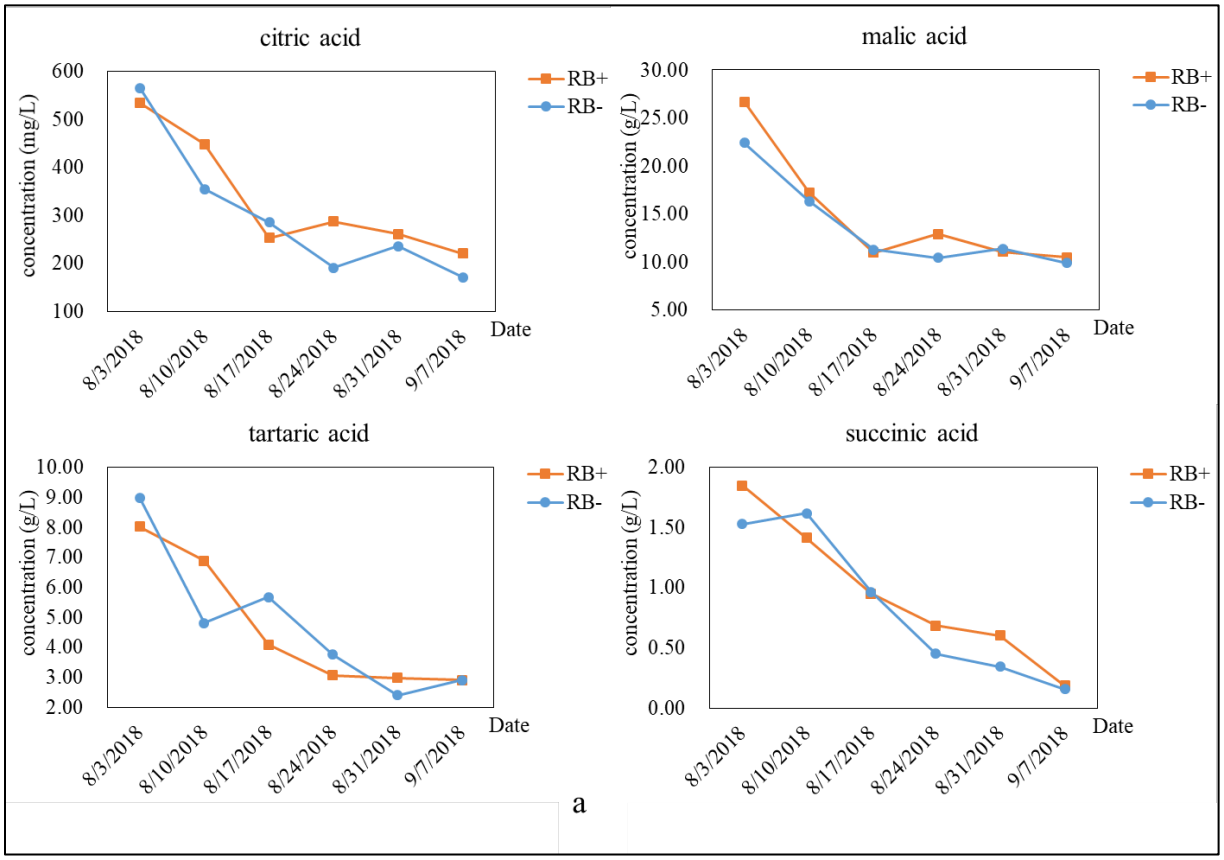


Figure 3a. Organic acids of grape juice, for grapes harvested in 2018 (n = 4).

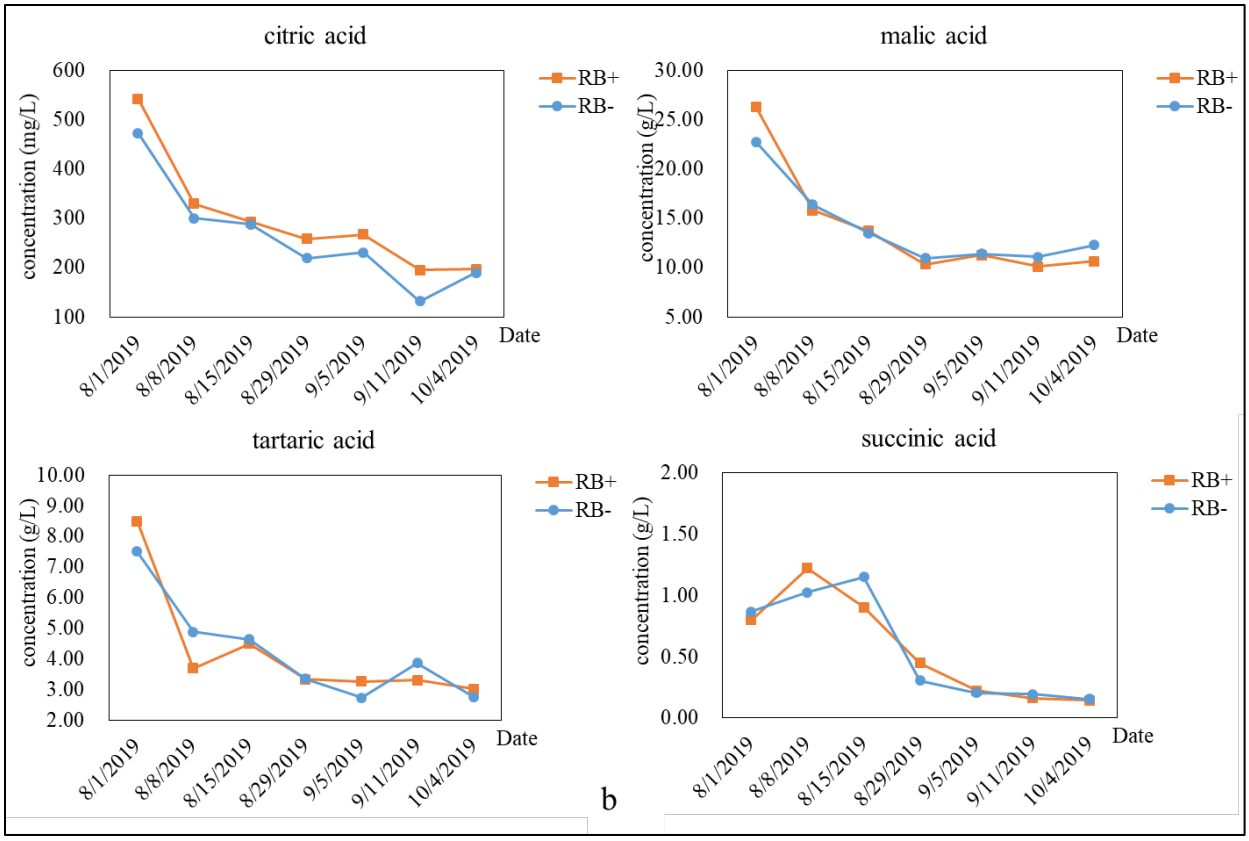


Figure 3b. Organic acids of grape juice, for grapes harvested in 2019 (n = 4).

Objective 2. Wine Monomeric Anthocyanin and Total Phenolic Content

Monomeric anthocyanin and total phenolic content (**Table 2**) are higher in general for RB- wines. Wine from vines without wet treatment has the highest level of monomeric anthocyanin of the four treatment groups. RB+ wines show lower total phenolic content than RB- wines. Major phenolic compounds determined by high performance liquid chromatography are summarized in **Table 3**. Within each irrigation treatment, RB+ show lower concentrations of major phenolics than RB- wines, which indicates that disease status impacts the concentration of phenolic compounds regardless of irrigation practice.

Table 2. Monomeric anthocyanin and total phenolic content in red blotch wines (mg/l).

Compounds	D+	D-	W+	W-
monomeric anthocyanin	6.2±0.7	8.3±2.3	5.4±1.0A	14.0±0.8B
total phenolic content	1084±33	1181±33	1041±52A	1410±141B

Different letters indicate statistical significance ($P < 0.05$) between means (n = 3) of RB+ and RB- for each irrigation treatment, with D = dry treatment, and W = wet treatment.

Table 3. Concentration of major phenolic compounds with irrigation treatment (mg/l).

Compounds	D+	D-	W+	W-
caffeoyltartaric acid	30.0±0.8a	34.0±0.3b	29.6±1.6A	33.2±0.9B
catechin	21.2±1.4a	22.4±0.1b	19.2±1.1A	29.1±1.2B
cafferic acid	2.4±0.1a	3.4±0.3b	3.0±0.2A	3.6±0.1B
epicatechin	27.4±2.7a	38.4±1.9b	24.9±0.4A	30.9±1.2B
malvidin-3-monoglucoside	12.1±0.7a	15.6±1.4b	8.6±0.4A	45.8±1.9B

Different letters indicate statistical significance ($P < 0.05$) between means (n = 3) of RB+ and RB- for each irrigation treatment.

Objective 3. Wine Volatile Profiles

The total concentration of different classes of volatile compounds in wine samples (D+, D-, W+, W-), including esters, acids, alcohols, ketones, and terpenes, is shown in **Figure 4**. The total esters concentration of D+ is significantly higher than D- at the 95% level, while levels of total acids, alcohols, ketones, and terpenes are neither significant between RB+ and RB- wines nor between different irrigation treatments.

Irrigation treatments have different impacts on the concentration of volatile compounds of wines with two disease states (**Tables 4a to 4d**). Wet treatment increased the levels of phenethyl acetate and (E)-2-hexen-1-ol and decreased the concentration of ethyl decanoate, ethyl phenylacetate, phenyl alcohol, γ-decalactone, and δ-undecalactone in RB+ wines, compared to dry treatment. Wet treatment also revealed higher concentrations of ethyl butanoate, isobutyl acetate, ethyl 3-methylbutanoate, isoamyl acetate, and hexanoic acid, but lower ethyl decanoate, 6-methyl-5-hepten-2-one, and 1-octen-3-ol concentrations in RB- wines than dry treatment. Within the dry treatments, D+ resulted in higher levels of isobutyl acetate, ethyl 3-methylbutanoate, isoamyl acetate, ethyl octanoate, ethyl decanoate, ethyl phenylacetate, ethyl dodecanoate, nerol, γ-decalactone, and δ-undecalactone but lower levels of 1,1,6,-trimethyl-1,2-dihydronapthalene (TDN) (after hydrolysis). RB+ wines with wet treatment revealed higher levels of isobutyl acetate and ethyl decanoate, whereas phenyl alcohol, 3-isopropyl-2-methoxypyrazine (IPMP), vitispirane (after hydrolysis), and TDN (after hydrolysis) were lower compared to the RB- wines (W-). Overall, both different irrigation treatments and red blotch disease states have influence on only a few volatile aroma compounds.

CONCLUSIONS

Red blotch disease is a growing concern to the wine industry, with loss of crop and large associated costs. Investigating the impact of red blotch disease on grape development and resulting wine quality are the main objectives of this study. Grape berries were assessed by measuring TSS and several organic acids. Analysis of volatile profiles, anthocyanin, and total phenolics content were used for criteria to assess the resulting wine quality of control and red blotch affected crops in two different irrigation conditions. RB+ grapes showed a lower level of TSS and lower total phenolic content in the resulting wine, while most volatile compounds did not show statistical difference between RB+ and RB- wines. Different irrigation treatments had great impact on wine aroma, as well as anthocyanin and phenolics compounds.

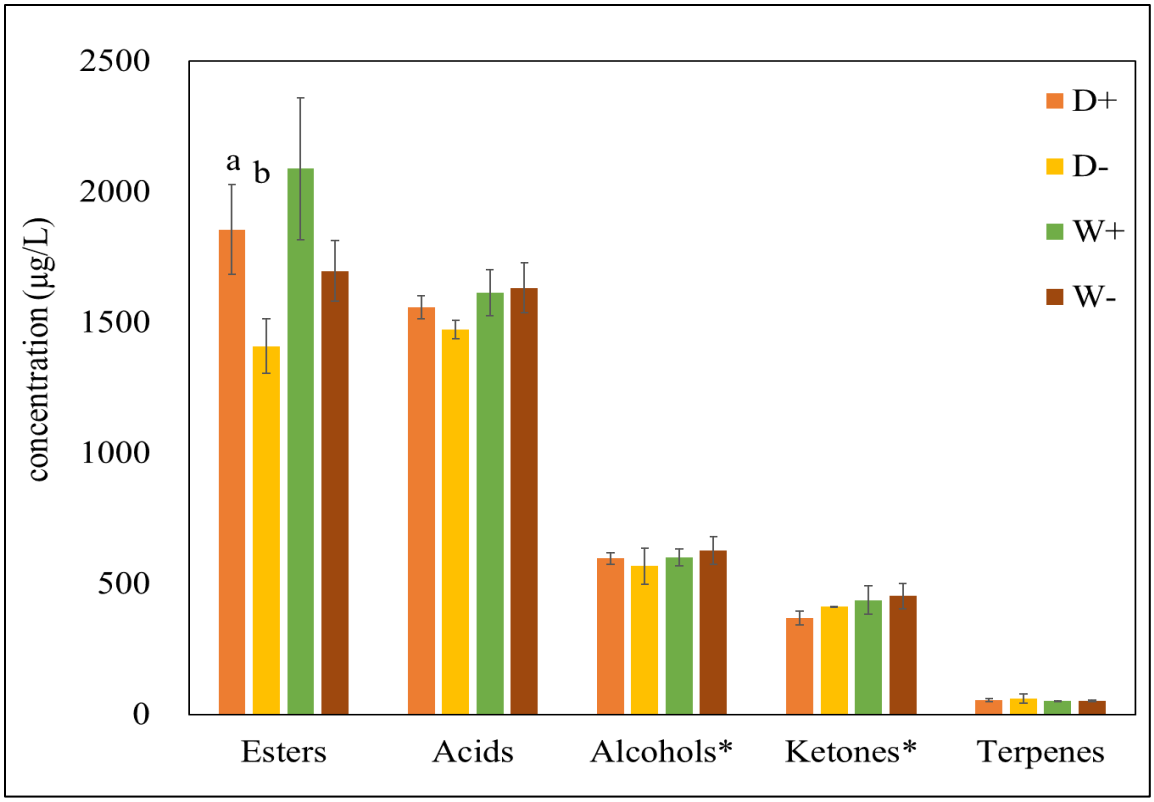


Figure 4. Total concentration of esters, acids, alcohols*, ketones*, and terpenes in wines with different treatments. Units = µg/l, except * = mg/l. D = dry conditions; W = wet conditions; + = red blotch affected wine; - = red blotch nonaffected wine. Different letters indicate statistical significance ($P < 0.05$) between means (n = 3) of RB+ and RB- for each irrigation treatment.

Table 4a. Concentration of esters of wines with irrigation treatment (µg/l).

Compounds	D+	D-	W+	W-
ethyl acetate*	37.3±2.4	37.6±1.1	40.9±6.8	39.5±2.7
ethyl propionate	75.1±5.5	77.7±2.2	77.3±3.8	76.5±4.6
ethyl 2-methylpropanoate	89.8±11.8	74.7±3.0	85.8±3.2	79.1±6.9
ethyl butanoate	108±7	105±2c	131±12	113±4d
isobutyl acetate	175±31a	121±13bc	215±26a	156±13bd
ethyl 3-methylbutanoate	2.30±0.35a	1.65±0.15bc	1.66±0.43	2.06±0.16d
isoamyl acetate	914±134a	616±104bc	1151±193	869±119d
ethyl 2-methylbutanoate	2.44±0.44	1.91±0.1	2.34±0.6	2.11±0.26
ethyl hexanoate	286±15	274±34	292±39	284±22
hexyl acetate	7.5±6.6	7.3±0.4	17.3±3.9	15.3±2.7
ethyl octanoate	149±9ac	106±19b	98±6d	85±6
ethyl decanoate	42.8±12.0ac	20.8±2.3bc	19.4±0.4ad	15.3±1.0bd
ethyl phenylacetate	1.21±0.33ac	0.54±0.15b	0.29±0.15d	0.56±0.20
phenethyl acetate	8.5±1.1c	11.1±2.2	14.8±1.1d	14.0±2.9
ethyl dodecanoate	0.24±0.05a	0.12±0.02b	0.17±0.03	0.14±0.03

Table 4b. Concentration of ketones, aldehydes, and alcohols of wines with irrigation treatment (μg/l).

Compounds	D+	D-	W+	W-
ketone & aldehyde				
acetaldehyde*	389±26	441±51	378±49	423±30
1-octen-3-one	ND	ND	ND	ND
6-methyl-5-hepten-2-one	1.08±0.39	1.41±0.38c	0.50±0.03	0.41±0.08d
alcohol				
propanol*	25.3±1.4	23.4±0.8	32.1±4.4	25.0±1.0
isobutyl alcohol*	199±17	165±26	197±4	200±17
isoamyl alcohol*	334±7	328±44	318±8	368±26
2-heptanol	10.2±0.7	11.2±1.6	10.2±2.3	8.7±1.0
1-hexanol	1496±312	1629±458	1661±293	1434±94
(E)-3-hexen-1-ol	86.4±22.9	109.1±46.3	91.2±21.9	74.1±24.5
(Z)-3-hexen-1-ol	16.7±1.5	21.6±3.4	20.3±2.3	20.3±1.0
(E)-2-hexen-1-ol	11.7±0.2c	11.8±0.2	13.1±0.5d	12.0±0.6
1-octen-3-ol	4.29±0.89	4.02±1.29c	2.53±0.66	1.69±0.16d
benzyl alcohol	328±81	394±60	381±25	373±8
phenyl alcohol*	36.8±1.6c	41.1±2.3	33.0±1.7ad	39.1±1.1b

Table 4c. Concentration of terpenes, lactones, acids, and methoxypyrazines of wines with irrigation treatment (μg/l).

Compounds	D+	D-	W+	W-
terpene				
linalool	4.49±0.41	4.99±1.35	3.61±0.84	4.28±1.65
α-terpinol	1.40±0.28	2.04±0.42	1.30±0.25	1.22±0.49
citronellol	10.1±1.2	12.3±3.9	9.5±0.4	10.4±0.9
nerol	4.31±0.97a	2.51±0.57b	4.13±0.81	3.25±0.66
β-damascenone	4.57±0.44	5.96±2.34	3.81±1.06	4.11±0.33
geraniol	28.5±2.5	32.5±11.2	27.5±2.3	27.7±3.9
β-ionone	0.33±0.04	0.32±0.08	0.40±0.12	0.30±0.03
lactone				
γ-decalactone	6.44±1.24ac	2.35±0.56b	2.56±0.77d	2.13±0.23
δ-undecalactone	1.91±0.06ac	1.17±0.34b	1.02±0.36d	0.88±0.20
acid				
hexanoic acid	609±9	580±20c	614±30	633±26d
octanoic acid	857±45	797±32	896±54	894±69
decanoic acid	91.8±2.3	94.3±11.7	103.0±17.8	104.7±9.1
methoxypyrazine				
IPMP**	1.05±0.09	0.95±0.13	1.02±0.15A	1.15±0.10B
SBMP**	18.3±3.7	17.3±0.9	14.8±1.7	18.0±4.9
IBMP**	1.77±0.08	1.97±0.23	1.48±0.03	1.73±0.15

Table 4d. Concentration of bound form C13-norisoprenoids of wines with irrigation treatment (µg/l).

Compounds	D+	D-	W+	W-
vitispirane A #	7.77±0.32c	8.00±0.62C	6.24±0.38Ad	9.41±0.48BD
vitispirane B #	6.16±0.20	6.35±0.79	5.25±0.54A	7.61±0.20B
TDN	6.72±0.28a	8.77±0.70b	6.17±0.22A	8.17±0.17B
β-damascenone	11.97±0.94	12.23±0.94	11.17±1.08	10.91±0.73
β-ionone	0.41±0.06	0.41±0.06	0.45±0.05	0.41±0.02

Notes for Tables 4a to 4d: * = mg/l; ** = ng/l; # = β-damascenone equivalence; ND = not detected; IPMP = 3-isopropyl-2-methoxypyrazine; SBMP = sec-butyl-methoxypyrazine; IBMP = 3-isobutyl-2-methoxy-pyrazine; TDN = 1,1,6,-trimethyl-1,2-dihydronapthalene. Different letters represent significantly ($P < 0.05$) different in means (n = 3).

REFERENCES CITED

Al Rwahnih M, Dave A, Anderson MM, Rowhani A, Uyemoto JK, Sudarshana MR. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California, *Phytopathology* 103: 1069-76.

Bahder BW, Zalom FG, Jayanth M, Sudarshana MR. 2016. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* as a vector of grapevine red blotch-associated virus. *Phytopathology* 106: 1223-30.

Blanco-Ulate B, Hopfer H, Figueroa-Balderas R, Ye Z, Rivero RM, Albacete A, Perez-Alfocea F, Koyama R, Anderson MM, Smith RJ, Ebeler SE, Cantu D. 2017. Red blotch disease alters grape berry development and metabolism by interfering with the transcriptional and hormonal regulation of ripening, *Journal of Experimental Botany* 68: 1225-38.

Cieniewicz EJ, Pethybridge SJ, Gorny A, Madden LV, McLane H, Perry KL, Fuchs M. 2017. Spatiotemporal spread of grapevine red blotch-associated virus in a California vineyard. *Virus Research* 241: 156-62.

Cieniewicz EJ, Pethybridge SJ, Loeb G, Perry K, Fuchs M. 2018. Insights into the ecology of grapevine red blotch virus in a diseased vineyard. *Phytopathology* 108: 94-102.

Eridon SS. 2017. Assessing the effect of different percentages of red blotch affected fruit on wine composition for Cabernet Sauvignon. University of California, Davis.

Krenz B, Thompson JR, McLane HL, Fuchs M, Perry KL. 2014. Grapevine red blotch-associated virus is widespread in the United States. *Phytopathology* 104: 1232-40.

Lim S, Igori D, Zhao F, Moon JS, Cho IS, Choi GS. 2016. First report of grapevine red blotch-associated virus on grapevine in Korea. *Plant Disease* 100: 1957-57.

Poojari S, Alabi OJ, Fofanov VY, Naidu RA. 2013. A leafhopper-transmissible DNA virus with novel evolutionary lineage in the family geminiviridae implicated in grapevine redleaf disease by next-generation sequencing, *PLOS ONE* 8: e64194.

Ricketts KD, Gómez MI, Fuchs MF, Martinson TE, Smith RJ, Cooper ML, Moyer MM, Wise A. 2017. Mitigating the economic impact of grapevine red blotch: Optimizing disease management strategies in U.S. vineyards, *American Journal of Enology and Viticulture* 68: 127-35.

Tardaguila J, Martinez de Toda F, Poni S, Diago MP. 2010. Impact of early leaf removal on yield and fruit and wine composition of *Vitis vinifera* L. Graciano and Carignan. *American Journal of Enology and Viticulture* 61: 372-81.

Xiao H, Shabanian M, Moore C, Li C, Meng B. 2018. Survey for major viruses in commercial *Vitis vinifera* wine grapes in Ontario. *Virology Journal* 15: 127.

FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board (for July 2019 thru June 2021) and the American Vineyard Foundation (for July 2018 through June 2019.

STRUCTURE-FUNCTION STUDIES ON *GRAPEVINE RED BLOTCH VIRUS* TO ELUCIDATE DISEASE ETIOLOGY

Project Leader: Christopher D. Rock | Department of Biological Sciences | Texas Tech University | Lubbock, TX 79409 | chris.rock@ttu.edu
Co-Project Leader: Sunitha Sukumaran | Department of Biological Sciences | Texas Tech University | Lubbock, TX 79409 | sunitha.sukumaran@ttu.edu
Cooperator: Rhonda J. Smith | Cooperative Extension | University of California | Santa Rosa, CA 95403 | rhsmith@ucanr.edu
Cooperator: Achala KC | Extension Service | Oregon State University | Central Point, OR 97502 | achala.kc@oregonstate.edu
Cooperator: David M. Tricoli | Plant Transformation Facility | University of California | Davis, CA 95616 | dmtricoli@ucdavis.edu

Reporting Period: The results reported here are from work conducted July 1, 2019 to October 2019**.**

ABSTRACT
Grapevine red blotch virus (GRBV) is a serious threat to North American vineyards that the Pierce's Disease and Glassy-winged Sharpshooter Board is addressing by investing in applied research focused on vectors, epidemiology, ecology, and field transmission. An understanding of the molecular mechanisms evolved by GRBV to mount successful infection is essential to developing resistance strategies against the virus. Consistent with geminiviruses, open reading frame (ORF) predictions confirm that transcription of six GRBV genes is bidirectional, but experimental elucidation of gene function is lacking. RNA silencing has evolved as a major host defense mechanism against the invasive pathogens. The presence of a robust viral counter-defense machinery is underscored by the ubiquitous presence of one or more silencing suppressor proteins in plant viral genomes. The arms race between silencing and silencing suppression results in resistance or susceptibility to the pathogen. We are taking a comprehensive approach by cloning all the viral ORFs from GRBV-infected vines to test for potential GRBV silencing suppressor proteins. We have identified two silencing suppressor proteins (C2 and V2) encoded by GRBV. We have made recombinant hairpin vectors targeting C2 and V2, which will be used to generate stably transformed transgenic grapevine plants which will be tested for GRBV resistance. To identify the host targets of the viral suppressor proteins we have cloned the suppressor protein genes in expression vector pMAL-c5X and yeast two-hybrid bait vector pGBTK7-BD.

LAYPERSON SUMMARY
The etiology of grapevine red blotch virus (GRBV) on the host plant is completely unknown. We submit that understanding the viral gene functions and effects on host physiology and molecular mechanisms is necessary to effectively combat red blotch disease. Understanding how GRBV causes disease can present cogent strategies for mitigating this threat to a multibillion-dollar industry. Degradation of viral transcripts (RNA silencing) has evolved as a major host defense mechanism against invasive pathogens. Viruses counter the plant defense mechanisms by evolving one or more "silencing suppressor" proteins. The efficacy of host silencing versus viral silencing suppression results in resistance/tolerance or susceptibility to the pathogen. The

anthocyanin levels in dicot leaves are under a tightly controlled regulatory mechanism involving endogenous small RNAs (sRNAs). The red patches in the interstitial lamina of GRBV-infected leaves and in petioles and veins are caused by deranged anthocyanin accumulation, a well-known stress response in plants. We hypothesize the viral suppressor protein(s) of GRBV interfere with the anthocyanin regulatory pathways and result in uncontrolled anthocyanin accumulation in vegetative tissues, thus serving as a visual cue for feeding by the assumed arthropod vector capable of transmitting the viruses. Thus, identifying the GRBV viral suppressor proteins and host targets is an essential objective for developing disease resistance strategies involving engineering and/or breeding for virus resistance going forward.

INTRODUCTION

Geminiviruses are single-stranded (ss) DNA viruses that cause major losses to many crops throughout the world (1-3). *Geminiviridae* constitutes the second largest family of plant viruses. Geminiviruses are characterized by small, circular, ssDNA genomes encapsidated in twinned (hence, the name *Gemini*) icosahedral particles (4-6). They are vector-transmissible and infect both monocotyledonous and dicotyledonous plants (7). The genomes are either monopartite or bipartite with circular DNA molecules of 2.5 to 3 kilobases. Geminiviruses possess a highly conserved common region (CR) of ~200 nucleotides containing an inverted repeat that forms a hairpin loop with an invariant 9-nt 5'-TAATATT↓AC-3' that acts as the origin of virion (V) strand DNA replication. The viral gene products are required for its replication and transmission.

Grapevine red blotch virus (GRBV) is a monopartite, grapevine-infecting grablovirus causing grapevine red blotch disease and was first observed in California in 2008 (8). Bahder et al. (9) identified the three-cornered alfalfa hopper (*Spissistilus festinus*) as the candidate vector that transmits GRBV under laboratory conditions. Disease symptoms manifest as red patches in the middle of the grapevine leaf and in veins and petioles, which coalesce at the end of the growing season (10). GRBV infection results in delayed and uneven berry ripening, higher titratable acids, reduced sugar, and reduced anthocyanin content in the berry (11), impairing fruit quality for both table grape and wine industries (12).

Consistent with geminiviruses, GRBV possesses the conserved nonanucletide sequence and transcription is bidirectional (10). GRBV encodes three open reading frames (ORFs) in the virion strand (*V1*, *V2*, and *V3*) and three in the complementary strand (*C1*, *C2*, and *C3*; **Figure 1**). Similar to mastrevirus (a monopartite geminivirus), GRBV complementary-sense ORF *C1* encodes replication-associated protein (RepA). Another spliced transcript encompassing the *C1* and *C2* ORFs encodes the replication protein (Rep) (10, 13-15). GRBV virion-sense strand ORFs *V2* and *V3* are predicted to encode movement proteins, whereas *V1* ORF encodes coat protein.

The functions of the predicted GRBV ORFs are yet to be elucidated experimentally. Understanding the molecular mechanisms by which the virus mounts a successful infection is fundamental and essential to developing cogent engineered resistance strategies. A practical issue is that the few proteins encoded by geminiviruses are multifunctional and likely modulate several host regulatory genes, a mechanism uniquely evolved by the viruses to balance the genome size-constraint emplaced by the capsid. A comprehensive "omics" profiling experiment on berry development and select metabolite and enzyme quantitations in GRBV-infected grapes from two different vineyards suggested several host regulatory pathways, in particular

phenylpropanoids, are impacted by the virus (28). GRBV infection results in deranged expression of host post-transcriptional machinery, transcription factors, and several hormone biosynthesis and response pathways. Post-transcriptional gene silencing (PTGS) processes involving microRNAs (miRNAs) and small interfering RNAs (siRNAs) are known to regulate host immune responses to viruses and microbes, as well as normal plant development and hormonal signaling (29, 30).

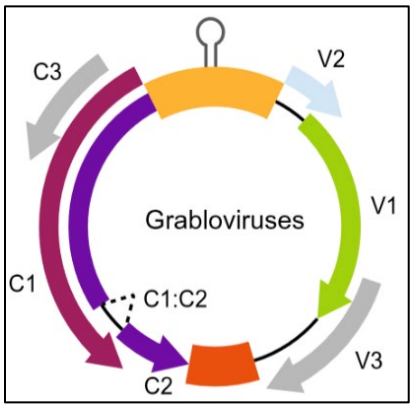


Figure 1. Genome organization of GRBV, previously called grapevine red blotch-associated virus (GRBaV).

PTGS has evolved as a major host defense mechanism against invasive pathogens, including viruses. The presence of a robust viral counter-defense mechanism is underscored by the ubiquitous presence of one or more silencing suppressor proteins in the genomes of many plant viruses. The "arms race" between host silencing of pathogen transcripts and silencing suppression by pathogen gene products results in resistance or susceptibility to the pathogen. Numerous geminiviruses encode silencing suppressor proteins that target PTGS, transcriptional gene silencing (TGS), and cellular regulatory genes (**Table 1**).

Transgenic approaches involving overexpression of viral coat protein has been very successful in developing commercially produced papaya ring spot virus (PRSV) resistant papaya (31, 32), potato virus X and Y resistant potato (33), and squash mosaic virus resistant squash (34). The only successful report of engineering geminiviral resistance using coat protein was in tomato against TYLCV (35). Transgenes of RepA protein have been successfully deployed to generate geminivirus resistance (36-38). Mutants of many geminiviral genes have been evaluated for *trans*-dominant negative inhibition of geminivirus replication and movement (39-42). The major limitation of introducing geminivirus sequences into transgenic plants was that in several cases the transgenic protein facilitated viral replication (43, 44). The expression of *gene5* protein (g5p) from *Escherichia coli* phage M13 (45) and transgenic expression of *Agrobacterium Vir*E2 (46-48), an ssDNA binding protein essential for virulence, are resistance strategies deployed against a broad spectrum of DNA viruses. Thus, the strategy of expressing non-viral proteins overcomes the limitations of functional/mutant viral proteins by not contributing to viral replication and can confer broad-spectrum resistance to other geminiviruses.

Table 1. Suppressor proteins characterized in geminiviruses and their plant targets.

Virus*	Suppressor	Suppressing PTGS	Suppres-sing TGS	Cellular Pathways
MYMV	AC2	Upregulate host suppressor protein WEL1 (16)		
TGMV BCTV	AL2 L2	Inactivate adenosine kinase (17, 18)		Inactivate a serine-threonine kinase SnRK1 (19)
BSCTV	C2	Stabilize S-adenosyl methionine decarboxylase1 (SAMDC1) (20)		
TGMV CaLCuV BCTV	AL2 AL2 L2		Inactivate adenosine kinase and stabilize SAMDC1 (21) Inhibit histone Me-transferase SUVH4/KYP (22)	
TGMV SCTV	AL2 C2			Elevation of cellular cytokinin levels (23)
TYLCSV	C2			Interact with CSN5 and inhibit jasmonate signaling (24)
ACMV	AC4	Binds ss miRNA (25)		
WDV	Rep	Binds ss-and duplexed 21 and 24 nt siRNAs (26)		
TYLCV	V2		Compete *NbMET1* for binding to histone deacetylase6 (27)	

*Acronyms
ACMV = African cassava mosaic virus; BCTV = beet curly top virus; BSCTV = beet severe curly top virus; CaLCuV = cabbage leaf curl virus; MYMV = mungbean yellow mosaic virus; SCTV = spinach curly top virus; TGMV = tomato golden mosaic virus; TYLCSV = tomato yellow leaf curl Sardinia virus; TYLCV = tomato yellow leaf curl virus; WDV = wheat dwarf virus.

Early reports of engineered geminivirus resistance, which serendipitously involved host RNA silencing before its significance was understood, were by expressing sense and antisense viral RNAs in plants. Expression of *AC1* in antisense orientation conferred resistance against TGMV, BGMV, and TYLCV (49-52), whereas expression of various cotton leaf curl virus genes in antisense orientations in tobacco (*Nicotiana tabacum*) conferred resistance (53). Transient expression of the hpRNA gene of the MYMV bidirectional promoter (54), ACMV-[CM] *Rep* siRNA (55), and MSV *Rep* hpRNA gene (56) conferred resistance against the respective viruses. The hp*AC1*/*C1* genes conferred resistance against TYLCV in tobacco (57), BGMV in common bean (58, 59), and ACMV in cassava (60). Transgenic expression of hpRNA from the bidirectional promoter of ACMV in cassava (61) and TYLCV *CP* promoter in tomato (62) conferred resistance against the respective viruses. Silencing the suppressor protein by transgenic expression of hp*AC1* and hp*AC4* of ToLCV in tomato (63), hp*AC4* (64), and hp*AC2* of MYMV (65) have proven to be a very effective strategy in conferring resistance.

Previous work on the model plant *Arabidopsis* in the project leader's lab showed that altered source-sink distributions of sucrose and the stress hormone abscisic acid (ABA) (66) interact to regulate anthocyanin accumulation via miR828, *trans-acting small-interfering locus4 (TAS4),* and their target MYeloBlastosis viral oncogene-like (v-MYB) transcription factors, i.e., Vvi-MYBA6/7 and close homologues targeted by miR828 in grapevine (67, 68). We recently characterized (69) the conserved autoregulatory loop involving miR828 and *TAS4* that is induced in grape by ultraviolet light, similar to the ABA and sugar stress associated induction described in *Arabidopsis* (66). The loop is hypothesized to fine tune homeostatic repression of anthocyanin biosynthesis by RNA interference silencing of target *MYB* transcription factors. The recently published transcriptome profiling study of GRBV-infected host berries identified significant repression of rate-limiting ABA biosynthesis loci *NCED2/3* [first described by the project leader (70)] in infected berries (28).

Our working model is that GRBV infection interferes with the normal PTGS pathways of the host by the activity of viral-encoded suppressor proteins. miRNAs/tasi-RNAs/phasi-RNAs regulate a large array of host gene expression at the post-transcriptional and transcriptional levels. Viruses utilize plant miRNAs to facilitate pathogenesis, and plants have co-opted miRNAs for innate immunity (71-74). Their collective loss in virus-infected tissues that results in susceptibility (75, 76) demonstrates their functions as master regulators targeted by pathogens. Broader roles for plant small RNAs (sRNAs) in evolutionary adaptations (77, 78) may include virus vector feeding processes and olfactory preferences. We hypothesize the red blotch phenomena observed in GRBV-infected grape leaves is a consequence of viral suppressor proteins targeting the miR828/*TAS4/MYBA5/6/7* autoregulatory loop (66, 69) which fine tunes anthocyanin levels by a "rheostat" feedback (69).

A recent paper reported GRBV effects on berry development (28). **Table 2** provides preliminary evidence drawn from this publicly available berry transcriptome data which supports our model. As per our hypothesis, we observe a near-statistically significant downregulation of Vvi-*TAS*4c at veraison and post-veraison in GRBV-infected berries, indicating the miR828-*TAS4-MYB* pathway is a specific target of GRBV. This is supported by the strong upregulation of *MYBA6* at harvest, the target of a deeply conserved *TAS4c* tasi-RNA 3'D4(-) along with several other *MYB*s (68, 69) shown to function in the phenylpropanoid/flavonol pathway and targeted by miR828. Interestingly, we observe upregulation of *AGO, DICER* and *SUPPRESSOR_OF_GENE_ SILENCING3 (SGS3)* transcripts, all major effectors of the PTGS machinery and themselves subject to PTGS and spawning of amplified phasi-RNAs (79, 80) by unknown mechanisms. It will be very interesting to determine if transitivity of these loci and *MYBA5/6/7* is deranged by GRBV. One reason is because the "21_1 mechanism" of transitivity (81) in play with TAS4-3'D4 (-) and target *MYBA5/6/7* is novel and its significance is not understood, unlike the known "21_2" and "22_1 hit" mechanisms (81). We hypothesize a repression of silencing machinery upon virus infection, but the evidence is that the host is compensating by overexpressing PTGS effector pathways. These preliminary results underscore the need to perform transcriptome and sRNA analyses from different tissues of field-infected grapevines.

Table 2. Analysis of publicly available transcriptome data^ for GRBV-infected berries across development.

developmental stage:			pre-veraison		veraison		post-veraison		harvest	
target; sRNA effector	gene ID	Phase Score	beta ~LFC	pval	beta~LFC	pval	beta~LFC	pval	beta~LFC	pval
GRBaV genome	JQ901105.2	n.d.	6.26	1.91E-15	NA	NA	NA	NA	6.76	3.47E-32
Vvi-TAS4c; miR828	chr1:2961251:2961747	3375	NA	NA	-1.01	0.13	-1.01	0.13	0.38	0.53
AGO1a; miR168/530	VIT_17s0053g00680	n.d.	0.06	0.55	0.17	0.04	0.17	0.04	0.16	0.05
AGO1b; miR168/530	VIT_19s0014g01840	n.d.	0.26	0.47	0.43	0.04	0.43	0.04	0.08	0.75
MYBA6, TAS4	VIT_14s0006g01290	22.2	NA	NA	NA	NA	NA	NA	1.25	0.09
MYB^{PAL1}; miR828	VIT_00s0341g00050	476	0.52	0.01	0.12	0.39	0.12	0.39	0.13	0.31
MYB; miR828	VIT_17s0000g08480	1330	0.62	0.09	0.33	0.35	0.33	0.35	NA	NA
MYB; miR828	VIT_04s0079g00410	24.6	0.39	0.01	0.17	0.04	0.17	0.04	-0.06	0.46
AGO2a; miR403	VIT_10s0042g01180	50	0.61	0.02	0.36	0.07	0.36	0.07	0.82	0.02
AGO2b; miR403	VIT_10s0042g01200	n.d.	0.04	0.81	0.03	0.81	0.03	0.81	-0.16	0.29
DCL2; unknown	VIT_04s0023g00920	33.8	0.39	0.25	0.47	0.03	0.47	0.03	0.11	0.57
SGS3; unknown	VIT_07s0130g00190	177.4	0.04	0.69	0.23	0.01	0.23	0.01	0.16	0.06
DCL1; miR162	VIT_15s0048g02380	n.d.	-0.05	0.62	0.05	0.54	0.05	0.54	-0.21	0.15

^ Oakville vineyard dataset (ref. 25) analysed by kallisto/sleuth.

OBJECTIVES

1. Validate the identified candidate GRBV suppressor proteins C2 and V2.
2. Elucidate by a systems approach the molecular mechanisms by which GRBV causes symptoms from genome-wide analyses of host miRNAs, *trans*-acting small interfering (tasi-) RNAs, phased-tasi-RNAs (phasi-RNAs), and effects on host target mRNAs by RNA sequencing (RNA-seq) and degradome analyses of (a) field samples, and (b) tobacco genotypes over-expressing GRBV C2 and V2 suppressor proteins and an effector of anthocyanin, *AtMYB90/PRODUCTION_ OF_ ANTHOCYANIN2(PAP2*).
3. Identify the host grapevine targets of GRBV suppressor proteins C2 and V2.
4. Initiate transgenic grapevine experiments to test disease resistance of transgenic grape expressing hairpin silencers directed to GRBV suppressor protein transcripts.

RESULTS AND DISCUSSION

Objective 1. Validate the Identified Candidate GRBV Suppressor Proteins C2 and V2

GRBV C2 and V2 proteins were identified as candidate suppressor proteins, and methods [transfer DNA (T-DNA) binary effector constructs pCAM-C1-gus and pCAM-V2-gus] and evidence presented in the final report for CDFA agreement number 18-0296-000-SA. This preliminary result has been repeated (data not shown), providing compelling evidence for *C2* and *V2* functioning as GRBV silencing suppressor proteins. RNA and sRNA blot analysis of agroinfiltrated leaf tissues using a green fluorescent protein (*gfp*) probe is in progress. The presence of full-length *GFP* transcript and absence of *GFP* siRNAs in C2 and V2 test samples (not shown), like previously demonstrated in the final report for HcPro positive controls, will independently validate suppression of PTGS by C2 and V2.

Objective 2. Elucidate by a Systems Approach the Molecular Mechanisms by Which GRBV Causes Symptoms

Field Samples. We completed deep sequencing of GRBV-infected and control healthy grapevine leaf sRNA libraries and mRNA transcriptome libraries. Genome-wide systems analysis of datasets can reveal the specific host genes in vegetative tissues deranged by the pathogen and provide leads for understanding the underlying mechanisms, e.g., specific miRNA effectors of host gene regulatory networks controlling plant immunity. We used GRBV-infected and control samples from the Calle Contento Vineyard (cultivar Merlot) in Temecula, California, and from Pinot Noir cultivars collected in Jacksonville, Oregon in July 2018 for sRNA library preparation. As we were unable to obtain high quality RNA [RNA integrity number (RIN) value: 8] from the Calle Contento vineyard samples, we prepared six RNA libraries from polymerase chain reaction (PCR)-confirmed Pinot Noir infected but asymptomatic and uninfected cultivars collected in Jacksonville, Oregon. Grape RNA-seq libraries were filtered for adapter presence. The reads were then sequentially mapped using Bowtie against the grape non-coding RNA (ncRNA) in the Rfam database to remove ncRNAs in the data, and were also mapped against the GRBV genome (**Table 3**). The grape filtered reads of RNA-seq were mapped using kallisto to the *Vitis vinifera* 12X complementary DNA (cDNA) sequence. The differential expression analysis of target genes was done using DeSeq2. We obtained 27 differentially expressed genes (2 up, 25 down) with expression above a threshold (> 30 reads mapped to a transcript per library on average), and multiple-testing Bonferroni-Hochberg adjusted $p < 0.05$ for statistical significance (**Table 4**). PCR-positive infected asymptomatic samples used for RNA-seq library prep had very few reads when mapped against the GRBV genome (**Table 3**). This might be the cause for the very few significantly differentially expressed genes we obtained (**Table 4**). We will repeat RNA-seq library prep and analysis with the symptomatic samples we collected in 2019.

Table 3. Sequentially mapped RNA-seq reads using Bowtie against the grape ncRNA in the Rfam database.

Sample	Condition	Total Reads	rRNA cleaned	snRNA cleaned	Intron, ribozyme, riboswitch etc. cleaned	tRNA cleaned	Reads mapped to GRBV genome
47-33	Control	57834077	57309876 (99.09%)	57163193 (99.74%)	48752860 (85.29%)	48752860	0.12%
47-68	Control	47754306	47552212 (99.58%)	47539744 (99.97%)	45903110 (96.56%)	45903110	0.0%
46-3	Control	68751746	68339073(99.40%)	68241262 (99.86%)	60918778 (89.27%)	60918778	0.06%
47-41	Infected	53684675	53507593 (99.67%)	53498138 (99.98%)	51360661 (96.00%)	51360661	0.06%
46-22	Infected	51217871	51046772 (99.67%)	50965171 (99.84%)	49299402 (96.73%)	49299402	0.01%
47-9	Infected	28678554	28470915 (99.28%)	28457967 (99.95%)	26601010 (93.47%)	26601010	0.02%

We prepared four sRNA libraries from infected and uninfected samples from the Calle Contento Vineyard (cultivar Merlot) in Temecula, California and five libraries from Pinot Noir infected and uninfected cultivars collected in Jacksonville, Oregon. The quality assessment of the raw reads was done by FastQCv0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and adaptor sequences were trimmed using fastx_clipper (http://hannonlab.cshl.edu/fastx_ toolkit/index.html). Reads longer than 18 nucleotides (nt) were retained and were sequentially

mapped to Rfam13.0 grapevine non-*MIRNA* sequences using bowtie to remove reads matching the same in respective libraries made from grapevine (**Table 5)** samples. ShortStack (version 3.8.5) was used to map respective sRNA-seq datasets to the reference *V. vinifera* 12X genome sequence. The reads were also mapped against the GRBV genome.

Table 4. DESeq2 imputed differential expression mRNAs in GRBV infected vs. control samples from Oregon.

Target_ID	log2Fold Change	Padj	Gene Annotation
VIT_13s0106g00200	3.16	0.000004	Unknown
VIT_13s0106g00080	2.83	0.000004	Ankyrin repeat
VIT_02s0025g02790	-1.13	0.008	Starch synthase
VIT_18s0001g08320	-1.36	0.014	IMP dehydrogenase/GMP reductase
VIT_18s0001g09910	-1.37	0.01	L-asparaginase
VIT_07s0129g01050	-1.69	0.0008	Myb domain protein 73
VIT_02s0012g01570	-1.83	0.02	Cinnamoyl-CoA reductase
VIT_06s0004g04700	-1.90	0.004	Outer envelope protein 16
VIT_01s0011g02960	-1.98	0.02	Leucoanthocyanidin reductase 1
VIT_04s0023g03370	-2.03	0.004	Flavonone- 3-hydroxylase
VIT_04s0008g01120	-2.11	0.006	Glutaredoxin
VIT_03s0017g01210	-2.14	0.0008	Phosphate-induced protein 1
VIT_03s0038g03860	-2.26	0.0003	Phosphate-induced protein 1
VIT_05s0094g01590	-2.34	0.006	Kelch repeat-containing F-box
VIT_02s0025g03260	-2.53	0.01	Aquaporin NIP5;1
VIT_05s0136g00260	-2.54	2.1E-10	Chalcone synthase
VIT_03s0088g00260	-2.54	0.004	Serine carboxypeptidase S10
VIT_02s0025g04720	-2.56	0.0008	Leucoanthocyanidin dioxgenase
VIT_06s0061g00730	-2.65	0.00001	Aquaporin GAMMA-TIP3/TIP1;3
VIT_03s0091g01290	-2.89	0.002	Serine carboxypeptidase S10
VIT_17s0000g08960	-2.96	0.0001	Raffinose synthase
VIT_05s0020g03930	-3.04	0.0001	Sulfate transporter 3.1 (AST12)
VIT_17s0119g00080	-3.04	0.001	Organic cation transport OCT1
VIT_18s0001g09400	-3.42	0.004	Cytochrome b5 DIF-F
VIT_03s0091g00040	-3.58	0.00001	Limonoid UDP-glucosyltransferase
VIT_04s0008g04050	-3.84	0.004	RD22
VIT_05s0077g00770	-4.85	8.8E-09	Unknown

Similar to RNA-seq libraries (**Table 3**), the infected samples from Oregon were asymptomatic and control samples had several reads that mapped to the GRBV genome (**Table 5**). Hence only Temecula, California libraries were taken for downstream DESeq analysis. We obtained 104 differentially expressed sRNAs (95 up, 9 down) after multiple-testing Bonferroni-Hochberg adjusted $p < 0.05$ for statistical significance (**Table 6**). We observed significant upregulation of vvi-MIR399i and homolog of ath-miR8175 in infected samples while vvi-MIR166h, vvi-156f, vvi-MIR396c, vvi-MI398a, and vvi-MIR3633a were downregulated. Several sRNA reads corresponded to the transcripts involved in anthocyanin biosynthesis pathway (**Table 6**). We will repeat sRNA-seq library prep and analysis with the symptomatic samples we collected in 2019.

Table 5. Sequentially mapped sRNA-seq reads using Bowtie against the grape ncRNA in the Rfam database.

Sample	Condition	Total Reads	tRNA cleaned	rRNA cleaned	snRNA cleaned	Intron, ribozyme, riboswitch etc. cleaned	Reads mapped to GRBV genome
C2	Control	17483878	16951677 (96.96%)	12703398 (74.94%)	12332068 (97.08%)	12318555 (99.89%)	43 (0.00%)
PD3	Infected	13637586	13311618 (97.61%)	9709177 (72.94%)	9536072 (98.22%)	9528101 (99.92%)	13 (0.00%)
47-68	Control	17812652	17260243 (96.90%)	11767896 (68.18%)	11396866 (96.85%)	11377002 (99.83%)	1299 (0.01%)
C3	Control	20008110	19563792 (97.78%)	14286629 (73.03%)	13979781 (97.85%)	13966455 (99.90%)	78 (0.00%)
46-3	Control	23275341	22643431 (97.29%)	16107062 (71.13%)	15352738 (95.32%)	15332821 (99.87%)	64754 (0.42%)
47-9	Infected	18909351	18536835 (98.03%)	12535797 (67.63%)	12148456 (96.91%)	12133839 (99.88%)	51577 (0.43%)
46-22	Infected	25127437	24692550 (98.27%)	15668865 (63.46%)	15221645 (97.15%)	15208863 (99.92%)	56545 (0.37%)
47-33	Control	37562970	37313031 (99.33%)	22883232 (61.33%)	21261140 (92.91%)	21237891 (99.89%)	665805 (3.13%)
PD8	Infected	8540662	8195148 (95.95%)	5959639 (72.72%)	5731517 (96.17%)	5724536 (99.88%)	30 (0.00%)

Figure 2. Axenically established control and transgenic tobacco plants for super-transformation.

Table 6. DESeq2 imputed differential expression of selected sRNAs in GRBV-infected vs. control leaf samples from Temecula, California.

	log2Fold Change	pvalue	padj	miRNA		mRNA
Cluster_277231	9.4	5.4E-07	0.001		VIT_06s0004g02620	Phenylalanine ammonia-lyase
Cluster_367876	-6.3	2.6E-06	0.002	vvi-MIR166h		
Cluster_240796	7.5	4.4E-06	0.002		VIT_05s0136g00260	Chalcone synthase
Cluster_277235	8.4	1.2E-05	0.004		VIT_06s0004g02620	Phenylalanine ammonia-lyase
Cluster_282987	8.4	1.2E-05	0.004		VIT_06s0004g04650	Putative uncharacterized; Metallothionein-like protein 1
Cluster_486421	8.3	1.8E-05	0.005		VIT_10s0116g00760	GPT2
Cluster_940630	-7.5	2.4E-05	0.005		VIT_13s0101g00220	Putative uncharacterized
Cluster_198654	6.7	3.9E-05	0.007		VIT_04s0023g03370	Naringenin,2-oxoglutarate 3-dioxygenase
Cluster_442312	7.9	5.0E-05	0.008		VIT_09s0002g05710	ADP,ATP carrier protein
Cluster_949593	7.7	7.6E-05	0.012		VIT_18s0001g12800	Dihydroflavonol-4-reductase
Cluster_819730	-7.4	9.8E-05	0.013		VIT_13s0019g02630	Photosystem Q(B)
Cluster_65933	7.9	1.2E-04	0.014	vvi-MIR399i		
Cluster_1134142	7.5	1.2E-04	0.014		VIT_00s0480g00040.	PPO1_KFDV domain-containing protein
Cluster_953005	7.6	1.5E-04	0.017		VIT_18s0001g14310	Fe2OG dioxygenase domain-containing protein
Cluster_696167	7.5	1.8E-04	0.019		VIT_13s0156g00590	Receptor-like serine/threonine-protein kinase
Cluster_768263	-7.3	1.9E-04	0.019	vvi-miR156f		
Cluster_435602	7.4	2.0E-04	0.020		VIT_09s0002g02970	Putative uncharacterized
Cluster_881643	7.4	2.0E-04	0.020		VIT_17s0000g01280	WRKY-type DNA binding protein 1
Cluster_290627	5.9	2.3E-04	0.020		VIT_06s0004g08150	Trans-cinnamate 4-monooxygenase
Cluster_412132	7.3	2.3E-04	0.020		VIT_08s0007g01430	Usp domain-containing protein
Cluster_943186	7.4	2.3E-04	0.020		VIT_18s0001g09850	Transcription factor MYB44
Cluster_947193	4.9	2.8E-04	0.023		VIT_18s0001g11630	Allene oxide synthase 1, chloroplastic
Cluster_934231	7.2	3.2E-04	0.025		VIT_18s0001g06120	UDP-glycosyltransferase activity
Cluster_1133450	6.4	3.5E-04	0.026		VIT_00s0324g00060	Glycosyltransferase
Cluster_572593	6.4	4.4E-04	0.030		VIT_11s0052g00030	RNA-binding KH domain-containing protein PEPPER
Cluster_824966	6.4	4.4E-04	0.030		VIT_15s0046g03090	CAP10 domain-containing protein
Cluster_818880	7.0	5.1E-04	0.032		VIT_15s0048g02990	AAA domain-containing protein
Cluster_442308	6.9	6.3E-04	0.038		VIT_09s0002g05710	ADP,ATP carrier protein
Cluster_72078	6.9	8.2E-04	0.042		VIT_02s0012g00760	Haloacid dehalogenase-like hydrolase domain-containing protein Sgpp
Cluster_113124	6.8	7.7E-04	0.042		VIT_03s0063g01870	Cold-regulated 413 plasma membrane protein 2
Cluster_624929	6.8	7.8E-04	0.042		VIT_12s0034g02390	Putative uncharacterized
Cluster_763992	5.2	7.4E-04	0.042		VIT_14s0068g00920	Chalcone synthase
Cluster_1082478	6.9	8.1E-04	0.042		VIT_00s1372g00020	GTP-binding protein YPTM2
Cluster_831310	6.0	9.6E-04	0.046		VIT_16s0039g02040.	Coumaroyl-CoA ligase
Cluster_77639	6.7	9.9E-04	0.048		VIT_02s0012g02190.	Cellulose synthase-like protein D3
Cluster_66376	4.5	1.1E-03	0.049		VIT_02s0025g04720	Leucocyanidin oxygenase
Cluster_16513	6.7	1.1E-03	0.051		VIT_01s0137g00230	Ultraviolet-B receptor UVR8
Cluster_737269	6.6	1.4E-03	0.054	ath-miR8175-iso		
Cluster_162997	-4.0	1.1E-02	0.133	vvi-MIR396c		
Cluster_1783	-4.2	1.9E-02	0.183	vvi-MIR398a		
Cluster_892853	-3.0	2.8E-02	0.247	vvi-MIR3633a		

Tobacco Genotypes. As a proof of concept of our hypothesis that GRBV suppressor proteins target the *MIR828-TAS4-MYBA5/6/7* autoregulatory loop, we proposed to super-transform with GRBV suppressor protein C2- and V2-expressing T-DNA binary effector constructs pCAM-C1-gus and pCAM-V2-gus (prior completed Objective 1, described in the final report for CDFA agreement number 18-0296-000-SA), a bialaphos-resistant transgenic tobacco line that overexpresses the *Arabidopsis* target of *TAS4* siRNA: *AtMYB90/PRODUCTION_OF-ANTHOCYANIN_PIGMENT2* (82). Towards this we have established axenic tissue-cultured control, hemizygous, and homozygous transgenic plants (**Figure 2**) which will be super-transformed with constructs in hand by selection with kanamycin as the next step.

Objective 3. Identify the Host Grapevine Targets of GRBV Suppressor Proteins C2 and V2

To understand if the mechanism of silencing suppression is by binding miRNA/siRNA, we have proposed to purify the suppressor proteins C2 and V2 using the pMAL™ Protein Fusion & Purification System (New England Biolabs). Towards this we have PCR-amplified the GRBV C2 and V2 genes as a blunt end fragment in the 5' end and with *Sbf*I restriction site in the 3' end. The PCR product was digested with *Sbf*I and was cloned into the pMAL-c5X vector digested with *Xmn*I and *Sbf*I. The clones were confirmed by restriction digestion (**Figures 3a** and **3b**) and sequencing. We will retransform the clone into *E. coli* strain ER2523 (NEB express) for protein expression. The induced protein will be purified and will be used for ss- and dsDNA binding assays as the next step.

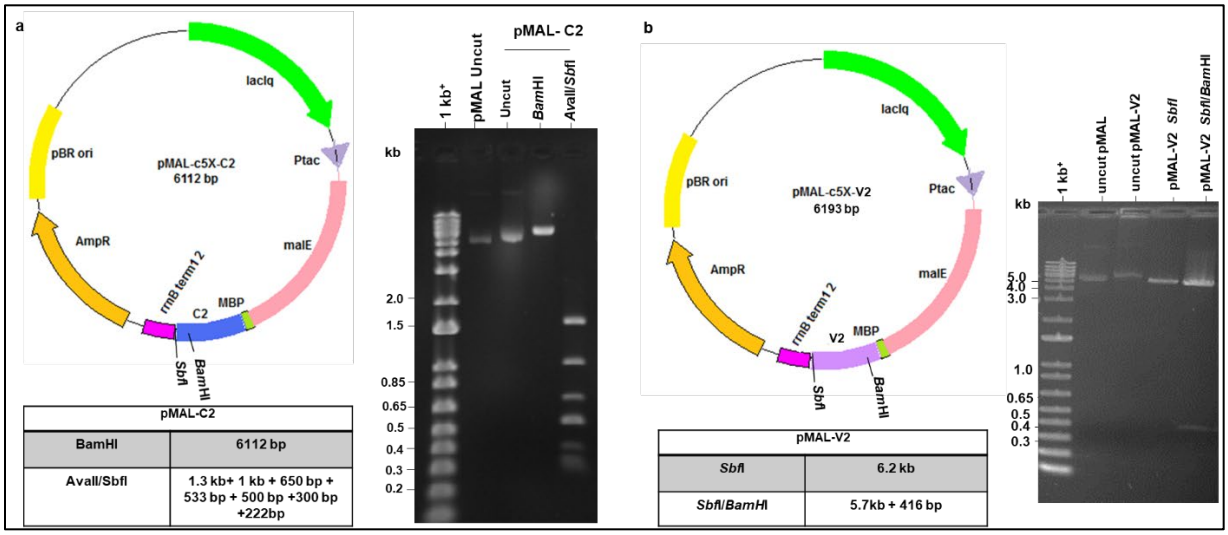


Figure 3. Restriction enzyme digestion. (a) pMAL-C2; (b) pMAL-V2.

We proposed yeast two-hybrid (Y2H) screen as an unbiased alternative approach to discover host proteins that bind physically to GRBV C2 and V2. Towards this objective suppressor proteins C2 and V2 were cloned in a bait vector pGBTK7-BD. GRBV C2 and V2 were PCR amplified with primers flanking *Nde*I and *Eco*RI restriction sites and were introduced into the corresponding sites of pGBTK7-BD vector. The clones were confirmed by restriction analysis (**Figures 4a** and **4b**) and sequencing. We are in the process of making a grape cDNA library

using the Mate & Plate library system (Takara). We will identify the targets of GRBV C2 and V2 in grape cDNA library by Y2H screening as the next step.

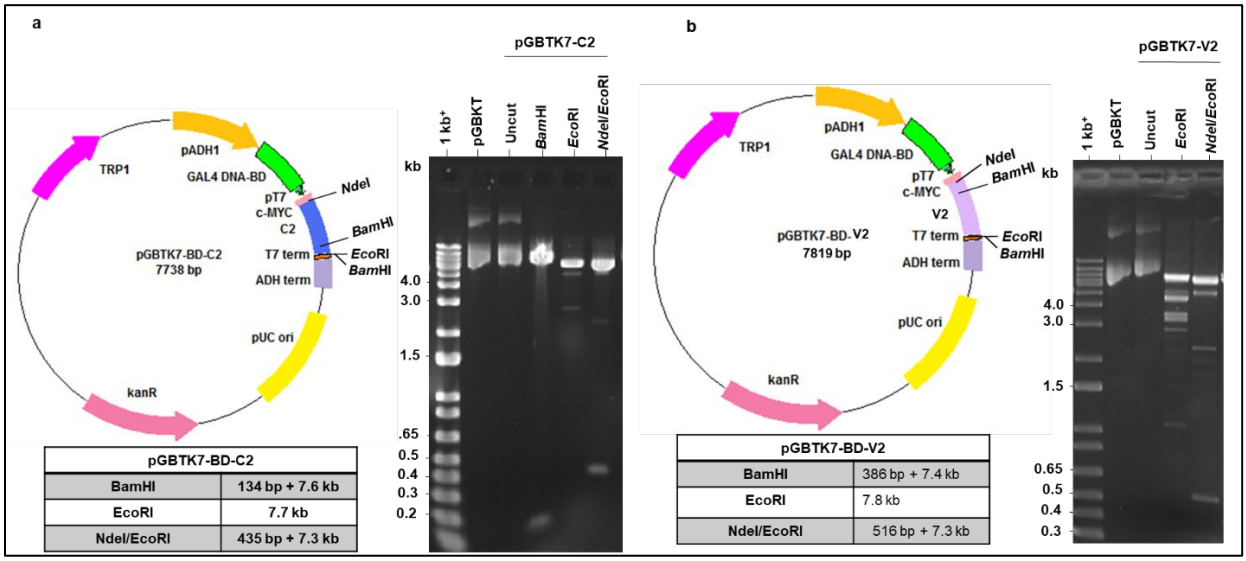


Figure 4. Restriction enzyme digestion. (a) pGBTK7-BD-C2; (b) pGBTK7-BD-V2 **EcoR*I enzyme digestion displayed star activity.

Objective 4. Initiate Transgenic Grapevine Experiments to Test Disease Resistance of Transgenic Grape Expressing Hairpin Silencers Directed to GRBV Suppressor Protein Transcripts

Several reports have demonstrated that PTGS of viral suppressor proteins is an effective strategy for engineering viral resistance. We proposed to construct hpRNA vectors targeting GRBV *C2* and *V2* genes. Towards this we PCR amplified *C2* and *V2* genes by introducing *Xho*I and *Kpn*I sites in the primers and cloned the digested PCR product in the corresponding sites of the pHANNIBAL vector (84) to obtain the sense orientation clone (pHANNIBAL-C2/ pHANNIBAL-V2) (**Figures 5a** and **5b**). The clones were confirmed by restriction digestion analysis.

To clone the antisense arm of the hairpin vector, we PCR amplified *C2* and *V2* with primers flanked by *Cla*I and *Xba*I restriction sites. The digested PCR products were cloned in the corresponding sites of pHANNIBAL-C2/pHANNIBAL-V2 to obtain the hpRNA vector pHANNIBAL-hpC2 or pHANNIBAL-hpV2. The clones were confirmed by restriction digestion analysis (**Figures 6a** and **6b**) and sequencing.

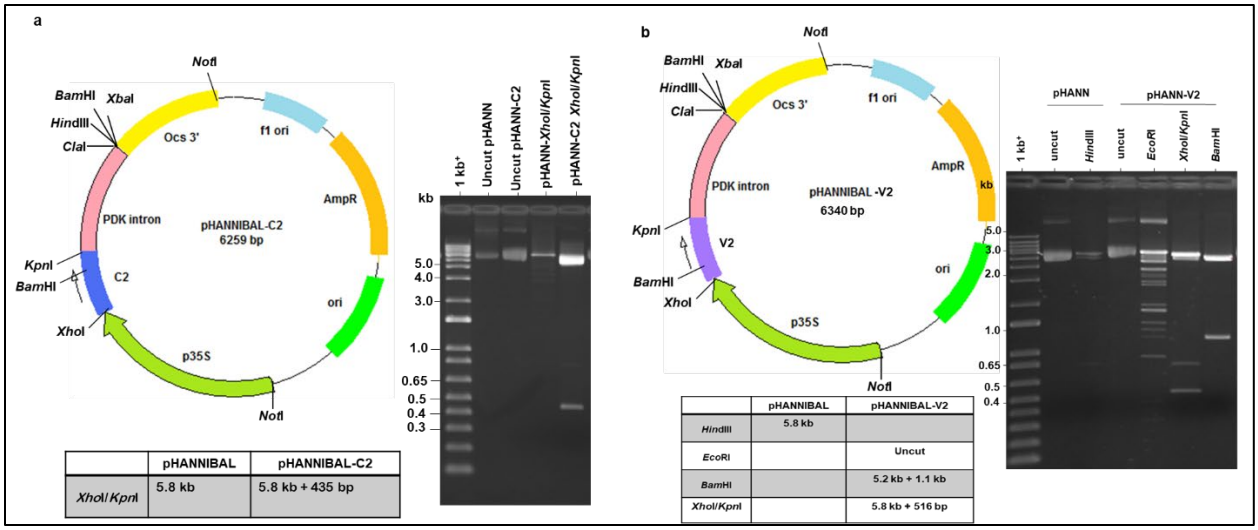


Figure 5. Restriction enzyme digestion. (a) pHANNIBAL-C2; (b) pHANNIBAL-V2 **Eco*RI and *Kpn*I enzyme digestion displayed star activity.

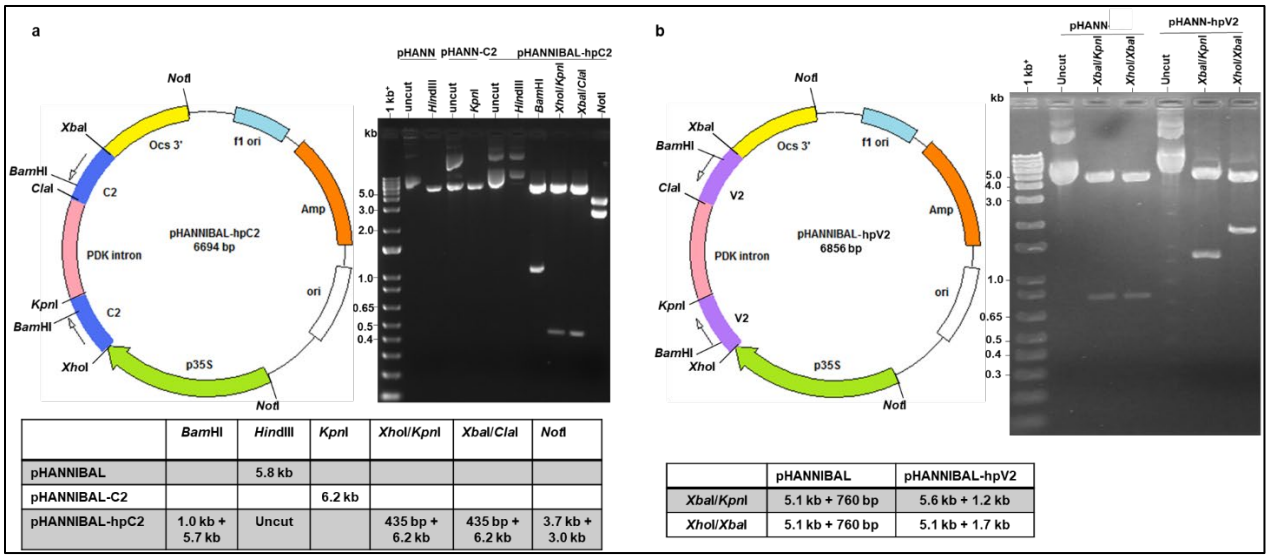
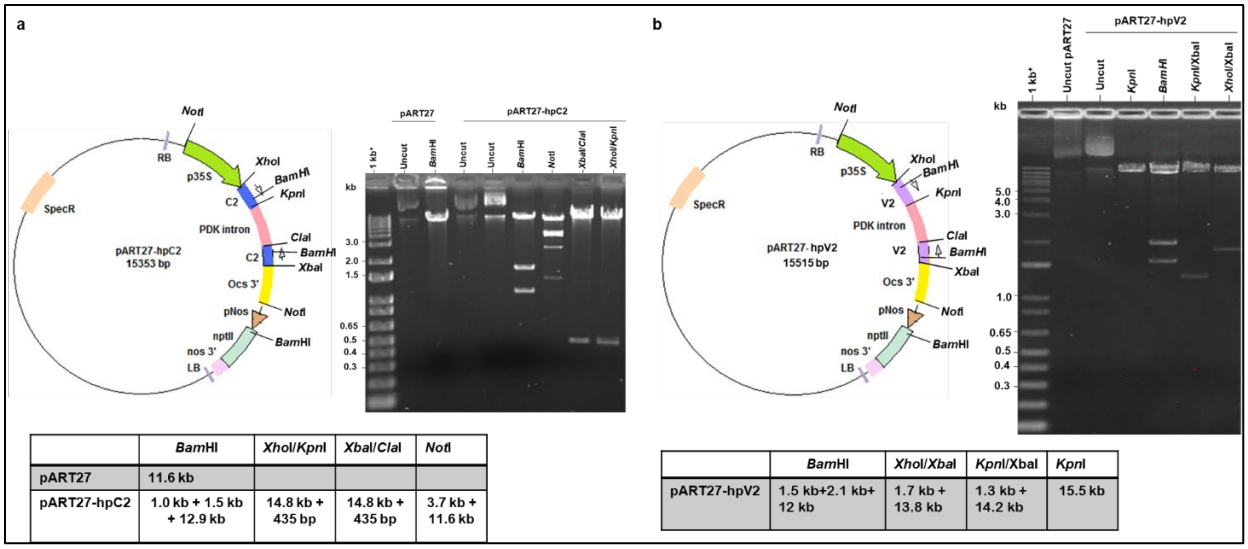


Figure 6. Restriction enzyme digestion. (a) pHANNIBAL-hpC2; (b) pHANNIBAL-hpV2.

The hpRNA gene cassette comprising the hpC2 or hpV2 was excised as a *Not*I fragment and cloned in the *Not*I site of T-DNA binary vector pART27 (83), which harbors the neomycin phosphotransferase gene as the plant transformation marker under the nopaline synthase promoter and terminator. The clones were confirmed by restriction analysis (**Figures 7a** and **7b**). The binary vector will be mobilized into the *A. tumefaciens* strain EHA105 and used for transforming embryogenic callus derived from anthers of grapevine rootstock 101-14 by cooperator D. Tricoli as the next step.



	BamHI	XhoI/KpnI	XbaI/ClaI	NotI
pART27	11.6 kb			
pART27-hpC2	1.0 kb + 1.5 kb + 12.9 kb	14.8 kb + 435 bp	14.8 kb + 435 bp	3.7 kb + 11.6 kb

	BamHI	XhoI/XbaI	KpnI/XbaI	KpnI
pART27-hpV2	1.5 kb+2.1 kb+ 12 kb	1.7 kb + 13.8 kb	1.3 kb + 14.2 kb	15.5 kb

Figure 7. Restriction enzyme digestion. (a) pART27-hpC2; (b) pART27-hpV2 **Not*I enzyme digestion displayed star activity.

CONCLUSIONS

1. We are in the process of completing molecular validation of suppression by GRBV proteins.
2. We have completed sRNA and RNA-seq library sequencing and analysis of samples collected from the field in 2018. We are in the process of completing sequencing and analysis of libraries made from 2019 field samples.
3. We have cloned the suppressor proteins in the pMAL-c5X vector and in the pGBTK7-BD vector for protein purification and Y2H assay, respectively.
4. We have completed the binary vector cloning of the hpRNA vector targeting the GRBV suppressor proteins.

REFERENCES CITED

1. Boulton MI. 2003. Geminiviruses: Major threats to world agriculture. *Annals of Applied Biology* 142: 143-143.
2. Moffat AS. 1999. Geminiviruses emerge as serious crop threat. *Science* 286: 1835-1835.
3. Rojas MR, Hagen C, Lucas WJ, Gilbertson RL. 2005. Exploiting chinks in the plant's armor: Evolution and emergence of geminiviruses. *Annual Review of Phytopathology* 43: 361-394.
4. Hanley-Bowdoin L, Settlage SB, Orozco BM, Nagar S, Robertson D. 1999. Geminiviruses: Models for plant DNA replication, transcription, and cell cycle regulation. *Critical Reviews in Plant Sciences* 18: 71-106.
5. Lazarowitz SG, Shepherd RJ. 1992. Geminiviruses: Genome structure and gene function. *Critical Reviews in Plant Sciences* 11: 327-349.
6. Palmer KE, Rybicki EP. 1998. The molecular biology of mastreviruses. Maramorosch K, Murphy FA, Shatkin AJ (eds.). *Advances in Virus Research.* Academic Press, pp. 183-234.
7. Ninth International Committee on Taxonomy of Viruses. 2012. Report: Family Geminiviridae. King AMQ, Lefkowitz E, Adams MJ, Carstens EB (eds.). *Virus Taxonomy.* Elsevier, pp. 351-373.

8. Calvi BL. 2011. Effects of red-leaf disease on Cabernet Sauvignon at the Oakville experimental vineyard and mitigation by harvest delay and crop adjustment. MS thesis, University of California, Davis.
9. Bahder BW, Zalom FG, Jayanth M, Sudarshana MR. 2016. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* as a vector of grapevine red blotch-associated virus. *Phytopathology* 106: 1223-1230.
10. Sudarshana MR, Perry KL, Fuchs MF. 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Phytopathology* 105: 1026-1032.
11. Oberhoster A, Girardello RC, Lerno LA, Eridon S, Cooper MLY, Smith RH, Brenneman CA, Heymann H, Sokolowsky M. 2016. Impact of red blotch disease on grape and wine composition. *Workshop on Recent Advances in Viticulture and Enology.* UC Davis, Dec 9, 2016. Available at http://ucanr.edu/repository/fileaccess.cfm?article=162938&p=YQCEFO.
12. Rwahnih MA, Rowhani A, Golino DA, Islas CM, Preece JE, Sudarshana MR. 2015. Detection and genetic diversity of grapevine red blotch-associated virus isolates in table grape accessions in the National Clonal Germplasm Repository in California. *Canadian Journal of Plant Pathology* 37: 130-135.
13. Dekker EL, Woolston CJ, Xue YB, Cox B, Mullineaux PM. 1991. Transcript mapping reveals different expression strategies for the bicistronic RNAs of the geminivirus wheat dwarf virus. *Nucleic Acids Research* 19: 4075-4081.
14. Wright EA, Heckel T, Groenendijk J, Davies JW, Boulton MI. 1997. Splicing features in maize streak virus virion- and complementary-sense gene expression. *Plant Journal* 12: 1285-1297.
15. Krenz B, Thompson JR, McLane HL, Fuchs M, Perry KL. 2014. Grapevine red blotch-associated virus is widespread in the United States. *Phytopathology* 104: 1232-1240.
16. Trinks D, Rajeswaran R, Shivaprasad PV, Akbergenov R, Oakeley EJ, Veluthambi K, Hohn T, Pooggin MM. 2005. Suppression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes. *Journal of Virology* 79: 2517-2527.
17. Bisaro DM. 2006. Silencing suppression by geminivirus proteins. *Virology* 344: 158-168.
18. Wang H, Hao L, Shung CY, Sunter G, Bisaro DM. 2003. Adenosine kinase is inactivated by geminivirus AL2 and L2 proteins. *Plant Cell* 15: 3020-3032.
19. Hao L, Wang H, Sunter G, Bisaro DM. 2003. Geminivirus AL2 and L2 proteins interact with and inactivate SNF1 kinase. *Plant Cell* 15: 1034-1048.
20. Zhang Z, Chen H, Huang X, Xia R, Zhao Q, Lai J, Teng K, Li Y, Liang L, Du Q, et al. 2011. BSCTV C2 attenuates the degradation of SAMDC1 to suppress DNA methylation-mediated gene silencing in *Arabidopsis*. *Plant Cell* 23: 273-288.
21. Buchmann RC, Asad S, Wolf JN, Mohannath G, Bisaro DM. 2009. Geminivirus AL2 and L2 proteins suppress transcriptional gene silencing and cause genome-wide reductions in cytosine methylation. *Journal of Virology* 83: 5005-5013.
22. Castillo-González C, Liu X, Huang C, Zhao C, Ma Z, Hu T, Sun F, Zhou Y, Zhou X, Wang XJ, et al. 2015. Geminivirus-encoded TrAP suppressor inhibits the histone methyltransferase SUVH4/KYP to counter host defense. *eLife* 4: e06671.
23. Baliji S, Lacatus G, Sunter G. 2010. The interaction between geminivirus pathogenicity proteins and adenosine kinase leads to increased expression of primary cytokinin responsive genes. *Virology* 402: 238-247.
24. Lozano-Durán R, Rosas-Díaz T, Gusmaroli G, Luna AP, Taconnat L, Deng XW, Bejarano ER. 2011. Geminiviruses subvert ubiquitination by altering CSN-mediated derubylation of

SCF E3 ligase complexes and inhibit jasmonate signaling in *Arabidopsis thaliana*. *Plant Cell* 23: 1014-1032.
25. Chellappan P, Vanitharani R, Fauquet CM. 2005. MicroRNA-binding viral protein interferes with *Arabidopsis* development. *Proceedings of the National Academy of Sciences USA* 102: 10381-10386.
26. Wang Y, Dang M, Hou H, Mei Y, Qian Y, Zhou X. 2014. Identification of an RNA silencing suppressor encoded by a mastrevirus. *Journal of General Virology* 95: 2082-2088.
27. Wang B, Yang X, Wang Y, Xie Y, Zhou X. 2018. Tomato yellow leaf curl virus V2 interacts with host histone deacetylase 6 to suppress methylation-mediated transcriptional gene silencing in plants. *Journal of Virology* 92: e00036-00018.
28. Blanco-Ulate B, Hopfer H, Figueroa-Balderas R, Ye Z, Rivero RM, Albacete A, Pérez-Alfocea F, Koyama R, Anderson MM, Smith RJ, et al. 2017. Red blotch disease alters grape berry development and metabolism by interfering with the transcriptional and hormonal regulation of ripening. *Journal of Experimental Botany* 68: 1225-1238.
29. Vaucheret H. 2006. Post-transcriptional small RNA pathways in plants: mechanisms and regulations. *Genes and Development* 20: 759-771.
30. Weiberg A, Bellinger M, Jin HL. 2015. Conversations between kingdoms: Small RNAs. *Current Opinions in Biotechnology* 32: 207-215.
31. Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL, Sanford JC. 1992. Virus resistant papaya plants derived from tissues bombarded with the coat protein gene of papaya ringspot virus. *Bio/Technology* 10: 1466-1472.
32. Ferreira SA, Pitz KY, Manshardt R, Zee F, Fitch M, Gonsalves D. 2002. Virus coat protein transgenic papaya provides practical control of papaya ringspot virus in Hawaii. *Plant Disease* 86: 101-105.
33. Jongedijk E, Huisman MJ, Cornelissen BJC. 1993. Argonic performance and field resistance of genetically modified, virus-resistant potato plants. *Seminars in Virology* 4: 407-416.
34. Hu JS, Pang SZ, Nagpala PG, Siemieniak DR, Slightom JL, Gonsalves D. 1993. The coat protein genes of squash mosaic virus: Cloning, sequence analysis, and expression in tobacco protoplasts. *Archives of Virology* 130: 17-31.
35. Kunik T, Salomon R, Zamir D, Navot N, Zeidan M, Michelson I, Gafni Y, Czosnek H. 1994. Transgenic tomato plants expressing the tomato yellow leaf curl virus capsid protein are resistant to the virus. *Bio/Technology* 12: 500-504.
36. Prins M, Laimer M, Noris E, Schubert J, Wassenegger M, Tepfer M. 2008. Strategies for antiviral resistance in transgenic plants. *Molecular Plant Pathology* 9: 73-83.
37. Shepherd DN, Martin DP, Thomson JA. 2009. Transgenic strategies for developing crops resistant to geminiviruses. *Plant Science* 176: 1-11.
38. Vanderschuren H, Stupak M, Fütterer J, Gruissem W, Zhang P. 2007. Engineering resistance to geminiviruses: Review and perspectives. *Plant Biotechnology Journal* 5: 207-220.
39. Brunetti A, Tavazza R, Noris E, Lucioli A, Accotto GP, Tavazza M. 2001. Transgenically expressed T-Rep of tomato yellow leaf curl Sardinia virus acts as a *trans*-dominant-negative mutant, inhibiting viral transcription and replication. *Journal of Virology* 75: 10573-10581.
40. Hanson SF, Maxwell DP. 1999. *trans*-Dominant inhibition of geminiviral DNA replication by bean golden mosaic geminivirus *rep* gene mutants. *Phytopathology* 89: 480-486.
41. Chatterji A, Beachy RN, Fauquet CM. 2001. Expression of the oligomerization domain of the replication-associated protein (Rep) of tomato leaf curl new delhi virus interferes with

DNA accumulation of heterologous geminiviruses. *Journal of Biological Chemistry* 276: 25631-25638.
42. Lucioli A, Noris E, Brunetti A, Tavazza R, Ruzza V, Castillo AG, Bejarano ER, Accotto GP, Tavazza M. 2003. Tomato yellow leaf curl Sardinia virus Rep-derived resistance to homologous and heterologous geminiviruses occurs by different mechanisms and is overcome if virus-mediated transgene silencing is activated. *Journal of Virology* 77: 6785-6798.
43. Hayes RJ, Buck KW. 1989. Replication of tomato golden mosaic virus DNA B in transgenic plants expressing open reading frames (ORFs) of DNA A: Requirement of ORF AL2 for production of single-stranded DNA. *Nucleic Acids Research* 17: 10213-10222.
44. Hanley-Bowdoin L, Elmer JS, Rogers SG. 1990. Expression of functional replication protein from tomato golden mosaic virus in transgenic tobacco plants. *Proceedings of the National Academy of Sciences USA* 87: 1446-1450.
45. Padidam M, Beachy RN, Fauquet CM. 1999. A phage single-stranded DNA (ssDNA) binding protein complements ssDNA accumulation of a geminivirus and interferes with viral movement. *Journal of Virology* 73: 1609-1616.
46. Sunitha S, Marian D, Hohn B, Veluthambi K. 2011. Antibegomoviral activity of the agrobacterial virulence protein VirE2. *Virus Genes* 43: 445.
47. Resmi TR, Hohn T, Hohn B, Veluthambi K. 2015. The *Agrobacterium tumefaciens* Ti plasmid virulence gene virE2 reduces Sri Lankan cassava mosaic virus infection in transgenic *Nicotiana benthamiana* plants. *Viruses* 7: 2641-2653.
48. Yousaf S, Rasool G, Amin I, Mansoor S, Saeed M. 2015. Evaluation of the resistance against begomoviruses imparted by the single-stranded DNA binding protein VirE2. *Pakistan Journal of Agricultural Science* 52: 887-893.
49. Day AG, Bejarano ER, Buck KW, Burrell M, Lichtenstein CP. 1991. Expression of an antisense viral gene in transgenic tobacco confers resistance to the DNA virus tomato golden mosaic virus. *Proceedings of the National Academy of Science USA* 88: 6721-6725.
50. Aragão FJL, Ribeiro SG, Barros LMG, Brasileiro ACM, Maxwell DP, Rech EL, Faria JC. 1998. Transgenic beans (*Phaseolus vulgaris* L.) engineered to express viral antisense RNAs show delayed and attenuated symptoms to bean golden mosaic geminivirus. *Molecular Breeding* 4: 491-499.
51. Bendahmane M, Gronenborn B. 1997. Engineering resistance against tomato yellow leaf curl virus (TYLCV) using antisense RNA. *Plant Molecular Biology* 33: 351-357.
52. Yang Y, Sherwood TA, Patte CP, Hiebert E, Polston JE. 2004. Use of tomato yellow leaf curl virus (TYLCV) *Rep* gene sequences to engineer TYLCV resistance in tomato. *Phytopathology* 94: 490-496.
53. Asad S, Haris WAA, Bashir A, Zafar Y, Malik KA, Malik NN, Lichtenstein CP. 2003. Transgenic tobacco expressing geminiviral RNAs are resistant to the serious viral pathogen causing cotton leaf curl disease. *Archives of Virology* 148: 2341-2352.
54. Pooggin M, Shivaprasad PV, Veluthambi K, Hohn T. 2003. RNAi targeting of DNA virus in plants. *Nature Biotechnology* 21: 131-132.
55. Vanitharani R, Chellappan P, Fauquet CM. 2003. Short interfering RNA-mediated interference of gene expression and viral DNA accumulation in cultured plant cells. *Proceedings of the National Academy of Sciences USA* 100: 9632-9636.

56. Owor BE, Martin DP, Rybicki EP, Thomson JA, Bezuidenhout ME, Lakay FM, Shepherd DN. 2011. A rep-based hairpin inhibits replication of diverse maize streak virus isolates in a transient assay. *Journal of General Virology* 92: 2458-2465.
57. Fuentes A, Ramos PL, Fiallo E, Callard D, Sánchez Y, Peral R, Rodríguez R, Pujol M. 2006. Intron–hairpin RNA derived from replication associated protein *C1* gene confers immunity to tomato yellow leaf curl virus infection in transgenic tomato plants. *Transgenic Research* 15: 291-304.
58. Aragão FJL, Faria JC. 2009. First transgenic geminivirus-resistant plant in the field. *Nature Biotechnology* 27: 1086-1088.
59. Bonfim K, Faria JC, Nogueira EOPL, Mendes ÉA, Aragão FJL. 2007. RNAi-mediated resistance to bean golden mosaic virus in genetically engineered common bean (*Phaseolus vulgaris*). *Molecular Plant-Microbe Interactions* 20: 717-726.
60. Vanderschuren H, Alder A, Zhang P, Gruissem W. 2009. Dose-dependent RNAi-mediated geminivirus resistance in the tropical root crop cassava. *Plant Molecular Biology* 70: 265-272.
61. Vanderschuren H, Akbergenov R, Pooggin MM, Hohn T, Gruissem W, Zhang P. 2007. Transgenic cassava resistance to African cassava mosaic virus is enhanced by viral DNA-A bidirectional promoter-derived siRNAs. *Plant Molecular Biology* 64: 549-557.
62. Zrachya A, Glick E, Levy Y, Arazi T, Citovsky V, Gafni Y. 2007. Suppressor of RNA silencing encoded by tomato yellow leaf curl virus-Israel. *Virology* 358: 159-165.
63. Praveen S, Ramesh SV, Mishra AK, Koundal V, Palukaitis P. 2010. Silencing potential of viral derived RNAi constructs in tomato leaf curl virus-AC4 gene suppression in tomato. *Transgenic Research* 19: 45-55.
64. Sunitha S, Shanmugapriya G, Balamani V, Veluthambi K. 2013. Mungbean yellow mosaic virus (MYMV) AC4 suppresses post-transcriptional gene silencing and an AC4 hairpin RNA gene reduces MYMV DNA accumulation in transgenic tobacco. *Virus Genes* 46: 496-504.
65. Shanmugapriya G, Das SS, Veluthambi K. 2015. Transgenic tobacco plants expressing siRNA targeted against the mungbean yellow mosaic virus transcriptional activator protein gene efficiently block the viral DNA accumulation. *VirusDisease* 26: 55-61.
66. Luo Q-J, Mittal A, Jia F, Rock CD. 2012. An autoregulatory feedback loop involving *PAP1* and *TAS4* in response to sugars in *Arabidopsis*. *Plant Molecular Biology* 80: 117-129.
67. Rock CD. 2013. *Trans-acting small interfering RNA4*: Key to nutraceutical synthesis in grape development? *Trends in Plant Science* 18: 601-610.
68. Pantaleo V, Szittya G, Moxon S, Miozzi L, Moulton V, Dalmay T, Burgyan J. 2010. Identification of grapevine microRNAs and their targets using high-throughput sequencing and degradome analysis. *Plant Journal* 62: 960-976.
69. Sunitha S, Loyola R, Alcalde JA, Arce-Johnson P, Matus JT, Rock CD. 2019. The role of UV-B light on small RNA activity during grapevine berry development. *G3: Genes | Genomes | Genetics* 9: 769-787.
70. Rock CD, Zeevaart JA. 1991. The *aba* mutant of *Arabidopsis thaliana* is impaired in epoxy-carotenoid biosynthesis. *Proceedings of the National Academy of Sciences USA* 88: 7496-7499.
71. Padmanabhan C, Zhang XM, Jin HL. 2009. Host small RNAs are big contributors to plant innate immunity. *Current Opinions in Plant Biology* 12: 465-472.

72. Zhang XM, Zhao HW, Gao S, Wang WC, Katiyar-Agarwal S, Huang HD, Raikhel N, Jin HL. 2011. *Arabidopsis* Argonaute 2 regulates innate immunity via miRNA393*-mediated silencing of a Golgi-localized SNARE gene, MEMB12. *Molecular Cell* 42: 356-366.
73. Wang X-B, Jovel J, Udomporn P, Wang Y, Wu Q, Li W-X, Gasciolli V, Vaucheret H, Ding S-W. 2011. The 21-nucleotide, but not 22-nucleotide, viral secondary small interfering RNAs direct potent antiviral defense by two cooperative Argonautes in *Arabidopsis thaliana*. *Plant Cell* 23: 1625-1638.
74. Schott G, Mari-Ordonez A, Himber C, Alioua A, Voinnet O, Dunoyer P. 2012. Differential effects of viral silencing suppressors on siRNA and miRNA loading support the existence of two distinct cellular pools of ARGONAUTE1. *EMBO Journal* 31: 2553-2565.
75. Li F, Pignatta D, Bendix C, Brunkard JO, Cohn MM, Tung J, Sun H, Kumar P, Baker B. 2012. MicroRNA regulation of plant innate immune receptors. *Proceedings of the National Academy of Sciences USA* 109: 1790-1795.
76. Shivaprasad PV, Chen HM, Patel K, Bond DM, Santos B, Baulcombe DC. 2012. A microRNA superfamily regulates nucleotide binding site-leucine-rich repeats and other mRNAs. *Plant Cell* 24: 859-874.
77. Seifi A. 2011. Write 'systemic small RNAs': Read 'systemic immunity.' *Functional Plant Biology* 38: 747-752.
78. Boyko A, Kovalchuk I. 2011. Genetic and epigenetic effects of plant-pathogen interactions: An evolutionary perspective. *Molecular Plant* 4: 1014-1023.
79. Zhai J, Jeong D-H, De Paoli E, Park S, Rosen BD, Li Y, Gonzalez AJ, Yan Z, Kitto SL, Grusak MA, et al. 2011. MicroRNAs as master regulators of the plant NB-LRR defense gene family via the production of phased, *trans*-acting siRNAs. *Genes and Development* 25: 2540-2553.
80. Turner M, Yu O, Subramanian S. 2012. Genome organization and characteristics of soybean microRNAs. *BMC Genomics* 13: 169.
81. Axtell MJ, Jan C, Rajagopalan R, Bartel DP. 2006. A two-hit trigger for siRNA biogenesis in plants. *Cell* 127: 565-577.
82. Velten J, Cakir C, Youn E, Chen J, Cazzonelli CI. 2012. Transgene silencing and transgene-derived siRNA production in tobacco plants homozygous for an introduced *AtMYB90* construct. *PLOS ONE* 7: e30141.
83. Gleave AP. 1992. A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Molecular Biology* 20: 1203-1207.
84. Wesley SV, Helliwell CA, Smith NA, Wang M, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM. 2001. Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant Journal* 27: 581-590.

FUNDING AGENCIES

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

ACKNOWLEDGEMENTS

The authors thank Texas Tech University Vice President for Research Joseph Heppert for supplemental travel funds, Bob Martin of USDA-ARS in Corvallis, Oregon for coordination in

obtaining samples from Willamette Valley vineyards, Satyanarayana Tatineni of the USDA-ARS and University of Nebraska, Lincoln for the plasmid constructs pCASS4-TriMV-P1, pCASS4-TuMV HC-Pro, pCASS4, and pZAP-ssGFP, David Baulcombe of Cambridge University and Herman Scholthof of Texas A&M University for seeds of *Nicotiana benthamiana* GFP reporter line 16c, and K. Veluthambi of Madurai Kamaraj University for *Agrobacterium* strains EHA105, LBA4404, C58C1, Ach5, and pTi358.

UNDERSTANDING SYMPTOMOLOGY AND PHYSIOLOGICAL EFFECTS OF RED BLOTCH DISEASE IN VINEYARDS IN OREGON'S WILLAMETTE VALLEY

Project Leader: Patty Skinkis | Department of Horticulture | Oregon State University | Corvallis, OR 97331 | patricia.skinkis@oregonstate.edu
Co-Project Leader: Robert R. Martin | Horticultural Crops Research Unit | USDA Agricultural Research Service | Corvallis, OR 97330 | bob.martin@usda.gov

Reporting Period: The results reported here are from work conducted from July 1, 2018 to October 15, 2019.

ABSTRACT
Grapevine red blotch disease is an important grapevine virus that is a major concern for the United States wine industry, in part due to the potential for reduced fruit and wine quality. Much of the information about the virus has been shared from virus biology and insect vector work that has been conducted in recent years, but information is lacking on how the virus impacts grapevine growth, productivity, and fruit composition. We designed a two-year study to evaluate the impacts of grapevine red blotch virus on grapevines in the cool climate of Oregon's Willamette Valley. This study determines the impacts of the virus on vine growth, photoassimilation, water status, vine nutrient status, and fruit composition. To date, we have found little to no virus impact on vine growth, vine water status, leaf photoassimilation, or nutrient status at veraison. Symptoms of the virus first appear at the start of veraison in lower leaves, but are not easily visible. Leaves remain green and do not have leaf chlorosis until harvest or post-harvest. Virus-infected vines maintain their leaves longer post-harvest than healthy vines. The impact of virus on fruit composition was site dependent, with the older, dry-farmed, lower-vigor site having a greater virus impact on Brix than the younger, irrigated site. Results of this project will be used to design future research projects to ameliorate the impacts of the disease in the vineyard.

LAYPERSON SUMMARY
Grapevine red blotch disease is a newly identified virus of grapevines that is causing substantial concern for commercial grape producers, as it is thought to reduce fruit and wine quality. Many grape producers fear that infected vineyards will require removal and replacement, which comes at a substantial cost and may not be economically feasible. This research was conducted to better understand how the virus affects vine growth and fruit composition on a practical level for the producer. This is an important first step towards understanding how to manage infected vines, if possible. To date we have found no impact on vine health or productivity and limited or no impact on fruit composition at harvest under the cool climate conditions of Oregon's Willamette Valley. However, the symptoms were more severe in the lower vigor vineyard in the drier of the two growing seasons, which suggests the role of abiotic stressors (soil, water, and nutrient) and suggests that maintaining vine health is a means to ameliorate virus impacts. Further analysis of the data collected in the second year will help determine consistency of vine responses to the virus that will suggest future studies into best-suited vineyard management practices.

INTRODUCTION

Grapevine red blotch disease has recently become a major concern for winegrape producers in Oregon and other areas of the United States. The causal agent of the disease, grapevine red blotch virus (GRBV), was first identified by researchers in California and New York (Al Rwahnih et al., 2013; Krenz et al., 2014). The disease has been at the forefront of industry concern during a time of significant industry expansion (vineyard planting) since spread has primarily been through infected nursery stock (NCPN, 2017).

Anecdotal information from industry indicate that fruit stops ripening in the most severe cases. Studies indicate that sugar levels can lag by 1 to 2.7 °Brix (Shudarshana et al., 2015), and that fruit lack normal ripening as a result of altered secondary metabolite production that is important for wine quality (Blanco-Ulate et al., 2017). The lack of fruit ripening is a major concern for premium winegrape producers in cool climate regions such as the Willamette Valley, where ripening can be a challenge in some years due to the limited season heat accumulation and length.

There is significant research underway to understand the virus biology and to identify insect vectors of the virus. While researchers in virology and entomology have made great strides in a matter of a few years to understand the virus-insect complex (Bahder et al., 2016), there still is little definitive evidence of the impacts of the virus on vine physiology, and more research projects are needed to understand the productivity and fruit quality effects on grapevines.

As we seek to provide management options for growers, we need information about how the virus is affecting vine growth and fruit ripening under the current climatic conditions and production systems. We have information from an industry survey that indicates that GRBV exists in most of Oregon's production regions, and some growers report that the virus has little to no impact while others claim that their vineyards are no longer economically viable. The best advice to date is to remove vines that are infected and replant with "clean" plant material, but the cost of removal and replacement may not be economically feasible (Ricketts et al., 2017), particularly if there is no effect on the vines and/or little risk of spread.

OBJECTIVES

1. Determine vine growth and physiology effects related to grapevine red blotch disease in vineyards in Oregon's Willamette Valley.
2. Determine the effects of grapevine red blotch disease on fruit ripening for vineyards in Oregon's Willamette Valley.

RESULTS AND DISCUSSION

Objective 1. Determine Vine Growth and Physiology Effects Related to Grapevine Red Blotch Disease in Vineyards in Oregon's Willamette Valley

Two Pinot Noir vineyards were used for research during summer 2018 to 2019 for collecting symptom and vine physiological response data based on GRBV status. We had originally planned to work with Pinot Noir and Chardonnay vineyards, but we were not able to find a Chardonnay site with GRBV that was causing concerns for vineyards/wineries in the region. Due to the economic impact of Pinot Noir on the Oregon wine industry, we decided to use two Pinot Noir vineyards to evaluate how the disease is affecting vines under two different vineyard

conditions and across two American Viticultural Areas (AVAs). Vineyard 1 is located in the Eola-Amity Hills AVA near Amity, Oregon and is planted (2007) to Pinot Noir clone 828 grafted to Riparia Gloire rootstock, and it is irrigated. Vineyard 2 is located in the Dundee Hills AVA near Lafayette, Oregon and is planted (2002) to Pinot Noir clone 777 grafted to 101-14, and is dry farmed (no irrigation). We used these vineyards because of the prior knowledge of GRBV being present, with symptom mapping data from the collaborating vineyard managers. We were able to find a mix of virus positive and negative vines within confined sections of vineyard blocks that were used to evaluate vine physiological responses in both Vineyard 1 and Vineyard 2. Data collection began earlier for Vineyard 1 in 2018 since we knew the virus status based on polymerase chain reaction (PCR) testing conducted using leaf tissues in summer 2017. Vineyard 2 was a new vineyard site for 2018. We did not receive virus detection results from an August leaf sampling until September 28, 2018, just before harvest. Therefore, summer 2018 data for Vineyard 2 was collected based on visual symptoms only. A total of 20 vines were used for all vine growth measures in Vineyard 1, including 10 GRBV+ and 10 GRBV-, and we further classified the vines based on symptom expression (asymptomatic vs. symptomatic). For Vineyard 2, 24 vines were selected based on symptoms to focus on late season vine physiological measures. However, the PCR testing came back with 18 vines being GRBV+ and six vines being GRBV-. We limited our vine numbers for this study based on the duration of time required to measure physiological responses such as photoassimilation and stomatal conductance using an infra-red gas analyzer in a timely fashion to ensure sound data. We retested all vines in Vineyards 1 and 2 using dormant tissue samples at pruning in December 2018 to double-check the virus status, as some of the vines that were found GRBV- had symptoms in 2018. These dormant tissue results showed that 13 vines were GRBV+ and seven vines were GRBV- in Vineyard 1, and all 24 vines at Vineyard 2 were GRBV+. The results presented in this report reflect the initial virus testing and symptom classifications given in 2018. However, as we finalize analyses in fall/winter 2019/2020, we are reclassifying the experimental units based on the most consistent testing results for those vines.

Vine growth measures included vine leaf area, fruit yield, and dormant pruning weights. Leaf area was measured at veraison on two shoots per vine using a non-destructive field sampling method (Navarrete, 2015). Research suggests that leaf chlorophyll begins to decline before visual symptoms are observed, so we monitored leaf chlorophyll with a SPAD meter (SPAD-502, Minolta) using leaves from three zones within the canopy (basal, middle, and upper canopy) on a biweekly basis, beginning in July each year. A pressure chamber was used for determining midday leaf water potential on several dates in Vineyard 1 during July and August 2018. To determine the impacts of the virus on photoassimilation and stomatal conductance, an infrared gas analyzer (LiCor 6400) was used on fully exposed leaves within vine canopies on clear, cloudless days, during late summer (July-September) each year. Vine tissue samples (leaf and petiole) were collected at veraison and analyzed for macro- and micronutrients by Fruit Growers Lab. Symptoms of GRBV were monitored throughout summer and continued biweekly or more frequently as we conducted growth and physiology measures. We documented canopy symptoms, fruit development symptoms, and timing of leaf fall post-harvest each year. During 2019, we monitored vines for incidence and severity of symptoms throughout the entire season. Whole vine yield weights and cluster counts were measured at harvest each year, and pruning weights were measured during dormancy following the crop year. Both yield and pruning weight were used to quantify impacts on vine growth and determine the yield to pruning weight ratios.

Vineyard 1 Results. Leaf SPAD data (an indicator of chlorophyll) was monitored in Vineyard 1 from mid-July through late August 2018 and first began to show lower SPAD in GRBV+ compared to GRBV- vines by August 7, 2018 (berry touch stage) in basal leaves only and was consistent through the following sample date (August 22, 2018). Leaves in the mid- to upper-canopy did not differ in SPAD readings, indicating a similar level of leaf greenness throughout summer. During the 2019 growing season vines were monitored from late July to late August and readings between GRBV+ and GRBV- did not differ during these dates. In general, SPAD readings were high, averaging ~41, and the minimum value reported (of basal leaves) was 26. The vines were vigorous and healthy with sufficient canopy greenness throughout summer. Vine leaf blade nitrogen was high (2.4 to 2.5% N) for both GRBV+ and GRBV- vines, and there were no differences by virus status. Leaf blade potassium was higher in asymptomatic vines than symptomatic vines at 0.99 and 0.79% K, respectively. There were no other nutrient differences for macro- or micro-nutrients for leaf blades analyzed at veraison in 2018. Samples for veraison nutrient status were collected on August 28, 2019, and statistical analysis is underway.

The first virus-associated symptoms in Vineyard 1 were observed in leaves at veraison each year, starting with interveinal reddening of the most basal leaves. There was little to no leaf chlorosis during pre-veraison or post-veraison (**Figure 1**). A slight leaf chlorosis was visible by harvest (September 28, 2018), and red leaf symptoms were visible primarily in basal leaves with some occasional mid- and upper-canopy leaves having interveinal reddening. Post-harvest observations revealed the majority of GRBV+ vines had delayed leaf yellowing and senescence compared to GRBV- vines in late October and early November. There were no differences in timing of bud break or bloom in either year.

Leaf photoassimilation and stomatal conductance were measured on 20 individual vines on seven dates from July 5, 2018 to September 6, 2018, and in 2019 on three dates between July 26 and August 12, to detect differences based on virus or symptom status. Leaves in two zones were measured on each vine, including basal leaves and mid-upper canopy leaves. Photoassimilation and stomatal conductance gradually declined as the season advanced, as expected with increasing seasonal temperatures, soil moisture deficit, and vine water status. There was rarely a difference in photoassimilation or stomatal conductance of the mid-upper canopy leaves. However, asymptomatic vines had higher basal leaf photoassimilation and stomatal conductance than symptomatic vines for three of the seven dates measured in 2018 and there were no differences found in 2019. There were no photoassimilation or stomatal conductance differences based on virus status for any of the dates measured in 2018 and 2019.

Leaf and stem water potential were measured during three dates in August 2018 (pre-veraison and at veraison). There were no differences in leaf or stem water potential based on virus or symptom status that year. This is an irrigated vineyard, and drip irrigation was applied judiciously only when vines experienced stress late season. Across the three dates measured, mean leaf water potential was -0.74 megapascals (MPa), -1.3 MPa, and -0.96 MPa on August 1, August 8, and August 20, 2018, respectively. No water potential measures were taken in 2019 due to limitation on clear, sunny days that would allow measurements, and we prioritized leaf photoassimilation and stomatal conductance measures on those days.

There were no differences between vine virus status or symptom status for vine growth as measured by vine leaf area, yield, or dormant pruning weight in 2018 and 2019. Vines were vigorous with high cane weights (> 100 g) and low yield to pruning weight ratios from 2.2 to 2.9 in 2018.

Vineyard 2 Results. Leaf greenness data (SPAD) were collected on three dates in late summer 2018 and three dates in 2019. There were lower SPAD readings (lower chlorophyll) in basal leaves of GRBV+ vines on August 28 and September 6, 2018, but no differences were found for 2019. Furthermore, there were no differences in SPAD readings in the mid-upper canopy leaves for any date. This accurately reflects the visual symptom expression at that stage since only the basal leaves were showing red coloration, and mid-upper leaves had not yet shown these symptoms. In general, the SPAD readings were lower than Vineyard 1, and canopies were visibly less vigorous (low to moderate vine vigor by comparison to Vineyard 1), with 69% smaller leaves than in Vineyard 1 in 2018.

Photoassimilation and stomatal conductance data were collected on the same three dates from veraison to harvest as the SPAD data in the mid-upper and basal leaves within the canopy in 2018 and on three dates in 2019. There was lower photoassimilation and stomatal conductance in the basal leaves of GRBV+ vines and vines showing symptoms for the latest sample date in 2018 (September 6, 2018) and 2019 (August 26, 2019). There were no differences in photoassimilation and stomatal conductance of any mid-upper canopy leaves on any of the three late season dates in 2018 and 2019. These data match with the leaf chlorophyll data (SPAD readings). The photoassimilation and stomatal conductance rates decreased for all vines as the season advanced, as expected. However, photoassimilation rates began to drop to a low level (2-3 µmol CO2/m2/s in 2018 and 7-10 µmol/CO2/m2/s in 2019) while stomatal conductance was high enough to not be considered under significant water stress (> 130 mmol H20/m2/s).

The first virus-associated symptoms in Vineyard 2 were observed in leaves before veraison (early August 2018 and late July 2019), starting with interveinal reddening of the most basal leaves. There was no leaf chlorosis during the pre-veraison or post-veraison period (**Figure 1**). However, leaf chlorosis was visible by harvest (October 1, 2018, September 30, 2019). By harvest, red leaf symptoms were visible primarily in basal leaves with some occasional mid- and upper-canopy leaves having interveinal reddening. Since this vineyard had more abiotic stress prior to harvest than Vineyard 1, leaf fall occurred earlier in 2018, and there were no differences based on virus or symptom status in timing of leaf abscission post-harvest. In hindsight, this may be due to all of the vines being infected with virus, as noted by our winter tissue testing during dormancy.

There were no differences by virus status for whole vine yield or yield components (cluster count, cluster weight, or berry weight) at harvest in 2018 and 2019. Vine pruning weights were indicative of moderate vigor vines (~ 40 g canes, and yield to pruning weight ratios of 5.1-5.9) in 2018. Vine vigor was improved in 2019 as the season was milder (more rainfall and cooler temperatures), which resulted in visibly more healthy canopies and less intense symptom expression in 2019 (**Figure 1**). The pruning weights will be measured this December 2019.

Figure 1. Pinot Noir vines with GRBV from Vineyard 1(top) and Vineyard 2 (bottom) on September 19, 2019. Vineyard 1 has a higher vigor and no visible symptoms compared to the lower vigor and appearance of red blotches and veins at Vineyard 2 (appearing almost purple in the photos). Overall, symptoms were hard to detect in vines during 2019 due to cool summer temperatures and ample seasonal rainfall, which is typical for the Willamette Valley.

Objective 2. Determine the Effects of Grapevine Red Blotch Disease on Fruit Ripening for Vineyards in Oregon's Willamette Valley

To determine impacts of grapevine red blotch virus on berry ripening, we monitored the progression of veraison between healthy and infected vines at both vineyards. We initially intended to sample vines for fruit ripening up to harvest, but we were unable to do so given the limited amount of fruit on individual vine experimental units. Also, we wanted to obtain unamended yield weights at harvest. Therefore, we waited until the block was to be harvested commercially and came in one to two days earlier to obtain cluster counts, yield weights, and cluster samples. At harvest, we collected two 5-cluster samples from each vine. One of the samples was processed and analyzed for the following: cluster weight, berry count, and berry size, and then pressed to juice for analysis of total soluble solids (TSS, °Brix), pH, and titratable acidity. Another 5-cluster sample from each vine was stored at -80°C until analysis for total anthocyanin using the pH-differential method (Lee et al., 2005), total phenolics using the Folin-Ciocalteu method (Waterhouse, 2002), and total tannins using the methyl cellulose precipitation method (Sarneckis et al., 2006) could be conducted.

Vineyard 1 had no differences in the start or progression of berry coloration through veraison based on virus or symptom status in 2018 or 2019. By harvest, there were no differences in total soluble solids based on virus status or symptom, but GRBV+ vines produced fruit with higher pH ($p = 0.0098$) and lower titratable acidity ($p = 0.0113$) than GRBV- vines in 2018. Furthermore, the fruit from GRBV+ and GRBV- vines did not differ in total phenolics concentration, including total anthocyanins, total phenolics, and total tannins. These vines had sufficient canopy growth, vine nutrient status, and leaf greenness, and a lack of differences in vine water status, leaf photoassimilation, and stomatal conductance (noted in Objective 1 results), so these vines likely had sufficient capacity to ripen fruit. Fruit composition is still pending for Vineyard 1 as of this report, as harvest was recently completed.

Vineyard 2 did not differ in the start or progression of berry coloration during veraison. By harvest, fruit from GRBV+ vines had 2.1°Brix lower than fruit from GRBV- vines (23.7 and 25.8 °Brix, respectively) ($p = 0.0008$). However, there were no differences in pH (3.21) and titratable acidity (6.7 g/L) in 2018. Fruit from GRBV+ vines had 14% lower total anthocyanins ($p = 0.0287$) but 6% higher total phenolics than GRBV- fruit ($p = 0.0144$). Fruit from vines that were asymptomatic had 28% higher total phenolics than those with symptoms ($p = 0.0157$). However, there were no differences in total tannin concentration by virus or symptom status. The vines in Vineyard 2 had less green canopies by harvest and lower vine vigor than Vineyard 1, and these factors may account for differences observed at this site. Since the winter virus testing revealed that all of the vines in Vineyard 2 are GRBV+, it is likely that differences in the results are based on symptom severity and higher virus concentration that led to earlier virus detection. We will be exploring differences further in 2019 based on symptoms rather than virus in this block, and fruit composition is underway at present.

CONCLUSIONS

Based on the two years of data, we find that it is possible for infected vines to have limited visual symptoms and minimal or no impact on fruit quality at harvest. Between the two sites and the two vintages, we observed that visual symptoms are more pronounced when there are more abiotic stressors. The 2019 season had less symptom severity for both vineyards, and the site

with irrigation had lower overall virus impact in both years. Vine water status and photoassimilation were not reduced based on virus status. Given that there were few, if any, differences in nutrient status, this suggests that the virus may not be ameliorated by a specific nutrient fertilization program. Further analysis of 2019 data will clarify differences in the symptom expression and physiology of the virus under different environmental conditions.

REFERENCES CITED

Al Rwahnih M, Dave A, Anderson M, Rowhani A, Uyemoto JK, Sudarshana MR. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology* 103: 1069-1076.

Bahder BW, Zalom FG, Jayanth M, Sudarshana MR. 2016. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* as a vector of grapevine red blotch-associated virus. *Phytopathology* 106: 1223-1230.

Blanco-Ulate B, Hopfer H, Figueroa-Balderas R, Ye Z, Rivero RM, Albacete A, Perez-Alfocea F, et al. 2017. Red blotch disease alters grape berry development and metabolism by interfering with the transcriptional and hormonal regulation of ripening. *Journal of Experimental Botany* 68(5): 1225-1238.

Krenz B, Thompson JR, McLane HL, Fuchs M, Perry KL. 2014. Grapevine red blotch-associated virus is widespread in the United States. *Phytopathology* 104: 1232-1240.

Lee J, Durst RW, Wrolstad RE. 2005. Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: Collaborative study. *Journal of AOAC International* 88: 1269-1278.

National Clean Plant Network (NCPN). 2017. *Fact Sheet: Grapevine Red Blotch Disease*.

Ricketts KD, Gómez MI, Fuchs MF, Martinson TE, Smith RJ, Cooper ML, Moyer MM, Wise A. 2017. Mitigating the economic impact of grapevine red blotch: Optimizing disease management strategies in U.S. vineyards. *American Journal of Enology and Viticulture* 68: 127-135.

Sarneckis CJ, Dambergs RG, Jones P, Mercurio M, Herderich MJ, Smith PA. 2006. Quantification of condensed tannins by precipitation with methyl cellulose: Development and validation of an optimized tool for grape and wine analysis. *Australian Journal of Grape and Wine Research* 12: 39-49. http://onlinelibrary.wiley.com/doi/10.1111/j.1755-0238.2006.tb00042.x/full.

Sudarshana MR, Perry KL, Fuchs MF. 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Phytopathology* 105: 1026-1032.

Waterhouse AL. 2002. Polyphenolics: Determination of total phenolics. In: Wrolstad RE (ed.). *Current Protocols in Food Analytical Chemistry*. Wiley, Hoboken, NJ, pp. 463-470.

FUNDING AGENCIES

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

IMPROVED UNDERSTANDING OF VIRUS TRANSMISSION OF GRAPEVINE RED BLOTCH VIRUS

Project Leader: Vaughn Walton | Department of Horticulture | Oregon State University | Corvallis, OR 97331 | vaughn.walton@oregonstate.edu
Co-Project Leader: Rachele Nieri | Department of Horticulture | Oregon State University | Corvallis, OR 97331 | nierir@oregonstate.edu
Co-Project Leader: Rick Hilton | Southern Oregon Research and Extension Center | Oregon State University | Central Point, OR 97502 | richard.hilton@oregonstate.edu

Reporting Period: The results reported here are from work conducted July 1, 2019 to October 23, 2019.

ABSTRACT
This project has the goal of testing plant materials subjected to feeding by two treehopper species known to occur in vineyards where grapevine red blotch virus (GRBV) is spreading. Plant materials were collected and transmission biology tests were performed during 2016 to 2018. All of these plant materials are currently available for testing after the initial trials were performed. These plant materials are being tested for transmission of GRBV using quantitative polymerase chain reaction (qPCR) and primers specific to GRBV. Samples resulting in a measurable cycle threshold (Ct) value will be subjected to droplet digital PCR (ddPCR), which is believed to add sensitivity because each 20-µL sample is partitioned into up to 20,000 one-nL droplets, each individually assayed for the presence of virus particles.

LAYPERSON SUMMARY
We will test vine samples that were subjected to treehopper transmission studies for three years of transmission (2016 to 2018).

INTRODUCTION
Vineyard managers and winemakers are concerned about grapevine virus diseases in all Western production regions due to impacts on grape berry quality. Growers and scientists have noticed a consistently lower °Brix at harvest of virus-infected vines (Al Rwahnih et al., 2013, 2015). The reduced grape berry quality resulted in the removal of symptomatic vines from vineyards in production regions ranging from California into Oregon. Grapevine red blotch virus (GRBV) is a newly identified virus that is spreading in Oregon. We showed up to a tenfold increase of virus incidence across three seasons (Dalton et al., 2019). One species of treehopper, the three-cornered alfalfa hopper (*Spissistilus festinus*), has been identified as an insect vector in California (Bahder et al., 2016; Cieniewicz et al., 2017). This species has been found in the majority of Pacific grapevine production regions. Other treehoppers, particularly in the genus *Tortistilus* (**Figure 1**), are indigenous to the Pacific Northwest (Yothers, 1934) and are likely vectors of GRBV. From 2016 to 2018, *Tortistilus* species were consistently found feeding on grape shoots and leaves in Oregon vineyards where virus spread is documented. Evidence from controlled transmission experiments strongly suggests that these insects are vectors of GRBV. Refined protocols are needed to confirm this evidence. Work is currently being conducted through a grant funded by CDFA's Pierce's Disease and Glassy-winged Sharpshooter Board (July 2017 to June 2019) to follow insect vector distribution and biology, but this funding does not allow for

additional work beyond June 2019. Work funded by CDFA suggests that these insects are distributed along vineyard edges and are closely associated with both perennial and annual non-crop host plants. Research conducted during 2017 to 2018 described insect movement from alternate host plants into vineyards as soon as the annual species dry out during the hot summer months.

Figure 1. Locally abundant *Tortistilus* spp. (left) are likely vectors of GRBV. These insects feed on canes and leaves, resulting in distal flagging (arrow, right). Spread of GRBV was documented in southern Oregon and in the Willamette Valley (Dalton et al., 2019).

OBJECTIVES

1. Determine the virus status of plants subjected to transmission biology assays from 2016 to 2018.

We believe that it will be essential to continue to test the infection status of plants that underwent transmission biology experiments during 2016 to 2018 in order to confirm preliminary results gained during testing in 2018. This is particularly important, as the current understanding is that there is a significant latent period from initial transmission to positive detection of approximately two to three years (Fuchs et al., 2018). Although the majority of our virus-subjected plants have currently tested negative for GRBV, we believe that follow-up testing is essential given the suspected latent period.

Objective 1. Determine the Virus Status of Plants Subjected to Transmission Biology Assays from 2016 to 2018

Approximately 1,200 plant samples will be assayed using established real-time quantitative polymerase chain reaction (qPCR) procedures (Dalton et al., 2019). Petioles, leaves, cane scrapings, or root material will be harvested from greenhouse-maintained test plants and homogenized under sterile conditions in the laboratory. Sample nucleic acid will be extracted in guanidine thiocyanate buffer and purified for analysis by qPCR using primers specific to GRBV. Samples resulting in a measurable cycle threshold (Ct) value will be subjected to droplet digital PCR (ddPCR) analysis using a QX 200 droplet reader (Bio-Rad). ddPCR analysis has added sensitivity because each 20-µL sample is partitioned into up to 20,000 one-nL droplets, each individually assayed for presence of virus particles. ddPCR samples will be prepared as for

qPCR (above) with the exception that two dilutions of purified nucleic acid will be used for positive and negative controls, as well as for the subjected plant samples: concentrations of 100% and 1% nucleic acid diluted in MilliQ H_2O.

Timetable for Project

July-November: Determine virus infection status of plants subjected to transmission biology experiments from 2016 to 2018.
October-June: Hold winter seminars with grower organizations.

RESULTS AND DISCUSSION

Currently the tests are being performed and it is not possible to provide results.

CONCLUSIONS

No conclusions have been reached at this time.

REFERENCES CITED

Al Rwahnih M, Rowhani A, Golino D. 2015. First report of grapevine red blotch associated virus in archival grapevine material from Sonoma County, California. *Plant Disease* 99(6): 895.

Al Rwahnih MA, Dave A, Anderson MM, Rowhani A, Uyemoto JK, Sudarshana MR. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology* 103: 1069-1076.

Bahder BW, Zalom FG, Jayanth M, Sudarshana MR. 2016. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* as a vector of grapevine red blotch-associated virus. *Phytopathology* 106: 1223-1230.

Cieniewicz E, Pethybridge S, Gorny A, Madden L, Perry KL, McLane H, Fuchs M. 2017. Spatiotemporal spread of grapevine red blotch-associated virus in a California vineyard. *Virus Research* 230: 59-62.

Dalton DT, Hilton RJ, Kaiser C, Daane KM, Sudarshana MR, Vo J, Zalom FG, Buser JZ, Walton VM. 2019. Spatial associations of vines infected with grapevine red blotch virus in Oregon vineyards. *Plant Disease*. doi: 10.1094/PDIS-08-18-1306-RE.

Fuchs M, Perry K, Golino D. 2018. Ecology of grapevine red blotch virus. *Proceedings of the 2018 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA, pp. 205-214.

Virant-Doberlet M, King RA, Polajnar J, Symondson WOC. 2011. Molecular diagnostics reveal spiders that exploit prey vibrational signals used in sexual communication. *Molecular Ecology* 20: 2204-2216. doi: 10.1111/j.1365-294X.2011.05038.x.

Yothers MA. 1934. Biology and control of the tree hoppers injurious to fruit trees in the Pacific Northwest. *U.S. Department of Agriculture Technical Bulletin 402*, 46pp.

FUNDING AGENCIES

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

BIOLOGY AND ROLE OF TREEHOPPERS IN GRAPEVINE RED BLOTCH DISEASE

Project Leader: Frank Zalom | Department of Entomology and Nematology | University of California | Davis, CA 95616 | fgzalom@ucdavis.edu
Co-Project Leader: Mysore R. Sudarshana | USDA-ARS and Department of Plant Pathology | University of California | Davis, CA 95616 | mrsudarshana@ucdavis.edu
Cooperator: Kent Daane | Department of Environmental Science, Policy, and Management | University of California | Berkeley, CA 94720 | kdaane@ucanr.edu
Cooperator: Rhonda Smith | Cooperative Extension | University of California | Santa Rosa, CA 95403 | rhsmith@ucanr.edu
Cooperator: Kaan Kurtural | Department of Viticulture and Enology | University of California | Davis, CA 95616 | skkurtural@ucdavis.edu
Cooperator: Lynn Wunderlich | Cooperative Extension | University of California | Placerville, CA 95667 | lrwunderlich@ucanr.edu
Cooperator: Vaughn Walton | Department of Horticulture | Oregon State University | Corvallis, OR 97333 | vaughn.walton@oregonstate.edu

Reporting Period: The results reported here are from work conducted July 2018 to October 2019.

ABSTRACT

The results of this project are expected to better define the possible role of the three-cornered alfalfa hopper (*Spissistilus festinus*; 3CAH) and other vineyard treehoppers in the epidemiology of grapevine red blotch virus, including management of virus spread, by determining their feeding on grapevines seasonally and their phenology in relation to cover crops and non-crop vegetation in and around vineyards. Possible transmission by other treehoppers found in vineyards where grapevine red blotch virus is spreading will also be confirmed. This essential information will contribute to the management of grapevine red blotch disease by cultural methods such as reducing plant hosts favorable to sustaining vector populations, or precise treatment timings based on treehopper biology in vineyards and when transmission is most likely to occur.

LAYPERSON SUMMARY

The results of this project are expected to better define the possible role of the three-cornered alfalfa hopper (*Spissistilus festinus*; 3CAH) and other vineyard treehoppers in the epidemiology of grapevine red blotch virus, including management of virus spread, by determining their feeding on grapevines seasonally and their phenology in relation to cover crops and non-crop vegetation in and around vineyards. Possible transmission by other treehoppers, planthoppers, psyllids, and phloem-feeding treehoppers found in vineyards where grapevine red blotch virus is spreading will also be confirmed. This essential information will contribute to the management of grapevine red blotch disease by cultural methods such as reducing plant hosts favorable to sustaining vector populations, or precise treatment timings based on treehopper biology in vineyards and when transmission is most likely to occur.

INTRODUCTION

A single-stranded DNA virus, grapevine red blotch virus (GRBV; family Geminiviridae), associated with grapevine red blotch disease (Al Rwahnih et al., 2013; Sudarshana et al., 2015), is now recognized as the causal agent of grapevine red blotch disease (Cieniwicz et al., 2017). Because of its adverse effect on wine quality and resulting revenue loss, GRBV is becoming one of the most intensely studied grapevine viruses in California. A recent analysis on the economic impact indicated that the disease can cause economic losses of as much as $30,000 per acre in North Coast vineyards (Rickett et al., 2016).

Among the several insect species found in commercial vineyards with red blotch disease, the three-cornered alfalfa hopper (*Spissistilus festinus*; 3CAH) was found to be capable of transmitting GRBV under greenhouse conditions (Bahder et al., 2016). In studies conducted in California by Cornell University virologists, spatial patterns of red blotch distribution and 3CAH adults caught on yellow sticky traps that tested positive for GRBV by polymerase chain reaction (PCR) indicated that this membracid is the most likely vector of significance to virus epidemiology (Cieniewicz et al., 2018). However, our studies on GRBV transmission using 3CAH have not produced consistent results. We are curious whether this inconsistency might be due to the fact that different sources of field-collected insects have been used in different studies and different years, raising the possibility of differential transmission specificity among 3CAH populations or even the existence of potential cryptic species, such as is the case with sweet potato whitefly (*Bemisia tabaci*), the vector of the geminivirus tomato yellow leaf curl virus (Polston et al., 2014).

There remains a need to study related treehopper species (especially in the treehopper genus *Tortistilus*) that have been found in vineyards where virus spread is occurring in Oregon and California (Dalton et al., 2019), and other members of the Hemiptera suborder Auchenorrhyncha, for their possible role in GRBV transmission. In spring 2017 and again in spring 2018 we made extensive collections of *Tortistilus* adults from a Napa County vineyard, and found morphs of brown and green color both with and without suprahumeral horns from the same host plants on the same day. The insects had previously been identified as *T. albidosparsus*, *T. pacificus*, and *T. wickhami* primarily based on the presence or absence of "horns." We are collaborating with Dennis Kopp, an expert on Membracidae at the Smithsonian Natural History Museum in Washington D.C., to unravel the identification of *Tostistilu*s treehoppers, study their seasonal biology, and determine their possible role in GRBV transmission.

In summary, this project is intended to unravel the role of treehoppers (Hemiptera: Membracidae) and related Auchenorrhyncha found in California vineyards in the spread of GRBV, and develop sustainable management guidelines by building on our recently completed studies (Preto et al., 2019b) involving the population dynamics of 3CAH in vineyards (Preto et al., 2019a), the suitability of grapevines as a reproductive host for 3CAH (Preto et al., 2018b), and cover crops and common weeds that serve as their feeding and reproductive hosts (Preto et al., 2018a).

OBJECTIVES

The specific objectives of this project are:

1. Determine the timing of treehopper girdling in relation to red blotch incidence in vineyards.
2. Conduct field and greenhouse GRBV transmission studies using 3CAH and *Tortistilus* spp. treehoppers collected from vineyards with grapevine red blotch disease, and detect GRBV presence in the salivary glands of insects collected.
3. Confirm the taxonomic identification and monitor *Tortistilus* spp. populations in California vineyards and surrounding landscapes over the season.

RESULTS AND DISCUSSION

Objective 1. Determine the Timing of Treehopper Girdling in Relation to Red Blotch Incidence in Vineyards

We documented 3CAH phenology from 2016 to 2018 in a GRBV-infected block of Cabernet Sauvignon at the UC Davis Oakville Experimental Station and published the results of the study, including the incidence of treehopper girdling in the block (Preto et al., 2019a). In 2017, we also began sampling a commercial Cabernet Sauvignon vineyard west of California State Route 29 near Oakville (Oakville 1) for treehopper girdles every two weeks, as well as a Cabernet Sauvignon research vineyard at the UC Davis Armstrong Tract in Solano County. The latter block has a three-meter wide strip of alfalfa planted adjacent to the southern edge that serves as a reservoir and source for natural migration of 3CAH to the vineyard. In 2018, we continued to monitor and count girdles in both the Armstrong and Oakville 1 vineyards in order to obtain a second year of data. We also began to count girdles in a replanted Cabernet Sauvignon vineyard located along Oakville Cross Road just east of Oakville (Oakville 2) that is close to a riparian area. Girdle counts from all three vineyards were taken every two weeks, beginning three weeks after bud break until fall leaf drop, from six rows containing five vines each located within the same vineyard. Girdles were documented as being located on the apical shoot or leaf petiole, and counted only if necrosis extended around the entire petiole or shoot. **Figure 1** presents the results of our girdle sampling in the Oakville 1 vineyard. Similar to our results from 2017, girdles were first observed in June with peaks of new girdles occurring in July and late September, coinciding with emergence of adult 3CAH. More girdles were found on petioles than apical shoots. **Figure 2** presents the results of our girdle sampling in the UC Davis Armstrong Tract vineyard. The seasonal occurrence of new girdles was similar to what was observed in 2017, with the first girdles observed in late July and a single peak of new girdles occurring in late September into October. The number of petiole girdles was similar to the number of apical shoot girdles.

In May 2018, at the Oakville 2 site that is adjacent to a vineyard that had been removed due to a high level of GRBV infection, we planted fifteen four-year-old recipient Cabernet Sauvignon grapevines [quantitative PCR (qPCR) tested GRBV negative] between the established field vines. The fifteen interplanted and adjacent vines were sampled for treehopper girdles every two weeks (data presented in **Figure 3**). The first girdle was found on June 22 (on a young vine), with peak new girdles occurring in late September. In general, more girdles were found on the older established vines than the younger interplanted vines, but this could simply be due to their relative size difference. The occurrence of girdles on each of the young vines is now known and will provide some background on when treehopper feeding occurred should GRBV be detected in one of these previously-tested GRBV negative vines.

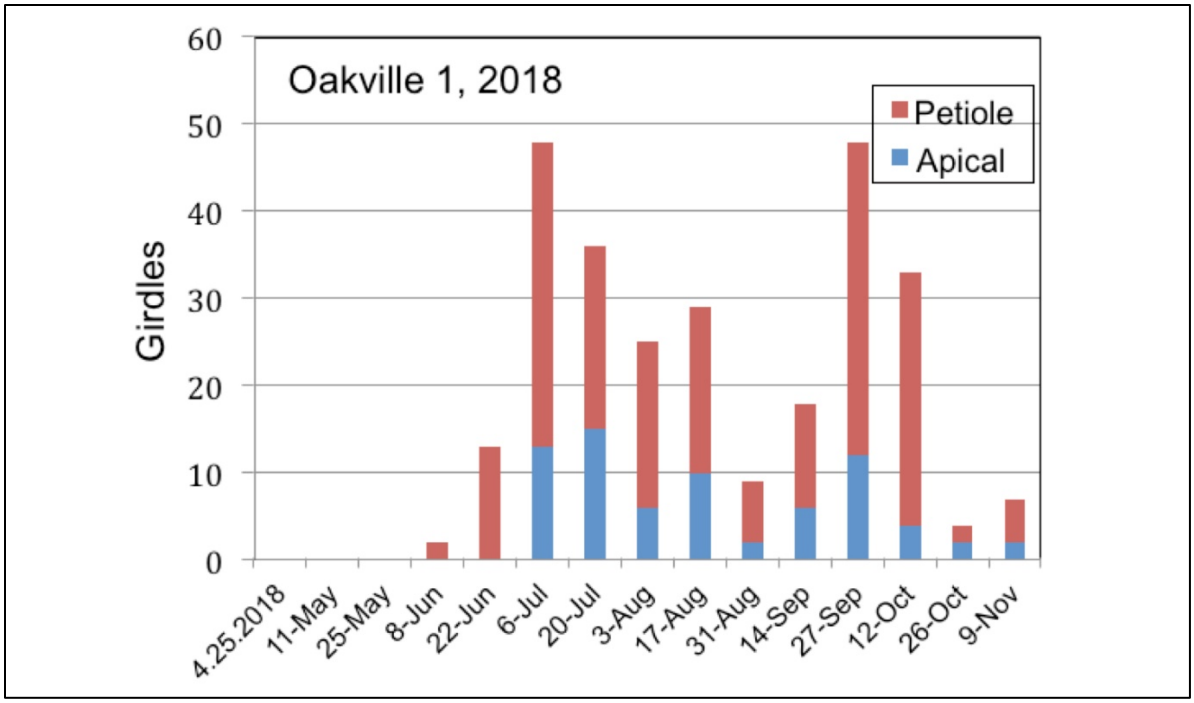


Figure 1. Total new petiole and apical girdles found on 30 vines at the Oakville 1 site in 2018. Sampling was terminated following the November 9 sampling date because this entire block was removed due to high grapevine red blotch disease incidence.

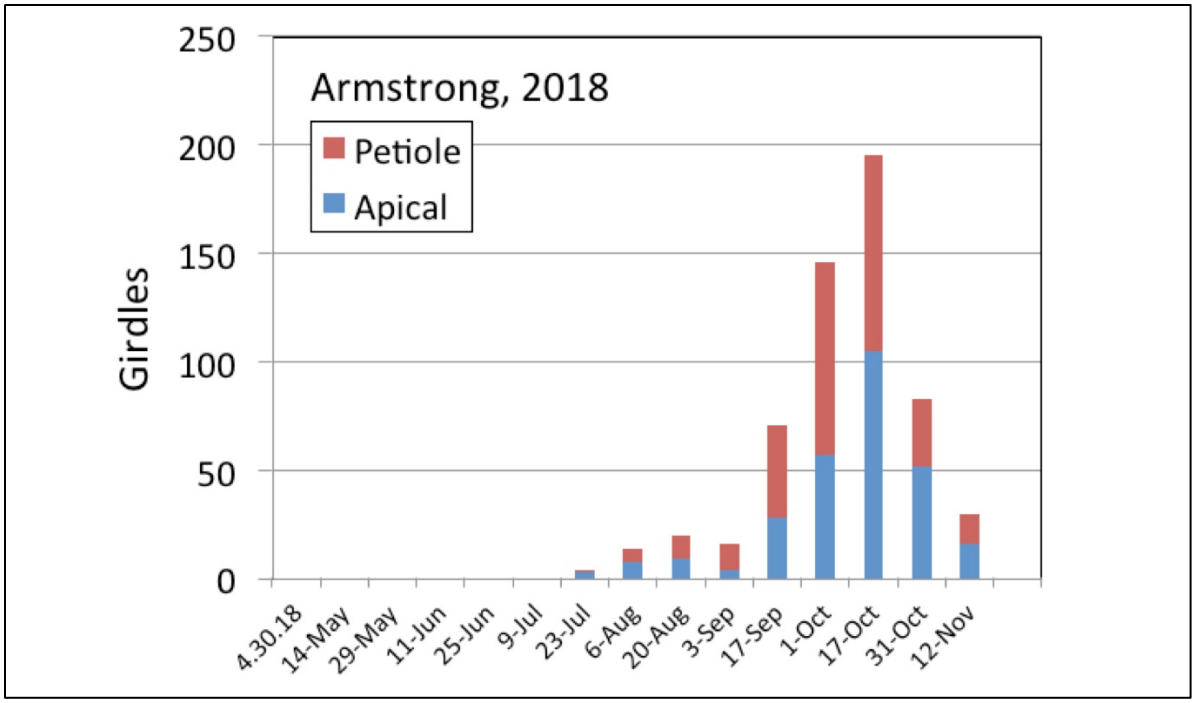


Figure 2. Total new petiole and apical girdles found on 30 vines at the UC Davis Armstrong site in 2018. Sampling was terminated following a severe wind storm that occurred on October 30 that removed most of the remaining leaves from the vines.

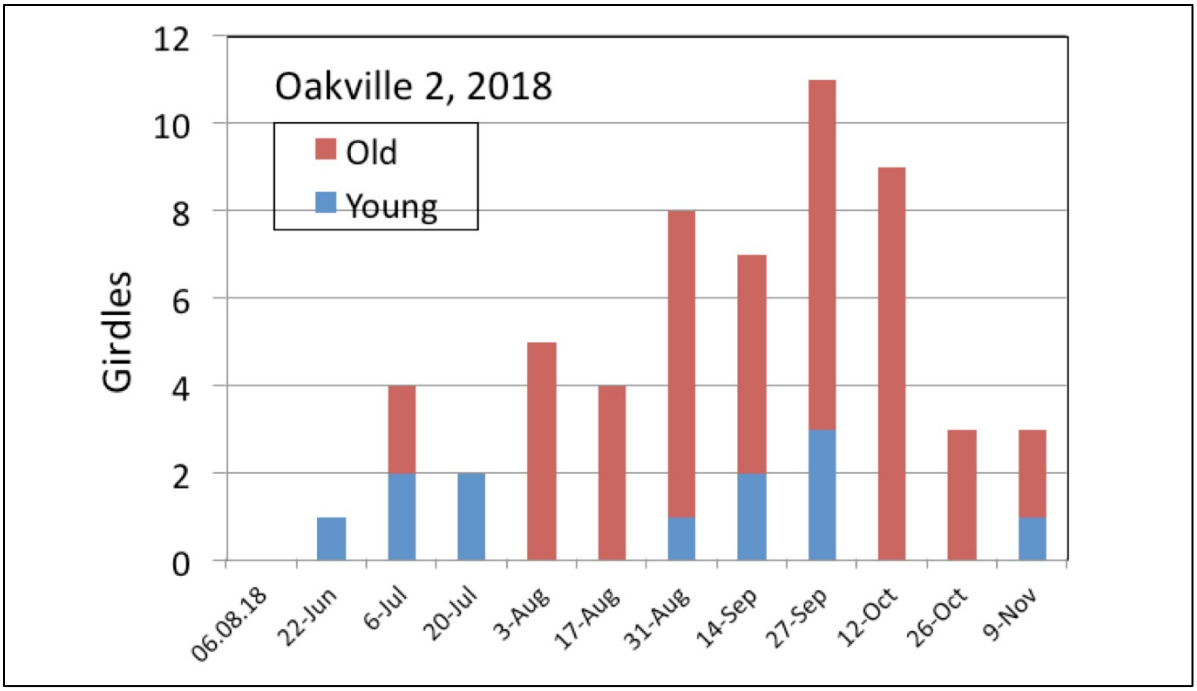


Figure 3. Total new petiole and apical shoot girdles found on established (old) and interplanted (young) vines at the Oakville 2 vineyard.

Objective 2. Conduct Field and Greenhouse GRBV Transmission Studies Using 3CAH and *Tortistilus* spp. Treehoppers Collected from Vineyards with Grapevine Red Blotch Disease, and Detect GRBV Presence in the Salivary Glands of Insects Collected

In December 2017, we collected and subsequently rooted cuttings from qPCR-tested GRBV-infected grapevines from a Zinfandel vineyard in Amador County that we had studied for the previous three years and had documented GRBV spread for use in our transmission studies during the first year of this project (Wunderlich et al., 2017). Sequencing of amplicons obtained by qPCR assays on these GRBV isolates indicates that they belong to clades I (ACU-1) and II (ACU-II).

Field Spread and Transmission Studies

UC Davis Armstrong Tract Spread Study. We have previously established a Cabernet Sauvignon vineyard using qPCR-tested GRBV negative vines at the UC Davis Armstrong Tract in Solano County for use in field transmission experiments. The vineyard consists of 30 vine rows with 55 vines per row, is oriented east-to-west, and has an established alfalfa strip along the southern edge of the vineyard that became naturally infested with 3CAH. Twenty grapevines each infected with GRBV clade-I (CS-337-1) and clade-II (CF-214-1) isolates were planted along the southernmost vine row when the vineyard was established three years ago. The vines have been tested for GRBV by qPCR annually since then, but no virus spread has been detected to date. On September 8, 2018, we planted ten rooted GRBV clade I (ACU-1) and II (ACU-II) Zinfandel vines (five of each clade) from the Amador County vineyard in the third vine row north of the alfalfa strip, and an additional ten infected vines were transplanted in the same vine row in July 2019. We will continue testing vines in this block throughout this project to determine if GRBV spread is occurring.

UC Davis Oakville Experimental Station Spread Study**.** In fall 2017, we mapped a newly-planted Cabernet Sauvignon block (A block) at the UC Davis Oakville Experimental Station (1,066 vines of CS clone 7 on C3309) that is adjacent to blocks that had a history of grapevine red blotch disease occurrence, and found that a third of the vines had girdling damage (**Figure 4**). None of the vines had grapevine red blotch disease symptoms at that time. In fall 2018, we qPCR-tested all 1,066 grapevines for GRBV. We intend to continue monitoring symptoms and testing symptomatic vines for GRBV during the course of this project.

Row/Vine	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	V16	V17	V18	V19	V20	V21	Total
R1			G		G	G											G					4
R2		G	G										G						G		G	5
R3		G	G		G	G					G										G	6
R4		G		G	G		G	*		*	G		G				G	G			G	9
R5										G								G				2
R6	G	G							G		G	G			G			G		G	G	9
R7						G		G		G		G							G		G	6
R8							G	G	G	G				G			G			G		7
R9	G			G				G	G				G					G		G	G	8
R10	G											G		G						D		3
R11	G	G		G	G		G	G			G				G						G	9
R12	G	G	G	G											G	G	G	G	G		G	10
R13	G	G	G	*			G					G			G					G		7
R14	G	G			G	G			G		G		G							G	G	9
R15	G							G	G				G	G							G	6
R16	G	G	G	G	G		G	G	G	G		G						G				11
R17										G					G						G	3
R18	G		G	G			G	G				G		G	G	G					G	10
R19											G	G	G	G		G					G	6
R20											G											1
R21	G						G	G	G	G						G	G	G	G		G	10
R22					G					G	G					G	G		G		G	7
R23	G				G	G			G		G			G	G	G	G				G	10
R24	G	G	G	G	G			G	G		G						G	G	G	G	G	13
R25	G			G		G	G		G	G		G				G	G				G	10
R26	G						G		G			G			G			G	G	G	G	9
R27					G								G							G		3
R28					G				G						G							3
R29	G				G	G									G		G				G	6
R30			G		G		G								G							4
R31			G				G	G	G	G	G					G	G	G				9
R32			G				G		G		G					*		G		G	G	7
R33	G		G	G	G				G		G	*	G			G			G			9
R34	G				G		G	G						G	G						G	7
R35	G		G	G							G				G							5
R36					G		G			G	G			G					G			6
R37				G	G		G	G	G					G			G	G	G	G	G	11
R38		G	G	G		G	G	G					G		G			G	G			10
R39						G		G	G			G			G	G	G			G		8
R40	G	G	G		G	G			G		*	G									G	8
R41	G	G				G		G		G	G		G		G		G	G	G			11
R42				G	*			G												G	G	4
R43				G				G	G	G	G			G	G				G	G	G	10
R44	G			G			G		G		G		G	G		G		G			G	10
R45	G			G		G	G	G	G		G	G			G				G			10
R46					G	G									G						G	4
R47	G			G	G							G					G	G				6
R48			G	G	G		G				G						G					6
R49																						0
R50			G															G		G		3
R51					G			*						*					*	G	G	3
	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	V16	V17	V18	V19	V20	V21	353

Figure 4. Map of treehopper girdles on a new Cabernet Sauvignon block (A block) at the UC Davis Oakville Experimental Station. (G = vine with girdles, * = rootstock only, and dark rectangle = missing vine.)

Tortistilus albidosparsus Gordon Valley (Napa County) Transmission Study. In June 2018, we began working in a Sauvignon Blanc vineyard in the Gordon Valley area of Napa County that had a large resident population of *T. albiodosparsus* treehoppers. We tested all of the vines at the easternmost edge of the most heavily insect-infested area of the vineyard blocks for GRBV infection by qPCR, and mapped which vines tested positive for the virus. Third and fourth instar *T. albidosparsus* nymphs were sweep-netted from vetch growing ~700 meters from a Pope Valley, Napa County vineyard in early May 2019 and returned to our UC Davis greenhouse, where they were raised on potted vetch plants to adults. The cytochrome oxidase 1 (CO1) gene, a mitochondrial gene used in insect taxonomy and identification, was used to confirm that the Pope Valley insects were the same species as those that were collected at the Gordon Valley field site. We used these insects as the uninfected controls for our June 2019 transmission study because *T. albidosparsus* collected at the study site could have potentially fed on GRBV-infected vines prior to the study. The insects used for our transmission study were sweep-netted directly off of GRBV-positive vines. Our study used 15 tested GRBV-free field grapevines at the field site as replicates for untreated controls, and 15 GRBV-positive grapevines as source vines for acquisition by the treehoppers. The GRBV status of these vines was reconfirmed by qPCR testing in April 2019. Individual mesh cages containing 10 insects each were placed onto each of our healthy (**Figure 5a**) and GRBV-positive block vines (**Figure 5b**). All insects were given a 48-hour acquisition access period (AAP) and then immediately placed onto potted, mesh-caged, tested GRBV-free recipient vines that were placed directly beneath all negative control and treatment vines. Insects were then given a 48-hour inoculum access period (IAP) in the field before all vines were brought back to our greenhouse. These will be tested for GRBV infection status by qPCR beginning in January 2020.

Figures 5a (left) and 5b (right). qPCR-tested grapevines that were uninfected (left) or GRBV-infected (right), showing AAP and IAP cages with *T. albidosparsus*.

To confirm that GRBV was present on the canes where the insects were caged for acquisition, we removed the nearest leaves distal to the cages and tested them using qPCR. All leaves from the 15 GRBV-positive vines tested GRBV positive and all leaves from the 15 GRBV-negative vines tested GRBV negative except one that had a high cycle threshold (Ct) value, which might be interpreted as a "potential" positive. Another transmission study was conducted

simultaneously with this experiment using the same methods as described, but did not have a set AAP for *T. albidosparsus.* Instead, adult insects that were feeding on tested GRBV infected and uninfected field vines were transferred directly to potted caged recipient vines. The rationale for the companion study without a defined AAP was to reduce the amount of handling of the insects, to avoid injuring their mouthparts or otherwise harming them in a way that could potentially inhibit virus transmission. Testing of these plants for GRBV by qPCR will also begin in January 2020.

Greenhouse Transmission Studies

3CAH Greenhouse Transmission Studies. On September 9, 2018, 500 adult 3CAHs were collected from an organic alfalfa field near Davis, California. These insects were then divided into three groups and transferred to insect cages. One cage contained a GRBV clade-I vine (ACU-I), one cage contained a GRBV clade-II vine (ACU-II), and one cage contained a tested (GRBV-free) vine. After a 48-hour AAP, insects were transferred individually into clip cages fastened onto the oldest leaf of tested GRBV-negative recipient vines. Ten replicate grapevines for each of the three treatments were established. These vines are currently being maintained and are being qPCR tested at six months post-AAP.

T. albidosparsus Transmission Study Using GRBV Clade Ghv-392 and Ghv 377 as GRBV Source Vines. In June 2018, 500 adult *T. albidosparsus* were collected from vetch growing 30 meters away from GRBV-infected grapevines in Pope Valley, Napa County. These insects were separated into four groups and transferred into four separate insect cages. One cage contained a GRBV-infected Ghv-392 vine, one cage contained a GRBV-infected Ghv-377 vine, one cage contained a tested GRBV-positive wild grapevine, and one cage contained a tested GRBV-negative vine. These insects were given a 48-hour AAP, then transferred to tested GRBV-negative recipient vines and allowed a 48-hour IAP. There were 15 replicates from each of the four groups, and samples from all 60 of these recipient vines have been collected and are being qPCR tested for GRBV. qPCR-testing of these vines was initiated in March 2019, but no GRBV has been detected up to this time.

Methodology for GRBV Detection in Salivary Glands. Dissection of insect abdomens, mouthparts, and salivary glands followed by qPCR testing to detect virus presence is a useful tool for identifying promising vector candidates. If insects are actively feeding on known GRBV-infected vines, then detecting the virus in these body parts would assist in defining circulative or noncirculative transmission. In August 2018, we collected hundreds of 3CAHs, as well as Virginia creeper leafhoppers (*Erythroneura ziczac*; VCLH) to serve as negative controls, from GRBV-free hosts. After starving the insects for three hours, half of the collected insects of each species were placed into mesh cages containing a GRBV-infected source vine (Ghv-392), and the other half of the insects of each species were placed into a mesh cage containing a GRBV-free healthy (Ghv-35) source vine.

All insects were allowed an AAP of 48 hours. Beet leafhopper (*Circulifer tenellus*; BLH) adults from a laboratory colony provided by the R. Gilbertson lab at UC Davis functioned as an internal positive control. These leafhoppers were fed on sugar beet plants infected with beet curly top virus (BCTV), a single-stranded DNA geminivirus. Individual 3CAH, VCLH, and BLH were aspirated from their hosts, placed singly into 1.5 ml centrifuge tubes containing one ml of 20%

bleach solution, and vortexed on high for five seconds. Individual insects were then placed into another 1.5 ml centrifuge tube containing one ml sterile Millipore water and vortexed again for five seconds. Insects were then removed from the centrifuge tubes using a #3 Bioquip insect pin and placed ventral side up onto a sterile Petri dish situated directly under a Leica 12.5 stereo-microscope. Two flame-sterilized insect pins were used to extract salivary glands from each insect. The first pin was used to press against the insect, stabilizing it so that the second pin could easily locate the area between the insect's first and second coxae. Once this area was located, the second pin was pressed through the entire insect, effectively severing the head/first coxae region from the rest of the insect's body. After the insect heads were removed, the heads were placed onto another sterile Petri dish and a single drop of Millipore filtered water was pipetted onto them. Entirely immersing an insect's head in fluid facilitates salivary extraction. After teasing out the salivary glands with insect pins, a sterile 10 uL pipette tip attached to a 0.1 uL to 20 uL pipette was used to sever the salivary glands from the insect's head. The salivary glands were individually placed into a 1.5 mL centrifuge tube containing 180 uL ATL buffer (Qiagen Inc.) and 20 uL proteinase K, and incubated for 30 minutes at 65°C. In this particular assay, none of the salivary gland extracted from 3CAHs or VCLHs tested positive for GRBV by qPCR tests. However, extracts from 9 of 10 beet leafhoppers fed on BCTV-infected sugar beets tested positive for BCTV.

This method was subsequently evaluated on 20 adult *T. albidosparsus* collected directly off of the tested GRBV-negative controls and 20 adults collected off of GRBV-positive grapevines at the Gordon Valley Road field site. qPCR tests indicated that the abdomen and salivary glands from one of the 20 adult *T. albidosparsus* collected from a positive grapevine tested positive for GRBV, while no virus was detected in any collected from the GRBV-negative grapevines, suggesting a rather low 5% possibility of GRBV vector competence. This method can be used to identify other potential GRBV vector candidates as well.

Objective 3. Confirm the Taxonomic Identification and Monitor *Tortistilus* spp. Populations in California Vineyards and Surrounding Landscapes Over the Season

In spring 2016, we found colonization of grapevines by treehoppers that we identified to belong to the genus *Tortistilus* in vineyards where virus spread was occurring. *Tortistilus* treehoppers had not been associated with grapevines prior to that time, although there was mention of the "buffalo treehopper" which actually belongs to a different treehopper genus (*Stictocephala*), as feeding on California grapevines in Smith (2013). Later that year, V. Walton's lab at Oregon State University also found *Tortistilus* treehoppers in Oregon vineyards where grapevine red blotch disease was spreading (Dalton et al., 2019). Both 3CAH and *Tortistilus* spp. belong to the Ceresini tribe of Membracidae. In spring 2017, we made an extensive collection of *Tortistilus* adults from a Napa County vineyard and found morphs of brown and green color both with and without horns from the same host plants on the same day (**Figure 6**). These insects were tentatively identified as *T. albidosparsus*, *T. pacificus*, and *T. wickhami* based on the presence or absence of a suprahumeral horn characteristic and to some extent their coloration. That three closely-related species would seemingly occupy the same feeding niche at the same time and location seemed odd to us, so we sent them to a specialist on the family Membracidae, D. Kopp at the Smithsonian Natural History Museum. He identified the four morphs (brown horned, green horned, brown hornless, and green hornless) as being the same species based on microscopic observations of genitalia and the characteristic spots on the front of their head.

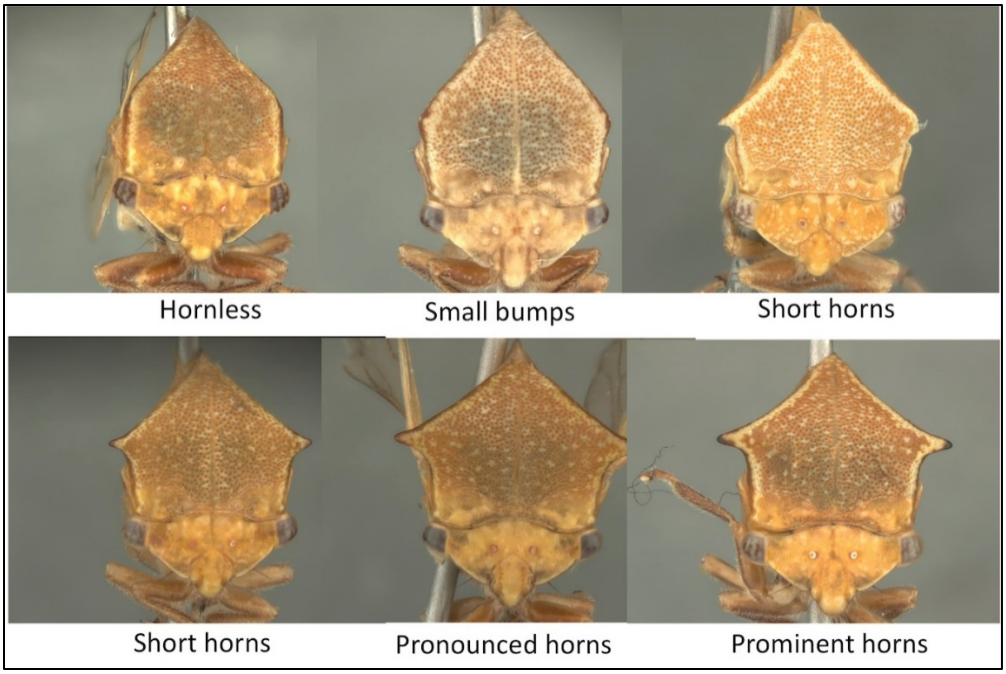


Figure 6. Clinal variation of suprahumeral horns in females from a single collection of *T. albidosparsus* from a Napa County vineyard.

Taxonomic Identification of Male *T. albidosparsus* via Sequencing and Auto-Montage. In 2018, we performed shotgun DNA sequencing on eight of these morphs, all collected on the same hosts on the same date and from the same Napa County vineyard, and found them to possess the identical cytochrome oxidase 1 gene (CO1), a mitochondrial gene used in insect taxonomy and identification (**Table 1**). The sequence reads described were deposited at the National Center for Biotechnology Information (NCBI) under bioproject (BIOPROJ00090900) as the first genomic resource for the genus *Tortistilus*. These results indicate that the morphs indeed all belong to *T. albidosparsus*.

Table 1. Sample information and sequence reads obtained by Illumina sequencing of DNA from four morphotypes of insects now described as *T. albidosparsus* and collected in a grape vineyard in Napa County, California.

Sample ID	Code	Color	Horned	Illumina reads (million)	Coverage Gbp)
DS17-01	BH-	Brown	No	46.0	13.8
DS17-02	BH-	Brown	No	44.8	13.4
DS17-03	GH-	Green	No	47.3	14.2
DS17-04	GH-	Green	No	45.8	13.7
DS17-05	BH+	Brown	Yes	43.9	13.2
DS17-06	BH+	Brown	Yes	45.9	13.8
DS17-07	GH+	Green	Yes	44.5	13.4
DS17-08	GH+	Green	Yes	45.3	13.6

Traditionally, identification of *Tortistilus* species was made exclusively by morphological characters of male genitalia. However, taxonomic determination of the *Tortistilus* species of interest to us as potential GRBV vectors was met with challenges, as the original descriptions of these particular insects were only accompanied by hand-drawings. In order to examine the male genitalia more thoroughly, we used high resolution Leica auto-montage to create photo images of the brown horned, green horned, brown hornless, and green hornless morphotypes of the *Tortistilus* we had sequenced. The auto-montage images revealed that they had identical genitalia (**Figures 7A-H**), confirming our biological observations as well as the results of the CO1 sequencing, and adding further confirmation that they represent a single species (*T. albidosparsus*).

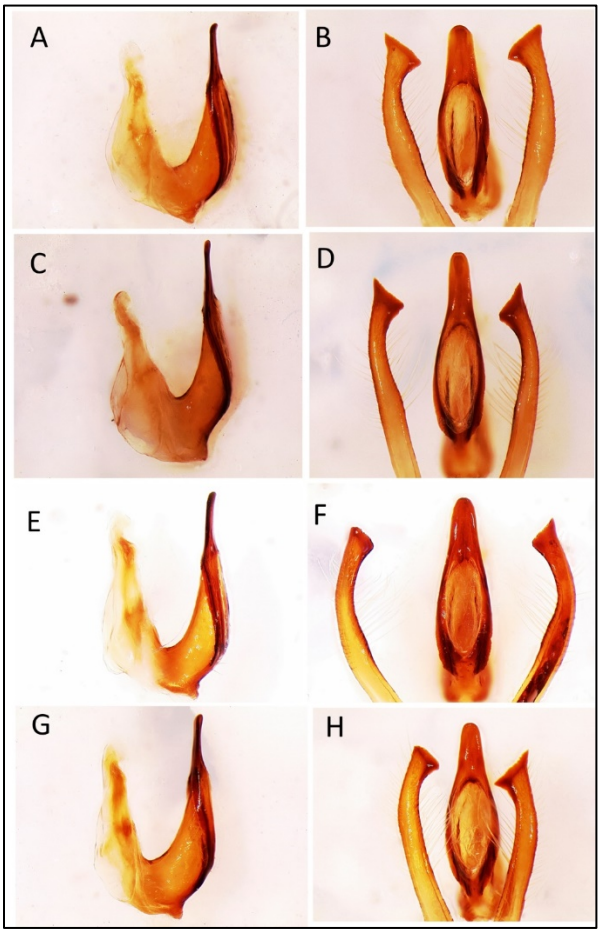


Figure 7. A, C, E, and **G** are profiles of male *T. albidosparsus* aedeagus posterior and anterior arms; **B, D, F**, and **H** are caudal views of male *T. albidosparsus* posterior aedeagus and posterior style arms. **A** and **B** are horned brown, **C** and **D** are horned green, **E** and **F** are hornless brown, and **G** and **H** are hornless green. All *Tortistilus* male genitalia were dissected and lysed using 180 uL ATL buffer and 20 uL proteinase K in a 1.5 ml centrifuge tube at an incubation temperature of 80ºC for 40 minutes. Images were taken with a digital JVC camera mounted onto a Leica MZ 16A dissecting microscope at 110X magnification.

Mating Study with *T. albidosparsus*. Morphotypes. On April 25, 2018, we collected third and fourth instar *T. albidosparsus* nymphs from common vetch growing in a riparian area 30 meters removed from a confirmed GRBV-infected vineyard in Pope Valley, Napa County. The nymphs were then returned to UC Davis, where they were transferred into individual clip cages placed on potted vetch plants in field cages. As the treehoppers emerged as adults, they were placed into separate clip cages in various combinations of color and horn morphotypes. These morphotypes were subsequently placed in various combinations according to their morphotype in cages on oak plants that were grown from acorns in one-gallon pots. Of six successful mating pair combinations, three samples had oviposition scars (**Figure 8**) that could indicate successful mating and egg-laying by the treehoppers. In late April, the mating pairs consisting of four hornless males and five horned females produced nymphs that were reared to adults, resulting in one horned brown female and one hornless brown female morphotypes. The mating pairs consisting of four hornless males and five hornless females produced nymphs, one of which survived to emerge as an adult horned brown female.

Figure 8. *T. albidosparsus* oviposition scar on oak twig in the mating study using both horned and hornless insects.

CONCLUSIONS

We have made substantial progress in addressing the objectives of this project. We completed the second year of monitoring the seasonal occurrence of petiole and apical shoot girdles in Napa County vineyards and in our UC Davis experimental vineyard. Girdling is an indication of feeding by treehoppers and suggests a time during which GRBV transmission could occur. First girdles were found in mid-June at the Napa County sites and somewhat later at UC Davis, and new girdles were found until the beginning of November. We believe that virtually all of the girdles found at these sites were caused by 3CAH feeding. We continued spread studies at our UC Davis vineyard and at other sites we have been monitoring in Amador and Santa Barbara counties, and initiated new monitoring at two additional sites in Napa County. We initiated greenhouse transmission studies with 3CAH using new isolates of GRBV clades 1 (ACU-I) and 2 (ACU-II) from an Amador County Zinfandel vineyard where we had been monitoring spread

for the previous three years, and with *T. albidosparsus* using GRBV clade Ghv-392 and Ghv 377 as GRBV source vines. A large semi-field transmission study with *T. albidosparsus* conducted in a Gordon Valley vineyard using a 48-hour AAP on GRBV source vines and a second study with *T. albidosparsus* that were collected directly from tested GRBV-positive vines and transferred directly to recipient vines brought to the field site were initiated as well. We improved and confirmed our methodology for dissection of insect abdomens, mouthparts, and salivary glands followed by qPCR testing to detect virus presence, and are applying this tool to identify additional promising vector candidates. Finally, we used molecular and auto-montage techniques to confirm the identity of the *Tortistilus* species treehoppers that are commonly found in certain North Coast vineyards. The brown horned, green horned, brown hornless, and green hornless treehopper morphotypes are in fact a single species, *T. albidosparsus*. Mating studies documented that crossing the horned and hornless morphotypes resulted in viable offspring. These accomplishments set the stage for further research on the possible role of treehoppers in GRBV transmission.

REFERENCES CITED

Al Rwahnih, M, Dave A, Anderson M, Rowhani A, Uyemoto JK, Sudarshana MR. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology* 10: 1069-1076.

Bahder BW, Zalom FG, Jayanth M, Sudarshana MR. 2016. Phylogeny of Geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* as a vector of grapevine red blotch-associated virus. *Phytopathology* 106(10): 1223-1230.

Cieniewicz EJ, Pethybridge SJ, Loeb G, Perry K, Fuchs M. 2018. Insights into the ecology of grapevine red blotch virus in a diseased vineyard. *Phytopathology* 108: 94-102.

Dalton DT, Hilton RJ, Kaiser C, Daane KM, Sudarshana MR, Vo J, Zalom FG, Buser JZ, Walton VM. 2019. Spatial associations of vines infected with grapevine red blotch virus in Oregon vineyards. *Plant Disease* 103: 1507-1514.

Polston JE, De Barro P, Boykin LM. 2014. Transmission specificities of plant viruses with the newly identified species of the *Bemisia tabaci* species complex. *Pest Management Science* 70: 1547-1552.

Preto CR, Sudarshana MR, Zalom F.G. 2018a. Feeding and reproductive hosts of *Spissistilus festinus* (Hemiptera: Membracidae) found in Californian vineyards. *Journal of Economic Entomology* 111(6): 2531-2535.

Preto CR, Sudarshana MR, Bollinger ML, Zalom FG. 2018b. *Vitis vinifera* as a reproductive host of *Spissistilus festinus* (Say) (Hemiptera: Membracidae). *Journal of Insect Science* 18(6): 20. https://doi.org/10.1093/jisesa/iey129s.

Preto CR, Bahder BW, Bick EN, Sudarshana MR, Zalom FG. 2019a. Seasonal dynamics of *Spissistilus festinus* (Say) (Hemiptera: Membracidae) in a Californian vineyard. *Journal of Economic Entomology* 112(3): 1138-1144.

Preto CR, Sudarshana MR, Zalom FG. 2019b. A grapevine red blotch virus vector: Three-cornered alfalfa hopper in Napa vineyards. *American Vineyard Magazine* 28(2): 14-18.

Ricketts KD, Gomez MI, Fuchs MF, Martinson TE, Smith RJ, Cooper ML, Moyer M, Wise A. 2016. Mitigating the economic impact of grapevine red blotch: Optimizing disease management strategies in U.S. vineyards. *American Journal of Enology and Viticulture* 68: 127-135.

Smith R. 2013. Three-cornered alfalfa hopper. Bettiga LJ (ed.). *Grape Pest Management, Third Edition*. University of California, Division of Agriculture and Natural Resources, Publication 3343, pp. 286-287.

Sudarshana MR, Perry KL, Fuchs MF. 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Phytopathology* 105: 1026-32.

Wunderlich L, Bollinger ML, Shaffer M, Preto CR, Bahder B, Zalom FG, Sudarshana MR. 2017. Investigating the spread and effect of grapevine red blotch virus in California-grown Zinfandel. *Proceedings of the 20th International Meeting of Viticulture GiESCO* 20: 215-219.

FUNDING AGENCIES

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

MONITORING VINE MEALYBUG RESISTANCE TO IMIDACLOPRID

Project Leader: Mark Sisterson | San Joaquin Valley Agricultural Sciences Center | USDA Agricultural Research Service | Parlier, CA 93648 | mark.sisterson@usda.gov
Co-Project Leader: Lindsey Burbank | San Joaquin Valley Agricultural Sciences Center | USDA Agricultural Research Service | Parlier, CA 93648 | lindsey.burbank@usda.gov
Co-Project Leader: Rachel Naegele | San Joaquin Valley Agricultural Sciences Center | USDA Agricultural Research Service | Parlier, CA 93648 | rachel.naegele@usda.gov
Co-Project Leader: Christopher Wallis | San Joaquin Valley Agricultural Sciences Center | USDA Agricultural Research Service | Parlier, CA 93648 | christopher.wallis@usda.gov
Cooperator: David Haviland | Cooperative Extension | University of California | Bakersfield, CA 93307 | dhaviland@ucdavis.edu
Cooperator: Kent Daane | Department of Environmental Science, Policy, and Management | University of California | Berkeley, CA 94720 | kdaane@ucanr.edu

Reporting Period: The results reported here are from work conducted July 2019 to October 2019.

ABSTRACT

The vine mealybug (*Planococcus ficus)* represents a worldwide threat to wine and table grape production. Contact insecticides have limited efficacy because vine mealybugs are often found under bark or concealed in grape clusters. As a result, systemic insecticides are a popular choice for mealybug control. Imidacloprid, a neonicotinoid, was registered for use in the United States in 1994 and is commonly applied in vineyards to suppress a wide range of insect pests including mealybugs. Baseline testing for vine mealybug susceptibility to imidacloprid by Prabhaker et al. (2012) between 2006 and 2008 provided no evidence of resistance. As it has been a decade since mealybug populations were screened for resistance to imidacloprid, testing is underway to determine if susceptibility has changed. For screening, a modified version of the assay used by Prabhaker et al. (2012) was developed. The modified assay consists of placing a four-inch grape seedling in a water pick with a known concentration of imidacloprid. The seedling is then infested with ten second instar vine mealybugs and survival at six days quantified. High performance liquid chromatography testing confirmed that imidacloprid content of grape leaves and stems reflected the concentration of imidacloprid placed in the water pick. In August and September of 2019, mealybugs were collected from seven conventional vineyards and four organic vineyards. Four collections yielded no mealybugs. The remaining seven collections (three organic and four conventional) yielded mealybugs that were transferred to squash to initiate colonies for testing. Testing to estimate the LC50 and LC99 of each population will begin once colonies reach a stable population size.

REFERENCES CITED

Prabhaker N, Gispert C, Castle SJ. 2012. Baseline susceptibility of *Planococcus ficus* (Hemiptera: Pseudococcidae) from California to select insecticides. *Journal of Economic Entomology* 105: 1392-1400.

FUNDING AGENCIES
Funding for this project was provided by the Consolidated Central Valley Table Grape Pest and Disease Control District, and by the USDA Agricultural Research Service, appropriated project 2034-22000-012-00D.

ABBREVIATIONS

3CAH	three-cornered alfalfa hopper
AAP	acquisition access period
ABA	abscisic acid
AChE	acetylcholinesterase
ACMV	African cassava mosaic virus
AIGC	area under the insect growth curve
ANOVA	analysis of variance
APHIS	Animal and Plant Health Inspection Service
ARS	Agricultural Research Service
AUDPC	area under the disease progress curve
AVA	American Viticultural Area
BAC	bacterial artificial chromosome
BAP	benzylaminopurine
BC	backcross
BCTV	beet curly top virus
BGSS	blue-green sharpshooter
BLH	beet leafhopper
bp	base pair
BSA	bovine serum albumin
BSCTV	beet severe curly top virus
CAD	cadaverine
CaLCuV	cabbage leaf curl virus
CAP	chimeric antimicrobial protein
Cas9	CRISPR-associated protein 9
CB	cecropin B
CDFA	California Department of Food and Agriculture
cDNA	complementary DNA
cfu	colony-forming unit
Chr	chromosome
cM	centimorgan
CO1	cytochrome oxidase 1
CP	coat protein
CRISPR	clustered regularly interspaced short palindromic repeats
Ct	cycle threshold
CTAB	cetyl trimethylammonium bromide
DAB	3,3’-diaminobenzidine

DAMP	damage-associated molecular pattern
DCeN	dynamically co-expressed neighborhood
ddPCR	droplet digital PCR
DE	differentially expressed
DNA	deoxyribonucleic acid
DSF	diffusible signal factor
dsRNA	double-stranded RNA
DVC	Davis Virus Collection
Ec	embryogenic culture
EDS	enhanced disease susceptibility
ELISA	enzyme-linked immunosorbent assay
ERF	ethylene response factor
ETc	crop evapotranspiration
FPS	Foundation Plant Services
GC	gas chromatography
GFP	green fluorescent protein
GGVA	grapevine geminivirus A
GlcNAc	N-acetylglucosamine
GLD	grapevine leafroll disease
GLRaV	grapevine leafroll-associated virus
GMB	grape mealybug
GRBD	grapevine red blotch disease
GRBV	grapevine red blotch virus
GVA	grapevine virus A
GWSS	glassy-winged sharpshooter
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high-performance liquid chromatography
HS	headspace
IAP	inoculation access period
IPMP	isopropyl methylphenol
JA	jasmonic acid
Kb	kilobase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LAMP	loop mediated isothermal amplification
LC	liquid chromatography
LPS	lipopolysaccharide
LS	least squares
MAMP	microbe-associated molecular pattern

MAPK	mitogen-activated protein kinase
MAS	marker-assisted selection
MB	meristematic bulk
MDS	multi-dimensional scaling
MES	2-(N-morpholino)ethanesulfonic acid
miRNA	microRNA
MRM	multiple reaction monitoring
MS	mass spectrometry
MYMV	mungbean yellow mosaic virus
NAA	1-naphthaleneacetic acid
nAChR	nicotinic acetylcholine receptor
NCBI	National Center for Biotechnology Information
ncRNA	non-coding RNA
NGS	next generation sequencing
NMR	nuclear magnetic resonance
nt	nucleotide
ORF	open reading frame
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PCA	pest control advisor
PCR	polymerase chain reaction
PD	Pierce's disease
PDR	Pierce's disease resistance
PGIP	polygalacturonase inhibitory protein
PM	powdery mildew
PMR	powdery mildew resistance
POI	point of inoculation
PRSV	papaya ringspot virus
PTGS	post-transcriptional gene silencing
PTI	PAMP-triggered immunity
PUT	putrescine
qPCR	quantitative PCR
QTL	quantitative trait locus
QTOF	quadropole time-of-flight
RACE	rapid amplification of cDNA ends
RGA	resistance gene analog
RIN	RNA integrity number
RNA	ribonucleic acid

RNAi	RNA interference
RNA-seq	RNA sequencing
ROS	reactive oxygen species
RT-PCR	reverse transcription PCR
SA	salicylic acid
SAR	systemic acquired resistance
SARE	Sustainable Agriculture Research and Education
SCTV	spinach curly top virus
siRNA	small interfering RNA
SJVASC	San Joaquin Valley Agricultural Sciences Center
SNP	single-nucleotide polymorphism
SPD	spermidine
SPM	spermine
SPME	solid phase micro-extraction
SRA	Sequence Read Archive
sRNA	small RNA
ss	single-stranded
STL	Stags' Leap
STSS	smoketree sharpshooter
SWUS	southwestern United States
TAP	Tree Assistance Program
TCAH	three-cornered alfalfa hopper
TDN	1,1,6-trimethyl-1,2-dihydronaphthalene
T-DNA	transfer DNA
TDZ	thidiazuron
TGMV	tomato golden mosaic virus
TGS	transcriptional gene silencing
TOF	time-of-flight
TSS	total soluble solids
TYLCSV	tomato yellow leaf curl Sardinia virus
TYLCV	tomato yellow leaf curl virus
U.S.	United States
UC	University of California
UHPLC	ultra-high-performance liquid chromatography
UPLC	ultra-performance liquid chromatography
USDA	United States Department of Agriculture
VCLH	Virginia creeper leafhopper
VMB	vine mealybug

WAG	wheat germ agglutinin
WDV	wheat dwarf virus
WPM	woody plant medium
wt	wild-type
WVV1	wild *Vitis* virus 1
Xf	*Xylella fastidiosa*

AUTHOR INDEX