

Pierce's Disease Control Program  
California Department of Food & Agriculture

# SYMPOSIUM PROCEEDINGS

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

VIRTUAL EVENT | DECEMBER 14-15, 2021



# **Proceedings of the 2021 Pierce's Disease Research Symposium**

- December 2021 -

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**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The reports in this document have not been peer reviewed.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**

- **Modeling of *Xylella fastidiosa* Transmission and Grapevine Susceptibility Using Fluid Dynamic Simulations**  
Rodrigo P.P. Almeida, Andrew J. McElrone, Leonardo De La Fuente,  
and Elizabeth G. Clark .....2
- **Progression of Pierce's Disease Symptoms and *Xylella fastidiosa* Colonization of Grapevines Under Field Conditions**  
Rodrigo P.P. Almeida .....8
- **Management of the Federal Permits for Multi-Investigator Field-Testing of Transgenic Grapevine Rootstocks in California**  
Abhaya M. Dandekar .....17
- **Systemic Formulations of Antibacterial Nanoparticles for Pierce's Disease Management**  
Leonardo De La Fuente, Lindsey Burbank, and Swadeshmukul Santra .....21
- **Field Evaluation of Cross-Graft Protection Effective Against Pierce's Disease by Dual DNA Constructs Expressed in Transgenic Grape Rootstocks**  
David Gilchrist.....31
- **Transgenic Rootstock-Mediated Protection of Grapevine Scion by Dual Stacked DNA Constructs**  
David Gilchrist and James Lincoln.....41
- **Optimizing Biological Control of Pierce's Disease with *Paraburkholderia phytofirmans***  
Steven Lindow and Caroline Roper.....52
- **Geographic Distribution of Isolate Virulence in *Xylella fastidiosa* Collected from Grape in California and Its Effect on Host Resistance**  
Rachel P. Naegele, Lindsey Burbank, and Leonardo De La Fuente.....67
- **Generating Pierce's Disease Resistant Grapevines Using CRISPR/Cas9 and Traditional Transgenic Approaches**  
Caroline Roper and Dario Cantu.....75
- **Development of a Gene Editing Technology for Grapevines Using Plant Protoplasts**  
David Tricoli.....81



- **Breeding Pierce's Disease Resistant Winegrapes**  
Andrew Walker .....91
- **Molecular Breeding Support for the Development of Pierce's Disease Resistant Winegrapes**  
Andrew Walker and Dario Cantu .....107

## **ABSTRACTS**

- **Isolation and Whole Genome Sequencing of *Curtobacterium* sp. Strain TXMA1 from a Grapevine Showing PD-like Symptoms in Texas, U.S.A.**  
Jianchi Chen, Craig Ledbetter, and Michael O'Leary .....121
- **Isolation and Characterization of Grapevine *Trichoderma* spp. for Biological Control of Vineyard Diseases**  
Christopher Wallis, Zachary Gorman, and Jianchi Chen .....123

<p><b>Section 2:</b> <b>Glassy-winged Sharpshooter</b></p>
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## **REPORTS**

- **CRISPR-Mediated Genome Modification of *Homalodisca vitripennis* for the Genetic Control of Pierce's Disease**  
Peter W. Atkinson, Richard A. Redak, Linda L. Walling, and Jason E. Stajich .....125
- **GWSS Perform Fewer *Xf*-inoculating Behaviors on Resistant *Vitis champinii* grapevines than on susceptible *Vitis vinifera* cv. 'Chardonnay'**  
Elaine A. Backus .....138
- **Riverside County Glassy-winged Sharpshooter Program: Temecula Valley and Coachella Valley**  
Matt Daugherty, Carmen Gispert, and Thomas Perring .....151

**Section 3:  
Other Pests and Diseases of Winegrapes**

**REPORTS**

- **Monitoring Grapevine Red Blotch Virus at Russell Ranch Foundation Vineyard**  
Maher Al Rwahnih, Kent Daane, Neil McRoberts, Deborah Golino,  
and Vicki Klaassen .....157
- **Genomics Based Technology for Identification, Tracking, Insecticide Resistance  
Surveillance, and Pest Management of Vine Mealybug in Vineyards**  
Lindsey Burbank, Rachael P. Naegele, Dario Cantu, and Mark Sisterson .....170
- **Improving Extension Outcomes: Identifying Drivers and Barriers to Adoption of  
Management Practices Using Leafroll and Red Blotch Disease as Model Systems**  
Monica L. Cooper and Malcolm B. Hobbs .....175
- **Improved Decision-Making for Grapevine Leafroll and Red Blotch Diseases Using  
Rapid Identification Tools and a Regional Approach to Monitoring and Management**  
Monica L. Cooper and Jennifer K. Rohrs .....185
- **Spotted Lanternfly Outreach and Mapping the Risk-Prone Areas in California**  
Surendra K. Dara .....198
- **Developing a GMO-Free RNA Interference Approach to Mitigate Red Blotch Negative  
Impacts on Grape Berry Ripening**  
Laurent Deluc.....211
- **Ecology of Grapevine Red Blotch Virus**  
Marc Fuchs and Keith Perry .....227
- **Resistance to Grapevine Leafroll-Associated Virus 3 and its Major Mealybug Vectors**  
Marc Fuchs and Greg Loeb.....240
- **Virus-Based Delivery of Interfering RNAs Targeting Grapevine Leafroll-  
Associated Virus(es) and Associated Mealybugs**  
Yen-Wen Kuo and Bryce W. Falk .....252
- **Identification of Grape Cultivars and Rootstocks with Resistance to Vine  
Mealybug**  
Rachel P. Naegele .....264
- **Evaluation of *Vitis* Rootstocks with Partial Resistance to Vine Mealybug**  
Rachel P. Naegele .....270

- **Investigation of the Impact of Grapevine Red Blotch Virus on Grape Skin Cell Wall Metabolism and Soluble Pathogenesis-Related Proteins in Relation to Phenolic Extractability**  
Anita Oberholster .....273
- **Effects of Grapevine Red Blotch Disease on Flavor and Flavor Precursor Formation in the Grape and on Wine Quality**  
Michael Qian and Alexander D. Levin .....280
- **Structure-Function Studies on Grapevine Red Blotch Virus to Elucidate Disease Etiology**  
Christopher D. Rock and Sunitha Sukumaran .....300
- **Improved Understanding of Virus Transmission of Grapevine Red Blotch Virus**  
Vaughn Walton .....335
- **Biology and Role of Treehoppers in Grapevine Red Blotch Disease**  
Frank Zalom and Mysore R. Sudarshana.....344

#### **ABSTRACTS**

- **Monitoring Vine Mealybug Resistance to Imidacloprid**  
Mark Sisterson, Lindsey Burbank, Rachel P. Naegele, and Christopher Wallis .....366

**ABBREVIATIONS** .....368

**Section 1:**

***Xylella fastidiosa***

**and**

**Pierce's Disease**



## MODELING OF *XYLELLA FASTIDIOSA* TRANSMISSION AND GRAPEVINE SUSCEPTIBILITY USING FLUID DYNAMICS SIMULATIONS

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**Reporting Period:** This report describes work conducted from September 2021 to October 2021.

### ABSTRACT

*Xylella fastidiosa*, which causes Pierce's disease of grapevines, is a xylem-limited bacterium transmitted by insect vectors. Several insects are known to transmit *X. fastidiosa* with different transmission efficiencies and the biomechanics underlying the transmission process are not well understood. Here, we are performing computational fluid dynamics simulations to model bacterial transmission during vector feeding and *X. fastidiosa* colonization. We hypothesize that morphological features of the functional foregut and plant vasculature are crucial and overlooked components in transmission efficiency; hypothesis testing will be performed by integrating 3D digital models from synchrotron-based tomography with downstream computational fluid dynamics simulations.

### LAYPERSON SUMMARY

*Xylella fastidiosa* is transmitted from plant to plant by insects feeding predominantly on xylem. However, little is known about how bacteria are transferred from the infected insect to the plant during the probing and feeding process. This is due to the fact that much remains to be understood regarding how insects feed on xylem sap. Here, we are integrating 3D digital models with tools to simulate fluid and particle transmission to infer how bacterium transmission occurs. This may reveal how and why some insects are more effective at transmitting *X. fastidiosa* than others.

### INTRODUCTION

Xylem sap-feeding insects transmit bacteria that cause Pierce's Disease of grapevines. This bacterium is limited to the xylem and clog the vessels in the network, limiting effective water

transport in the plants (Hopkins 1989; Newman et al. 2003). Xylem sap-feeding insect vectors that transmit *X. fastidiosa* include spittlebugs (Cercopidae) and leafhoppers (family Cicadellidae) (Almeida and Purcell 2006). As xylem is a nutritionally poor food source (Raven 1983), xylem sap-feeding insects must ingest a large amount of sap in order to obtain sufficient nutrients. The rate of xylem sap ingestion has been estimated to be up to 1m/s (Ranieri et al. 2020), and daily ingestion has been inferred to approach 1,000x their body mass (Mittler, 1967).

Xylem sap-feeding insects have a specialized feeding complex to aid in sap extraction and ingestion. The distalmost part of the feeding apparatus is made of interlocking maxillary segments forming the food and salivary canals which are surrounded by two mandibular stylets (Backus 1985). Proximally, the stylets diverge to surround the distal end of the hypopharynx (Almeida and Purcell 2006). The hypopharynx and the epipharynx are fused to form the precibarium, which houses the precibarial valve (Ruschioni et al. 2019). The precibarial valve controls the flow of fluid from the stylet into the cibarium (Backus and McLean 1982). Contraction of the basin-like muscle (blm) attached to the epipharynx opens a flap in the precibarial valve that enables flow of fluid through the precibarial valve and into the cibarium (Ruschioni et al. 2019). The cibarium is a chamber connecting the precibarium and the esophagus (Nault and Ammar 1989). Contraction of the cibarial muscle creates negative pressure which pulls fluid through the distal part of the system and into the cibarium; extension pushes the fluid in the cibarium through the esophagus to the esophageal valve and into the midgut. However, many aspects of the process of *X. fastidiosa* transmission and inoculation during feeding remain unknown. Here, we apply synchrotron-based micro-CT imaging, 3D digital modeling and computational fluid dynamics to illuminate the factors that influence *X. fastidiosa* transmission.

## OBJECTIVES

A thorough understanding of xylem sap-feeding mechanics is integral to inferring the processes underlying the inoculation and transport of xylem-limited bacteria in plants. Through this research project, we will:

- (1) Simulate fluid dynamics in the functional foreguts of vectors during feeding.
- (2) Simulate fluid dynamics in grapevine xylem during vector feeding.
- (3) Simulate fluid dynamics in grapevine xylem during *X. fastidiosa* colonization.

To achieve this, for the first component of this research project, we are currently:

1. Creating 3D models of the functional foregut in disparate *X. fastidiosa* vectors
2. Visualizing the structure of grapevine xylem *in vivo* in 3D with synchrotron imaging
3. Integrating computational fluid dynamics simulations with these 3D morphological reconstructions to test hypotheses regarding *X. fastidiosa* colonization.

## RESULTS AND DISCUSSION

### Progress on Objective 1: A 3D comparison of the feeding complex in disparate *X. fastidiosa* vectors

The morphology of the functional foreguts of *X. fastidiosa* vectors have been inferred through dissection with traditional microscopy, SEM, and micro-CT imaging; however, the 3D structure of key anatomical features such as the pre-cibarial valve have not been reconstructed. Obtaining

an accurate, high-resolution visualization of the underlying morphology of the fluid ingestion complex is a critical step towards understanding how *X. fastidiosa* is transmitted. Synchrotron-based micro-CT imaging has been used to illuminate key morphological features in several invertebrate taxa and is a promising tool for addressing questions here. For instance, micro-CT imaging represents the only non-destructive technique for visualizing internal anatomy, allowing for both the morphology and the position between structures to be visualized and maintained. High-resolution synchrotron-based tomography permits the visualization of sub-micron sized features that would be difficult to prepare using traditional methods. Digitization can be used to create 3D digital or physical models that can be used for hypothesis testing through a variety of downstream analyses. We performed 3D imaging to visualize the anatomy of feeding structures in several *X. fastidiosa* vectors. We have applied several different tomography imaging tools and preparation techniques to develop optimal parameters for these and future analyses. This imaging will illuminate the disparate 3D morphology of feeding structures *in situ* for comparative analyses between taxa, highlighting differences in morphology and illuminating factors that influence *X. fastidiosa* transmission.

### **Progress on Objective 2: Visualization of xylem vessels in vivo during and post-ingestion**

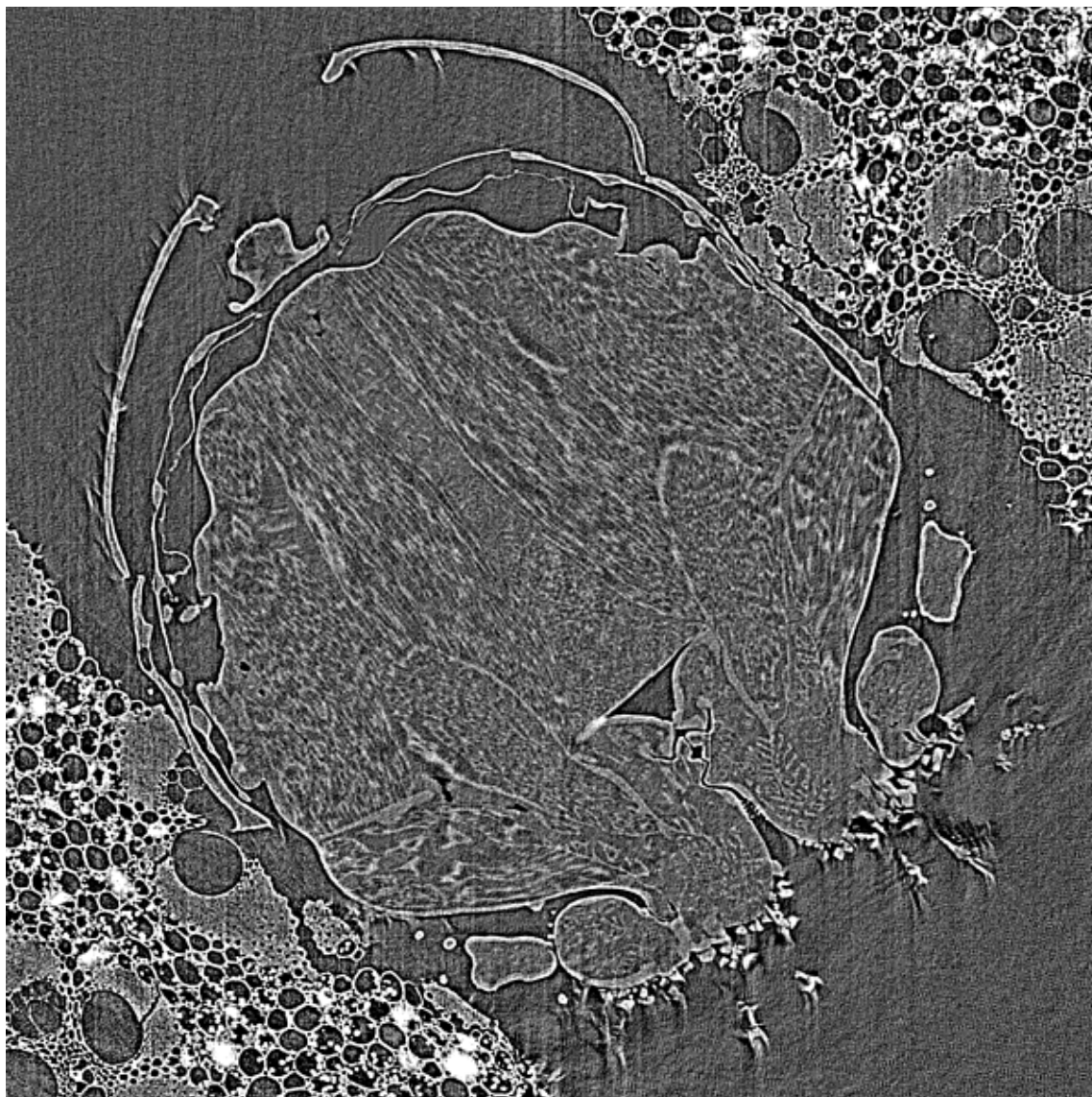
A critical step towards understanding the mechanics of xylem sap ingestion is to reconstruct the structure within the plant where the xylem is being ingested from. Here, we used synchrotron tomography to visualize the internal structure of live plants that have recently undergone xylem sap ingestion. This technique permits sub-micron level image resolution and has been used to visualize the internal structure of plant vasculature (e.g., Brodersen et al. 2019; Wason et al. 2019). We are using this strategy to pinpoint the vessels probed for sap ingestion and highlight the consequences of probing on water conductance.

### **Progress on Objective 3: Integrating computational fluid dynamics to test hypotheses regarding *X. fastidiosa* colonization**

Sap ingestion relies on the transport of fluid from the plant to the insect. The fluid dynamics underlying the total system, i.e. the integration of the insect feeding complex and associated plant vasculature, have yet to be investigated. 3D digital reconstructions created as part of objective 1 and 2 are being integrated to model the mechanics of transport through the plant vessels and into the insect feeding complex.

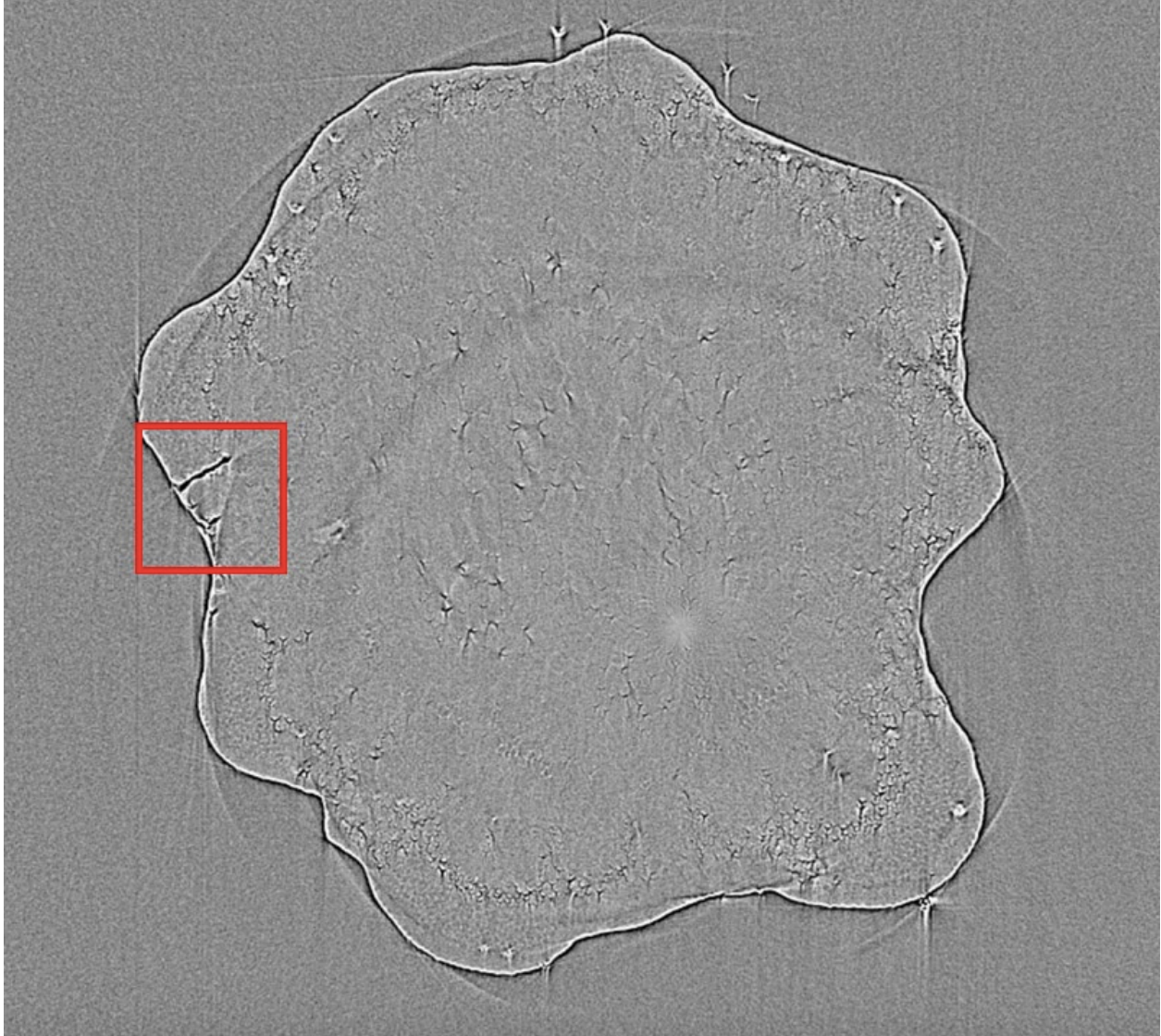
## **CONCLUSIONS**

3D imaging and computational fluid dynamics have been demonstrated as powerful tools for examining the relationship between structure and function in a variety of organisms (e.g., Rahman et al. 2015; Darroch et al. 2017; Ranieri et al. 2019; Clark et al. 2020a,b). Here, we are applying these techniques to examine the mechanics underlying sap ingestion and transmission of *X. fastidiosa*. We are approaching this through performing several different experiments integrating synchrotron-based micro-CT and digital modeling. Preliminary results suggest that this approach will be an effective technique to illuminate critical structural aspects of xylem sap-feeding insects and plants susceptible to Pierce's Disease.



**Figure 1.** Reconstructed 3D image showing a cross-section through a blue-green sharpshooter. Generated using synchrotron tomography at beamline 8.3.2 at the Advanced Light Source of Lawrence Berkeley National Laboratory. Custom imaging setup created by beamline scientist Harold S. Barnard and Dula Y. Parkinson of Lawrence Berkeley National Laboratory.





**Figure 2.** Cross-section of a stem of a grapevine generated using synchrotron tomography. Red box indicates location of stylet probing.

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## PROGRESSION OF PIERCE'S DISEASE SYMPTOMS AND *XYLELLA FASTIDIOSA* COLONIZATION OF GRAPEVINES UNDER FIELD CONDITIONS

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**Reporting Period:** The results reported here are from work conducted July 2021 to October 2021.

### ABSTRACT

This project is studying Pierce's disease symptom progression after *X. fastidiosa* infection under field conditions using an experimental vineyard 10 years old with 14 wine grape varieties. In addition, the research hopes to better understand grapevine overwinter recovery from *X. fastidiosa* infection. This report summarizes research done in 2021, when the plants were inoculated with *X. fastidiosa*.

### LAYPERSON SUMMARY

Despite years of study, we do not understand how mature grapevines respond to *X. fastidiosa* infections under field conditions. There are two reasons for this: first, infecting mature vines with these pathogens presents risks to commercial agriculture that are difficult to mitigate, and second, the time it takes to establish an experimental vineyard for this purpose is prohibitive in areas where wine grapes are commercially grown. Because of this, we have very limited understanding of disease progression in commercially relevant field conditions. This is a remarkable knowledge gap, both from an industry and academic perspective. In this project we use a 10-year-old vineyard with 14 wine grape cultivars grown under commercially relevant conditions at a UC research station to study the progression of Pierce's disease in inoculated vines. We infected grapevines with *X. fastidiosa* in May 2021 and report on the results of those infections.

### INTRODUCTION

Pierce's disease of grapevines (PD) is an economically important vector-borne disease caused by *Xylella fastidiosa* affecting the grape industry in California, USA, and other regions of the world. There is substantial accumulated information about the biology and epidemiology of PD and its etiological agent. However, there are also very noteworthy knowledge gaps. One important, persistent knowledge gap is the lack of studies on how pathogens colonize plants and how the disease progresses over time. More specifically, there are only a couple of very small-scale studies in which researchers reported when and where pathogens were inoculated that used mature grapevines grown in commercially relevant regions. Recently we inoculated *X. fastidiosa* into established grapevines in Napa Valley (Almeida and Cooper unpublished); we noted a lack of obvious PD foliar symptoms until one year post infection, which was unexpected given rapid colonization of vines. Furthermore, early symptoms were shriveled berries rather than foliar symptoms at the inoculated cane, which was also unexpected. However, the Napa study had a small number of vines to begin with, and vineyard managers removed heavily symptomatic

plants (unfortunately, despite early agreement that plants would remain in the field), further reducing the sample size. In other words, we still lack robust information on how *X. fastidiosa* colonizes grapevines and how PD symptoms develop under realistic and relevant field conditions. This information is critical to understanding the disease and developing and delivering science-based management guidelines.

In addition to clarifying symptom progression, this project offers an opportunity to test hypotheses about *X. fastidiosa* overwintering and climate adaptation. We showed that *X. fastidiosa* populations infecting grapevines in California may be adapted to regional climates (Vanhove et al. 2020). Furthermore, we know that grapevines may recover from *X. fastidiosa* infections based on time of infection and winter temperatures (Purcell 1980, Feil et al. 2003), although it is possible that historical recovery trends are shifting due to climate change (Sisterson et al. 2020). Historically, the northern limit of PD in California has been Napa and Sonoma, attributed to *X. fastidiosa*'s intolerance to cold winter temperatures. However, in 2020 our team isolated *X. fastidiosa* from multiple sites near Hopland, where the field site is located (Mendocino County). We hypothesize that these strains may have adapted to successfully overwinter in cooler areas of Northern California. Our experimental design aims to address questions regarding overwinter recovery of vines as well.

## OBJECTIVES

The Objectives of this research proposal are to follow the *X. fastidiosa* grapevine colonization patterns and Pierce's disease symptom progression under relevant field conditions using mature grapevines in an experimental vineyard. A particularly powerful component of the work is the fact that 14 varieties will be included in the study so that comparative analyses will be performed.

Objective 1. Progression of Pierce's disease in multiple varieties in field conditions

Objective 2. Overwinter recovery rates of distinct strains of Pierce's disease in multiple varieties

## RESULTS AND DISCUSSION

We visited four commercial vineyards in Hopland, CA in September 2020 to sample and isolate local *X. fastidiosa* strains. In the spring of 2021, we extracted DNA and performed whole-genome sequencing on 53 strain isolates from Hopland. The strain library is currently frozen for long-term storage both at UC Berkeley and with USDA Biologist Dr. Lindsey Burbank at Parlier, CA. Preliminary phylogenetic analysis of the Hopland *X. fastidiosa* genomes indicates that there are two genetically distinct populations. One cluster includes both Hopland and Sonoma strains, whereas the second cluster only includes Hopland strains. This latter cluster indicates a population that may be genetically isolated and locally adapted.

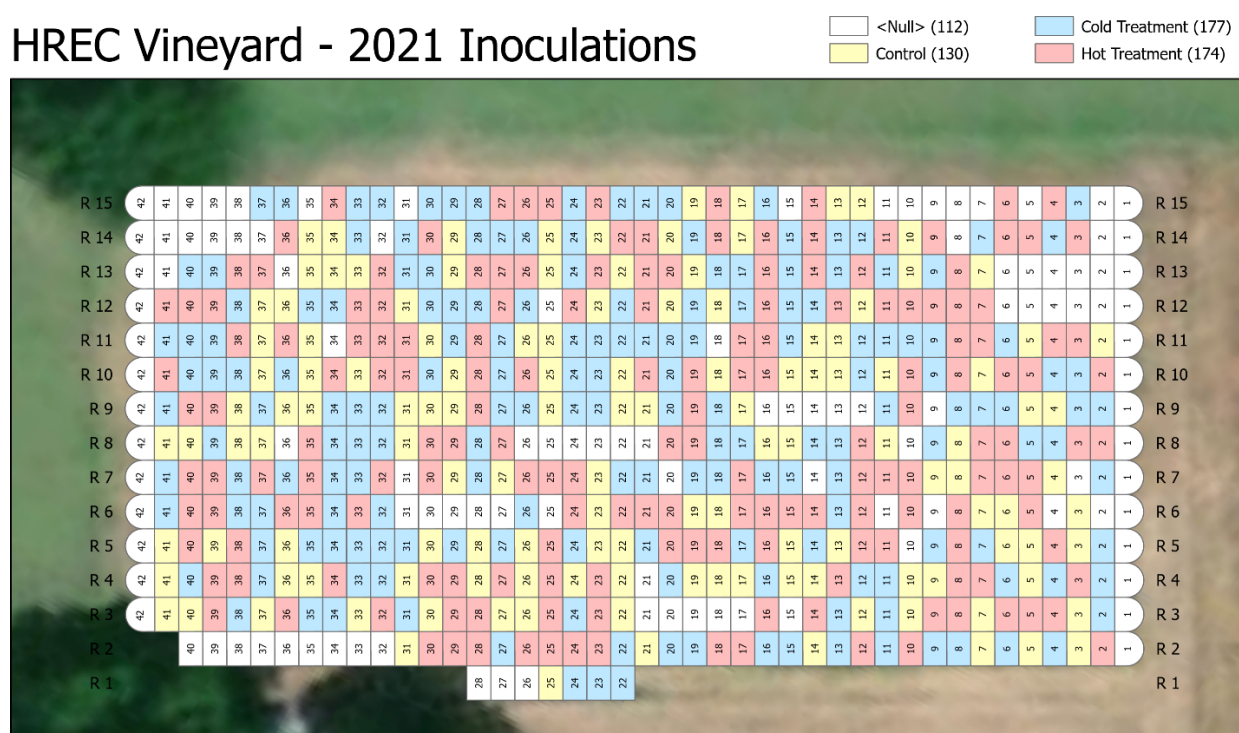
We conducted the inoculation trial in an experimental vineyard at the UC ANR Hopland Research & Education Center (HREC), in a plot planted in 2011 to 14 unique wine grape cultivars on 101-14 rootstock. The experiment included three inoculation treatments, a 'cold' strain from Hopland, a 'hot' strain from Temecula, and negative controls. Treatments were randomly assigned within each of 13 cultivars. The 14<sup>th</sup> cultivar was excluded because too few vines remained. Additional, poorly growing vines of other cultivars were also excluded. The



remaining 13 cultivars (n=40 vines per cultivar) were randomly assigned to 10 control, 15 hot, and 15 cold (Figure 1), and all vines were color-coded with flagging tape prior to inoculations.

On May 2021 we inoculated 481 vines at HREC. We prepared cell suspensions of the hot and cold strains in sterile Phosphate Buffered Saline (PBS), placing small aliquots of each cell suspension on dry ice to bring back to the lab for quantification. Based on qPCR of inoculum samples, we have confirmed that all inoculum contained at least  $10^9$  CFU / ml. For control vines, we inoculated with only PBS. Each vine was inoculated on the distal shoot (Western cordon) with two 10 microL droplets of the cell suspension. We took photos of a representative vine for each cultivar, as some cultivars (Tannat, Tinta Francisca, Mencia, Petit Manseng) had smaller shoots, whereas others (Teroldego, Greco di Tufo) had vigorous shoots.

## HREC Vineyard - 2021 Inoculations



**Figure 1.** Inoculations included 130 controls, 177 cold and 174 hot treatments across 13 cultivars.

A)



B)



C)

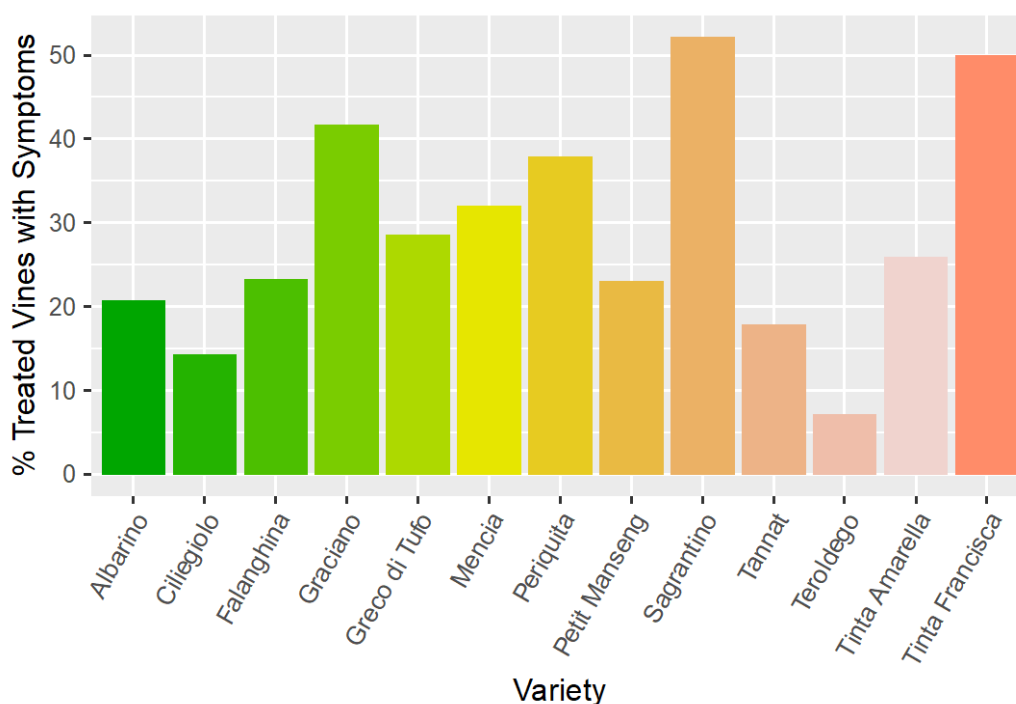


**Figure 2.** Inoculations at (A) HREC (B) Tinta Francisca and (C) Teroldego

### Symptom Evaluation

We evaluated all 481 vines for symptoms on August 25<sup>th</sup>, 2021 and September 24<sup>th</sup>, 2021. We recorded presence or absence (1/0) for five symptoms (leaf scorch, stunting, uneven lignification, matchstick petioles, and shriveled clusters), as well as notes of other observations. In all 13 cultivars, we observed symptoms in treated (inoculated) vines, although the cultivars varied in percent of symptomatic vines (Figure 3). Symptoms were observed only on the Western side of the vine, which includes the inoculated shoot.

The cultivars showed different Pierce's Disease symptoms, including some novel observations. Multiple cultivars showed foliar symptoms and shriveled clusters. Several cultivars (Falaghina, Graciano, Sagrantino, Tinta Amarella) also had leaves on the inoculated shoot that were dried out, especially on the edges (Figure 4, Figure 5). Additionally, one cultivar—Tinta Francisca—had inoculated shoots that were severely stunted and almost completely defoliated (Figure 6). Close to 40% of the inoculated Tinta Francisca vines displayed this symptom.



**Figure 3.** Percentage of symptomatic Vines in each of 13 cultivars. Control vines for each cultivar were not included in calculating the percent symptomatic.





**Figure 4.** Leaf Scorch and drying on the inoculated cane on Graciano



**Figure 5.** An example of the dried inoculated shoot on Tinta Amarella. There are also shriveled clusters on the Western cordon.





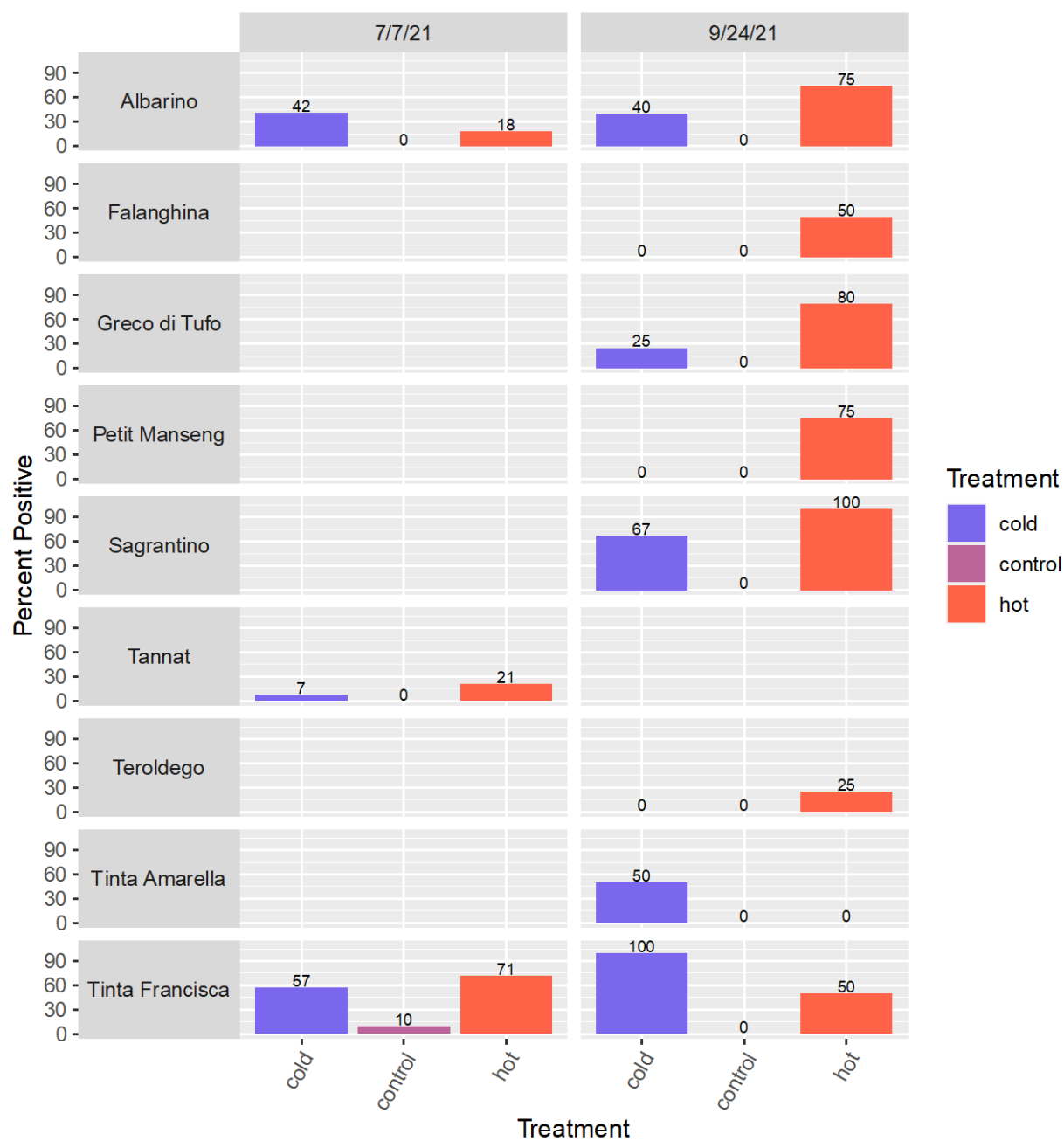
**Figure 6** Examples of the stunting and defoliation on the inoculated shoot on Tinta Francisca.

### **Molecular Detection of *X. fastidiosa* in Field Samples**

We continued to subsample three varieties (Albarino, Tannat, and Tinta Francisca) at four points across the vine to assess pathogen movement from the inoculation point. These varieties were sampled in June, July, and September, while the other ten varieties were sampled in September. So far, we completed DNA extractions and qPCR detection for all samples of the inoculated shoot from July (n=108) and 74 of 481 samples from the inoculated shoot from September. Percentage of positive vines by treatment (cold, hot, and control) and variety are summarized in Figure 7. The percentages will change as the sample size increases, and some varieties are not included in the initial results. Still, the initial results show that the inoculations were successful and suggest varietal differences in susceptibility that matches our observation from the symptom data.

### **CONCLUSIONS**

We are still processing samples but the preliminary data show that the *X. fastidiosa* field infections were successful. We have also observed early disease symptoms in some varieties in the inoculated canes; interestingly, disease symptoms varied substantially based on variety. These early results also suggest that varieties differ in their responses to *X. fastidiosa* infection.



**Figure 7** Percentage of positive vines by treatment, variety, and sample date.

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## FUNDING AGENCIES

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

## MANAGEMENT OF THE FEDERAL PERMIT FOR FIELD TESTING TRANSGENIC GRAPEVINE ROOTSTOCKS IN CALIFORNIA

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**Reporting Period:** The results reported here are from work conducted October 2020 to October 2021.

### ABSTRACT

This project manages the APHIS-BRS federal permit that facilitates the multi-investigator field-testing of transgenic grapevine rootstock and scion varieties in California. The federal permit that was effective from April 1, 2018 to March 31, 2021 was for a 5.4-acre field site that includes the original 2-acre field site and the adjacent field site consisting of an additional 3.4 acres for testing all transgenic rootstocks with improved PD-resistance that we and other investigators developed. The transgenic rootstocks include the commercially relevant rootstocks 101-14 and 1103, which are being field tested for their ability to protect the sensitive wild type scion variety Chardonnay from developing PD. The federal permit was amended to include five *Vitis vinifera* cultivated varieties (Cabernet Sauvignon, Merlot, Pinot Noir, Sauvignon Blanc, and Zinfandel) to be planted as untransformed controls, self-rooted plants or as scions grafted to the permitted rootstocks. A federal permit renewal application was submitted on November 21, 2020, approved with an effective date of April 1, 2021 and an end date of April 1, 2022. APHIS-BRS inspection was conducted on December 8, 2020 and the field site complied with the federal permit requirement. "Final Field Test" and "Final Volunteer Monitoring" reports for the original federal permit were submitted to APHIS-BRS on September 1, 2021. A "No-Planting" report for the new Solano County permit, effective from April 1, 2021 to April 1, 2022, was submitted on September 30, 2021.

### LAYPERSON SUMMARY

This is an ongoing project that manages the Federal permit that enables the multi-investigator field-testing of transgenic grapevines in California. The APHIS BRS federal permit for 5.4 acres that is currently being used to test transgenic grapevines was effective from April 1, 2018 to March 31, 2021. This permit accommodates transgenic rootstock genotypes 101-14 and 1103 grafted to the sensitive scion variety Chardonnay for validating their efficacy in protecting the scion from developing PD. Also permitted after an amendment of the existing permit to include five *Vitis vinifera* varieties, which are Cabernet Sauvignon, Merlot, Pinot Noir, Sauvignon Blanc, and Zinfandel that can be planted as untransformed controls, either as self-rooted plants or as scions grafted to the permitted rootstocks. Currently, all planted grapevines in this site use a new federal permit effective from April 1, 2021 to April 1, 2022. Timely reporting and inspections are conducted on an ongoing basis to maintain compliance with federal permit conditions. Regulatory compliance is enforced by working closely with the participant investigators, the field coordinators, and their crews.

## INTRODUCTION

This is a continuing project that manages the APHIS-BRS federal permit that facilitates a multi-investigator field-testing of transgenic grapevine rootstock and scion varieties in California. An earlier expanded APHIS-BRS federal permit effective from April 1, 2018 to March 31, 2021 was approved for the entire 5.4-acre field site that included the original 2-acre and adjacent 3.4-acre field site and has been in use for testing all transgenic rootstocks with improved PD-resistance developed recently. These transgenic rootstocks incorporate the commercially relevant rootstock genotypes 101-14 and 1103, which are being field tested for their ability to protect the grafted sensitive wild type variety Chardonnay from developing PD. An application to amend the federal permit to include five *Vitis vinifera* varieties. The five cultivated varieties of *Vitis vinifera* include Cabernet Sauvignon, Merlot, Pinot Noir, Sauvignon Blanc, and Zinfandel to be planted as untransformed controls, self-rooted plants or as scions grafted to the permitted rootstocks. A new permit renewal application to continue this trial past the march 31, 2021 was approved with an effective date of April 1, 2021 and an end date of March 31, 2022. APHIS-BRS inspection was conducted on December 8, 2020 and the field site complied with the federal permit requirement. "Final Field Test" and "Final Volunteer Monitoring" reports for the original federal permit were submitted to APHIS-BRS on September 1, 2021. A "No-Planting" report for the present field permit was submitted on September 30, 2021. All of the "Permit Renewals and Amendments," "Final Field Test", "Final Field Volunteer Monitoring" and "Field Planting or No-Planting" mandatory reports for the federal permits are submitted in a timely manner to comply with the APHIS-BRS requirements.

## OBJECTIVES

Objective 1. Management of existing USDA-APHIS field permits, maintenance of regulatory oversight, and compliance with permit reporting requirements

Activity 1: Manage the documentation and submission processes for the federal permit that enables field-testing at the 'ePermits' website.

Activity 2: Maintain regulatory oversight and compliance with reporting requirements and regulatory compliance inspections at the field.

## RESULTS AND DISCUSSION

**Activity 1. Manage the documentation and submission process for the federal permit that enables field-testing at the 'ePermits' website.**

An APHIS-BRS federal permit for the expanded Solano site to begin field-testing transgenic versions of the commercially relevant 101-14 and 1103 was approved on with an end date of March 31, 2021. APHIS BRS approved an amendment for the permit to include five *Vitis vinifera* varieties as untransformed controls, either as self-rooted plants or as scions grafted to the permitted rootstocks. The five cultivated varieties of *Vitis vinifera* are Cabernet Sauvignon, Merlot, Pinot Noir, Sauvignon Blanc, and Zinfandel. A new permit application to continue the Solano's field trial was submitted on November 1, 2020 and approved by APHIS BRS with an effective date of April 1, 2021 and an end date of March 31, 2022.

**Activity 2: Maintain regulatory oversight and compliance at both field locations, including reporting requirements and regulatory compliance inspections.**

Personnel from the Dandekar laboratory are maintaining regulatory oversight of the field. The issues requiring regulatory oversight compliance are listed in the approved permit and permit supplemental conditions. Timely reporting and inspections are conducted to maintain compliance with specific APHIS-BRS federal permit performance standards and supplemental conditions. Regulatory compliance is enforced by working closely with the participant investigators, the field coordinators, and their crews. PD field trial activity information is updated quarterly using the PI's activity monitoring logs. Two individuals from the Dandekar laboratory are entrusted with the tasks of documentation, training, and inspection to ensure regulatory compliance with the APHIS-BRS permit supplemental conditions (USDA- APHIS-BRS 2012a, 2012b and 2017). An APHIS-BRS inspection of the current permitted site was conducted on December 8, 2020 and the field site complied with the federal permit requirement. The purpose of the APHIS-BRS inspections is to verify that the field trials are following APHIS-BRS performance standards and permit supplemental conditions. The inspections included visiting the field trial and related facilities (e.g., buildings for equipment, greenhouses, processing, disposal, etc.) and reviewing associated records for the field trial. A "No-Planting" reports for the present federal permit was submitted to APHIS-BRS on December 8, 2020. Planting reports provide either planting, no-planting and construct data for the field site. The 2018-21 "Final Field Test" and the 2018-21 "Final Field Test" reports for the Solano trial were submitted to APHIS-BRS on September 1, 2021, 2021. The "Final Field Test" report provides methods of observation, resulting data, and analysis regarding all deleterious effects on plants, non-target organisms, or the environment observed during the trial lifetime. The "Final Field Volunteer Monitoring" report contains the dates when the field site and perimeter zone were inspected for volunteers, the number of volunteers observed each month during the trial lifetime. A "No-Planting" report was submitted to APHIS-BRS on September 30, 2021. Planting reports provide either planting or no-planting and construct data for the Solano site. Permit Renewals and Amendments," annual or final "Field Test," annual and final "Field Volunteer Monitoring" and "Planting" mandatory reports are submitted in a timely manner to comply with the APHIS-BRS requirements.

**CONCLUSIONS**

To facilitate the multi-investigator field-testing of transgenic grapevine rootstock and scion varieties in California a federal permit approved by APHIS-BRS from April 1, 2018 to March 31, 2021 for a 5.4-acre field site for testing all transgenic rootstocks with improved PD-resistance we developed. This site was planted with different transgenic versions of the commercially relevant rootstocks 101-14 and 1103, which will be field tested for their ability to protect the sensitive variety Chardonnay from developing PD. The federal permit was amended in 2019 to include five *Vitis vinifera* cultivated varieties (Cabernet Sauvignon, Merlot, Pinot Noir, Sauvignon Blanc, and Zinfandel) to be planted as untransformed controls, self-rooted plants or as scions grafted to the permitted rootstocks. A new federal APHIS BRS permit to continue this trial past the March 31, 2021 was approved with an effective date of April 1, 2021 and an end date of March 31, 2022. APHIS-BRS inspection was conducted on December 8, 2020 and the field site complied with the federal permit requirement. "Final Field Test" and "Final Volunteer Monitoring" reports for the original federal permit were submitted to APHIS-BRS on September 1, 2021. A "No-Planting" report for the present Solano County permit was submitted on September 30, 2021. The project personnel maintain regulatory oversight and conduct

regulatory compliance and inspections of field trials to maintain compliance with APHIS-BRS federal permit standard and supplemental conditions. This includes submitting “Permit Renewals and Amendments” applications, annual and final “Field Test”, annual and final “Field Volunteer Monitoring” reports and “Field Planting” mandatory reports for each federal permit in a timely manner to comply with the APHIS-BRS requirements.

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#### **FUNDING AGENCIES**

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



## SYSTEMIC FORMULATIONS OF ANTIBACTERIAL NANOPARTICLES FOR PIERCE'S DISEASE MANAGEMENT

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**Reporting Period:** The results reported here are from work conducted July 2021 to November 2021.

### ABSTRACT

This project was initiated in July 2021. In this report, we will be presenting preliminary results obtained in the last 4 months, as well as the general concept of this research. At present, this project was funded for one year out of the three years proposed. This project is based upon research conducted in the last years by our multidisciplinary research team that assessed the potential of a novel nano-sized ZnO formulation, Zinkicide<sup>®</sup>, to control the vascular phloem-limited pathogenic bacterium '*Candidatus* Liberibacter asiaticus' (CLAs) in citrus. In preliminary experiments, we tested the activity of Zinkicide<sup>®</sup> against the strain *Xylella fastidiosa* Temecula1 which causes Pierce's disease (PD) in grapes, and showed that under greenhouse conditions it has significant activity in the model plant tobacco and the important crop blueberry (against a *X. multiplex* strain). A significant reduction of symptoms and bacterial populations were observed in both plants treated with different concentrations of Zinkicide<sup>®</sup>. Nevertheless, we observed that the effective levels of this compound in plants grown in pots in the greenhouse are close to phytotoxic levels. Therefore, we proposed modifying the formulation to avoid phytotoxicity and improve the formulation by adding compounds with proven antibiofilm activity against *X. fastidiosa*. In the last months, we have been testing a modified form of Zinkicide<sup>®</sup>, termed "TMN111", that showed higher antibacterial activity in vitro. Preliminary tests in tobacco plants in the greenhouse showed reduced phytotoxicity and strong antibacterial activity, reducing populations of *X. fastidiosa* Temecula1 to near detection limits. In grape seedling assays in the greenhouse, TMN111 showed little to no phytotoxicity when added as a soil drench. Novel combinations of nano formulations are being developed and will be tested in planta.

### LAYPERSON SUMMARY

Disease management tools against Pierce's disease (PD) in grapes are very limited. The only chemical control options available against this disease, however, target the insect vectors and not the pathogen. There are no antibacterial compounds effective in planta against the pathogen *X. fastidiosa*, because of the difficulty of reaching the vascular system by spray applications. We have tested a novel nano-size formulation ("Zinkicide<sup>®</sup>") against a different vascular (phloem)

bacterial pathogen in citrus. That formulation showed effective reduction of Huanglongbing (HLB) symptoms in citrus in Florida in a field trial ongoing for five years. We have carried out preliminary tests of the same formulation against *X. fastidiosa* (the PD-causing pathogen) in the greenhouse with promising results in blueberry and tobacco. In our experiments, Zinkicide® significantly reduced symptoms and pathogen populations. With this preliminary information, we proposed to modify the chemical composition of the nano-formulation to improve performance against *X. fastidiosa* at lower doses and to test it in grapes. The availability of an antibacterial chemical treatment easily applied in the field by soil drench or foliar spray will be a useful tool to be adopted by growers to manage PD.

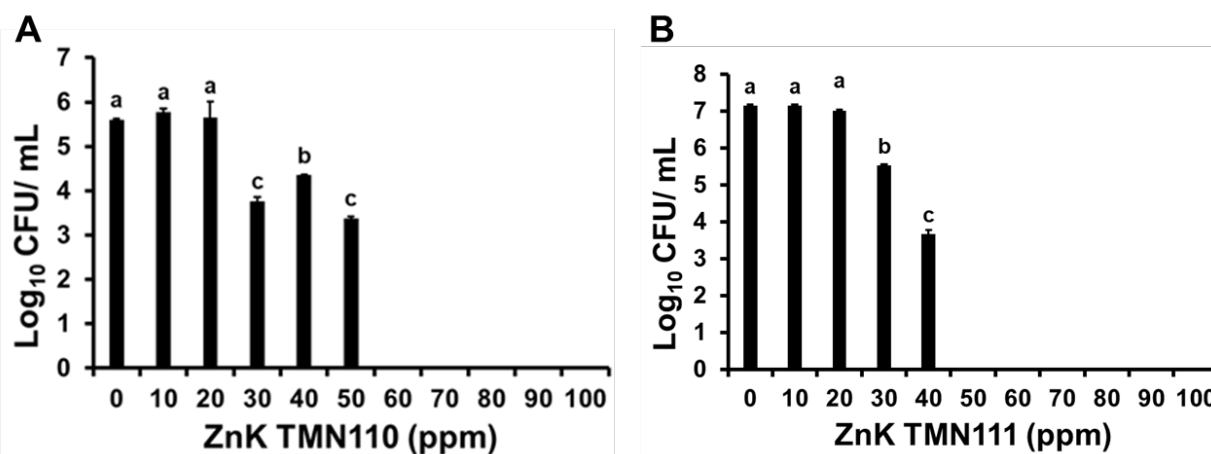
## INTRODUCTION

*Xylella fastidiosa* (*Xf*) is a pathogen endemic to the Americas causing diseases in grape (Pierce's disease, PD), almond, peach, olive, pecan, and blueberry among others (Chang et al. 2009; Hopkins and Purcell 2002; Purcell and Hopkins 1996). This vascular bacterium lives exclusively in the plant xylem and the foreguts of xylem sap-feeding insects (Chatterjee et al. 2008; Hopkins and Purcell 2002). Disease management options against PD rely mainly on insect vector control, a controversial approach due to the non-target effects on the environment by the use of neonicotinoids (Hladik et al. 2018). While antimicrobial management strategies have been a viable option for many bacterial plant diseases (Sundin et al. 2016), their use to manage vascular pathogens is challenging (Sundin et al. 2016; Yadeta and Thomma 2013), primarily due to the hurdles in delivering inhibitory concentrations to individual pathogen cells. In the case of *Xf*, the pathogen cells are protected within biofilm formed inside vascular vessels (Chatterjee et al. 2008). We have been interested in understanding the role of mineral elements during *Xf* infection (Cobine et al. 2013; Cruz et al. 2012; De La Fuente et al. 2016; De La Fuente et al. 2013; Oliver et al. 2014), and in particular, we have studied the role of Zn in *Xf* (Navarrete and De La Fuente 2014, 2015), showing that this element plays an important role during pathogen interactions with the plant. On the other hand, a recent study by our group showed that bulk CuSO<sub>4</sub> amendments to the soil slightly augmented *Xf*-caused leaf scorch disease severity in tobacco (Ge et al. 2020), instead of controlling the disease. Nevertheless, Cu-based microbiocides are among the most common compounds widely used in agriculture since the 1880s to control fungal (Brun et al. 1998; Rusjan 2012) as well as bacterial diseases (Lamichhane et al. 2018). The use of antimicrobial approaches for control of vascular pathogens is challenging due to the lack of availability of systemic compounds. For this reason, we will be testing a series of nano-formulations that, due to their small size, can be taken up by plant roots and become systemic.

Nanoparticles have been extensively studied in drug delivery systems, offering a wide spectrum of formulations with improved physicochemical characteristics and curative properties (Graham et al. 2016; Zaman et al. 2014). Among them, zinc oxide (ZnO) nanoparticles represent an important class of commercially available materials and have been widely applied in diagnostics, therapeutics, drug-delivery systems, and food preparation processes, among other fields (Dizaj et al. 2014). The nano-formulation "Zinkicide®" (ZnK) was developed by co-PI Santra and has been produced with industry-grade reagents by the company TradeMark Nitrogen (TMN). This is a novel antimicrobial composite that contains protein-size (~4.0 nm) ZnO nanoparticles coated with weak organic acids including salicylic acid, initially designed to control bacterial citrus diseases (Santra and Berroth 2018). The selection of Zn as the metal element of ZnK and the use of salicylic acid as a coat is supported by the preferential localization and transport of these two

compounds by the plant vasculature (Lu et al. 2013; Rocher et al. 2006; Tian et al. 2014; Xie et al. 2019). ZnK has been shown to have a two- to eight-fold lower minimum inhibitory concentration (MIC) than traditionally used Cu-based compounds against *Xanthomonas citri* subsp. *citri* and *X. alfalfae* subsp. *citrumelonis* (Graham et al. 2016). In field trials, ZnK significantly reduced the incidence of citrus canker after foliar sprays and showed lower phytotoxicity than commercially available Cu-based compounds (Graham et al. 2016).

Since ZnK is easily taken up by roots and moves systemically in the xylem, we performed preliminary experiments to assess the activity of ZnK against *Xf*. First, we used tobacco (De La Fuente et al. 2013; Francis et al. 2008) as a model plant for a series of experiments where different concentrations of ZnK were applied as a drench treatment, to determine concentrations that would not affect normal plant growth (viz., phytotoxicity). One-time applications or weekly doses were tested at multiple concentrations. After trying different concentrations and applications regimes, an optimal treatment was found. This treatment significantly reduced disease symptoms, from an area under the disease progress curve (AUDPC) for the control of ~200 to a value of ~10 for ZnK treated plants. *Xf* populations were reduced from log 4.5 CFU/mg of plant material in the infected control, to ~log 1 CFU/mg. Next, we tested the activity of ZnK against *Xf* subsp. *multiplex* strain AlmaEm3, which causes bacterial leaf scorch in blueberry. As with tobacco, a series of experiments were conducted using different doses of ZnK. Effective treatments were found that significantly reduced symptoms and *Xf* populations without phytotoxic effects. For this project, we are developing new nano-formulations with the goal of avoiding phytotoxicity effect and achieving bactericidal effect at lower concentrations, as well as adding anti-biofilm properties.



**Figure 1.** Nano formulations evaluated in vitro for bactericidal activity against *X. fastidiosa* subsp. *fastidiosa* TemeculaL (*Xf*). The experiment was conducted in 96 well plates, where *X. fastidiosa* was cultured in PD2 media (200  $\mu$ L) and exposed to nano formulation at different concentrations (n=6, three independent repetitions). Preliminary results were used to assess minimum bactericidal concentration (MBC) where 99.9% of the *Xf* population was killed. A) Zinkicide TMN110 (formulation used in the past), showed an MBC = 60 ppm. B) Zinkicide TMN111 is a modified version of TMN110 and showed an MBC = 50 ppm. Graph bars correspond to mean  $\pm$  SE, different letters on the bar indicate bactericidal significance according to Tukey-Kramer HSD at  $P \leq 0.05$  (JMP<sup>®</sup> 14.2.0, SAS Institute Inc., Cary, NC)

## OBJECTIVES

1. Modify nano-formulations for optimal antibacterial activity against *X. fastidiosa* and reduced phytotoxicity.
2. Characterize the response of California PD strains of *X. fastidiosa* to nano-formulations in vitro.
3. Evaluate the phytotoxicity threshold and plant uptake of nano-formulations in grapevine.
4. Assess disease control by nano-formulations in inoculated grapevines in the greenhouse.

## RESULTS AND DISCUSSION

### **Objective 1: Modify nano-formulations for optimal antibacterial activity against *X. fastidiosa* and reduced phytotoxicity.**

We have assessed so far, the efficacy of two ZnK formulations; ZnK -TMN110, used in our preliminary studies, and ZnK TMN111, and improved formulation. TMN 111 is the latest version of the ZnK manufactured by Trademark Nitrogen Inc. (TMN, Tampa, FL) that demonstrated better formulation stability over prior versions (such as TMN 110) due to the presence of additional agriculture grade stabilizer/dispersant (TMN proprietary information), without loss of antibacterial activity in vitro against several model plant pathogens. First, we tested their activity in vitro against strain *X. fastidiosa* TemeculaL. The minimum bactericidal concentration (MBC), which killed 99.9 % of culturable bacteria, was calculated from in vitro plate assay. The tested nano formulations MBC against *Xf* were as follows, ZnK TMN110 = 60 ppm, TMN111 = 50 ppm. The above-mentioned formulations were further tested in the greenhouse by foliar and soil drench application on tobacco model plants and grapes (Objective 3).

### **Objective 2: Characterize the response of California PD strains of *X. fastidiosa* to nano-formulations in vitro.**

See preliminary results in Objective 1.

### **Objective 3: Evaluate the phytotoxicity threshold and plant uptake of nano-formulations in grapevine.**

First, we assessed TM111 (the new ZnK formulation) in tobacco plants for phytotoxicity and activity against *Xf*. Plants were treated with a suspension of TMN111 either as foliar spray or drench application. Phytotoxicity was not observed with drench applications, up to a single application of 2500ppm (Figure 2 a,b). But when TMN111 was applied as a foliar spray, phytotoxicity was observed in leaves at applications of 750ppm and higher (Figure 2 d, e). With a single dose of 1000 (foliar spray Figure 2f) or 1500 ppm (drench, Figure 2c), *Xf* populations were significantly reduced, practically to near detection limit levels.

Next, to assess the phytotoxicity of the two formulations of ZnK in grapes, we conducted experiments in the greenhouse where seedlings were treated either with a single (Figure 3) or multiple (Figure 4) doses of the nano formulations applied as a soil drench. Plants were not inoculated with *Xf* for these first experiments. Plants treated with either TMN110 or TMN111 showed more leaves and were taller than untreated controls. Nevertheless, when these compounds were applied at the highest concentration tested here (1000ppm), plants were shorter than the ones treated with other ZnK concentrations, but not different from untreated control plants (Figure 3B, E). Some chlorosis was observed at the highest concentration of 1000ppm (Figure 3F). On the other hand, when ZnK was applied as weekly doses of 250 ppm each, no

phytotoxicity was observed up to 6 weeks of treatment ( Figure 4). Interestingly plants treated with ZnK show more leaves and were taller than untreated controls, starting after the second week of treatment (Figure 4).

**Objective 4: Assess disease control by nano-formulations in inoculated grapevines in the greenhouse.**

This objective has not been started, and it is contingent upon extension of funding. Plants are already growing in the greenhouse.

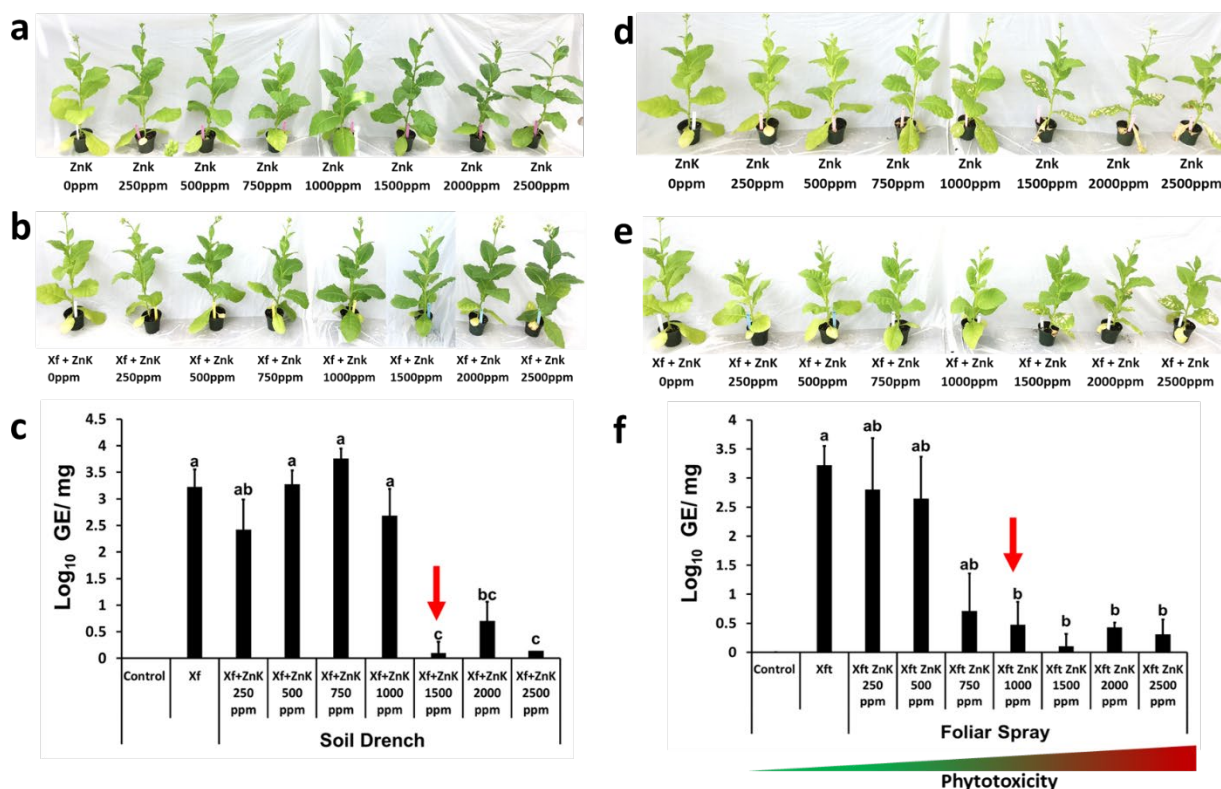
**CONCLUSIONS**

Preliminary results indicate that the new formulation TMN111 is slightly more active than TMN110 in vitro against *Xf*. Interestingly, both show a positive effect in tobacco reducing *Xf* populations, and in grape seedlings, increasing number of leaves and height of treated plants. Care needs to be taken to avoid high concentration doses since it can cause phytotoxicity, therefore a regime of multiple applications at lower concentrations seems more appropriate. New nano formulations are being developed and will be tested soon. We hope to conduct experiments with grapes infected with *Xf* and treated with different nano formulations next year.

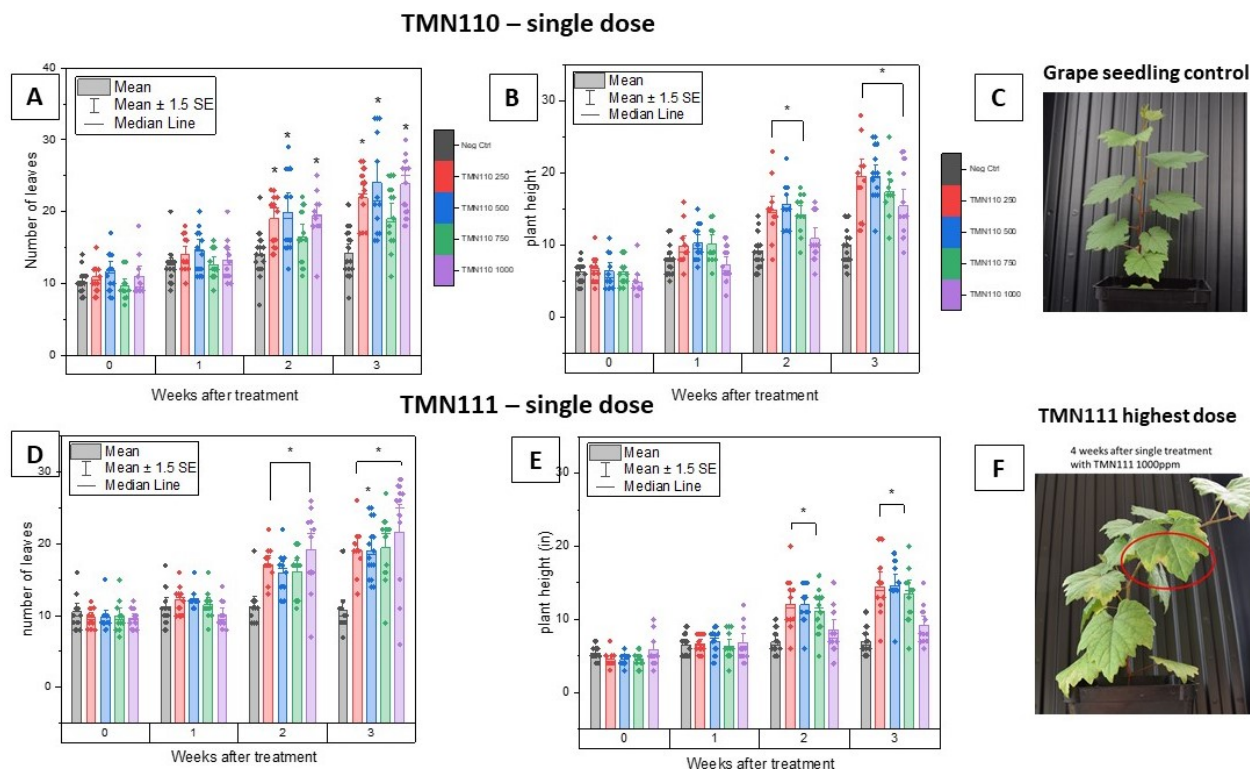
Recent presentations include:

Shantharaj, D., E. Naranjo, S. Swadeshmukul, and L. De La Fuente. 2021. Zinc oxide-based nanoformulation Zinkicide® reduces population and symptoms caused by the xylem-limited pathogen *Xylella fastidiosa* in tobacco and southern highbush blueberry. Poster presentation at the 1<sup>st</sup> Auburn University Postdoctoral Research Symposium. Sept 2-3, 2021.

Shantharaj, D., E. Naranjo, S. Swadeshmukul, and L. De La Fuente. 2021. Zinc oxide-based nanoformulation Zinkicide® reduces population and symptoms caused by the xylem-limited pathogen *Xylella fastidiosa* in tobacco and southern highbush blueberry. American Phytopathology Society, poster presentation at the Plant Health meeting held online. Aug 2-6, 2021.



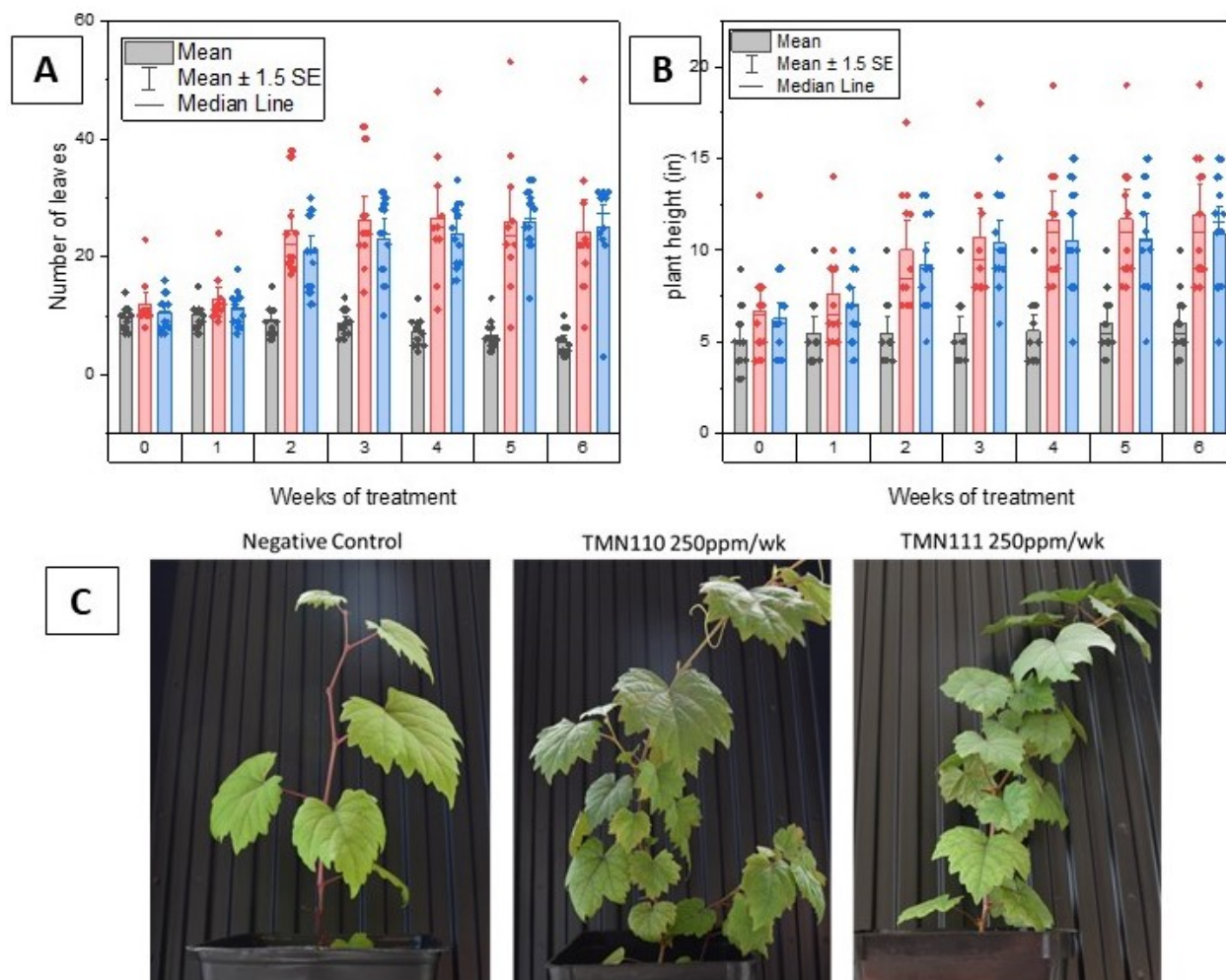
**Figure 2.** Zinkicide TMN111 in planta bactericidal activity. *Nicotiana tabacum* SR1 plants (15 day old) were infected with a *X. fastidiosa* subsp. *fastidiosa* TemeculaL (*Xf*) suspension in PBS ( $OD_{600} = 0.8$ ). Twenty microliters of the bacterial suspension were pinpricked to the petiole of 1<sup>st</sup>, 2<sup>nd</sup> 3<sup>rd</sup> plant leaves. Zinkicide TMN111 (ZnK) different concentrations (ppm) were applied one week after *Xf* inoculation to plants by soil drench (a-c) or foliar spray (d-f). Each experimental treatment included nine plant replicates. Experimental results were evaluated 5 weeks after *Xf* infection and 4 weeks after ZnK application. **a & b**) ZnK soil drench applications did not show phytotoxicity to plants. Plant chlorophyll content increased with the application of ZnK at higher concentrations (data not shown). **c**) *Xf* population was analyzed by qPCR. Petioles of 5<sup>th</sup> and 6<sup>th</sup> leaf from experimental treatments were excised and processed for DNA extraction (CTAB DNA extraction procedure). qPCR reactions were performed using the HL5/HL6 primers along HLP as TaqMan probe labeled with FAM (De La Fuente et al. 2013; Francis et al. 2008). *Xf* population was calculated as  $\log_{10}$  Genome equivalence (GE) / mg petiole tissue. ZnK at concentrations of 1500, 2000, 2500 ppm was able to reduce the *Xf* population significantly ( $p < 0.001$ ). **d & e**) ZnK foliar spray applications showed foliar phytotoxicity with increase in ZnK concentration; except for ZnK 250, 500 ppm concentrations which did not cause phytotoxicity. **f**) ZnK concentrations 1000, 1500, 2000, 2500 ppm were able to reduce *Xf* populations significantly ( $p < 0.001$ ) with foliar applications. *Xf* population analysis was represented as mean  $\pm$  SE ( $n = 4$ ). Statistical significance in reducing *Xf* populations among ZnK treatments was calculated by Tukey's HSD at  $P \leq 0.05$  (JMP® 14.2.0, SAS Institute Inc., Cary, NC).



**Figure 3.** Phytotoxicity assessment in grape seedlings. Grape seedlings were treated with a single dose of different concentrations of either ZnK formulation TMN110 (A, B), or the newest formulation TMN111 (D, E), applied as a soil drench. A, D) Number of leaves were counted weekly. N=10 plants per treatment. Gray = Negative (untreated) control, Red = 250ppm, Blue = 500ppm, Green = 750ppm, Purple = 1000ppm. B, E) Plant height measured in inches. N=10 plants per treatment. Gray = Negative (untreated) control, Red = 250ppm, Blue = 500ppm, Green = 750ppm, Purple = 1000ppm. \*Indicates treatments significantly different than negative control based on one-way ANOVA and Tukey's means comparison test ( $p < 0.05$ ). C) Picture of typical control seedling used in these trials. *Vitis vinifera* cv Chardonnay was grown from seeds in Sunshine Mix #2 (Sungro Horticulture) in 4-inch pots. Seedlings were not given additional fertilizer during the trials and were watered as needed. F) Some chlorosis potentially due to phytotoxicity was observed in seedlings treated with 1000ppm of TMN111. Chlorosis was not observed in any other treatments, including repeated doses of TMN111 250ppm (see Figure 4).



# TMN110/111 – weekly doses



**Figure 4.** Phytotoxicity assessment in grape seedlings treated with repeated doses of smaller concentrations of either ZnK formulation TMN110, or the newest formulation TMN111, applied as a soil drench. A) Number of leaves counted weekly. B) Height of plants measured weekly. Plants were treated every week with 250ppm. No fertilizer was applied during the trial which could explain slow growth of the controls. Gray = negative (untreated) control, red = TMN110, Blue = TMN111. \*Indicates treatments significantly different than negative control based on one-way ANOVA and Tukey's means comparison test ( $p < 0.05$ ). C) Control and treated seedlings after 5 weeks of weekly doses of 250ppm.

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## FUNDING AGENCIES

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

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

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**Reporting Period:** The results reported here are from work conducted October 2020 to August 2021, with historical information for context with a related ongoing experiment.

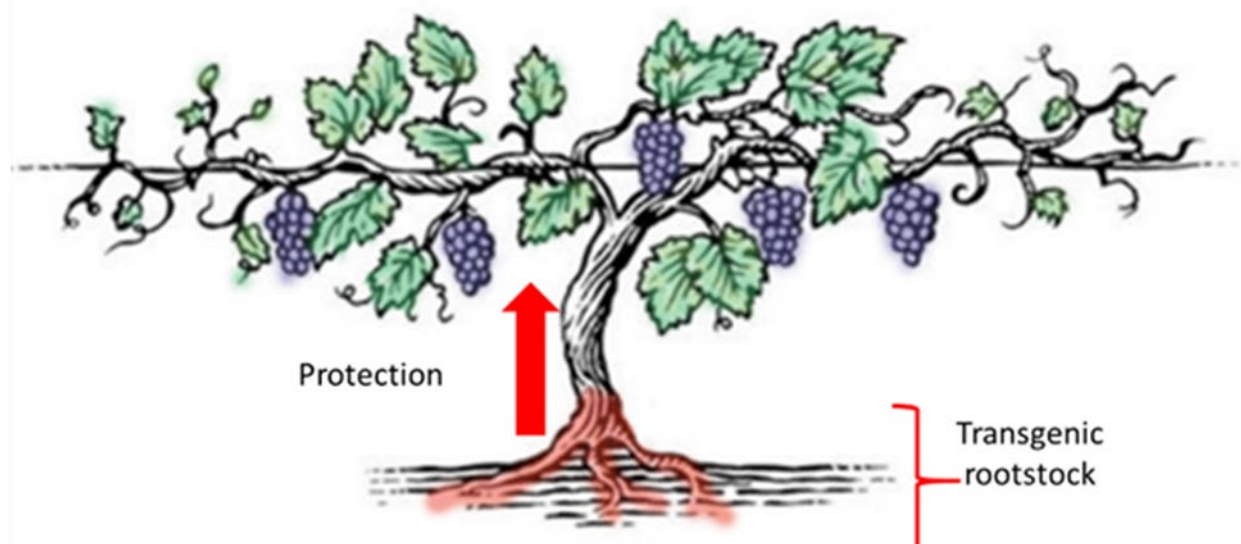
**ABSTRACT**

A field project began in 2010 to evaluate grapevines expressing potential Pierce's Disease (PD) suppressive transgenes expressing single DNA inserts of five genes from Dandekar, Lindow, Powell, and Gilchrist projects as whole plant transgenics in Thompson Seedless and Freedom rootstock plants. The disease was successfully introduced into the cordon trained plants by mechanical injection of *Xylella fastidiosa* (Xf) of 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 and untransformed control plants. All plants are located in a secured, USDA-APHIS-regulated field area. The results of these experiments confirmed the appearance of classic foliar symptoms of PD and cane death within 24 months in susceptible controls in contrast to the transgenic plants. Each of the transgenes suppress the symptoms of PD in inoculated vines to varying degrees (Gilchrist and Lincoln 2015, 2016, 2017, 2018a, 2018b; Gilchrist et al. 2015; Lincoln et al. 2018). There was no evidence of spread of the bacteria to uninoculated susceptible grape plants adjacent to infected plants over the duration of the test, confirming tight experimental control on the pathogen spread. This project was terminated in 2018 and the research advanced to field testing the potential for cross graft protection of an un-transformed scions grafted to transgenic rootstocks (Figure 1). The rootstocks were transformed to express pairs of the five disease suppressive genes in a gene stacking approach with the genes paired together by differential molecular function. Laboratory analysis confirmed each of the dual constructs was inserted in the rootstocks as required, before each was grafted to a PD susceptible non-transgenic Chardonnay. A total of 721 transgenic and untransformed control individuals were planted in 2019. All plants displayed normal growth and morphology and were inoculated with *Xylella fastidiosa* in July 2021. (Figure 2 and 3). This project funds the costs of planting, training the plants to commercial standards, and all field costs associated with tilling, pest management, irrigation and other requirements dictated by USDA-APHIS permit.

**LAYPERSON SUMMARY**

This project is for direct support for expenses for 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 costs and objectives for production transgenic rootstock and grafting to untransformed PD susceptible Chardonnay scions, inoculating the plants with *Xylella fastidiosa* (*Xf*) and subsequent analysis of bacterial dynamics and disease symptoms are funded by a separate grant entitled: Transgenic rootstock-mediated protection of grapevine scion against Pierce's Disease by dual stacked DNA constructs by CDFA project Number 20-0269-000-SA to PI Gilchrist. The new planting, managed to commercial standards, consists of untransformed PD susceptible Chardonnay scions grafted to transgenic rootstocks, (Paulsen 1103 and MGT 101-14) expressing the paired constructs. The field experiment, conducted in an USDA-APHIS-regulated Solano County site, includes mechanical inoculation of *Xf* that was used successfully in past field experiments. Pierce's Disease symptoms, bacterial movement, and fruit yield will be measured during the course of the experiment. A total of 721 transgenic and controls were planted in 2019 (Figure 2). All plants displayed normal growth and morphology and were inoculated with *Xf* in July 2021. (Figure 3). This project funds the costs of planting, training the plants to commercial standards, and all field costs associated with tilling, pest management, irrigation and other requirements dictated by USDA-APHIS permit.



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

## INTRODUCTION

This continuation project supports grape vine management, field supplies, irrigation, pest management, and labor required for the field research component of a dual gene transformed rootstock experiment designed to evaluate the potential of cross graft protection against Pierce's Disease (PD) in grape. The grape planting was completed in August 2019 in the USDA-APHIS regulated field site. The current support is for 3 years (2019-2022), which covers the period from

completion of planting of the stacked gene transformed rootstocks to the inoculation of the plants in July 2021, disease and bacterial analysis, to be followed by analysis of associated effects on grape yield and quality. The goal of this field research is to determine if pairs of five genes transformed into adapted rootstocks will act across a graft union to suppress Pierce's Disease in non-transgenic Chardonnay scions. Data on Pierce's Disease symptoms and bacterial dynamics will be collected shortly after the first inoculation with *Xf* that was completed July 2021 and is supported by a separate grant to PI Gilchrist (CDFA Number 20-0269-000-SA).

## **OBJECTIVES**

There are four principal objectives:

1. Complete preparation of a new planting area within the current 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 (Paulsen 1103 and MGT 101-14). These rootstocks will be grafted to Pierce's Disease (PD) susceptible Chardonnay 04 scions prior to field planting. The goal is to assess the potential of cross graft protection against PD of a non-transgenic scion. (Figure 1).
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. The plant and field management conducted by Field Supervisor Bryan Pellissier will include trimming, rototilling, weed control, irrigation and timely application of pesticides for powdery mildew and insects following the protocol specified by the USDA APHIS permit. This includes pressure washing of all equipment leaving the field.
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, bacterial dynamics (population and movement) beginning in 2021 following inoculation with *Xf* also is being funded by the separate grant to PI Gilchrist.

## **RESULTS AND DISCUSSION**

### **Objective 1. Completed the new planting area within the current APHIS approved site.**

Land preparation and configuration of the experimental 3.5-acre area by Bryan Pellissier is sufficient to accommodate and manage as many as 900 new plants was completed in 2019. Row spacing is 11 feet between rows with 7 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 APHIS permit. The planting pattern permits a 2-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. Experimental design is a complete randomized block with six (6) plants per each of six (6) entries (replications), including all controls. Each plant will be trained as a single trunk with 2 cordons.

The field planting of the stacked gene rootstock combinations was completed August 20, 2019 (Figure 2). The new planting within the current APHIS approved site consists of a new set of lines bearing paired PD suppressive, DNA constructs, referred to as stacked genes, in two adapted rootstocks (1103 and 101-14) (Gilchrist and Lincoln 2018a) These rootstocks were grafted to PD-susceptible un-transformed Chardonnay scions prior to field planting to assess

potential for cross-graft protection of the infected scion. The plantings were done by personnel from the Foundation Plant Services (FPS) that was directed by Josh Puckett from FPS (Figure 2) assisted by Bryan Pellissier. A total of 721 transgenic and control plants are in the field and growing normally (Figure 4).

All field activities are coordinated by PI Gilchrist, conducted by field superintendent Bryan Pellissier with assistance by Foundation Plant Services field personnel for plant pruning and training to commercial standards. All field expenses and personnel time are paid by this grant. Total fenced area occupied by plants and buffer zones as required by the APHIS permit is 3.4 acres. All plants are being maintained under a drip irrigation system.

**Objective 2. Train and manage the planting to conform to commercial standards activities**

The grafting, planting, and training of the vines was guided initially by Josh Puckett and Deborah Golino (FPS) working with PI Gilchrist and Bryan Pellissier. The FPS crew, now directed by Marcos Arriaga, will continue to provide personnel and guidance for trellising and plant management to reflect commercial production standards from funds provided by this grant. (Table 1). The field plot design enables experimental *Xf* inoculations, pathogen and disease assessments, as well as grape yield.

After the first year, the canes were tied down during the dormant season and trimmed to the appropriate length or shorter if the cane girth is not over 3/8" in diameter. The shoots that push were suckered to remove double shoots and to achieve a shoot (and hence spur position) spacing of about 4-5 inches between them. Figure 3 shows the plants on July 1, 2021, that have been trained as 2-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.

All field activities are coordinated by PI Gilchrist and conducted by Bryan Pellissier and personnel organized through FPS supported by funds from this grant.

Irrigation and pest management, primarily powdery mildew, other foliar pathogens, weeds and regular tilling and hand weeding will maintain a weed-free planting area. The APHIS permit number 7CFRE340 has strict requirements for regular reporting of all field activities and for movement of any plant material outside the fenced area and gated area. All pruning material is to be left between the rows to dry, then flail chopped and later rototilled to incorporate the residue per requirements as required by the APHIS permit. All equipment must be pressure washed prior to leaving the field as required by APHIS to ensure that no plant material leaves the field (Figure 5). Samples for laboratory analysis are transported in bags and then autoclaved after subsampling.

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 was observed with these practices throughout the past six growing seasons of the previous planting per the protocol carried out by Bryan Pellissier.





**Figure 2.** Planting of the dual constructs. This image illustrates the initial planting of the dual construct transformed rootstocks grafted with an untransformed clone of Chardonnay that was completed in summer of 2019.



**Figure 3.** Status July 1, 2021. Completed planting of transgenic rootstock with untransformed scions in 2019. Plants are all vigorous in 2021 and have been trained as described. under objectives pending inoculation, which will be completed in July 2021.



**Figure 4.** Image of a dual transformed rootstock grafted to untransformed Chardonnay scion. This photo shows a cordon trained plants prior to inoculation of individual canes on July 23, 2021.



**Figure 5.** Image of pressuring washing of field equipment prior to leaving the APHIS controlled field. area containing transgenic rootstock plants as required by the APHIS Permit

**Objective 3. Inoculate the individual cordon-trained vines in the July 2021, which is funded under CDFA Number 20-0269-000-SA. Described here to provide context to the total field experiment**

The following protocol, while not part of the field research effort funded by this grant, will be carried out by support from CDFA Number 20-06297-000-SA to PI Gilchrist 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 utilize the same protocol as 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 – 20,000 cells per injection site. Each site is tagged to enable assessment over time (Figure 6). 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 on the level of resistance to Pierce's Disease infection and the effect of the transgenes on the amount and movement of the bacteria in the non-transgenic scion area. 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 5-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.

**Objective 4. Quantitative data collection on disease rating, bacterial dynamics (population and movement) beginning in 2021 with fruit yield per plant in the third and fourth years will be conducted and is funded under CDFA Number 20-0269-000-SA.**

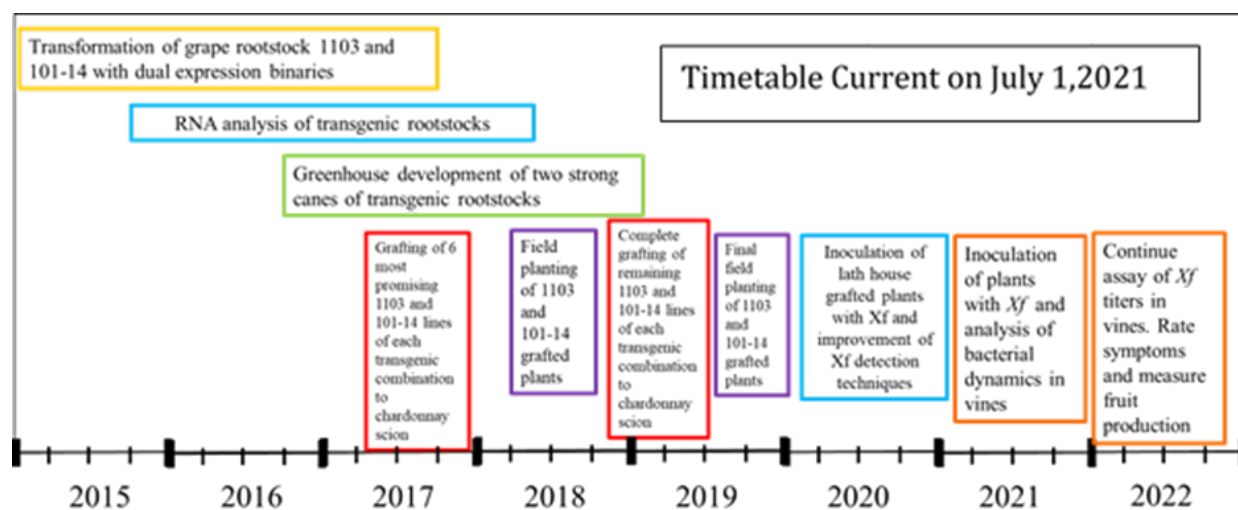
Detection of the amount and movement of the bacteria in plant tissues (mainly leaves and stems) by quantitative PCR (qPCR) assays in the Gilchrist lab, will be supported by the grant CDFA Number 20-0269-000-SA and is provided, here to illustrate the total context for all aspects of the field management and treatment of plants at this site. The inoculation and analysis has begun in 2021. Evaluation of the experimental plants for plant morphology, symptoms of Pierce's Disease 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 later leaf symptom severity as previously observed. The assessment format and data collected has been reported annually in the Pierce's Disease Symposium Reports (Gilchrist and Lincoln 2015, 2016, 2017, 2018a, 2018b; Gilchrist et al. 2015). Chardonnay fruit yield will be measured after third and fourth year by harvesting fruit from individual plants and combining by genotype should the disease differentials warrant.

Note: This project does not overlap but is interdependent on the proposal entitled "Transgenic rootstock-mediated protection of grapevine scion by single and stacked dual DNA constructs", which generated the experimental material described herein (CDFA Number 20-0269-000-SA). Separate funding to support management of the APHIS Permit is provided to Abhaya Dandekar as the holder of the Permit.





**Figure 6.** Red arrows point to inoculations sites on individual canes. Inoculations were completed on July 23, 2021. Picture taken November 2, 2021.



**Figure 7.** Research Timetable to accomplish the objectives of this field planting

## **CONCLUSIONS**

This field project began in 2018 to evaluate grapevines expressing pairs of five potential Pierce's Disease (PD) suppressive transgenes in two adapted rootstocks (Paulsen 1103 and MGT 101-14) grafted to a non-transformed PD susceptible scion (Chardonnay 04). The five transgenes from three investigators were shown in a previous field using single genes in fully transformed plants to suppress symptoms of PD to varying degrees over the duration of the experiment (Gilchrist and Lincoln 2015, 2016, 2017, 2018a, 2018b; Gilchrist et al. 2015; Lincoln et al. 2018). Consequently, the research of this project has moved forward to field testing a new generation of grape plants 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 test for potential cross graft protection against PD in the USDA-APHIS regulated field site. The planting of 721 experiment rootstock-scion combinations plus controls, completed in 2019, display normal growth and morphology as illustrated in July 2021 (Figure 3). The first set of inoculations were completed in July 2021. Four months after inoculation, tissue samples were taken from 3 random inoculation sites and extracted for DNA. The DNA was analyzed by qPCR and showed detectable *Xf* in each sample and confirms the transfer of *Xf* to the plants in the field. All timelines indicated in the schematic below have been completed within the proposed periods through 2021 in spite of constraints imposed by the Covid-19 virus due to UC Davis campus tight restrictions on laboratory activities, field research, and personnel interactions.

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**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

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**Cooperator:** Bryan Pellissier | Department of Plant Pathology | University of California | [bpellissier@ucdavis.edu](mailto:bpellissier@ucdavis.edu)

**Reporting Period:** The results reported here are from work conducted October 2020 to August 2021.

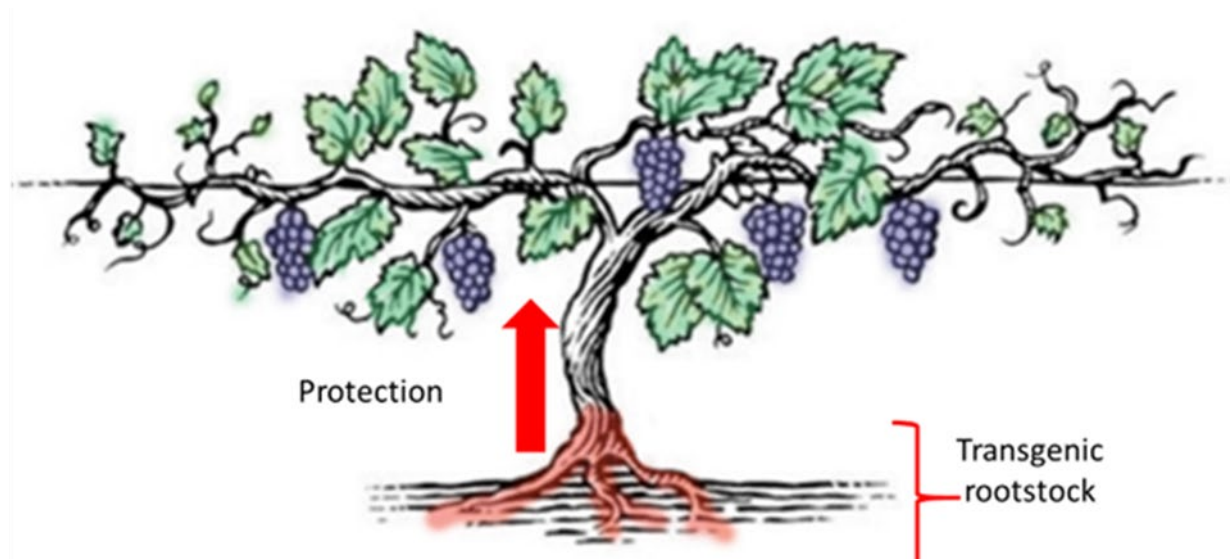
### ABSTRACT

Field studies to evaluate potential Pierce's Disease (PD) suppressive activity of transgenes introduced into PD susceptible grapevines were begun in 2010. DNA sequences, isolated from earlier laboratory and greenhouse projects by the Abhaya Dandekar, Ann Powell, Steve Lindow, and David Gilchrist (Table 1) were inserted individual into Freedom rootstocks and own rooted Thompson Seedless grape. The pathogenic bacteria, *Xylella fastidiosa* (*Xf*), was introduced into the cordon trained field plants by mechanical injection into young shoots resulting in classic PD symptoms and plant death within 24 months in the susceptible controls. Consistent protection against PD symptoms was recorded in whole plant transgenics for the 5 introduced genes over a 5-year period following infection. There was no movement of the bacteria from inoculated plants to the uninoculated control plants confirming tight experimental control on bacterial confinement to inoculated plants. This experiment was terminated in 2017 and a second phase field experiment wherein pairs of the five PD potentially suppressive genes were introduced into two adapted rootstocks to evaluate the potential for cross-graft protection of an untransformed PD susceptible Chardonnay scion was approved by The Product Development Committee of the Pierce's Disease Control and begun in 2018.

The Gilchrist lab developed transformation vectors containing pairs of the five PD suppressive transgenes, which were incorporated by the UC Davis Transformation Facility into two adapted rootstocks (Paulsen 1103 and MGT 101-14) and confirmed by molecular analysis by Gilchrist lab to contain the correct paired genes. The transformed rootstocks were bud grafted by the Foundation Plant Services (FPS) with PD susceptible non-transgenic Chardonnay 04 scions for field testing for cross-graft protection against PD. A total of 522 transgenic rootstock plants, bud grafted to a non-transgenic PD susceptible scion, along with un-transformed control plants for a total of 721 plants that were established in the secured, USDA-APHIS-controlled field in 2019. Canes on the transgenic plants and control plants were mechanically inoculated with *Xf* cells in July 2021. Subsequent laboratory analysis by PCR of random canes confirmed the bacteria were established in the inoculated tissue. Pathogen activity and disease expression in the transgenic vs analogous non-transgenic control plants will be assessed over time to determine if protection of the PD susceptible Chardonnay scion can move across the graft union (Figure 1)

**Table 1.** Genes selected to evaluate as dual genes in the 2<sup>nd</sup> generation field evaluation for suppression of Pierce's disease in grape. The table lists gene names, abbreviation used, and presumed function.

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



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

## LAYPERSON SUMMARY

The translational research described herein will test for potential cross-graft protection of a PD susceptible Chardonnay 04 scion against the development of Pierce's Disease symptoms by expression of dual combinations of five previously identified PD suppressive transgenes in two adapted rootstocks. The current experiment derives from an earlier field study, begun in 2010 in which five (5) different DNA constructs, expressed as single genes in whole plant transgenics, gave protection against classical PD symptoms and death in non-transgenic control vines within

24 months following mechanical inoculation of *Xylella fastidiosa* (*Xf*). The suppression of PD symptoms persisted over a five-year period compared with the non-transgenic vines until the experiment was terminated in 2017. A second field experiment began in 2018 to test potential cross-graft protection by these five genes expressed as paired (dual) combinations of the genes in two adapted rootstocks (Paulsen 1103 and MGT 101-14) grafted to a PD susceptible Chardonnay 04 scion. The successfully transformed rootstocks, confirmed by laboratory analysis to contain the desired gene pairs, were bud grafted to PD susceptible Chardonnay 04, and planted in USDA-APHIS-controlled area in 2018 and 2019. The current experiment is designed to assess both potential cross-graft protection of the non-transformed scion and the effect of the transgenes to protect the rootstocks against downward bacterial movement into the perennial tissue and plant death compared to equivalent combinations of untransformed rootstock/scion control combinations. The experiment protocol include planting in an USDA APHIS regulated field area, training the plants to commercial standards, and mechanically inoculating the plants with pathogenic *Xf*, as done in the previous experiment. The data to be collected will evaluate both disease and yield components in the PD susceptible scions. The first inoculation with *Xf* was completed in July 2021 and subsequent laboratory testing of random canes confirmed the bacteria were established in inoculated canes.

## INTRODUCTION

The long-term objective of this field and laboratory research on protection of grape against Pierce's disease was begun in 2010 following extensive laboratory and greenhouse studies designed to screen for and isolate plant DNA sequences that could suppress symptoms of Pierce's Disease (PD) following mechanical infection with *Xylella fastidiosa* (*Xf*). Genes identified by investigators Abhaya Dandekar, Ann Powell, Steve Lindow, and David Gilchrist were then expressed in PD susceptible Freedom and Thompson Seedless lines for testing in a USDA APHIS controlled field setting for response to mechanically inoculated pathogenic *Xf* cells to assess PD symptom expression and behavior of the bacteria in the tissue. The inoculated shoots expressed classic PD symptoms and death within 24 months in the susceptible controls. Consistent protection against PD symptoms was recorded in whole plant transgenics for the 5 introduced genes persisted over a 5-year period following infection until the experiment was terminated in 2017. A second field experiment was initiated in 2018 to test if expressing two genes simultaneously in stacked combination in two different adapted rootstocks would protect an untransformed and PD susceptible Chardonnay scion across the graft union (figure 1). 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 enhance protection and drastically reduce the probability of *Xf* overcoming the resistance.

## OBJECTIVES

1. Complete introduction of 10 combinations of protective paired constructs via the dual insert binary vector developed by Dr. James Lincoln in the Gilchrist lab into adapted grapevine rootstocks Paulsen 1103 and MGT 101-14 for a total of 20 types of transgenic lines to be evaluated with at least 10 paired combinations from each rootstock line delivered by the transformation facility, prepare them for bud-grafting and harden off for field planting
2. Field and laboratory analysis of potential transgenic rootstock cross-graft protection of an untransformed PD susceptible scion. Train and manage the vines to conform to commercial

- standards, to enable collection of fruit yield data as well as data on disease and bacterial dynamics as the infections proceed. A 2-bud pruned bilateral cordon system of sufficient lengths for inoculation to determine in real time the bacterial dynamics in inoculated untransformed Chardonnay scion.
3. Management of the experimental planting includes pruning, training, tilling and application of pesticides for foliar diseases, especially powdery mildew, and insects. These points are noted here for information only, since the costs associated with the field activities are funded by a separate grant to PI Gilchrist through 2023 (CDFA 19-0260-000SA).
  4. Production of the pathogenic *Xf* bacterial inoculum by the Gilchrist lab for inoculation of the transgenic grafted plants and susceptible controls in the field. Inoculum will consist of cells of culture-grown *Xf*, confirmed to be pathogenic in lath house tests of comparable genotypes to assure that the cultured bacterial will produce classical foliar PD symptoms prior to field inoculation.
  5. Inoculate the individual cordon-trained vines in 2021 when shoots have expanded to 8-10 inches with ~20,000 *Xf* bacterial cells per inoculation site by stem puncture through a 10-microliter droplet of inoculum. We anticipate inoculating each plant at 5 sites to ensure establishment of infection and provide infected tissue for subsequent analysis of bacteria within the vine.
  6. Quantitative data collection on bacterial dynamics (population and movement) beginning 3 months after first inoculation in 2021,
    - a. Vines of the representative transgenic rootstocks will be sampled for qPCR analysis at defined intervals to identify any differential in behavior of the bacteria in the untransformed Chardonnay scion grafted to the transgene modified rootstocks. Confirmation of infection is expected within 3-6 months following inoculation.
    - b. Systematic rating of *Xf* infected transgenic and control plants for foliar symptoms of PD disease and cane or plant death will be recorded when symptoms are expected to appear first at bud emergence and later in the season as foliar symptoms appear. Previous experience with this format indicates symptoms on susceptible plants will likely first appear within a 1-2-year period after infection, although bacterial sampling will begin 3 months after inoculation to establish presence of the bacteria, regardless of symptoms.
  7. The management of the plants to commercial standards will permit measuring fruit yield per plant in the third and fourth years if the disease and bacterial dynamic results warrant.

## RESULTS AND DISCUSSION

1. Completed introduction of 10 combinations of protective paired constructs via the dual insert binary vector developed by Dr. Lincoln into adapted grapevine rootstocks 1103 and 101-14 for a total of 20 types of transgenic lines to be evaluated with at least 10 paired combinations from each rootstock line delivered by the transformation facility (Table 2) (Ref 1,2).
2. A total of 6 independent transgenic lines of each dual construct in the two rootstocks were advanced to lath house for overwintering in 2017. In early spring, cuttings were made, rooted and bud grafted with non-transformed Chardonnay. Up to 6 copies of each rootstock/scion combination was prepared for field planting in the spring of 2018 at the USDA-APHIS approved site in Solano County. There were 464 vines planted in 2018 and 257 in 2019 for a total of 721 vines planted. Representatives of each paired combination were planted in each year, along with the non-transgenic control vines (Ref 3,4,5) (Table 2).



**Figure 2.** Planting of the dual constructs. This image illustrates the initial planting of the dual construct transformed rootstocks grafted with an untransformed clone of Chardonnay that was completed in summer of 2019.



**Figure 3.** image of 721 dual transformed rootstock grafted to untransformed Chardonnay scions and controls. Field planting completed in 2019. This photo shows the cordon trained plants prior to inoculation of individual canes in July. 2021



**Table 2.** August 20,2019. Updated list of transgenic paired genes and rootstock combinations including controls planted in the APHIS controlled 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
<b>Totals</b>				<b>149</b>	<b>721</b>

3. Established planting is shown in Figures 2 in 2019. Management of new planting was guided by PI Gilchrist with trellising and plant management to reflect commercial production standards conducted by Marcos Arriaga (FPS) and Bryan Pellissier. Plants are currently in excellent condition as shown in Figure 3.
4. The design will enable experimental *X. fastidiosa* inoculations, pathogen and disease assessments, as well as fruit yield. Row spacing is 11 feet between rows with 9 feet between plants. These spacing permits 32 rows of 30 plants and includes a 50-foot open space around the planted area as required by the USDA-APHIS permit. Experimental design is a complete randomized block with six (6) plants per each of six (6) entries (replications), including all controls. 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 were installed after the first year of growth and a 13-gauge wire supported the drip irrigation wire, about 18" off the ground.
  - a. After the first year, the canes were tied down during the dormant season and trimmed to the appropriate length or shorter if the cane girth is not over 3/8" in diameter. The shoots that pushed were suckered to remove double shoots and to achieve a shoot (and hence spur position) spacing of about 4-5 inches between them. Plant appearance in July 2021 is shown in Figure 4.

#### **Mechanical inoculation of *X. fastidiosa* into vegetative shoots**

Follows the same protocol used to effectively establish the pathogen in the plant tissue and elicit PD symptoms as done successfully in the previous planting on this field site and were completed in July 2021. (Gilchrist and Lincoln, 2016). Four months, after inoculation, tissue samples were taken from 3 random inoculation sites and extracted for DNA. The DNA was analyzed by qPCR and showed detectible *Xf* in each sample (10-50 *Xf* cells per gram of tissue) and confirms the transfer of *Xf* to the plants in the field.

#### **Molecular analysis of bacterial dynamics**

Each disease parameter will be determined overtime by visual monitoring of symptom development and detection of the amount and movement of the bacteria in plant tissues (mainly leaves and stems) by quantitative PCR (qPCR) assays. 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 (Gilchrist and Lincoln, 2018). 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 isolate by a reproducible CTAB-based extraction method. Quantitative detection of *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 untransformed grape scions and grape rootstocks.

#### **Symptom expression**

Both symptom expression and behavior of the inoculated bacteria will provide an indication on the level of resistance to Pierce's Disease infection and the effect of the transgenes on the amount and movement of the bacteria in the non-transgenic scion area and the movement into the rootstocks.



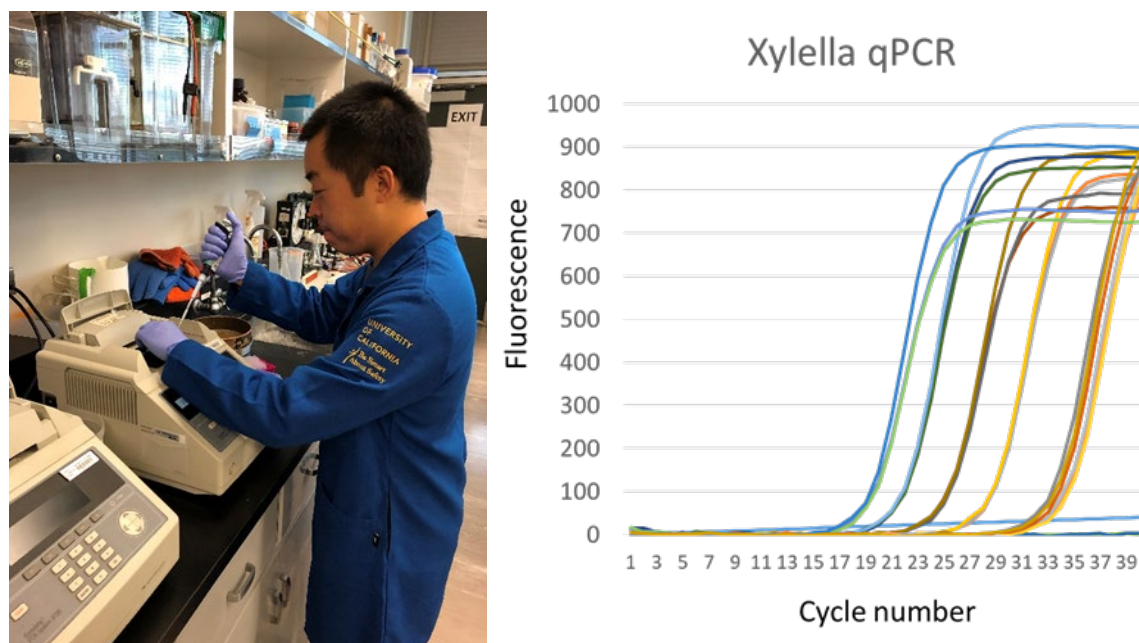
**Figure 4.** Image of a dual transformed rootstock grafted to untransformed Chardonnay scion. This photo shows a cordon trained plants prior to inoculation of individual canes on July 23, 2021.



**Figure 5.** Red arrows point to inoculations sites on individual canes. Inoculations were completed on July 23, 2021. Picture taken November 2, 2021.

### Plant and Pest Management

Irrigation and pest management, primarily powdery mildew, weeds and insects, will be coordinated by PI Gilchrist and conducted by Bryan Pellissier the Field Superintendent employed by the Department of Plant Pathology. The field crew work closely with PI Gilchrist to determine timing and need of each of the management practices, including pruning and thinning of vegetative overgrowth as necessary by a crew provided by Marcos Arriaga (FPS).

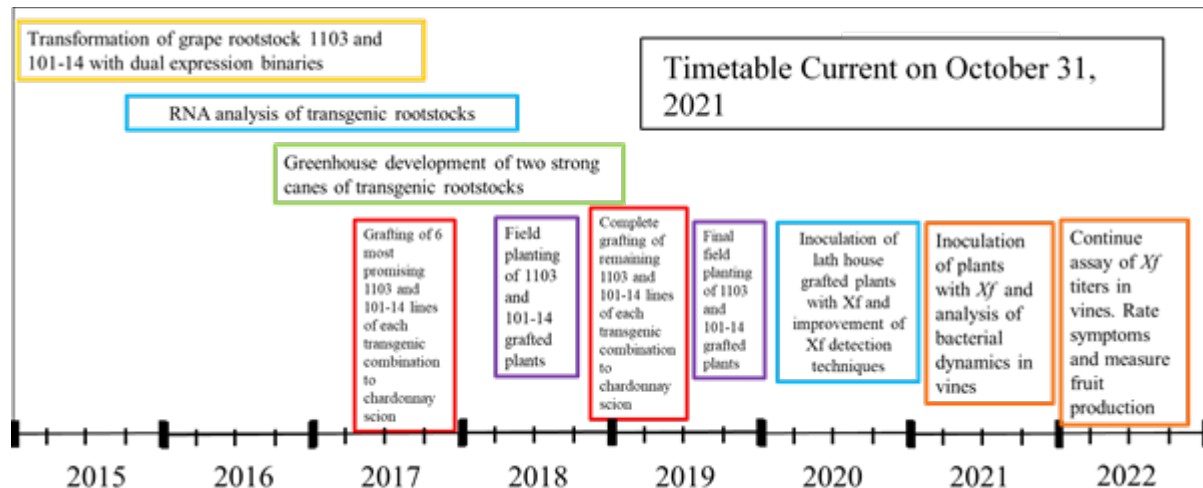


**Figure 6.** Illustration of PCR machine analysis of grape stem extracts for presence and amount of *Xylella fastidiosa* in field inoculated grape shoots and an example data set.

### Research Timetable

This project began with planting completed in 2019 (Figure 2). Inoculation and evaluation began in July 2021 and will continue annually until the field planting is terminated. 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 Professor Abhaya Dandekar. The protocols include plant management, inoculation with *Xylella fastidiosa*, assessment of classical symptoms of Pierce's Disease exhibiting the range from foliar symptoms to plant death and the assessment of protection by a set of transgenes selected by molecular techniques to suppress the symptoms of Pierce's Disease and/or reduce the ability of the pathogenic bacteria to colonize and move within the xylem of the grape plant. Management of the vines by commercial standards has been conducted by FPS personnel, Bryan Pellissier, and PI Gilchrist. While the Covid-19 virus has presented considerable challenge to maintaining progress due to UC Davis campus tight restrictions on laboratory research and personnel interactions, we have accomplished the objectives set forth through 2021, including the first inoculation the field plants in July 2021 and confirmed presence of the bacteria in the inoculated canes.





**Figure 7.** Research Timetable to accomplish the objectives of this field planting.

## CONCLUSIONS

This translational research described herein will test for potential cross-graft protection of a PD susceptible Chardonnay 04 scion against the development of Pierce's Disease symptoms by expression of dual combinations of five PD suppressive transgenes in two adapted rootstocks. The protocol includes planting, training, 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 effect of the transgenes to protect the rootstocks against bacterial movement and death compared to equivalent combinations of untransformed rootstock/scion control combinations. 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 Professor Abhaya Dandekar. The protocols for managing the existing and the new plantings with the dual constructs have been used successfully over the past 5 years (Gilchrist 2016). These protocols include plant management, mechanical inoculation with *Xylella fastidiosa*, development of classical symptoms of Pierce's Disease exhibiting the range from foliar symptoms to plant death and the assessment of protection by a set of transgenes selected by molecular techniques to suppress the symptoms of Pierce's Disease and/or reduce the ability of the pathogenic bacteria to colonize and move within the xylem of the grape plant. Management of the vines by commercial standards has been conducted by FPS personnel and PI Gilchrist. All timelines indicated in the schematic below have been completed within the proposed periods

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

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**Reporting period:** The results reported here are from work conducted July 1, 2019 to October 31, 2021

### ABSTRACT

*Paraburkholderia phytofirmans* strain PsJN is capable of extensive growth and movement within grape after both needle or spray inoculation. The population size of *X. 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 when inoculated at other locations in the plant either three weeks before or up to 6 weeks after that of the pathogen. This strain appears to induce disease resistance in the plant, causing eradication of the pathogen. Inoculation of plants with *P. phytofirmans* from three weeks before inoculation with the pathogen and up to six 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 six weeks. The levels of reactive oxygen species is elevated in plants inoculated with both *P. phytofirmans* and the pathogen and remain elevated for many days after co-inoculation of these strains into grape, both near the point of inoculation as well as at points many internodes distal to the site of initial inoculation, suggesting that the priming of disease resistance by *P. phytofirmans* is both relatively systemic and persistent in the plant. The changed patterns of gene expression in plants coinoculated with the pathogen and *P. phytofirmans* are also being assessed to understand the complexity of the induced disease resistance apparently conferred by *P. phytofirmans*. These promising preliminary results suggest that only a single applications of *P. phytofirmans* may be required for practical control under field conditions.

### 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 either before, or even several weeks 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 are being undertaken to addresses 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 to up to six weeks after inoculation with the pathogen provide equally great reductions in disease severity. These

findings suggest that a single application each year may provide substantial reduction in the likelihood of infection by the pathogen. Spray application of *P. phytofirmans* appears superior to that of direct inoculation into the plant, also indicating that biological control of disease with *P. phytofirmans* can be achieved by relatively simple application methods.

## INTRODUCTION

Unlike other bacteria that we recovered from the interior of grapes, the bacterium *Paraburkholderia phytofirmans* is able to grow to large population sizes and spread extensively within xylem vessels of mature grape (1). Surprisingly, while we considered it a possible surrogate bacterial host for expression of *X. fastidiosa rpfF* genes encoding DSF 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 *X. fastidiosa* (*Xf*) and very high levels of disease control is 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 are achieved (1). 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 is killed in plants that are also colonized by *Paraburkholderia*. Initial studies in the greenhouse revealed that plant disease resistance genes are induced when both *Xf* and *Paraburkholderia* are present in the plant (1). 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 (2). The presence of *Paraburkholderia* therefore seems to be priming plants for resistance reaction to *Xf* that would otherwise would not have occurred. These results are consistent with an observation by the Roper lab who found that the O antigen on LPS seems to mask it from perception by the plant, and thus the plant does not actively defend itself against *Xf* and that *Xf* is highly susceptible to the hydrogen peroxide and other defense chemicals produced by grape (2).

While inoculation of grape 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 - and population sizes of the bacteria exceeding  $10^6$  cells/g readily are achieved by topical application.

Remarkably, biological control of Pierce's disease can be achieved in the greenhouse by inoculation with *Paraburkholderia* at various times relative to that of inoculation with *Xf*. Surprisingly, disease control is poorer when *Paraburkholderia* was inoculated into the plants 3 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 pathogen. The highest levels of control were often 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 suggested that while *Paraburkholderia* can grow and spread rapidly in grape stems within 4 weeks after inoculation, its population sizes then subsequently decrease, and often become undetectably low by 8 to 10 weeks after inoculation. It is likely that a disease resistance reaction that is induced by *Paraburkholderia* causes it to succumb to the plant defenses. This current study is designed to provide a better understanding of the population dynamics of the biological control agent in grape under different conditions to better devise methods 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 strong evidence of its efficacy in controlling Pierce's disease revealed that the highest level of disease control was observed when plants were either co-inoculated with *Paraburkholderia* and *Xf*, or when *Paraburkholderia* was inoculated 3 weeks after that of the pathogen. The disease severity was 3 to 5- fold less 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 10-fold on plants co-inoculated at the same site with both the pathogen and *Paraburkholderia*.

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 grape after inoculation, thus exposing the plant to features of this bacterium such as lipopolysaccharide (LPS) and other so-called Microbe-Associated Molecular Patterns (MAMPs) that might be perceived as a potential pathogen. This study therefore is designed 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 the persistence of viable cells is necessary for it to suppress disease. This is being addressed in objectives #2 and #4. To effectively deploy *Paraburkholderia* in agricultural settings it is also important to know the spatial and temporal pattern of induced host disease resistance that occurs in grape. 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. 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 since in some plants, SAR/ISR is a transient event. The reduced efficacy of *Paraburkholderia* in conferring disease control when inoculated well before that of the pathogen suggests that systemic resistance that was induced may be transient, and thus ineffective if the pathogen is inoculated long after *Paraburkholderia*. These questions are being 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*. While we have demonstrated the apparent eradication of the pathogen 3 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 numbers 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 are being studied in objective #1. The topical application of *Paraburkholderia* with a penetrating surfactant appears particularly attractive as a means to inoculate grape. 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 are being explored in an attempt to improve disease control. These issues are being 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 is 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 infection can develop before eradication is no longer possible. Studies are being conducted to address these important issues.

## OBJECTIVES

- 1) Determine the amount of time after infection of grape 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 grape 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 grape 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.

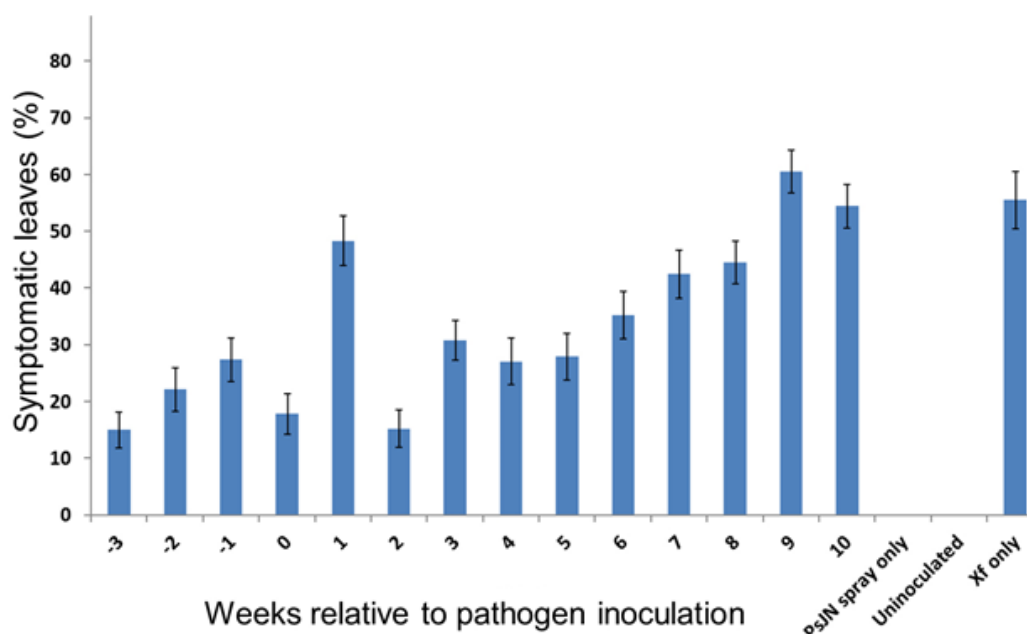
## RESULTS AND DISCUSSION

### Objective 1. Effect of inoculation time on efficacy of biological control

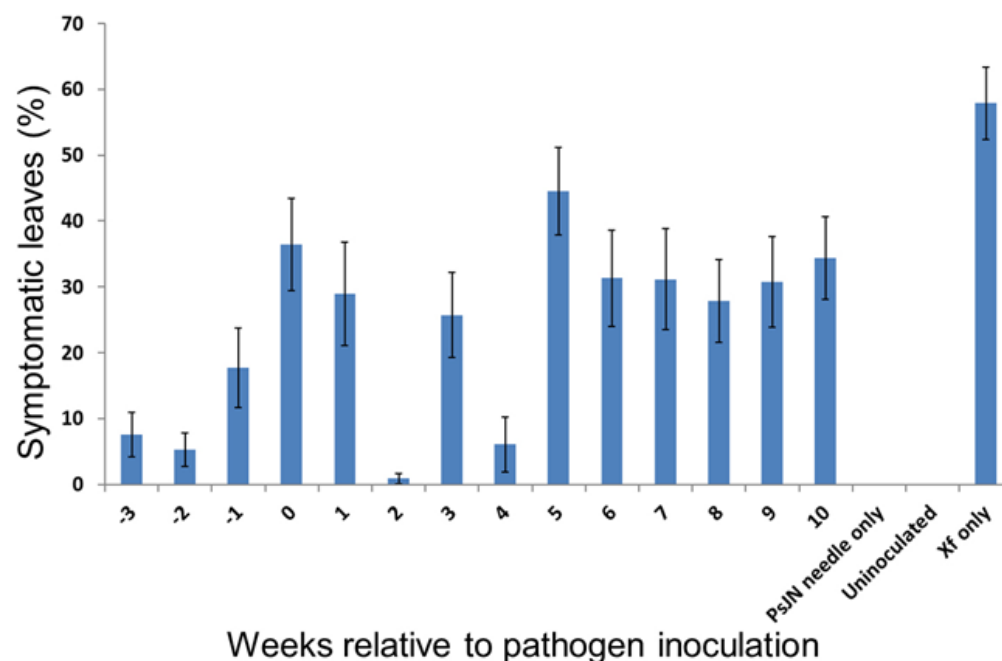
An extensive field study to address the efficacy of the application of *Paraburkholderia* at various times relative to that of the pathogen when applied either as a foliar spray with 0.2% Breakthru or by direct inoculation by needle puncture was conducted. This elaborate trial involved inoculation of several shoots on each 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 3 weeks prior to that of the pathogen, 2 weeks prior to that of the pathogen, 1 week prior to that of the pathogen, on the same day as that of the pathogen, and 1, 2,

3, 4, 5, 6, 7, 8, 9, and 10 weeks after plants were inoculated with the pathogen. Employing this experimental design, we therefore have applied *Paraburkholderia* at various times both before and up to several weeks after that of the pathogen. The goal of the study was 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 grape over a wide range of time relative to that of the pathogen - from three weeks before inoculation with the pathogen to as much as six weeks after the pathogen was inoculated into the plants (Figures 1 and 2). The efficacy of disease control decreased with increasing delay in inoculation time after inoculation with the pathogen after about six weeks. These promising preliminary results suggest that the number of applications of *P. phytofirmans* required for practical control under field conditions will be very limited and probably a single application early in the growing season – likely about 4 weeks after leaf emergence will provide season-long avoidance of disease.



**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 that of the pathogen is shown on the abscissa. 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 disease severity over 32 shoots for each timing assessed twice during the 2019 growing season. The vertical bars represent the standard error of the mean.

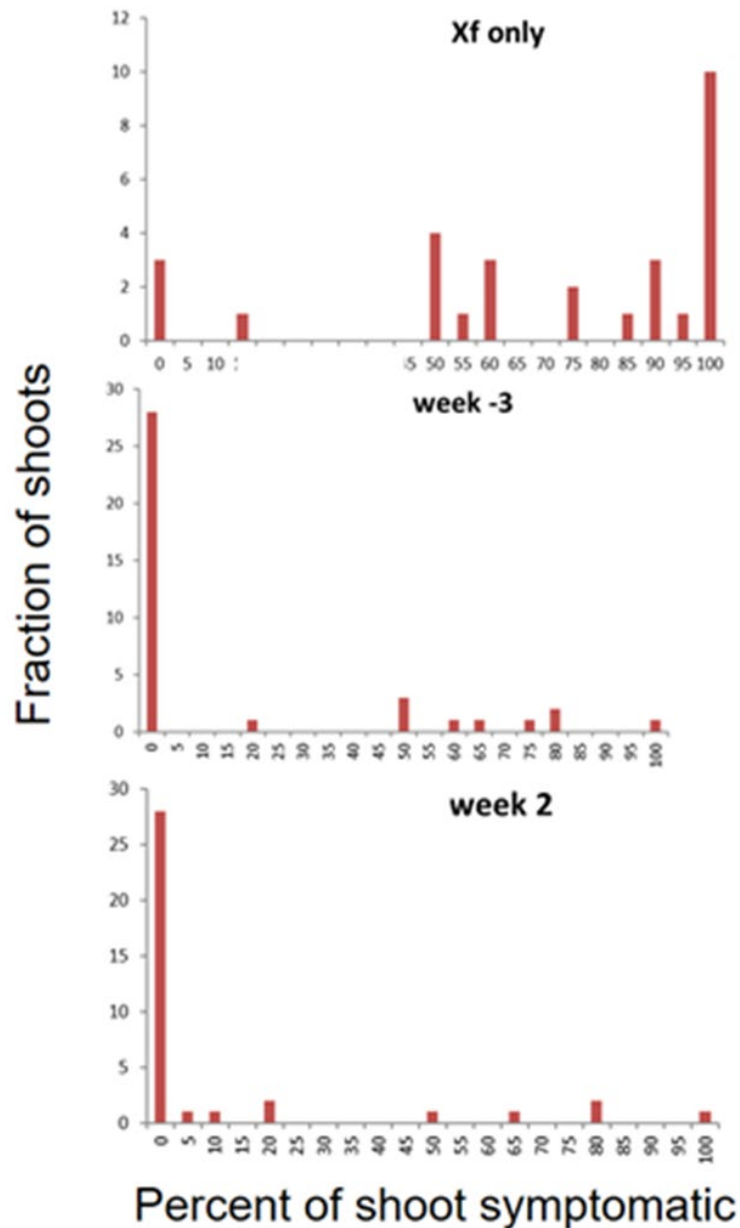


**Figure 2.** Disease severity of Chardonnay grape shown as the percentage of the leaves on a given shoot that were symptomatic on plants that were needle inoculated with *P. phytofirmans* at various times relative to that of the pathogen is shown on the abscissa. Negative values on the abscissa indicate 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 disease severity of 32 shoots for each timing assessed twice during the 2019 growing season. The vertical bars represent the standard error of the mean.

Since 30 individual shoots on the replicate plants in the field study that were treated with *Paraburkholderia* at various times before or after that of *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 (Fig. 3). In contrast, a very high proportion of the shoots that were inoculated with both *Paraburkholderia* and *Xf* at various times exhibited no evidence of disease, with a small proportion of vines exhibiting some disease. That is, inoculation of grape 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 eradicated 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. The likelihood that inoculation with *Xf* leads to infection was therefore reduced 4-fold or more - an outcome very distinct from, and much more practically important, than simply reducing the level

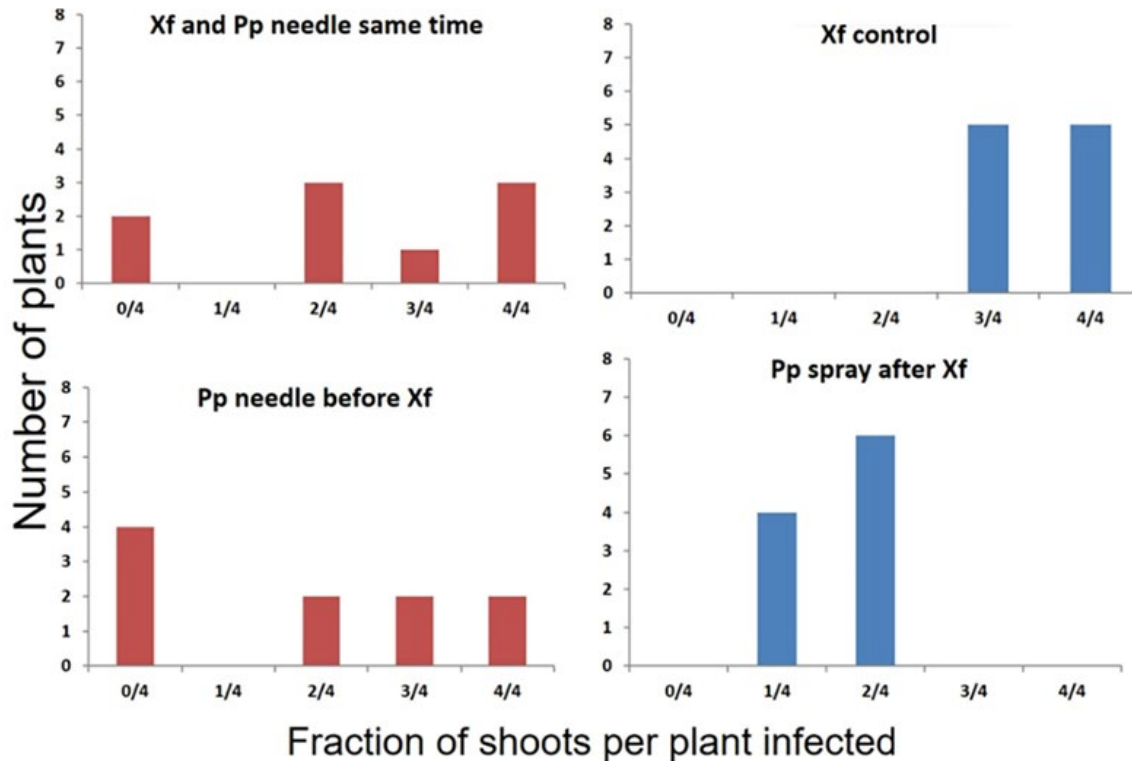


of symptoms that would have occurred in plants that would have become infected. It is very noteworthy that infection can be so dramatically reduced in these plants in the field despite the fact that they were inoculated with very high levels of the pathogen ( $>10^7$  cells/ inoculation site). We presume that viable cells of *Xf* were eliminated in those vines in which disease symptoms could not occur since symptoms never developed, even after prolonged observation throughout the summer.



**Figure 3.** Frequency histogram of the distribution of disease severity observed in 30 individual shoots inoculated only with *X. fastidiosa* (top panel), or inoculated with *Paraburkholderia* 3 weeks before (middle panel), or 2 weeks after (bottom panel) with *X. fastidiosa*.

The disease control results above suggests strongly that a given shoot in which both *P. phytofirmans* and *X. fastidiosa* are inoculated have a greatly reduced probability of expressing any symptoms (an all or none response to the presence of the *P. phytofirmans* that appears to act as a priming agent to induce plant disease resistance). However, since there are many shoots on an individual mature grape plant in the field, the question remained as to whether the priming of disease resistance was a systemic one that would spread to all of the shoots on a given plant. Since we had inoculated 4 individual shoots on a given grape plant in our field studies in 2018 and 2019 (one shoot on each cordon arm) we investigated the frequency of disease occurrence among the 4 shoots that had been inoculated with *X. fastidiosa*. If induced disease resistance had occurred throughout the whole plant due to disease resistance priming induced by *Paraburkholderia* in even one shoot, we would expect that all of the shoots inoculated with the pathogen on a given plant would show no disease, while many or all of the shoots on those on plants for which priming had not occurred would exhibit disease. We therefore developed histograms which showed the frequency with which the four shoots on a given plant that had been inoculated with the pathogen exhibited disease symptoms when considered over the ten plants receiving a given treatment of *Paraburkholderia*. It was apparent that on control plants that had been inoculated only with the pathogen that either all four of the shoots that had been inoculated became symptomatic or that three of the four shoots that had been inoculated became symptomatic (Figure 4). Only occasionally, were some shoots inoculated only with the pathogen not successfully infected using the droplet puncture method; in most cases the vines became infected and showed high levels of disease. In contrast, seldom did all four of the shoots inoculated with both the pathogen and *P. phytofirmans* at a given time exhibit disease symptoms among the 10 plants (Figure 4), consistent with the fact that many fewer shoots had become infected on plants inoculated with the biological control agent. However, on only relatively few plants were all of the shoots on plants in which shoots had been inoculated with both *P. phytofirmans* and the pathogen free of disease; often only one or two of the four inoculated shoots became symptomatic. Thus, while the likelihood that a given shoot that had been inoculated with the pathogen on plants that were also inoculated with *P. phytofirmans* was dramatically lower, the protection conferred by *P. phytofirmans* inoculation was not felt by all of the shoots on a given plant. Had apparent priming of disease resistance by *P. phytofirmans* been fully systemic throughout the plant, we would have expected many more plants to have been completely free of disease (none of the 4 shoots symptomatic). It thus appears that any systemic signal associated with priming of disease resistance occur systemically only within a given shoot, and there is apparently a limitation of the movement of such a systemic resistance signal between the shoots on these large woody plants. The architecture of vines, in which shoots emerge considerable distance from each another along a cordon may contribute to a blockage of full system movement of defensive chemicals in grape. Such a result suggests that it may be necessary to inoculate each of the shoots emerging from cordons to ensure induction of a systemic disease resistance. Spray application of *P. phytofirmans* may therefore be an optimum strategy of inoculation since it is easy to inoculate all of the emerging shoots with such a spray. Alternatively, inoculation of this biological control agent into the trunk of the plant may yield a systemic signal that would spread throughout the plant. These inoculation strategies are being examined further.

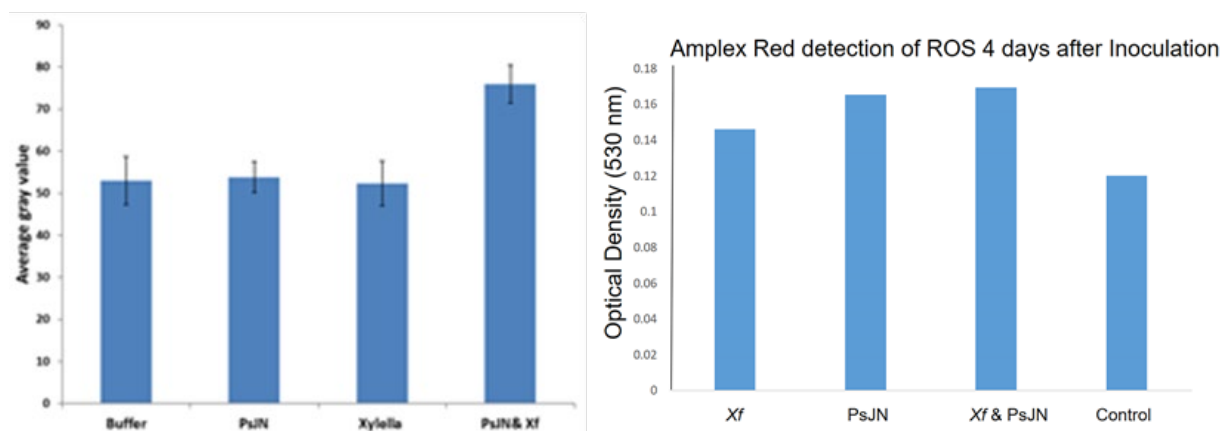


**Figure 4.** Frequency at which different numbers of the 4 shoots that had been inoculated with *X. fastidiosa* on a given plant exhibited disease symptoms when inoculated either with the pathogen alone or with *Paraburkholderia phytofirmans* at different times either before or after that of the pathogen.

### Objectives 2 and 3. Persistence and temporal dynamics of *P. phytofirmans* and induced reactive oxygen species

An extensive study of the population size of both *P. phytofirmans* and *Xf* at various locations within greenhouse Cabernet Sauvignon plants relative the point of inoculation with *P. phytofirmans*, in different ways and at different times relative to the inoculation with the pathogen that have been co-inoculated with these two species is underway and will be complete by February, 2022. A goal is to determine how long *P. phytofirmans* can maintain high population sizes within grape. Simultaneously, we are measuring the concentration of reactive oxygen species such as hydrogen peroxide at these same locations over time to ascertain whether this induced chemical stress is responsible for the reductions in pathogen viability seen in plants undergoing induced resistance. We have assessed two methods to quantify  $H_2O_2$  in the stems of grape. The method that worked the best for assessing stem concentrations of reactive oxygen species involves pressing cut vines onto nitrocellulose impregnated with 5 mg/mg 3,3'-diaminobenzidine HCL (DAB). 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 *X. fastidiosa* alone using this method (Figure 5). Importantly, a higher concentration of hydrogen peroxide was observed in petioles of plants that had been inoculated with both *Paraburkholderia* and *X. fastidiosa* (Figure 5). Estimates of relative ROS

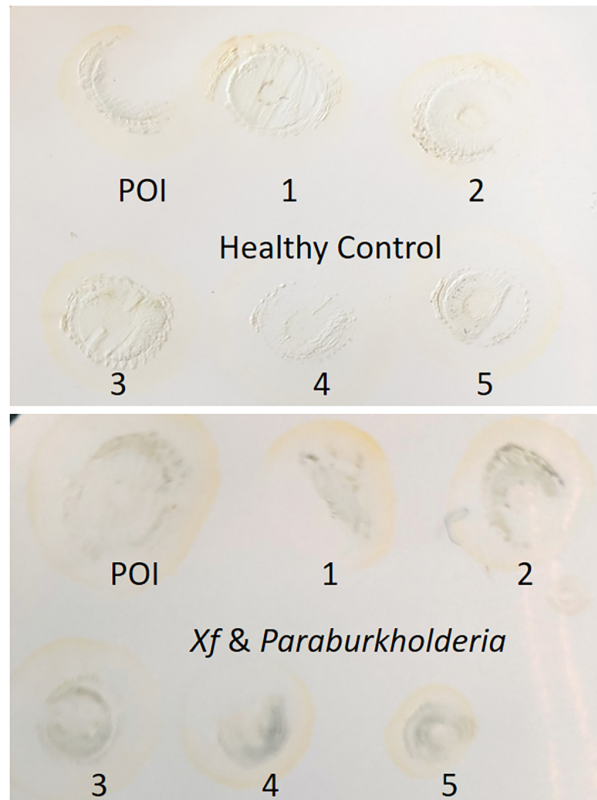
abundance by DAB printing were similar to more quantitative measures using an Amplex Red method (Figure 5) and allowed easy assessment of more replicate samples at different locations in the plant.



**Figure 5.** (Left panel). Estimations of reactive oxygen species in plants inoculated with buffer alone, *Paraburkholderia* alone, *X. fastidiosa* alone, and with both *Paraburkholderia* and *X. fastidiosa*. Stems were pressed onto DAB filters for 10 seconds and H<sub>2</sub>O<sub>2</sub> concentrations were determined after 30 minute incubation by quantifying the grey scale (combined RGB pixel intensity) intensity of the spots compared to background greyscale intensity of adjacent un-printed areas of the filter. The vertical bars represent the standard error. (Right panel) Estimates of relative ROS present in macerates of grape stems inoculated with the various bacteria estimated using Amplex Red. Shown is the Optical Density of buffer extracts assessed at a wavelength of 530 nm).

A focus of our current studies evaluating this method have addressed 1) the extent to which any H<sub>2</sub>O<sub>2</sub> that is produced by grape inoculated with both *Paraburkholderia* and *X. fastidiosa* extends beyond the immediate area to which both bacteria have been inoculated, 2) How soon after inoculation is H<sub>2</sub>O<sub>2</sub> detectable in plants, 3) Is the presence of H<sub>2</sub>O<sub>2</sub> in plants persistent after it has been induced in plants, and 4) to what extent is the amount of H<sub>2</sub>O<sub>2</sub> produced in plants inoculated with both *Paraburkholderia* and *X. fastidiosa* both greater or more persistent than that that may be induced by inoculation only with either *X. fastidiosa* or *P. phytofirmans*. These studies have involved droplet puncture inoculation of greenhouse grown Cabernet Sauvignon plants with individual bacteria or bacterial mixes followed by assessing ROS in stem cross sections sampled at various times and distances from the point of inoculation by pressing cut stems onto DAB-impregnated nitrocellulose filters. In this process, it became clear that H<sub>2</sub>O<sub>2</sub> is not uniformly distributed in vines inoculated with bacteria, particularly those inoculated with both *Xf* and *P. phytofirmans* (Fig. 6). While little or no brown pigmentation was produced in prints of healthy plants inoculated only with water when sampled 4 days after inoculation (Fig. 6 top panel), areas of brown pigment were apparent in all stem segments collected at various distances from the point of inoculation of plants in which *Xf* and *P. phytofirmans* were separately

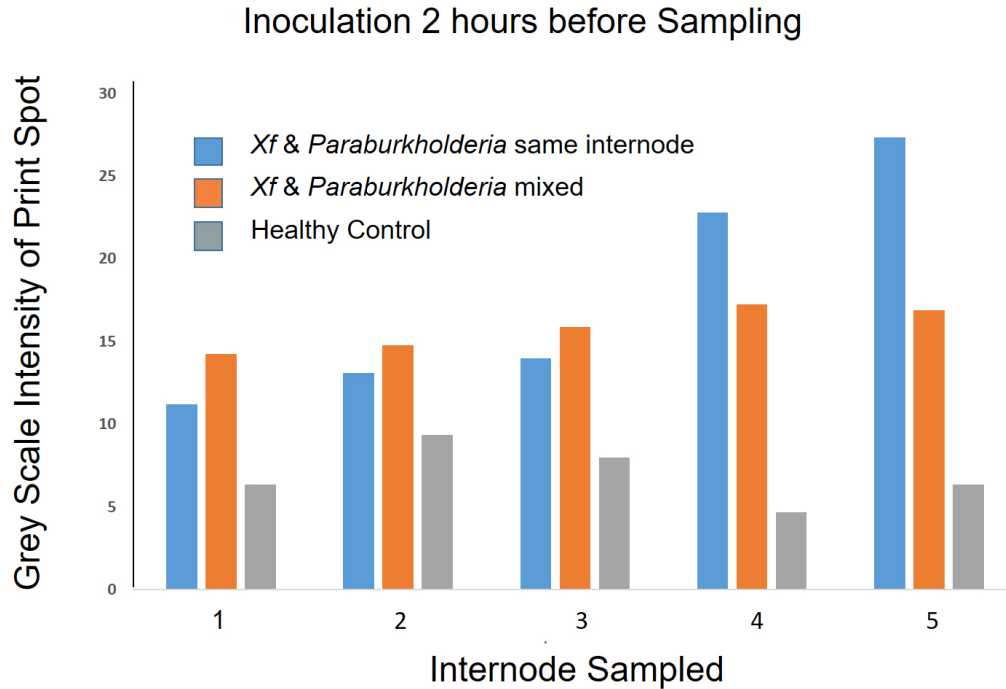
inoculated into separate but nearby locations at the base of the plant (Fig. 6 bottom panel). These brown areas, indicative of the presence of  $H_2O_2$  were found in only a small portion of the stem cross section (Fig. 6). Given that the stem is comprised of many xylem vessels and the  $H_2O_2$  is apparently associated with only a small subset of the xylem vessels and perhaps the associated xylem parenchyma tissues, the concentration in a stem segment as a whole is low because a large proportion of the vessels are not colonized by bacteria, and thus apparently do not accumulate  $H_2O_2$ .



**Figure 6.** Images of stem segments of healthy control plants (top panel) and plants in which *X. fastidiosa* and *P. phytofirmans* were separately inoculated into separate but nearby locations at the base of the plant that were printed 4 days after inoculation onto DAB-impregnated nitrocellulose filters. Stem sections were collected at the Point of Inoculation (POI) as well as at the number of internodes above the point of POI as indicated.

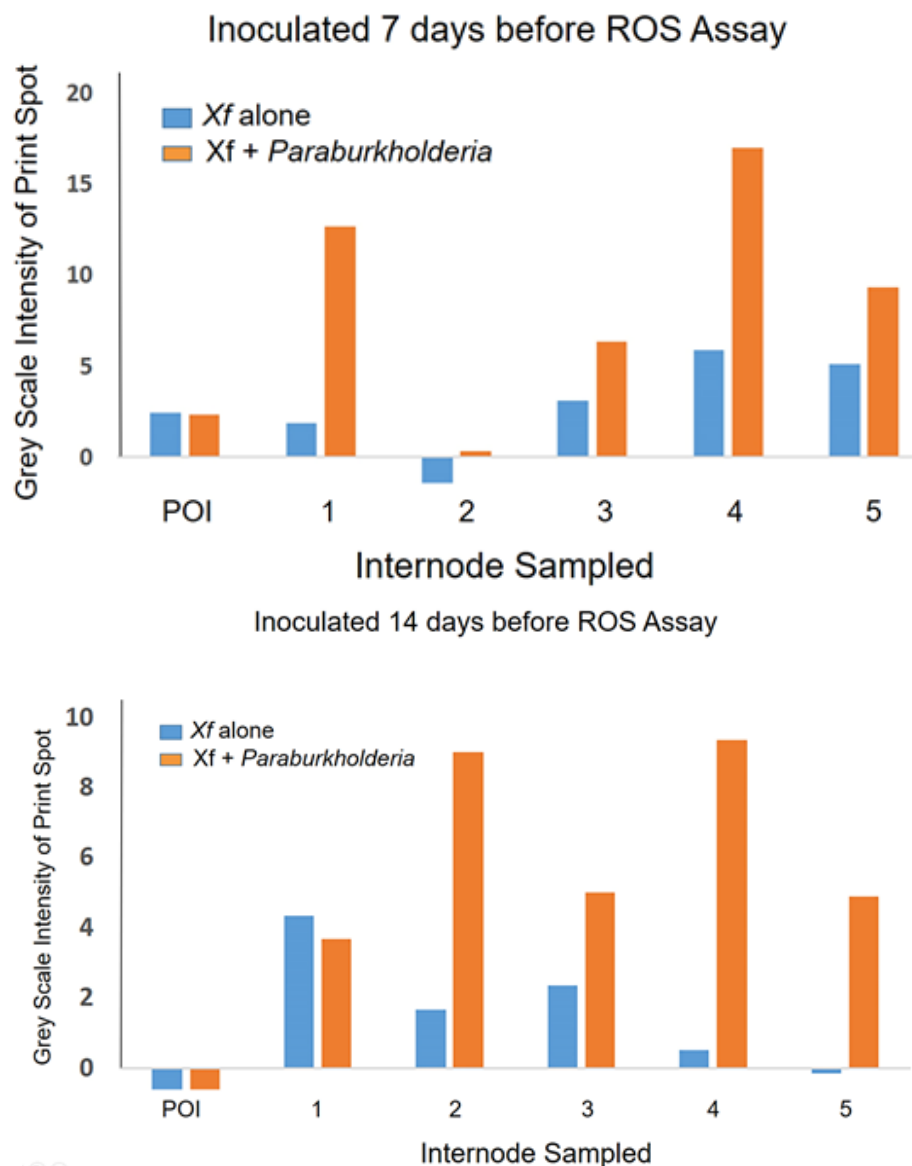
The speed at which ROS occur in plants after inoculation as well as its persistence and distribution after inoculation was assessed by sampling stem segments at different distances from the point of inoculation of in plants inoculated either with the pathogen alone or in plants in which *Xf* and *P. phytofirmans* were separately inoculated into separate but nearby locations at the base of the plant, at different times after inoculation. As expected, there was little detectable  $H_2O_2$  in any stem segments of healthy control plant irrespective of the distance from the point of

inoculation, as evidenced by little or no brown coloration of spotted areas on DAB filters (Fig. 7). Substantially higher levels of  $H_2O_2$  were detected in plants inoculated with both *Xf* and *P. phytofirmans*, irrespective of whether they were inoculated into the same localized site on stems or were inoculated in closely adjacent, but separate locations in the stem (Fig. 7). Surprisingly,  $H_2O_2$  was elevated throughout the stem, up to 5 internodes (ca. 50 cm) distal to the point of inoculation by 2 hours after inoculation. We are surprised by the rapid and apparent systemic occurrence of ROS in co-inoculated plants.



**Figure 7.** Quantification of relative  $H_2O_2$  concentrations in stem segments located at the number of internodes distal to the Point of Inoculation (shown on the abscissa) of Cabernet Sauvignon grape inoculated with *X. fastidiosa* and *P. phytofirmans* separately into separate but nearby locations at the base of the plant (blue bars), inoculated as a mixture into the same sites (orange bars) or inoculated with water (grey bars) when sampled 2 hours after inoculation. Shown is the differential average grey scale values determined from the entire area on which a stem segment was printed onto a DAB-impregnated nitrocellulose filter corrected for that of the average grey scale value of an adjacent area of the filter that was not printed.





**Figure 8.** Quantification of relative  $\text{H}_2\text{O}_2$  concentrations in stem segments located at the number of internodes distal to the Point of Inoculation (POI) (shown on the abscissa) of Cabernet Sauvignon grape inoculated with *X. fastidiosa* alone (blue bars) or *X. fastidiosa* and *P. phytofirmans* separately into separate but nearby locations at the base of the plant (orange bars), when sampled 7 days after inoculation (top panel) or 14 days after inoculation (bottom panel). Shown is the differential average grey scale values determined from the entire area on which a stem segment was printed onto a DAB-impregnated nitrocellulose filter corrected for that of the average grey scale value of an adjacent area of the filter that was not printed.

Additional studies were conducted to assess the presence of ROS in vines at various times after inoculation with either *Xf* alone or on plants in which *Xf* and *P. phytofirmans* were separately inoculated into separate but nearby locations at the base of the plant to determine how persistent the presence of H<sub>2</sub>O<sub>2</sub> was in plants and whether it was found preferentially in various places relative to the point of initial inoculation. While there was some variability in ROS at various locations in the plant, probably due to differences in the spatial location and colonization of the plant by the various bacteria, there was almost always a much higher level of H<sub>2</sub>O<sub>2</sub> detected in plants at all locations inoculated with both *Xf* and *P. phytofirmans* than in plants inoculated only with water or with the pathogen alone (Fig. 8). While relatively high levels of H<sub>2</sub>O<sub>2</sub> were found at all sampling site up to 5 internodes distal to the point of inoculation, very low amounts of H<sub>2</sub>O<sub>2</sub> were seen in most sample sites in plants inoculated with *Xf* alone, and the highest levels were generally observed only relatively close to the point of inoculation (Fig. 8). These observations are consistent with reports in the literature from the group of Caroline Roper, that there is generally a weak and delayed transcriptional response of grape to the presence of *Xf*, and little evidence of induced expression of genes conferring resistance such as those enabling the production of ROS, explaining why this pathogen is successful in colonizing grape. The widespread presence of ROS in plants inoculated with both *Xf* and *P. phytofirmans* supports our model that *P. phytofirmans* primes the plant for resistance to *Xf*, and the apparent persistent presence of ROS throughout the plant, while surprising, could explain the eventual decrease in populations of this beneficial bacterium with time after building to initially high numbers.

#### **Objective 4. Temporal and spatial patterns of altered grape gene expression in plants inoculated with *Paraburkholderia* and/or the pathogen**

Because of the substantial impact of the COVID-19 pandemic on hiring a scientist to pursue this objective as well as the impacts on laboratory activities at UC Berkeley early in the project, to make most rapid progress on this sub-objective, a subcontract for the project was established with approval of the CDFA Pierces Disease and Glassy-winged Sharpshooter Board with Professor Caroline Roper of the University of California, Riverside. Professor Roper has had extensive experience in measuring gene expression in grapes infected with *Xf*. The *Paraburkholderia* strain has been provided to her and plants have been inoculated with either it, *Xf* or both strains or a buffer only negative control. Professor Roper has harvested and flash frozen the petioles from all of the treatments. RNA has been extracted from the frozen petioles and the RNASeq libraries have been constructed. The libraries are now being quality checked and then will be sent for sequencing at the UC Davis Genomics Core facility. Once we receive the sequences back, Dr. Roper's group will perform the RNASeq to determine differential expression of genes in these treated plants, with special attention to those genes involved in induced resistance to plant pathogens.

#### **CONCLUSIONS**

Field trials reveal a very high degree of efficacy of *P. phytofirmans* for the control of Pierce's Disease when inoculated in 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 a 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.

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#### FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierces 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**

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**Reporting Period:** The results reported here are from work conducted November 2020 to November 2021.

**ABSTRACT**

Although virulence in different host plants has been evaluated for *Xylella fastidiosa* strains belonging to different subspecies, within subspecies variation has not been studied on any significant scale. This project assessed virulence differences between a large number of *X. fastidiosa* isolates causing Pierce's Disease (PD) of grapevine in California both in field and greenhouse-grown grapevines and in the model plant tobacco. Inoculation of more than 60 strains of *X. fastidiosa* in tobacco plants in the greenhouse have shown that PD strains differ in their virulence observed in this model system. In grapevine, some variation in virulence was observed in greenhouse grown grapevines as well as in grapevines inoculated in the field. However, in field inoculated plants there was also significant variation within cultivar treatments suggesting that plant and environmental factors are as important as isolate. Eight *X. fastidiosa* isolates were also tested in three commonly used sources of plant resistance to PD. No significant difference was found in resistance to the range of pathogen isolates indicating that if failures in resistance occur it is unlikely to be due to differences between isolates found in California.

**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 *Xf* if considered at the species level, often rely on multiple virulence and growth factors to colonize diverse host plants. Though *Xf* was the first plant pathogenic bacterium to have its full genome sequenced, (7,14,15) only a small number of studies have looked at virulence variation between bacterial strains in the same host plant variety (3,4,5,9,12,13). 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 between *X. fastidiosa* strains found in CA. Further evaluation of virulence differences in *Xf* strains from CA is needed to understand the relationship between genetics and virulence. In addition, virulence comparisons among *Xf* strains are useful to understand the biology of this pathogen. In *Nicotiana tabacum* (tobacco), different subspecies of *Xf* are capable of colonizing and causing leaf scorch symptoms (1,10), and show differences in host colonization and symptomatology (6, 13). Tobacco has been used as a model system to understand changes in host mineral and nutrient composition caused by *Xf* infection (13), bacterial gene function (2,11), and impact of new DNA acquired from natural competence and recombination (8). Using tobacco as a model system is beneficial for evaluating the relative virulence of diverse strains as well as differential host responses, as it can be accomplished with less greenhouse space and in about half the time compared with grapevine experiments. However, it is important to fully understand how well results obtained in tobacco will transfer to grapevine, in particular when considering larger vines grown under field conditions.

PD resistance has been identified in multiple *Vitis* species. How these sources differ in durability (sustainability of resistance when exposed to multiple pathogen strains) of resistance is unclear. A single source of resistance *PDR1* from *V. arizonica*, a wild southwestern grape, has been used to develop high quality wine grapes with PD resistance (breeding efforts by Andy Walker, UC Davis). Table grape 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 multiple *X. fastidiosa* isolates is unclear.

## OBJECTIVES

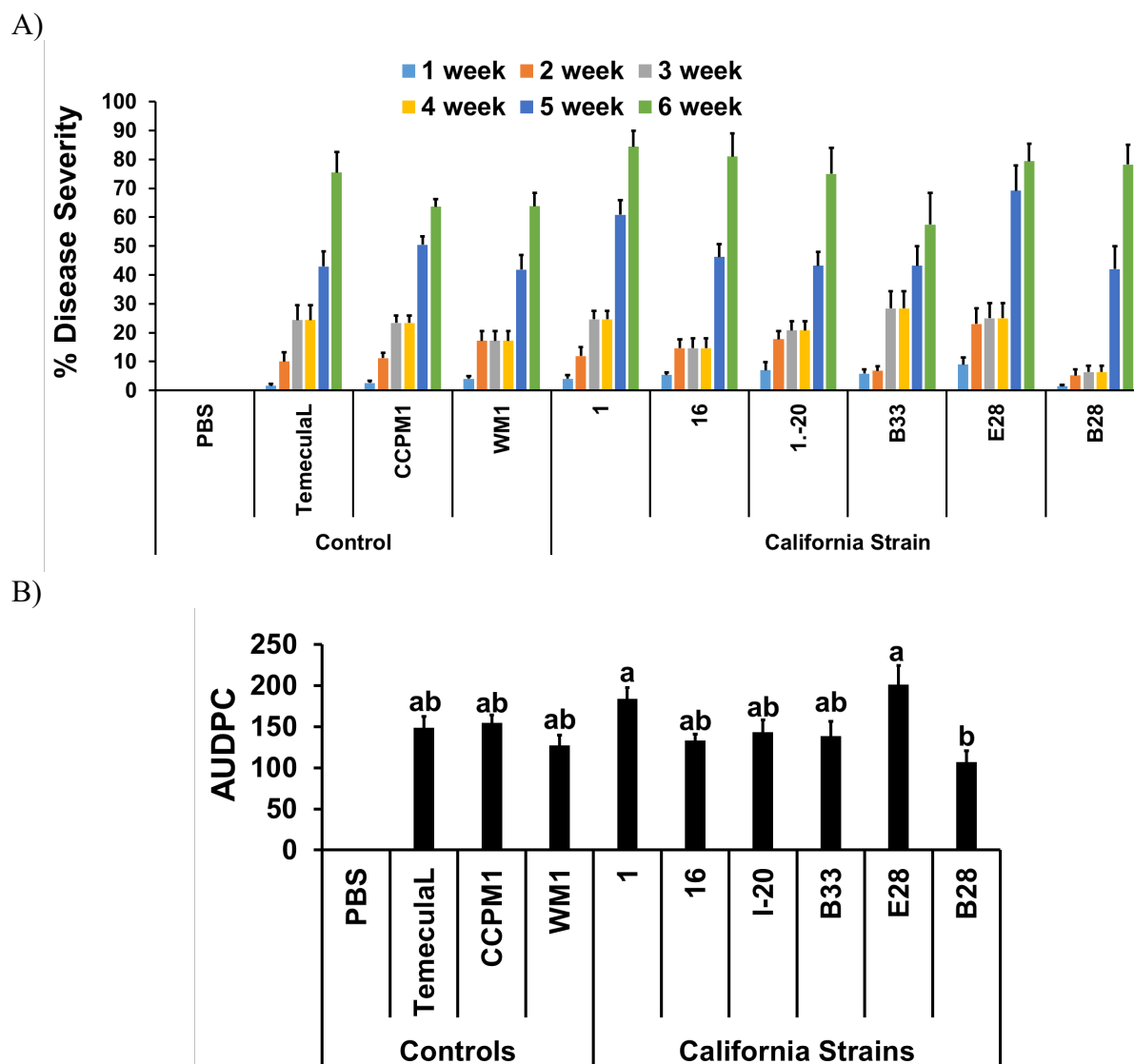
- 1. Evaluate the virulence diversity of *Xylella fastidiosa* strains from California**  
*Sub-objective a: Evaluate *Xf* isolate diversity in tobacco*  
*Sub-objective b: Evaluate *Xf* isolate diversity in grape*
- 2. Evaluate known grape sources of PD resistance against diverse strains of *Xylella fastidiosa*.**

## RESULTS AND DISCUSSION

**Objective 1: Evaluate the virulence diversity of *Xylella fastidiosa* strains from California**

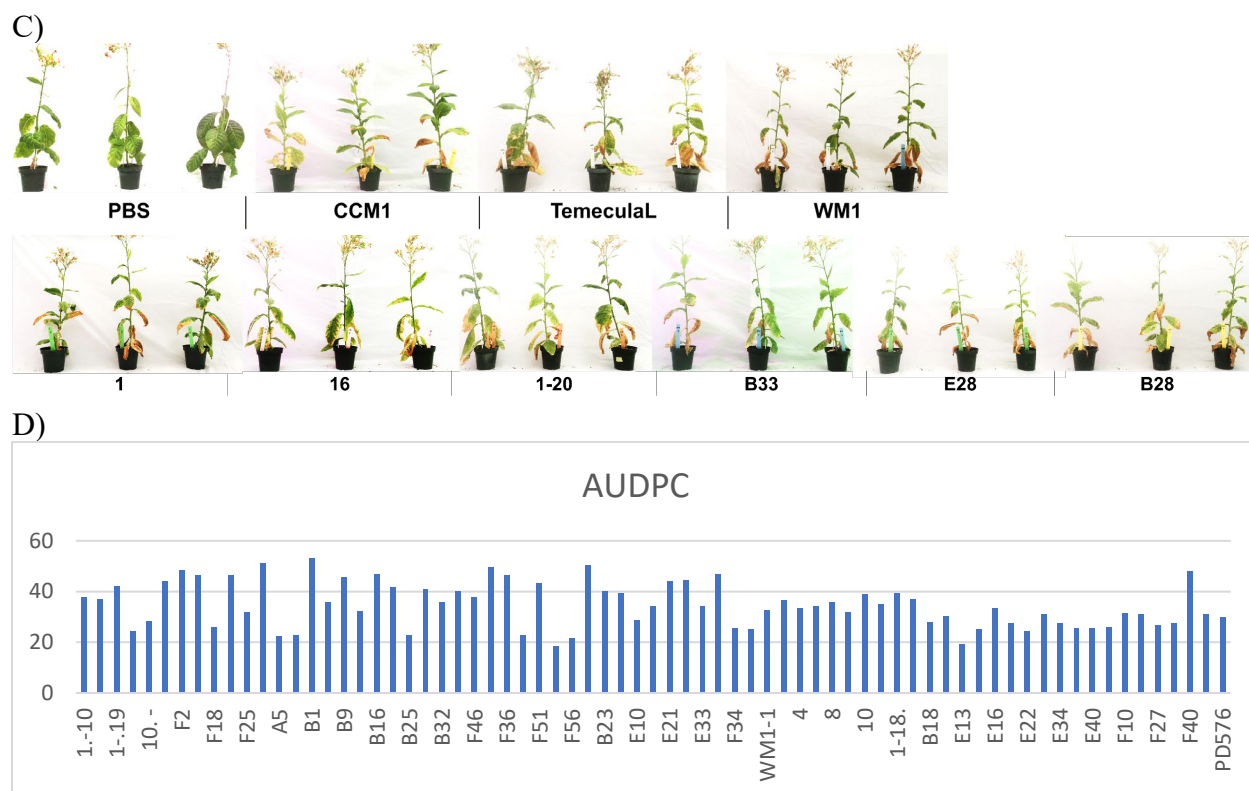
**Sub-objective a: Evaluate *Xf* isolate diversity in tobacco:** The virulence of additional six *X. fastidiosa* strains isolated from grapes in California (Castillo et al, 2021) was assessed in the tobacco plant model. The data presented here will be combined with our previous results that include more than 60 strains tested in total. Disease severity assessment based on AUDPC (Figure. 1) revealed that California strains *X. fastidiosa* 1 and E28 had the highest virulence ( $p < 0.001$ ); and strain B28 showed significantly less virulence ( $p < 0.001$ ) compared to other strains

used here. All California strains were compared to known control strains such as *X. fastidiosa* strains TemeculaL, CCPM1 and WM1-1 (the last two isolates are from grapes in Georgia), that did not show significant differences in virulence with the California strains according to Tukey-Kramer's HSD ( $P > 0.05$ ).



**Figure 1 A and B.** Virulence assessment of *X. fastidiosa* California strains isolated from grapes in tobacco plants. (A) Percent leaf scorch disease severity in tobacco plants infected with *X. fastidiosa* strains from California. Bars in graph represents mean and standard error of the means ( $n=10$ ). Data represented in the graph was collected from one experiment. (B) AUDPC of *X. fastidiosa* strains from California. AUDPC values were calculated based on leaf scorch disease severity per isolate during six weeks of the evaluation. Significance of the *X. fastidiosa* strain virulence in tobacco was compared with known control strains according to Tukey's HSD at  $P \leq 0.05$ .



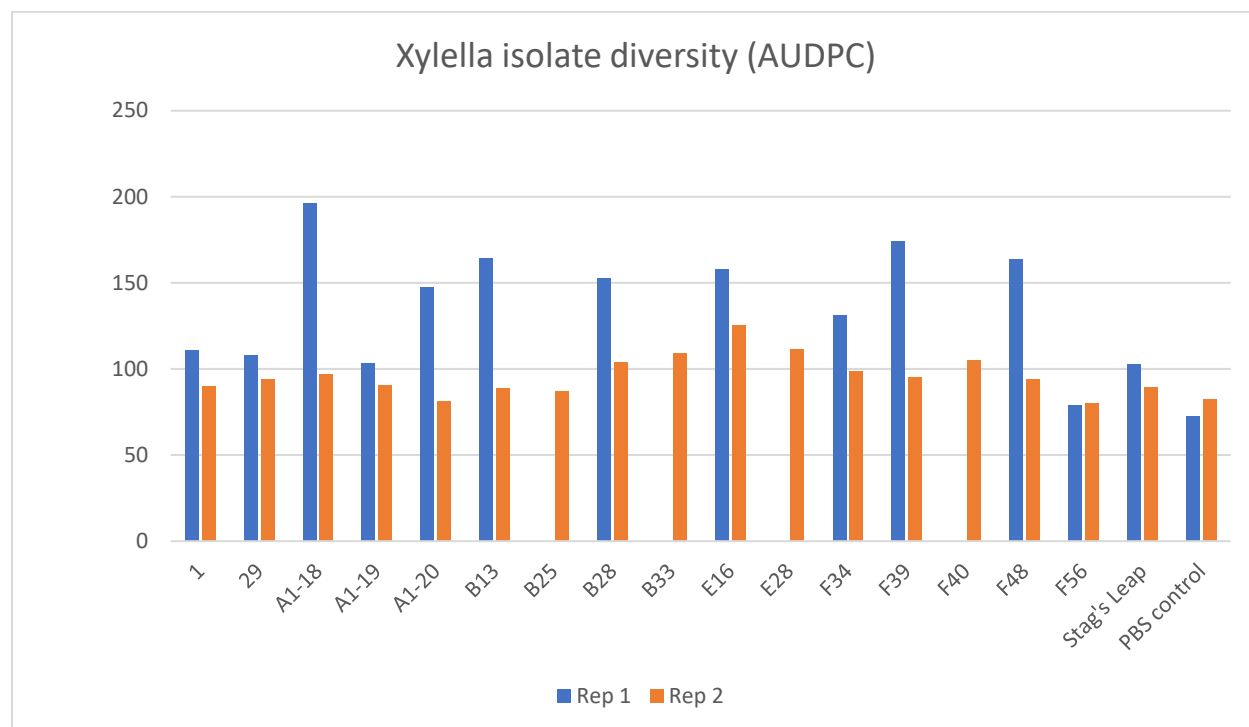


**Figure 1 C and D.** Virulence assessment of *X. fastidiosa* California strains isolated from grapes in tobacco plants. (C) Representative images (fifth-week disease severity rating) of tobacco plants infected with *X. fastidiosa* strains showing leaf scorch symptoms. D) AUDPC values of all isolates previously screened in tobacco.

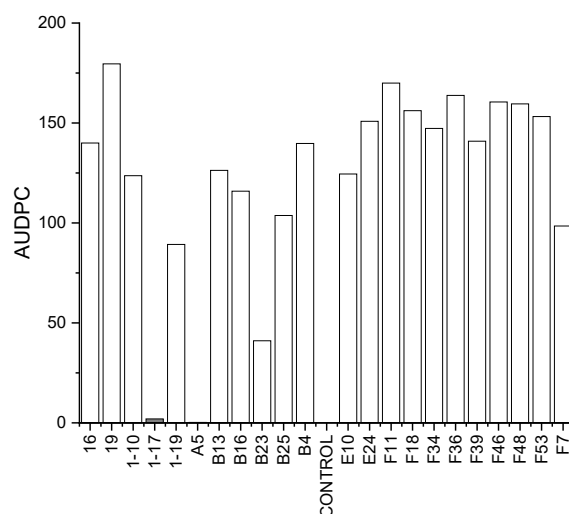
**Sub-objective b: Evaluate *Xf* isolate virulence diversity in grape:** Isolate virulence diversity (differences in disease severity caused among *Xf* isolates) was evaluated using potted Cabernet Sauvignon vines in the screenhouse. Eighteen isolates of *Xf* were evaluated for disease severity for approximately 8 weeks. Variability was seen among isolates with some isolates causing almost 30% more disease than others (Figure 2).

Isolate virulence diversity was also assessed in grapevines in the field. In 2020, approximately 400 plants were inoculated with 22 different *X. fastidiosa* isolates from California. Twenty plants were inoculated for each isolate and mock-inoculated plants were included as negative controls. Some of the isolates evaluated in the screenhouse were included to facilitate comparison between potted and field-grown vines. Plants were inoculated in June of 2020 and evaluated in October for presence of PD symptoms. Petiole samples were also collected from all vines in October 2020 to test for pathogen presence using qPCR. Plants remained in the field and were evaluated for occurrence and progression of PD symptoms the following year (July-October 2021). Petiole samples were collected again at the end of the second season. Majority of inoculated plants showed some degree of PD symptoms, and there was a high rate of severe disease for most *X. fastidiosa* isolates although a small number of isolates had significantly less disease (Figure 3.) For isolates 1-17 and A5, no disease symptoms were observed in 2021, however plants

inoculated with these strains also did not have significant disease symptoms the previous year suggesting a failure of the initial infection rather than loss of established infection. An additional 400 Cabernet Sauvignon vines were planted in spring of 2020, inoculated in June 2021 with the same isolates and are being evaluated for disease development in 2021-2022.



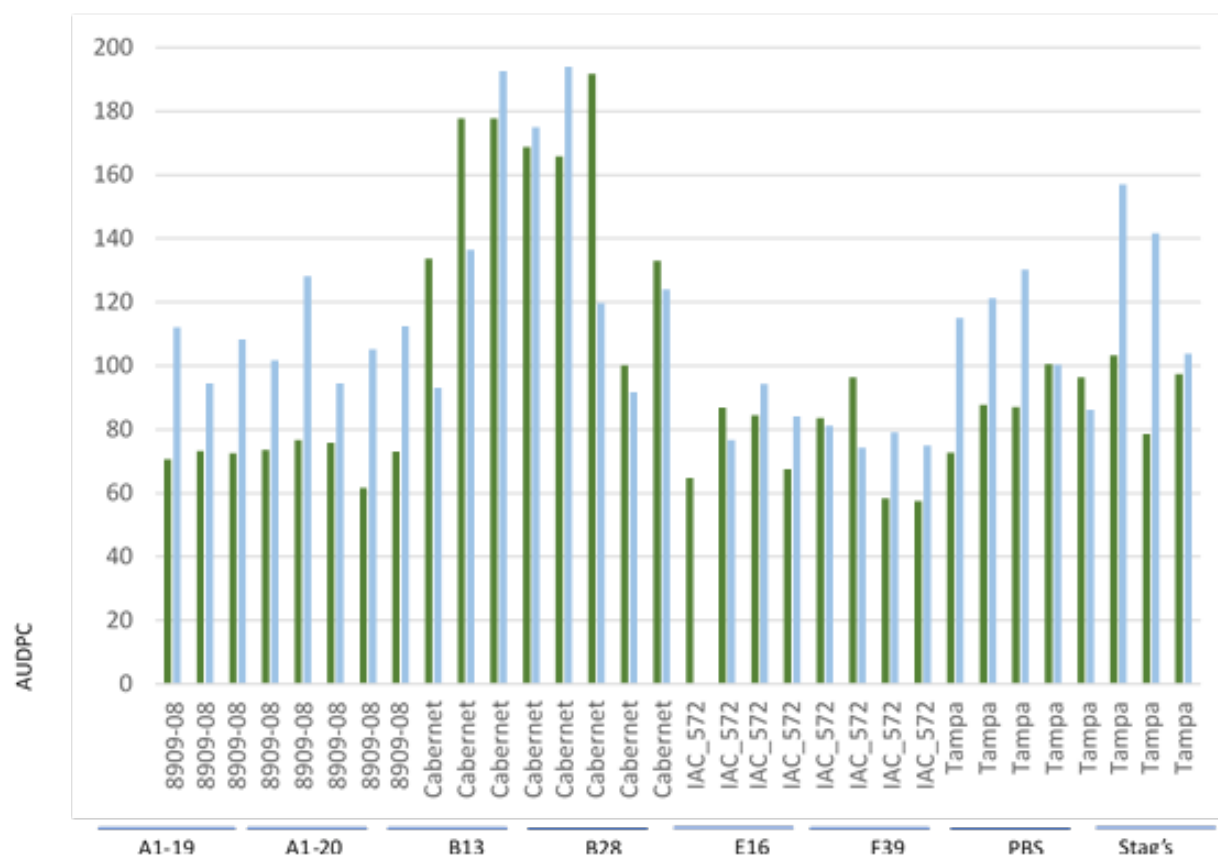
**Figure 2.** *Xylella fastidiosa* isolate diversity evaluated on Cabernet Sauvignon in the screenhouse on potted plants.



**Figure 3.** *Xylella fastidiosa* isolate diversity evaluated on Cabernet Sauvignon in the field, second year after inoculation. Area under the disease progress curve (AUDPC) is shown for each isolate based on 12 weeks of PD symptom ratings (n=20 inoculated plants per isolate).

**Objective 2: Evaluate known grape sources of PD resistance against diverse strains of *X. fastidiosa*.**

Three sources of resistance and a susceptible positive control (Cabernet Sauvignon) were evaluated for disease severity when inoculated with one of 7 isolates of *Xylella fastidiosa* or a PBS (negative control) in the greenhouse. Plants were evaluated based on disease ratings. All of the sources of resistance evaluated had some level of resistance to the isolates evaluated. Greater variability was observed among the isolates than among sources of resistance. Sources of resistance 8909 and IAC572 had lower disease than Tampa, consistent with previous data showing that Tampa is tolerant, but not resistant to *Xf*. All three sources of resistance showed low variability in disease response to each of the seven isolates of *Xf* and similar levels of leaf browning/death to those plants inoculated with PBS.



**Figure 4.** *Xylella* disease severity on three sources of resistance and the susceptible control (Cabernet Sauvignon) when inoculated with one of seven isolates of *Xf* or a negative control (PBS)

## CONCLUSIONS

Grapevine isolates of *X. fastidiosa* from California differ in virulence both in tobacco and in grapevine. Virulence is not necessarily correlated with geographic region of isolation, as more and less-virulent isolates were obtained from the same region. Particularly in field-grown grapevines, variability in disease between plants inoculated with the same isolate can be as high as between isolates, suggesting that other plant and environmental factors are equally important in determining disease outcomes in the field. Three different sources of PD resistance all showed consistently low disease across multiple isolates of *X. fastidiosa*.

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

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**Reporting Period:** The results reported here are from work conducted October 2020 to July 2021.

### ABSTRACT

There have been several recent advancements in molecular technology such as global transcriptomics (RNA-Seq), functional genomics, and analytical biochemistry. Using these techniques in conjunction with disease phenotyping has allowed us to discover a molecule (lipopolysaccharide (LPS)) on the cell surface of *Xylella fastidiosa* (*Xf*) that appears to elicit a potent grapevine immune response. We capitalized on this robust immune response to tease apart early elicitation of basal defense responses that leads to the activation of systemic and prolonged defense pathways against *Xf* in the grapevine (Rapicavoli et al, Nature Communications, 2018). Using this immune response information, our experimental goal is to develop grapevines that can effectively defend themselves against *Xf* infection. We will generate Pierce's disease (PD)-resistant vines through both a 1) traditional transgenic approach and 2) a new CRISPR/Cas9 approach. Thus far, we have developed three transgenic types of grapevines with several lines for each gene at the UCD Plant Transformation facility. We have started to test these vines for resistance to PD.

### LAYPERSON SUMMARY

*Xylella fastidiosa* (*Xf*) is a bacterial plant pathogen and the causal agent of Pierce's disease (PD) of grapevine, a destructive threat to the wine and table grape industry. Through advanced molecular techniques, we have identified a piece of *Xf* on its cell surface that acts as a strong elicitor of the grapevine defense response. Using this piece, we have stimulated the grapevine's immune system and determined which grapevine genes are involved in protecting the vines from future encounters with *Xf*. This protection includes significantly less bacterial colonization and significantly less disease symptoms in the vines. The goal of the proposed work is to use the information about the grapevine immune response to *Xf* to generate PD-resistant vines.

### INTRODUCTION

Through advanced molecular techniques, we have identified groups of grapevine genes, that when activated, may correspond to PD resistance, since they have been shown to induce a prolonged and systemic immune response when challenged with *Xf*. We have also identified groups of genes that may correspond to PD susceptibility. Through transgenesis, we have developed *Vitis vinifera* grapevines that overexpress genes associated with PD resistance, since they are thought to be linked to an effective immune response against *Xf* (Rapicavoli et al, 2018). Conversely, we will use a CRISPR/Cas9 approach to remove genes that we have found to be related to PD susceptibility. Vines developed using CRISPR/Cas9 are not considered to be genetically modified organisms.



Thus far, we have developed three transgenic grapevines with several lines for each gene. The plants have been analyzed at the UCD Plant Transformation facility to confirm the success of the transgenesis. They have been sent to UC Riverside where they have been rooted in soil and several of the lines have been clonally propagated. Overexpression of the genes has also been confirmed with qPCR. Two lines are currently being assessed for their relative resistance levels to PD through established disease phenotyping assays, as well as bacterial colonization levels (Roper et al, 2007; Clifford et al, 2013). Once the other lines are clonally propagated, we will also determine their relative resistance levels to PD through the same means. Additionally, we will perform microscopic phenotypic assessments of the responses of these vines' vasculature to *Xf* infection and assess the numbers of tyloses and occluded vessels in the xylem of the wild type parental vines and compare these to the numbers found in the vines produced through transgenesis. Tyloses, or outgrowths of xylem parenchyma cells into the xylem, are considered to be one of the major consequences of *Xf* infection and greatly exacerbate PD symptoms (Sun et al, 2013). We hypothesize that by preventing the production or reducing the number of tyloses in *V. vinifera* will result in PD resistance.

This project will allow us to determine which genes can contribute to the generation of PD resistant vines. This project derives from a key interaction between *Xf* and the grapevine host immune system and uses the data we have collected to develop vines resistant to PD. Through a myriad of molecular techniques such as global transcriptomics (RNA-Seq), functional genomics, analytical biochemistry and disease phenotyping, we identified a *Xf* cell surface molecule (lipopolysaccharide (LPS)) that elicits a potent response from the grapevine immune system. We used derivatives of this LPS as a tool to parse apart the grapevine's immune responses to *Xf* and found that early induction of the basal defense response leads to systemic and prolonged activation of defense pathways related to *Xf* perception in grapevine (Rapicavoli et al, 2018). Additionally, we have demonstrated that elicitation of defense pathways associated with a ROS burst and a salicylic acid-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). These are exciting and compelling findings that have identified key groups of the grapevine immune system involved in the response to *Xf* infection.

We are now ready and have started to test our hypothesis that specific important parts of the grapevine immune system involved in host recognition of *Xf* can be used to develop PD resistant grapevines. We define resistance as the ability of a plant to overcome or stop infection from an invading pathogen to some degree. We will generate vines, through 1) a transgenic approach that generates grapevines that overexpress genes that we have identified as corresponding to an effective immune response to *Xf* (Rapicavoli et al, 2018) and 2) a CRISPR/Cas9 approach that will generate deletions in genes that seem to correspond to PD susceptibility, that we are and will continue to test for PD resistance (Obj.1). We will then characterize these plants by using our established disease phenotyping assays and also look at their responses to *Xf* infection at the microscopic level in the vasculature (Obj. 2). Within Obj. 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 transcriptomic level to *Xf* compared to the wild type parental grape line. Thus far, we have successfully obtained three transgenic vines, with several more constructs in the pipeline.

## 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

#### Objective 1a. Overexpression of genes associated with elicitor activation of defense against *Xf*.

We have chosen genes of interest from our recent study that found a selection of genes that were significantly up-regulated early in the infection process in LPS-mediated elicited grapevines as compared to wild type inoculated plants (Rapicavoli et al, 2018). These genes include two Class III peroxidases, which are enzymes known to accumulate in abundance in xylem sap during colonization by vascular pathogens (Yadeta and Thomma, 2013; Chakraborty et al., 2016). These enzymes have multiple functions including 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 up-regulation 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).

Several genes encoding important parts of SA-mediated signaling pathways (*Enhanced disease susceptibility 1 (EDS1)* genes) were uniquely expressed both locally and systemically with LPS-mediated elicited immunity to *Xf* and had steady expression over time. *EDS1* genes encode proteins associated with the SA pathway and are associated with defense against pathogens such as powdery mildew. In addition, overexpression of *EDS1* in *Arabidopsis thaliana* has been shown to confer pathogen resistance against biotrophic pathogens (Cui et al., 2016). We will overexpress *EDS1* as well as one of the several Pathogen Resistance (*PR-1*) precursor genes found to be significantly upregulated as early as 8h 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. Furthermore, a thaumatin protein encoding gene was also significantly up-regulated in our immune stimulated grapevines that we found to be tolerant of *Xf*. Thaumatin is a PR protein that exhibits 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 thaumatin and defense against *Xf*. We will overexpress the thaumatin encoding gene.

Overall, we will be generating and testing eight genes through overexpression. In all cases, the resulting transformants will be clonally 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<sup>8</sup> cfu/ml *Xf* inoculum suspension or 1X PBS buffer only as described below in Obj. 2a. Control plants (non-transgenic Thompson seedless vines) will be used as positive controls for the experiment. Plants will be assessed for PD symptom development, *Xf* population numbers, and *Xf* movement as described in Obj. 2a,b.

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), and  
p35S:CP1-CDS (VvCabSauv08\_P0022F.ver1.0.g361850).

We initially tested these constructs in *Arabidopsis 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 *Vitis vinifera* (Thompson seedless) because of the time it takes to generate grape transformants (up to 6 months). These four constructs above have been successfully transformed into *A. thaliana* Col wild type. Transformants have been obtained and homozygous lines have been 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*. Additionally, we challenged these *Arabidopsis* transformants and *A. thaliana* Col wild type plants with *Xf* to assess how these genes may confer resistance to *A. thaliana* from *Xf*. Thus far, we have generated transgenic vines from two of the constructs and the remaining two have been unsuccessful. The transformation facility has reinitiated these unsuccessful attempts which are currently in progress. The first two aforementioned lines, (VvCabSauv08\_P0022F.ver1.0.g361850), p35S: EDS1-CDS, and (VvCabSauv08\_P0022F.ver1.0.g361850), p35S:CP1 like-gDNA, have been received at UC Riverside and have been rooted in soil for clonal propagation.

In addition, four more 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), and  
pCK4-CP1-CDS (VvCabSauv08\_P0022F.ver1.0.g361850).

All of these constructs have been transformed into *A. tumefaciens*. As described above, these have been initially 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). Again, we will challenge these *Arabidopsis* transformants and *A. thaliana* Col wild type plants with *Xf* to assess how these genes may confer resistance to *A. thaliana* from *Xf*. All of these constructs are in the transformation pipeline. Two have shown potentially positive embryo growth.

## **Objective 2. Evaluation of PD resistance: disease phenotyping and characterization of defense responses to *Xf* challenge**

Objective 2a. Disease phenotyping and bacterial colonization: So far, we have mechanically inoculated 8 wild type parental vines, 12 CP1-CDS line 003 vines, and 4 EDS1-CDS line 004 vines using the pin-prick method (Hill & Purcell, 1995). These plants will be visually examined for PD symptom development over the course of twelve weeks and rated on an arbitrary disease rating scale of 0-5 where 0=healthy and 5=dead or dying (Guilhabert and Kirkpatrick, 2005).

Future experiments will be replicated 3 times to allow for robust statistical evaluation. While these experiments cannot be completed until we have clonally propagated enough for a full virulence experiment, we have decided to use the material we have to run an experiment to start generating results. *Xf* titer will also be quantified using qPCR.

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 in the xylem. Tylose production is a result of *Xf* infection and has been shown to exacerbate PD symptom development (Sun et al, 2013). Therefore, preventing this host defense response may reduce or completely prevent PD symptoms occurrence. Stem sections of *V. vinifera* '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). These experiments will be completed when we have clonally propagated the transgenic lines we have obtained.

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. These experiments will also be completed when we have clonally propagated enough plants from the transgenic lines to conduct these experiments.

## CONCLUSIONS

Capitalizing on the information we have garnered about the grapevine defense response to LPS, we have developed eight constructs to transform Thompson Seedless grapevines through the Transformation Facility at UC Davis. Three transformants have been successfully developed and rooted in soil for clonal propagation. Two lines of these transgenic vines have been clonally propagated and will be assessed for disease symptom development and protection against *Xf* bacterial colonization. The rest of the transformants are still in this pipeline. This research will lead to the development of PD-resistant vines and contribute to the knowledge of overexpressing and silencing genes to produce disease resistant vines.

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## **DEVELOPMENT OF A GENE EDITING TECHNOLOGY FOR GRAPEVINES USING PLANT PROTOPLASTS**

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**Reporting Period:** The results reported here are from work conducted from July 2020 to November 2021.

### **ABSTRACT**

CRISPR-Cas gene editing technology allows for precise alterations in plant genomes. Protoplast culture provides one of the best avenues for producing non-chimeric gene edited plants especially in vegetatively propagated plants such as grapes. We have developed a protocol for isolating protoplasts from embryogenic cultures of Chardonnay, Colombard, Merlot, Thompson Seedless and 101-14 grapes. By encapsulating the protoplasts in calcium alginate beads and co-culturing them with grape cell suspension feeder cultures, the protoplasts divide to form callus colonies and eventually regenerate into embryos and ultimately whole plants. The ability to regenerate plants from protoplasts offers an avenue to employ gene editing techniques to grapevines using either plasmid DNA or Ribonucleoprotein (RNP)-Based Genome Editing. We intend to transfect protoplasts with plasmid DNA as well as RNPs to induce gene edits. In our initial experiments, we used plasmid DNA encoding for the scorable marker gene mCherry. This allowed us to monitor the effect of various parameters on transfection efficiency and protoplast viability. Once we optimized the parameters, we transfected protoplasts with guide RNAs targeting the conserved DELLA domain of the VvGAI1 gene of grape, which when edited will result in easily identifiable gibberellic acid insensitive phenotypes. We were able to regenerate hundreds of plants from transfected protoplasts. Sequence data showed that two of the 32 regenerated plants tested had edits in the DELLA domain. We will use these same gene editing targets, to compare the efficacy of generating non-integrated gene edits using plasmid DNA versus RNPs.

### **LAYPERSON SUMMARY**

CRISPR-Cas9 is a gene editing technology that allows one to make precise changes in a plant's genetic code. There are a number of methods for delivering the CRISPR-Cas9 into the animal cells. However, unlike animal cells, plants cells are incased 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. Once the cell wall is removed, plant cells can be transformed and edited using the same techniques used on animal cells. We have previously established a protocol to generate protoplasts from grape tissue and stimulate these protoplasts to reform whole plants. In this project, we plan to use this methodology to edit the genetic code of grape plants by treating them with DNA or Ribonucleoproteins (RNPs). We first used DNA called mCherry to test if we could successfully introduce DNA into the "naked" plant cell. If this DNA successfully makes its way into the protoplasts, it will cause the protoplasts to glow red under fluorescent light. Using this test DNA, we optimized the parameters for getting DNA into the protoplasts. Next we used



these parameters to introduce DNA into the protoplasts to target a gene that when edited will result in dwarf plants. We have identified our first successfully edited plants using this technique. When using DNA, a subset of the edited plants will be transgenic, since the CRISPR-Cas9 DNA can become incorporated into the cells genetic code. However, since RNPs are proteins and not DNA, no insertion of foreign DNA into the cells genetic code is possible and therefore the resulting edited plants will not be transgenic (non-GMO). We will be testing the use of RNPs to edit grape plants during the coming year. Successful editing and recovery of plants from grape protoplasts opens up a wide array of options for improving grape varieties for disease resistance, agronomic traits or quality improvement.

## 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, any integrated editing sequences, such as the CRISPR-Cas9, can be segregated out of the population in the subsequent seed generation and the null segregants containing only the desired gene edit advanced using traditional plant breeding. However, for clonally propagated plants like wine grapes, 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 a viable avenue for producing non-chimeric gene edited plants for clonally propagated species. CRISPR-Cas9 has been introduced into plant protoplasts using polyethylene glycol or electroporation and expressed transiently without integration of the CRISPR-Cas9 DNA. Protoplasts re-form cell walls within 48 to 72 hours and the edited cells can be stimulated to form callus colonies. However, routine regeneration of whole plants from protoplasts has not previously been achieved in grape (Zhu et al 1997, Xu et al., 2007). We have 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 protoplasts from grape embryogenic cultures, generate callus colonies from the protoplasts and regenerate whole plants from the callus. These developments have significant relevance to the PD/GWSS Research Community and the wine-grape industry. The ability to regenerate plants from protoplasts offers an avenue to employ gene editing techniques to grapevines using plasmid DNA as well as Ribonucleoprotein (RNP)-Based Genome Editing approaches. Using Cas9 RNP complexes eliminates any possibility of integration of foreign DNA into the plant genome, thereby allowing the generation of non-transgenic gene edited plants in vegetatively propagated crops. In this report, we first demonstrate efficient polyethylene glycol-mediated transfection of grape protoplasts using the scorable mCherry marker gene while maintaining protoplast viability. Next, we used that protocol to transform protoplasts with different CRISPR-Cas9 plasmids targeting the DELLA domain in VvGAI1 gene. Initial DNA sequencing of a small subset of the regenerated plants, revealed successful editing in the targeted region in two independent events.

## **OBJECTIVES**

1. Establish high efficiency polyethylene glycol-mediated transient transformation rates in grape protoplasts using a mCherry expressing reporter plasmid.
2. Produce gene edited plants from grape protoplasts transfected with a plasmid containing Cas9 and guide RNAs targeting the conserved DELLA domain of the grapevine VvGAI1 gene.
3. Produce gene edited plants from grape protoplasts using Ribonucleoprotein (RNP)-Based Genome Editing with guide RNAs targeting the conserved DELLA domain of the grapevine VvGAI1 gene.

## **RESULTS AND DISCUSSION**

### **Objective 1. Establish high efficiency polyethylene glycol-mediated transient transformation rates in grape protoplasts using a mCherry expressing reporter plasmid.**

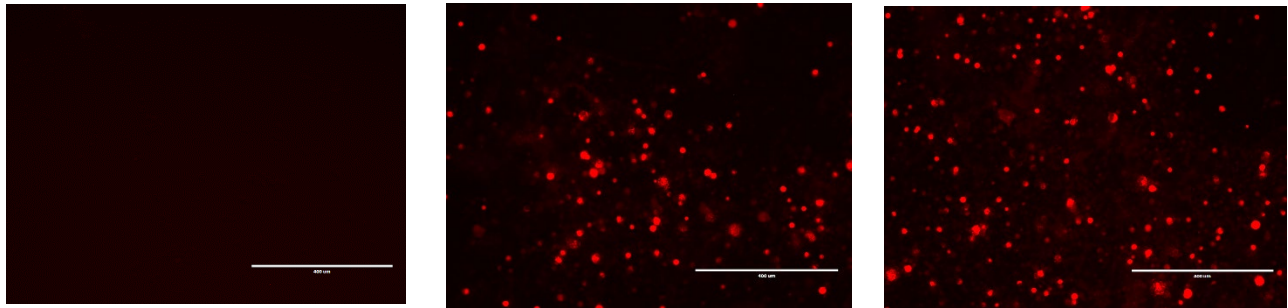
In order to monitor the effectiveness of grape protoplast transfection we used plasmid DNA coding for the scorable marker gene mCherry. This visual marker allowed us to monitor the effectiveness of various parameters on PEG-mediate transfection rates, including plasmid concentration, PEG concentration and time of transfection. We plated aliquots of cell suspension onto agar-solidified plates containing Lloyd and McCown Woody Plant Medium (WPM) (Lloyd, and McCown, 1981) supplemented with 20 g/l sucrose, 1 g/l casein, 1mM 2-(N-morpholino) ethanesulfonic acid (MES), 500 mg/l activated charcoal, 10 mg/l picloram and 2.0 mg/l thidiazuron (TDZ) (Pic/TDZ). We harvested embryogenic callus that developed on these plates and treated them in an enzyme solution consisted of filter sterilized 0.5% Onozuka Cellulase RS, 0.25% pectinase, 0.25% macerozyme R10, 0.6 M mannitol, 5 mM CaCl<sub>2</sub>, 10 g/l bovine serum albumin (BSA), 5 mM MES and 3 g/l glycine. We incubated the solution in the dark at 25 degrees centigrade on a platform shaker at 50 rpms. After approximately 16 to 24 hours incubation, we filtered the protoplast solution through a 40 um screen and collected the protoplasts by pelleting via centrifugation at 700 x g for 10 minutes. We washed the protoplasts twice in an osmotically adjusted wash solution containing 0.6 M mannitol, 2 mM CaCl<sub>2</sub>, 1 g/l BSA and 1,191 mg/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 3 g/l glycine (WS). We purified protoplasts using a dextran gradient consisting of 2 ml of a 13% dextran solution, overlaid with 1.5 ml of 0.6 M wash solution and harvested the protoplast band from the interface between the dextran and 0.6 M wash solutions.

We successfully transfected Thompson Seedless and 101-14 protoplasts with the mCherry plasmid. We isolated, purified and removed the protoplast band as described above and washed them by re-suspending in 4 ml of W5 solution (154 mM NaCl, 125 mM CaCl<sub>2</sub>, 5 mM KCL, 2 mM MES pH 5.7). We pelleted the protoplasts at 700 x g for 10 minutes, removed the supernatant and re-suspended the protoplasts in 1.0 ml aliquots of W5 solution at a cell density of  $5 \times 10^6$  cells per ml and held them on ice for 30 minutes. After 30 minutes, we pelleted the protoplasts and re-suspended them in 1.0 ml of MMG solution (4 mM MES, 0.4 M Mannitol, 15 mM MgCl<sub>2</sub>, pH 5.7) and transferred 200 ul ( $1 \times 10^6$  cells) to a 1.5 ml Eppendorf tube. We pelleted the protoplasts at 700 x g for 10 minutes and removed 150 of the 200 ul MMG supernatant. We added 0, 5, 10 or 20 ug of mCherry plasmid DNA directly to the pelleted protoplasts using a gentle swirling motion, followed by 200 ul of a freshly prepared PEG solution (40% w/v PEG 4000, 0.2 M mannitol, 0.1 M CaCl<sub>2</sub>). We then added 150 ul of MMG solution and inverted the tube gently to mix, then incubated the protoplasts at room temperature

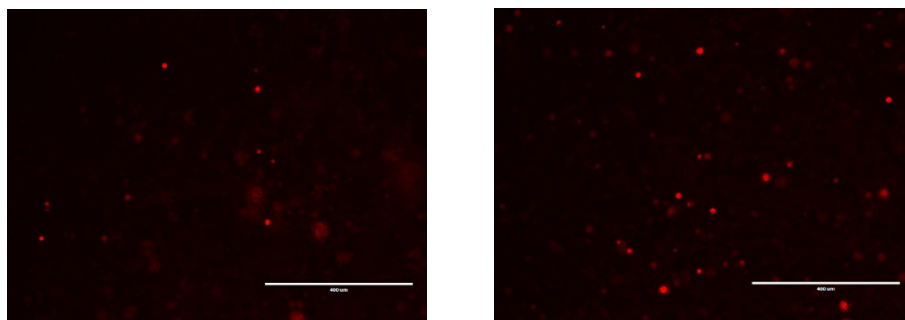
in the dark. After 15 minutes, we stopped the transfection by adding 880  $\mu$ l of W5 solution to each tube, inverting the tube gently to mix. We pelleted the protoplasts at 700 x g for ten minutes and washed the protoplasts in 4 ml W5 solution and pelleted them again. Finally, we re-suspended the pellet in 1 ml W1 solution and transferred 500  $\mu$ l to each well of a 24 well plate. We incubated the protoplasts in the dark at 40 rpms. After 72 hours, we viewed the protoplasts under a Life Technology EVOS inverted fluorescent microscope for mCherry expression.

In general, we observed increasing frequency of transfection with increasing amount of plasmid DNA up to the maximum 20  $\mu$ g tested. As expected, protoplasts which underwent mock transfection without the addition of plasmid DNA did not exhibit mCherry expression (Figure 1).

We also transfected the rootstock genotype, 101-14, with 0, 10 and 20  $\mu$ g of the mCherry plasmid and checked for mCherry expression after 72 hours. Again, we saw increasing transfection efficiency with increasing plasmid DNA (Figure 2).

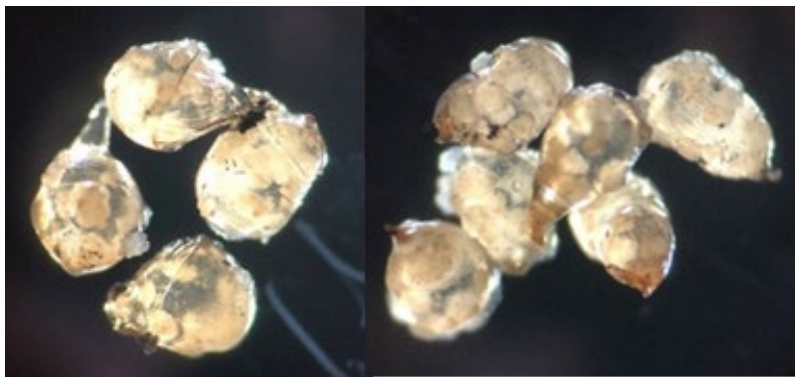


**Figure 1.** Transfection of Thompson Seedless protoplasts with 0  $\mu$ g (left), 10  $\mu$ g (middle) and 20  $\mu$ g (right) of the mCherry plasmid.



**Figure 2.** Transfection of 101-14 protoplasts with 10 $\mu$ g (left) and 20 $\mu$ g (right) of the mCherry plasmid.

After 72 hours, we collected the protoplasts from the 24 well plates and transferred them to 15 ml conical tubes, pelleted them at 700 x g for 10 minutes and re-suspended the protoplasts in 4 ml of WS solution. We pelleted the protoplasts again and re-suspended them in 0.5 ml of WS solution. We mixed the protoplasts solution with 0.5 ml of 3.2% sodium alginate solution composed of 72.87 g/L mannitol, 222 mg/l CaCl<sub>2</sub>, 1,191 mg/l HEPES and 3.2 g/l sodium alginate (adjusted to pH 5.7). We formed beads 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<sub>2</sub> solution composed of 72.87 g/L mannitol, 222 mg/l CaCl<sub>2</sub>, 1 g/l BSA and 1,191 mg/l HEPES (pH 5.7). After 30 minutes in the CaCl<sub>2</sub> solution, we washed the beads in 30 ml of WS solution and transferred them to 60 ml Nalgene jars containing 2.5 ml 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<sub>2</sub>, 1 g/l casein, 1,191 mg/l HEPES and 2 g/l activated charcoal, (0.4 M Pic/TDZ), which was further modified through the addition of 1.6 mM putrescines, 0.1 mM spermidine, 1.0 mM spermine, 15 ul of antioxidant solution (100 mg/ml ascorbic acid, 150 mg/ml citric acid, 100 mg/ml L-cysteine and 30 mg/ml reduced glutathione) and 3 g/l glycine . We then added 0.5 ml of a 7-day old suspension culture of 1103P cells which had been previously conditioned to grow in 0.4M Pic/TDZ medium. We cultured protoplasts at 25 degrees centigrade in the dark at 50 rpms. After 14 days, we added 3 ml of grape suspension culture medium without mannitol consisting of WPM medium supplemented with 20 g/l sucrose 10 mg/l Picloram, 2.0 mg/l TDZ, 0.0 g/L mannitol, 222 mg/l CaCl<sub>2</sub>, 1 g/l casein, 1,191 mg/l HEPES and 2 g/l activated charcoal, pH 5.7 (0.0 M Pic/TDZ) supplemented with 1.6 mM putrescines, 0.1 mM spermidine, 1.0 mM spermine, 15 ul of antioxidant solution and 3 g/l glycine to each jar, thereby reducing the starting mannitol concentration to 0.2 M. After an additional 14 days, we removed 3 ml of the suspension cultures from the jars and added 3 ml of 0.0 M Pic/TDZ medium with 1.6 mM putrescines, 0.1 mM spermidine, 1.0 mM spermine, 15 ul of antioxidant solution and 3 g/l glycine to the jars, thereby further reducing the starting mannitol concentration to 0.1 M. We saw a significant attrition in protoplast survival after transfection compared to controls. However, we observed callus colonies developing within the alginate beads six weeks after transfection. (Figure 3).



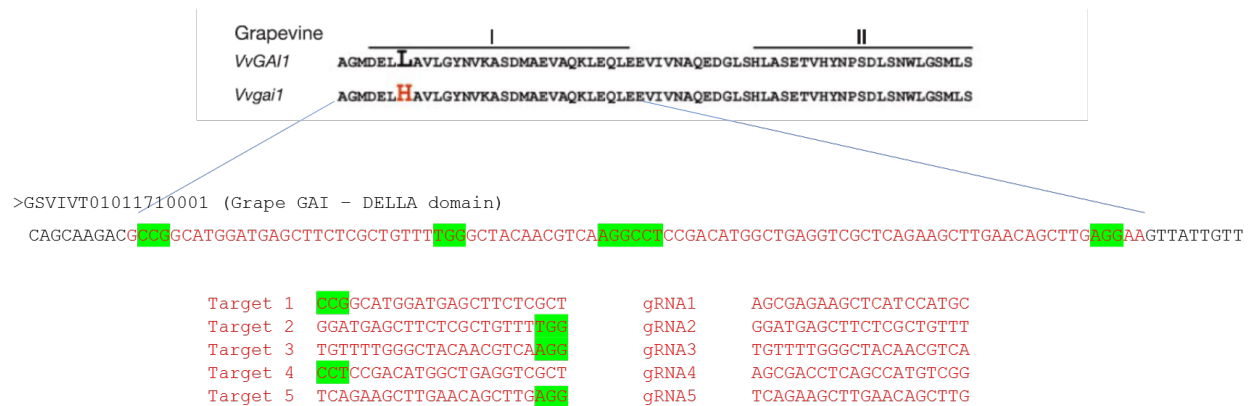
**Figure 3.** Colony formation within a calcium alginate bead from Thompson Seedless protoplasts after transfection with the mCherry plasmid.

## Objective 2. Produce gene edited plants from grape protoplasts transfected with a plasmid containing Cas9 and guide RNAs targeting the conserved DELLA domain of the grapevine VvGAI1 gene.

We proposed to target the DELLA domain of the VvGAI1 gene (GSVIVT01011710001) by CRISPR-Cas9 in order to generate GA insensitive alleles, that in grape result in dwarf plants that produce inflorescences where tendrils are normally formed (Boss and Thomas 2002). Based on the literature, multiple mutations disrupting the DELLA domain could potentially generate GA insensitive alleles [Amino acid changes (grape), premature stop codons (wheat), or small deletions (Arabidopsis or maize)]. Therefore, we selected five different target regions distributed along the sequence coding the DELLA domain of the VvGAI1 gene in order to identify the most active gRNAs (Figure 4). Moreover, we tested different combinations of gRNAs that could result in larger deletions in the target region.

We are testing the activity of the different gRNAs by transforming grape protoplasts with plasmid DNA harboring the editing machinery. We are using the vector pDIRECT\_10E designed by Daniel Voytas group, which was obtained from Addgene (Plasmid #91209). This vector allows one to clone one or multiple gRNAs. In the later, different gRNAs can be expressed using alternatives approaches (Csy4, tRNA or ribozyme arrays). In our case we cloned different combinations of gRNAs (1 and 3; 2, 4 and 5; 1 and 5) in tRNA arrays, since we have successfully used this approach in other species. After cloning and validating the sequences of the different constructs, we generated plasmid samples at a concentration of 1 ug/ul (Figure 4).

We initiated experiments to target the conserved DELLA domain of grapevines using the parameters determines in our mCherry transfections described above. To date, we initiated one experiment using 20 ug of plasmid DNA for each of the gRNAs combination (1 and 3; 2, 4 and 5; or 1 and 5) using protoplasts isolated from Thompson Seedless embryogenic callus. We also initiated six independent experiments in which we used all three of the gRNA combinations by mixing together 7 ug of each of the plasmids (Table 1).



**Figure 4.** Scheme showing the mutation in the DELLA domain in VvGAI1 gene (GSVIVT01011710001) associated with the dwarf phenotype (Adapted from Boss and Thomas 2002). In the middle, we show the coding region of the DELLA domain (in red) with the PAM sequences of the five target regions highlighted in green. The sequence of the five regions and the corresponding gRNAs are showed at the bottom.

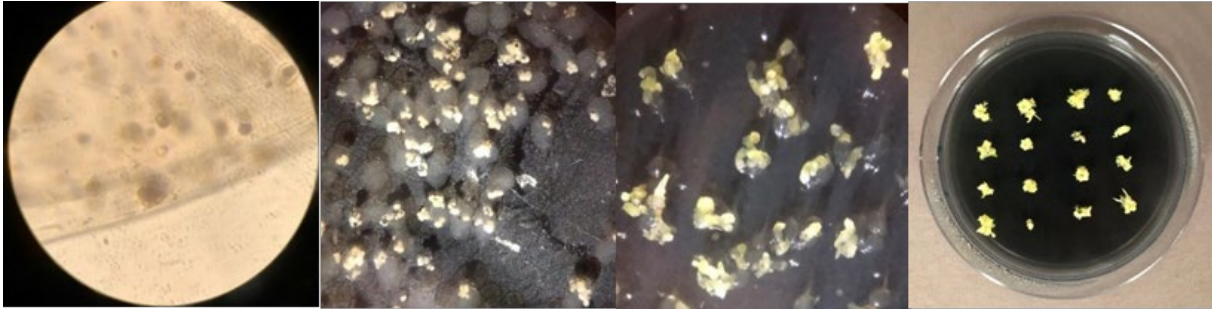
For each set of experiments, we also ran controls consisting of non-treated Thompson Seedless protoplasts which were prepared as the transfected protoplasts, but not treated with plasmid DNA or PEG. We saw a significant attrition in protoplast survival after transfection compared to controls. However, we observed cell division in alginate encapsulated transfected protoplasts. Once the dividing cells reach the 16-32 cell stage, we removed the beads from liquid culture and plated onto agar-solidified WPM medium supplemented with 20 g/l sucrose, 1 g/l casein, 1 mM MES, 500 mg/l activated charcoal, 0.5 mg/l BAP, 0.1 mg/l NAA (BN medium) to induce embryo formation (Figure 5). We have successfully regenerated very large numbers of plants from transfected protoplasts (Figure 6).

In order to explore for editing events in the regenerated plants we initially isolated genomic DNA from root tissue from a small group of 32 independent events generated in experiments: 21119 (x8 plants), 21133 (x8 plants), 21017 (x8 plants) and 21014 (x8 plants). We then performed a PCR with primers flanking the targeted region, and the PCR products were barcoded and submitted for CRISPR-Seq at the Massachusetts General Hospital CCIB DNA core.

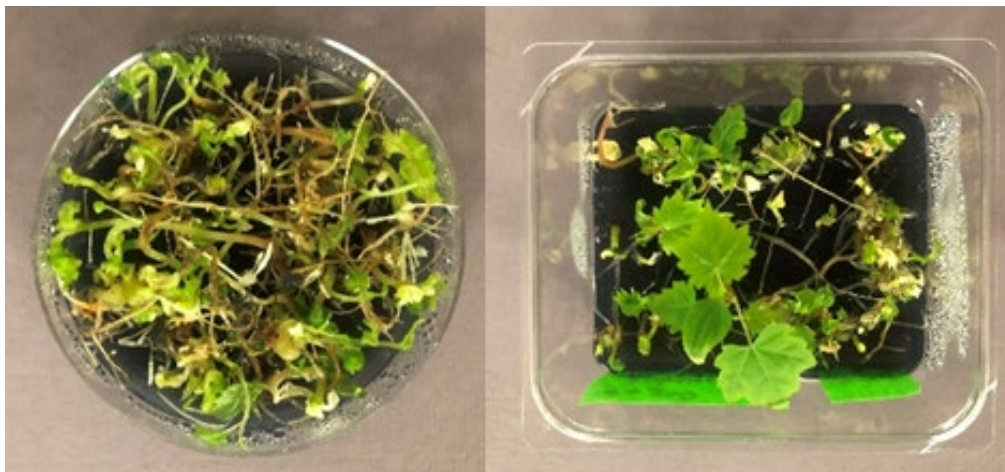
Analysis of the sequencing results revealed editing events in two independent regenerated plants (Figure 7A). A plant regenerated from experiment 21133 (T0#21133-2-3) was heterozygous harboring a mutant allele with an extra T (Allele-T) in the region targeted by gRNA#5. The second plant with edits was regenerated from experiment 21119 (T0#21119-1-5). This plant was also heterozygous, but interestingly, it harbored two different mutant alleles. Allele1 contains multiple mutations distributed in the target regions of all five gRNAs, while the second allele (Allele2) contains a large deletion between gRNAs #1 and #5. Analysis of the encoded proteins showed that the mutations in the three alleles may result in elimination of the DELLA domain from the VvGAI1 protein (Figure 7B). The mutations in Allele-T and the large deletion in Allele2 resulted in changes in the reading frame that introduce premature stop codons. In both alleles alternative ATG codons can be used to restore the reading frame, but they result in mutant proteins that include a short N-terminal peptide and lacks the DELLA domain. The plant with the Allele-T has a severely dwarfed phenotype (Figure 8). The mutations in Allele#1 also introduce frame changes that result in a premature stop codon in the normal reading frame. However, for this mutant allele the use of an alternative ATG codon result in a larger N-terminal deletion that includes the DELLA domain.

Although it is hard to predict the effect of these mutations on VvGAI1 activity, similar deletions and the use of alternative ATG codons that eliminate the DELLA domain were observed in GA-insensitive DELLA mutants in species like wheat and maize (Boss and Thomas 2002). So, it is possible that all three mutations would result in GA-insensitive VvGAI1 alleles. We are currently sequencing more independent events to get a better representation of the mutation frequency in the regenerated population. Also, we are analyzing in more detailed the phenotype of the two mutant plants, which could allow us to identify more independent events based on phenotype. Finally, from this preliminary analysis we were also able to get an approximation of the activity of the different gRNAs. The three alleles include mutations that indicate activity of gRNA#5, while Allele#1 and Allele#2 showed activity for gRNA#1. Allele#2 also has mutations that are likely generated by the other 3 gRNAs. Based on this preliminary analysis we plan to select gRNA#5 and gRNA#1 to test in RNP transfections in the third objective of this project.





**Figure 5.** Protoplast-derived colony formation and embryos developing from protoplasts after transfections with CRISPR-Cas9 and gRNAs designed to target the conserved DELLA domain of the grapevine VvGAI1 gene.



**Figure 6.** Protoplast derived Thompson Seedless embryos and small plants germinating from protoplasts transfected with CRISPR-Cas9 and gRNAs designed to target the conserved DELLA domain of the grapevine VvGAI1 gene.





**Figure 7. A.** DNA sequence of coding region of the DELLA domain (in red) with the PAM sequences of the five target regions highlighted in green. The Wild type (Wt) VvGAI1 sequence is indicated on the top, and mutant alleles below. Extra bases are highlighted in light blue, and deletions as (-). **B.** Amino acid sequence of the region including the DELLA domain (highlighted in yellow) in Wt (top) and the mutant alleles.



**Figure 8.** A plant regenerated from experiment 21133 (T0#21133-2-3) harboring a mutant allele with an extra T (Allele-T) in the region targeted by gRNA#5 is identified by the red circle in the far right image.

### Objective 3. Produce gene edited plants from grape protoplasts using Ribonucleoprotein (RNP)-Based Genome Editing with guide RNAs targeting the conserved DELLA domain of the grapevine VvGAI1 gene.

In the next step of this project we will begin testing RNP editing with guide RNA's targeting the conserved DELLA domain of the grapevine VvGAI1 gene. As indicated above we will initiate those experiments with RNPs including synthetic gRNA#1 and gRNA#5.

### CONCLUSIONS

We have been able to successfully transfected Thompson Seedless and 101-14 protoplasts with the mCherry plasmid. We are seeing high frequency transfection especially using 20 ug of plasmid DNA. Protoplasts survived the PEG treatment, were encapsulated in calcium alginate beads,

grown in medium containing grape feeder suspension and developed callus colonies within the alginate beads. We prepared plasmids containing Cas9 and guide RNAs targeting five different regions of the conserved DELLA domain of the grapevine VvGAI1 gene. We transfected protoplasts with various combination of gRNAs; 1 and 3, 1 and 5, 2, 4 and 5 as well as a mixture of all three combinations and regenerated plants from the transfected protoplasts. Although to date, we have only analyzed a small subset of the regenerated plants, we have shown that by transfecting protoplasts with a mixture of all three of the gRNA combinations we have been able to regenerate whole plants and verify that some contain edits in the DELLA domain.

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#### **FUNDING AGENCIES**

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## BREEDING PIERCE'S DISEASE RESISTANT WINEGRAPES

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**Reporting Period:** The results reported here are from work conducted July 2019 to June 2021.

### ABSTRACT

Breeding Pierce's disease (PD) resistant winegrapes continues rapid advancement aided 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* (Xf), 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. To date 20 scion and five PD resistant rootstocks based on *PdR1b* have been advanced to FPS for certification. Five of these selections are now available from certified grape nurseries and their patents completed. About 19,500 vines were sold in 2020, with the largest plantings across California and Texas. 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 *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. In the spring of 2019, the first three scion selections that employ both *PdR1* and *PdR2* resistance were delivered to FPS. Another was sent in the spring of 2020. Pierce's disease resistance from *V. shuttleworthii* and BD5-117 has also been pursued but progress has been limited by their complex multigenic resistance and the absence of associated genetic markers. An additional focus of our current PD breeding efforts is to stack powdery mildew (PM) resistance into our advanced selections. 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) and 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), and at both this and last year's Unified Symposium and at 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 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 (MAS) to screen for the DNA markers linked with these PD resistance genes to greatly accelerating our breeding progress. We have evaluated many thousands of resistant seedlings for horticultural traits and fruit quality. The best of these were advanced to greenhouse testing, where only those with the strongest PD resistance after multiple greenhouse tests, were 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 seven winters to verify their virus-free status. Five of them are available from certified grape nurseries and their patents completed. Four winegrape selections that have both *PdR1* and *PdR2* resistance were delivered to FPS in 2019 and 2020. Selections of other wild grape species are being studied and the best will be utilized in the PD resistance breeding program. An additional focus of our current PD breeding efforts is to stack natural powdery mildew resistance into our advanced selections. 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 use 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 five PD resistant rootstocks based on *PdR1b* have been advanced to FPS for certification. Five of them are available from certified grape nurseries and their patents granted. 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. Four winegrape selections that have both *PdR1* and *PdR2* resistance have been delivered to FPS over the past two springs. 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 2021 Unified Symposium.

The Walker lab is uniquely poised 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 *X. fastidiosa* under our greenhouse screen. Only those with the highest levels of resistance are advanced to small-scale winemaking trials by grafting them onto resistant rootstocks and planting six to eight vine sets on commercial spacing and trellising at Pierce's disease hot spots around California, where they continue to thrive. We have made wine from vines that are 94% *V. vinifera* level from the same resistance background for 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 *X. fastidiosa* 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 based on 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 marker-assisted selection (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 (Ch) 8, which we have called *PdR2*. In 2014, we advanced our *PdR1* x *PdR2* line to the 92% *vinifera* level and in spring 2016 made crosses to advance it to the 96% *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 (SEUS) 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

Our PD resistance breeding activities over the last two and a half calendar years are quantified and summarized in Table 1. We reached the 97% *vinifera* level in the *PdR1b* line in 2009 and finished planting out additional crosses at that level in 2011. A total of 2,911 genotypes were planted in the 2010-12 period. In 2016 we reached the 96% *vinifera* level in the *PdR1b* x *PdR2* stacked line with the planting of 126 marker tested genotypes having both *PdR1* and *PdR2*. In the spring of 2018 we planted another 328 seedlings incorporating a wider range of elite *vinifera* varieties in their parentage at this same backcross level from 2017 crosses. Plantings of the 2018 crosses in the spring of 2019 focused on combining various PD and PM resistance sources into individual lines. Our marker testing (1a) focused on eliminating as many individuals without resistance markers for both diseases which resulted in the relatively few genotypes that went to the field in 2019. Fruit evaluations (1c) include new stacked crosses but doesn't include spring evaluations for horticultural traits, flower sex or productivity. As we continue to advance the backcross level of various lines, especially in the absence of resistance markers for sources other than *PdR1* and *PdR2*, our greenhouse screening has steadily increased as we identify promising parents especially in lines without markers. In addition to scion genotypes, Table 1d includes rootstock breeding, mapping and germplasm testing but not any spacing or *Xf* strain trials, or the testing of biocontrol vine genotypes. As we identify particularly resistant individuals we test them multiple times (1e) to properly assess their level of resistance and insure that only the most resistant individuals are advanced. These tests are in addition to those listed in Table 1d immediately above. Seven selections were sent to FPS for certification over this period shown in the Table 1f and four PD x PM advanced selections are anticipated to be sent before the end of June.

**Table 1.** 2019-21 PD breeding activity summary. Numbers in italics are estimates.

Activity	Calendar Year			2019-2021 Total
	2019	2020	2021	
1a. # Genotypes MAS Tested	2,307	297	-	2,604
1b. # Genotypes Planted to Field	733	-	-	733
1c. # Genotypes Evaluated for Fruit	706	477	225	1,408
1d. # Genotypes Tested in GH	1,048	942	254	2,244
1e. # Genotypes Tested Multiple Times	101	69	77	247
1f. # Advanced Selections sent to FPS	3	4	4	11

To date over 700 wild accessions have been tested for PD resistance with the greenhouse screen, most of which were collected from the southwestern United States and Mexico. Our goal has been to identify accessions with the most unique PD resistance mechanisms. To do so we evaluated the genetic diversity of these accessions and tested them for genetic markers from chromosome (Ch) 14 (where *PdR1* resides; Riaz, 2016) to increase the likelihood that we were choosing genetically diverse resistance sources for population development and greenhouse screening efforts. Fifteen of the most unique accessions were used to develop F1 populations with *V. vinifera* to investigate the inheritance of PD resistance in their F1 progeny and the degree to which they resist *X. fastidiosa*. Despite the seeming diversity of sources, most of the resistance lines we have explored from the southwestern US have PD resistance associated with Ch 14 (albeit with different SSR marker sizes than b43-17). After years of testing and retesting an earlier mapping population based on b42-26, our mapping project identified *PdR2* on Ch 8. *PdR2* resistance although significant, generally doesn't confer as strong a resistance as *PdR1*. Table 2 summarizes the resistant loci location and progeny tested for the various resistance sources. Although resistance in the b46-43 source is dominated by LG14, the BC1 was explored for minor resistance genes. None were found (Huerta-Acosta, submitted?).

**Table 2.** Location of resistance loci and progeny screened for 15 accessions evaluated for PD resistance breeding.

Resistance source	Resistance loci	Unique progeny screened	Comments
A14	Not pursued.	28	Initial population too small.
A28	LG14	42	
ANU5	LG14	102	
ANU67	Undetermined	80	Not LG14 nor LG8
ANU71	LG14	30	
b40-29	LG14	78	
b41-13	LG14	360	Was inconclusive last reporting period
b43-57	LG14	51	
b46-43	LG14	378	
b47-32	LG14	89	Was inconclusive last reporting period
C23-94	LG14	44	
DVIT2236.2	LG14	30	
SAZ7	LG14	52	
SC36	LG14	35	
T 03-16	LG14	211	Was thought non-LG14 last reporting period



Another area of focus and one that should produce our next PD resistant wine grape selections for release are those that stack *PdR1b* resistance from b43-17 and *PdR2* resistance from b42-26. In spring 2017, from crosses made in 2016, we planted 126 seedlings from 4 different crosses that are 96% *vinifera* and have both resistance loci. In 2017 we expanded the diversity of elite *vinifera* parents used in the 96% *vinifera* *PdR1* x *PdR2* breeding line. These will give us varieties with a wide range of fruit and horticultural characteristics to present to the industry. Table 3 provides fruit characteristics and greenhouse test results from the first four selections sent to FPS for certification. Multi-vine trial have been established at UCD and the first 3 listed will provide enough fruit for small scale winemaking this year.

**Table 3.** Fruit characteristics and complete greenhouse screen results from the first four 96% *vinifera* *PdR1b* and *PdR2* selections sent to FPS.

Selection	Most recent <i>vinifera</i> parent	Color	Berry Wt (g)	Cluster Wt (g)	Average In cfu/ml	Times tested	Year Sent to FPS
16329-015	Primitivo	B	1.3	247	10.4	4	2019
16333-022	Cabernet Sauvignon	B	1.3	286	9.4	3	2019
16353-072	Chardonnay	W	1.0	191	11.8	4	2019
17706-041	Pedro Ximenez	W	1.3	199	9.7	3	2020

The main focus of our PD breeding efforts in 2018 was to stack PD resistance, either from *PdR1b* alone or in combination with b42-26 resistance, with one or more powdery mildew (PM) resistance sources in elite *vinifera* backgrounds. We have genetic markers for PM resistance derived from *V. vinifera* (*Ren1*), *V. rotundifolia* (*Ren4*), *V. piasezkii* (*Ren6*, *Ren7*), and two forms from *Muscadinia rotundifolia* (*Run1* and *Run2.1*). As usual we use MAS to advance only those progeny with resistance markers, the greenhouse screen to select only the most PD resistant and field and in vitro testing for PM resistance. Promising selections would be candidates for release (Table 12). With the exception of crosses made directly to elite *vinifera* cultivars, the challenges of most of these PD x PM crosses are both practical, as required for rapid advance of stacking and for inheritance of typical *vinifera* characteristics, and perceptual in terms of easier market acceptance, since they don't have a most recent elite *vinifera* parent to differentiate them. These factors will require a longer period of horticultural and enological evaluation than has been our experience to date with the crosses bred for PD resistance alone where the most recent parent has always been a *vinifera* cultivar.

Since Dr. Walker retired in June of this year, no PD or PD x PM crosses were made in 2019 or 2020. The remainder of this report will focus on the results of our greenhouse screens completed over the reporting period as we wind down the PD breeding program for now. Our rapid greenhouse screen is critical to our evaluation of PD resistance in wild accessions, new F1 and BC1 mapping populations and for selection of advanced late generation backcrosses for release. Table 4 provides a list of the PD greenhouse screens analyzed, initiated and/or completed over the reporting period.

**Table 4.** Greenhouse PD screens completed during 2019-2021 reporting period.

Group	Purpose	No. tested	Inoculation Date	ELISA Sample Date	PD Resistance Source(s)
4a	SWUS Species, <i>PdR1</i> x <i>PdR2</i> Test 10, b41-13	168	7/2/2019	10/1/2019	2018 PD x PM, 2017 Promising PD& PD x PM
4b	ANU67 F1, 96% <i>vin PdR1</i> x <i>PdR2</i> promising, 97% b40-14, 2017 PD x PM	255	9/12/2019	12/12/2019	Species, <i>PdR1b</i> x b42-26
4c	2016-17 96% <i>vin PdR1</i> x <i>PdR2</i> , 2015-17 PD x PM	115	10/10/2019	1/9/2020	<i>PdR1b</i> , <i>PdR1b</i> x b42-26
4d	2018 PD x PM, 2016-17 Promising PD & PD x PM, species	170	1/23/2020	3/19/2020	Species, <i>PdR1b</i> , <i>PdR1b</i> x b42-26
4e	2018 PD x PM, 2017 Promising PD& PD x PM	246	1/30/2020	4/30/2020	<i>PdR1b</i> , <i>PdR1b</i> x b42-26
4f	Retesting of promising 2016 and 2017 crosses	113	3/24/2020	6/23/2020	<i>PdR1b</i> , <i>PdR1b</i> x b42-26
4g	Species, retest promising 2017 seedlings, b46-43 BC2, vasculature sectioning	159	6/2/2020	9/1/2020	<i>PdR1b</i> , <i>PdR1b</i> x b42-26, b46-43
4h	2019 PD x PM OP Promising, Graft Saves Collection, Species	328	9/17/2020	12/17/2020	<i>PdR1b</i> , <i>PdR1b</i> x b42-26
4i	Promising and untested crosses from 2016-2018 Xs, mostly PD x PM	114	12/10/2020	3/11/2021	<i>PdR1b</i> , <i>PdR1b</i> x b42-26
4j	Multiple 5-vine reps of advanced selection, promising 2017 and 2018 crosses w/o 3 screens	70	12/29/2020	4/1/2021	<i>PdR1b</i> , <i>PdR1b</i> x b42-26
4k	2017 cross remnants, 2018 final tests, Graft Saves Collection, <i>Run1</i> x <i>Run2</i> w/o a PDR locus	240	3/4/2021	5/20/2021	<i>PdR1b</i> , <i>PdR1b</i> x b42-26, VR, <i>PdR1c</i>

Group 4a tested thirty-nine 96% *vinifera* *PdR1b* x *PdR2* genotypes from 2017 crosses. Elite *vinifera* parents Dolcetto, Refosco and Touriga Nacional were crossed to two 92% *vinifera* *PdR1* x *PdR2* seed parents. Although the sample sizes weren't large enough for rigorous analysis, a quick fit model test indicated that only the male parent mattered and that Refosco produced progeny with significantly higher *Xf* titers than the other two *vinifera* parents. Twenty-five *PdR1b* recombinants from 2018 crosses were tested for our companion mapping project; the R:S ratio was ~1:3. Tested for the same project were 17 b41-13 F1 genotypes which validated earlier findings that resistance resides on LG14.

Five sets were tested in Group 4b, two for our mapping project and three for advancing breeding lines. Sixty-two ANU67 resistance source F1 genotypes helped confirm that ANU67 resistance appears to not be located on either LG8 (*PdR2*) or LG14 (*PdR1*) making it unique among the 14 sources successfully tested. Seven *PdR1b* recombinants from 2018 crosses all tested susceptible (For more details see our companion mapping project). We also tested fifty-seven 96% *vinifera* *PdR1b* x *PdR2* genotypes descending most recently from 12 different elite *vinifera* parents. About 55% were resistant with 7% being highly resistant. The more resistant offspring had Dolcetto, Morrastel and Pedro Ximenez as their *vinifera* parents. Another 29 selections (similar to those sixteen in 4a above) with *PdR1b* enriched with b42-26 resistance were tested. Almost 90% were resistant and 14% were in the two most resistant categories. The best of both will be saved for use in the future breeding program. Of the thirty-five 97% *vinifera* *PdR1c* genotypes with resistance from wild accession b40-14, more than 75% were susceptible. This is consistent with what we have seen in previous generations in this line. One accession with good horticultural traits and high PD resistance were advanced to FPS. Fifty-two PD x PM selections from 2016 and 2017 crosses at the 82-97% *vinifera* level also completed testing. A total of 10 highly resistant selections were identified and underwent additional testing in other Table 4 trials.

In group 4c we tested 33 selections of the 2016-17 crosses that are 96% *vinifera* *PdR1* x *PdR2*. About 47% were resistant of which 5 were in the most resistant two categories. Three of these were confirmations of previous tests and resulted in the selection of two genotypes that went to FPS this past spring. Sixty PD x PM crosses at the 94-97% *vinifera* level were also tested. Nearly 75% tested susceptible with 7% in the two most resistant categories. Here as with other results discussed in previous reports, demonstrated that combining PD and PM resistance in the same genotype is clearly feasible but not quite as productive, in terms of recovery of highly resistant progeny, as breeding for PD resistance alone. Finally, a screen of 10 resistant females in the 94-97% *vinifera* level failed to identify any with enough resistance for use as future breeding stock.

The main focus of group 4e was a more extensive testing of our 2018 PD x PM dual resistant crosses with 172 tested in total. Details of the crosses and greenhouse screen results are shown in Table 5. Thirty of the selections were in the most resistant PD rating category and, if this assessment holds in future tests, some should offer promising candidates for release. With few exceptions, crosses of PD RxR parents produced a higher percentage of resistant progeny than crosses of resistant back to *vinifera*. The other major set tested involved 26 PD resistant genotypes at *vinifera* levels between 93-97% with 8 (~31%) in the most resistant two categories.

This is almost triple the percentage that was achieved in the resistant by *vinifera* crosses in Table 5 when PM resistance was also involved.

**Table 5.** Results from our 4e greenhouse screen of 172 PD x PM crosses made in 2018. For PDR type: A = *PdR1b*, B = *PdR1b*x**42-26**, C = *PdR1b*<sup>2</sup>x**42-26** and for PD Rating category -1 = S, 1 = R, 5 = very resistance and 10 = Xftiter below ELISA detectable level, no cane lignification symptoms and only minor leaf scorch. *Ren1* and *Ren4* are PM resistance (PMR) loci from *vinifera* and *V. rotundifolia*, respectively. *Run1* and *Run2.1* are PMR loci derived from *Muscadinia rotundifolia*.

Cross ID	Parentage	Cross PDR Type	Cross PM Type	% <i>vinifera</i>	PD Rating Category				Cross Total Tested
					-1	1	5	10	
18-312	16376-008 x 16382-034	A	<i>Ren1</i> x <i>Run1</i> <sup>2</sup>	94%	5	6			11
18-314	14305-078 x Fiano	B	<i>Ren4</i>	95%		5			5
18-315	14305-078 x Gouveio	B	<i>Ren4</i>	95%	1	9		1	11
18-316	14305-078 x Tinta Amarella	B	<i>Ren4</i>	95%		2			2
18-320	14305-078 x 09330-07	C	<i>Ren4</i>	93%				1	1
18-321	14305-078 x 09356-235	C	<i>Ren4</i>	93%		6	2	2	10
18-322	14305-078 x 10317-035	C	<i>Ren4</i>	93%		1	1	1	3
18-323	14305-078 x 09314-102	C	<i>Ren4</i>	93%		3	4	4	11
18-324	14305-078 x Alvarelhao	B	<i>Ren4</i>	95%		4	2		6
18-325	14305-078 x Pinot blanc	B	<i>Ren4</i>	95%	1				1
18-336	14305-078 x 14375-043	B	<i>Ren4</i> x <i>Run1</i>	93%	1	5		1	7
18-337	14305-078 x Bonarda	B	<i>Ren4</i>	95%	4	3	2		9
18-338	14305-078 x Teroldego	B	<i>Ren4</i>	95%		1			1
18-339	14305-078 x Tinta Cao	B	<i>Ren4</i>	95%		5			5

Cross ID	Parentage	Cross PDR Type	Cross PM Type	% <i>vinifera</i>	PD Rating Category				Cross Total Tested
					-1	1	5	10	
18-340	14305-078 x Cortese	B	<i>Ren4</i>	95%		14	1		15
18-342	14305-078 x 16382-034	B	<i>Run1</i> <sup>2</sup> <i>Ren4</i>	91%	1	7			8
18-370	16703-007 x 16344-003	C	<i>Run1</i> x <i>Ren1</i> <sup>3</sup> <i>Ren4</i>	94%	1	1			2
18-371	16703-007 x 16376-014	C	<i>Run1</i> x <i>Ren1</i> <sup>3</sup> <i>Ren4</i>	94%	3	16	1	1	21
18-377	16703-007 x 10317-035	A	<i>Run1</i> x <i>Ren1</i> <sup>2</sup> <i>Ren4</i>	95%	1	6			7
18-382	16703-007 x 16376-004	C	<i>Run1</i> x <i>Ren1</i> <sup>3</sup> <i>Ren4</i>	94%	1	7	3	1	12
18-384	15354-105 x 10317-035	A	<i>Ren1</i> x <i>Run2.1</i>	95%	2	6	1		9
18-390	14710-006 x 09314-102	A	<i>Run1</i>	97%	3	11	1		15
Resistance Category Total					24	118	18	12	172

Follow up screening of promising 2016 and 2017 PD crosses at the 93-96% *vinifera* level made up the majority of group 4f. Twenty-eight selections (~24%) were in the most resistant two categories. Three of 13 (~23%) were in the 97% *vinifera* *PdR1c* line; 7 of 19 (~37%) were at the 94% *vinifera* level and 4 of 11 (~36%) were at the 96% *vinifera* level. Should further testing confirm these results and with favorable field evaluations, some of these may be advanced.

Group 4g tested 61 b46-43 BC2 progeny to better explore this resistance source, tested or retested 32 promising selections from our 2017 PD and PD x PM crosses and 24 additional southwestern US *Vitis* species from our collection. For the b46-43 line, 23 tested as susceptible, 38 resistant but only 5 were in the two most resistant categories of which only 3 had fruit and horticultural characteristics sufficient to warrant keeping them. In the 2017 PD x PM crosses, all 32 tested as resistant and the 12 that were in the second highest category (none were in the highest category) were propagated again for follow on testing. The vascular sectioning part of the experiment for our companion mapping project involved multiple reps of 16 species with a range of PD resistance. After loading the greenhouse it was decided to abandon the section experiment so we've included them with the analysis of the *Vitis* species shown in Table 6 below. PD resistance for species was generally as expected with southern species like *arizonica*, *girdiana* and *treleasei* having accessions with quite low *Xf* titers and more northern and eastern species like *aestivalis*, *cinerea* and *rupestris* having accessions with higher minimum titers. It's interesting in the case of *girdiana* that some accessions had quite high titers but an examination of their origin revealed that the more susceptible accessions were from Nevada and Utah while the more resistant accessions were from Arizona.

**Table 6.** Forty accessions of 10 *Vitis* species tested in Group 4g.

Species	Minimum mean ln cfu/ml	Maximum mean ln cfu/ml	# accessions tested
<i>acerifolia</i>	14.8	14.8	1
<i>aestivalis</i>	13.1	14.7	3
<i>arizonica</i>	9.5	13.4	13
<i>berlandieri</i>	14.7	14.7	1
<i>californica</i>	12.7	12.7	1
<i>cinerea</i>	15.1	15.1	1
<i>girdiana</i>	9.3	15.5	14
<i>riparia</i>	14.1	14.1	1
<i>rupestris</i>	14.4	15.7	2
<i>treleasei</i>	9.2	14.6	3

Group 4h tested progeny from 19 different 2019 open pollinated crosses from promising *PdR1b* seed parents. Eleven selections were homozygous for *PdR1b* (RR) by MAS being tested as 5 vine reps and 86 single vine reps that were MAS tested R at the PdR1 loci. As shown in Table 7 below, being homozygous R at PdR1 was not sufficient to keep 1 selection from testing susceptible and failing to have even one selection in the most resistant rating category. For the single resistant allele group, about 31% were susceptible, 69 were resistant of which 16 (~19%) were in the two most resistant categories. Consistent with what we have reported earlier, having both resistant alleles does not confer additional levels of resistance and this trial further demonstrates the importance of our greenhouse screen in helping identify promising individuals once the presence of the PdR1 allele is confirmed. To better document resistance for the future breeder, we also tested 208 genotypes from previous PDR generations from our graft saves collection. Seven additional SEUS species were also tested to complete our documentation of resistance in our species collection.

**Table 7.** Counts of genotypes greenhouse screened in group 4h by *PdR1* marker status (r or RR) and PD rating category. Categories as in Table 5. Also shown is the PD rating distribution of a subset of our graft saves collection.

<i>PdR1b</i> MAS Type	PD Rating Category				Type Total Tested
	-1	1	5	10	
2019OP <i>PdR1</i> MAS RR	1	7	3		11
2019OP <i>PdR1</i> MAS R	27	43	5	11	86
Graft saves collection	48	120	35	5	208

Group 4i tested two main subgroups focused on promising and untested PD and PD x PM resistant selections from crosses made in 2016-2018. Table 8 gives an overview of the screen results. In the first group 11 selections were in the most resistant two categories and involved Dolcetto, Morrastel, Pedro Ximenez, Refosco, or Touriga Nacional as their most recent *vinifera* parent. These will offer a wide array of wine styles should these results continue to hold. In the PD x PM sub-group, 17 selections were in the most resistant two categories; were ~94% *vinifera*, derived their PD resistance from *PdR1b* enriched with b42-26 resistance in the absence of *PdR2*, and with their PM resistance predominantly from *Ren4*. Additional testing is required to confirm these latter encouraging results.

**Table 8.** Counts of genotypes greenhouse screened in group 4h by test subgroup and PD rating category. PD rating categories as in Table 5.

Test Group	PD Rating Category				Group Total Tested
	-1	1	5	10	
2017 PD only Promising & Untested	1	8	4	7	20
2016-18 PD x PM Promising & Untested	20	22	10	7	59

A summary of the selections tested in Group 4j are shown in Table 9. In the PD only subgroup, selections that needed one or more confirmatory tests were screened to make a determination as to whether to include these in the next vineyard blocks. Of the 5 in the two most resistant categories, three were possible breeding resources and two were *PdR1* x *PdR2* possible candidates for FPS awaiting spring field evaluations. For the 2017-18 PD x PM crosses, 12 of 32 (~37%) were in the two most resistant categories. Of these, 11 were at the 93-94% *vinifera* level and one at the 97% *vinifera* level. All are receiving a confirmatory screen in 4k and this season field evaluations are underway. We are continually trying to understand the variability in the results of our greenhouse screen for any particular selection tested. As previously reported, we've explored irrigation volume, light, humidity and temperature as possible sources of variation. In this trial we again looked at irrigation with normal and double volumes for 8 resistant genotypes and, as previously, found that the only effect that mattered was genotype, not irrigation volume and there was no interaction between the two effects. We also looked at 50-56 copies of each of our 5 PD releases distributed evenly on 4 benches that varied in temperature and relative humidity (RH) across the greenhouse where they received uniform light and irrigation volumes. Table 10 summarizes the results for two time periods, the first 15 days post inoculation and again for the whole trial. It's clear for both periods that the mean Xf titer goes up as temperature goes up and relative humidity goes down. Although from these results we can't say which period is the most important, in previous studies we've found the best correlation with whole trial average temperature.



**Table 9.** Counts of genotypes greenhouse screened in group 4h by test subgroup and PD rating category. PD rating categories as in Table 5.

Test Subgroup	PD Rating Category				Group Total Tested
	-1	1	5	10	
2016-17 PD only	2	4	1	4	11
2017-18 PD x PM	14	6	10	2	32
Graft Saves Collection	9	1	3	1	14

**Table 10.** Average temperature and relative humidity for the first 15 and all 93 days of the trial with bench ELISA ln mean cfu/ml Xf.

GH76 Logger Bench	Whole trial Avg Temp F	Whole trial Avg RH, %	First 15 days Avg Temp F	First 15 days Avg RH, %	Bench ln mean cfu/ml
B12	80.6	49.2	78.7	56.8	10.5a
B03	82.8	44.9	81.6	51.5	11.1b
B06	83.7	45.4	84.2	50.1	11.5b
B09	85.0	44.1	85.8	47.6	12.0c

We sampled our last greenhouse screen (4k) on 5/20/2021 and ELISA testing is in process. With the first 12 plates (~31%) complete, the test looks to be of high severity and results are expected to be representative. Phenotypic scores were taken but we've found that the correlation between them and the more important ELISA results are sufficiently variable to prevent making conclusions about the ultimate PD resistance of an individual selection using them alone. Table 11 below summarizes the subgroups to be discussed. In addition, there were 11 transformed genotypes for our companion genetics project and our usual 8 reference genotypes. Of the 14 in the PD only subgroup, 5 are in the b40-14 *PdR1c* line. Based on the ELISA results so far, no more than 3 are likely to be in the two most resistant categories. The same looks to be the case for the 7 in the *PdR1 x PdR2* line. In the PD x PM subgroup, it looks that as many as a third may be highly resistant and they range from 93-97% *vinifera*. The *Run1.1+Run2.1* without known PD resistance subgroup was tested to see if putting back the two major PM loci known to reside in *M. rotundifolia* might have a positive impact on PD resistance as well. With at least one sample from 16 of the 22 genotypes tested, we can be fairly confident that this doesn't appear to be the case. An additional 92 selections from previous PDR generations were tested to document resistance for the future breeder. Once these results are finalized in the next couple of weeks, we'll rogue out from the vineyard all but the most resistant PD and PD x PM selections for final field evaluations.

**Table 11.** Subgroups greenhouse tested with quantities as part of group 4k in Table 4.

Test Subgroup	Group Total Tested
2017 PD only Promising & Untested	14
2017-18 PD x PM Promising & Untested	94
<i>Run1.1+Run2.1</i> w/o known PDR	22
Graft saves collection	92

In late May we planted a multi-vine trial at UCD of 7 promising PD x PM selections. Selections were based on earlier greenhouse screen results and field evaluations over the last two or more seasons. Table 12 gives details on the selections:

**Table 12a.** Parentage, PD and PM resistance type, percent *vinifera*, average PD rating (as in Table 5) and vigor for the UCD multi-vine trial selections planted this May.

Genotype	Female Name	Male Name	PDR Type	PM Resistance	% <i>Vinifera</i>	Ave PD R-rating	Times tested	Vigor
18320-035	14305-078	09330-07	<i>PdR1bxb42</i> -26	<i>Ren4</i>	93.0%	10.0	1	MH
18323-062	14305-078	09314-102	<i>PdR1bxb42</i> -26	<i>Ren4</i>	93.0%	5.3	3	MH
18323-065	14305-078	09314-102	<i>PdR1bxb42</i> -26	<i>Ren4</i>	93.0%	5.3	3	M
18323-135	14305-078	09314-102	<i>PdR1bxb42</i> -26	<i>Ren4</i>	93.0%	5.5	2	VH
18371-001	16703-007	16376-014	<i>PdR1bxb42</i> -26	<i>Run1</i>	93.6%	2.3	3	H
18371-015	16703-007	16376-014	<i>PdR1bxb42</i> -26	<i>Run1</i>	93.6%	7.5	2	H
18373-040	16703-007	09330-07	<i>PdR1b</i>	<i>Run1</i>	95.3%	3.0	2	H

**Table 12b.** Fruit and horticultural details for UCD multi-vine trial selections planted this May.  
WF is well filled.

Genotype	color	Field Berry Wt (g)	Field Cluster Size (g)	Productivity	Fruiting Habit	Budbreak	Cane color	Cluster Compact	Comments
18320-035	B	1.2	100	Mod	SF	mid-late	very red	WF	Fruity
18323-062	W	1.1	125	High	SF	Early	mostly green	WF	
18323-065	W	1.1	150	High	SF	Early	mostly green	WF	
18323-135	B	1.1	100	Mod	SF	mid-late	light red	WF	
18371-001	W	1.3	100	M-H	SF	Early	red	Compact	Sprawling
18371-015	B	1.1	150	High	SF	Early	red	WF	Sl wild tst
18373-040	B	1.1	150	High	SF?	Early	red	V. cmpt	

## CONCLUSIONS

We have made rapid progress breeding PD resistant winegrapes through aggressive vine training, marker-assisted selection, and our rapid greenhouse screen procedures. These practices allowed us to produce four backcross generations with elite *V. vinifera* winegrape cultivars in our first 10 years. We have screened through thousands of seedlings that were 97% *V. vinifera* with the *PdR1b* resistance gene from *V. arizonica* b43-17 and selected only the most promising. Routinely, we first selected for fruit and vine quality and then have moved the best to greenhouse testing, where only those with the highest resistance to *X. fastidiosa*, after multiple greenhouse tests, were advanced to multi-vine wine testing at Davis and in PD hot spots around California. The best of these have been planted in vineyards at 50 to 1,000 vine trials with enough fruit for commercial scale winemaking. We have sent 20 advanced scion selections to FPS over the past five winters to begin the certification and release process. Five of them are available from certified grape nurseries and their patents granted. Five PD resistant rootstocks based on *PdR1b* were also sent to FPS for certification. Over 500 seedlings from our PdR1 x PdR2 stacked line have been evaluated with the four most promising sent of FPS for certification and multi-vine trials established in Davis for small lot winemaking. Pierce's disease resistance from *V. shuttleworthii* and BD5-117 were also being pursued, but progress and effort was limited because their resistance is controlled by multiple genes without effective resistance markers. Stacking of PD and PM resistance is well underway and the selections sent to FPS before the end of June. Other forms of *V. arizonica* were studied and the resistance of some was genetically mapped for future efforts to combine multiple resistance sources and ensure durable resistance. Very small-scale wines from 94% and 97% *V. vinifera* *PdR1b* selections have been very good, and have been received well at tastings in the campus winery, at public tastings throughout California, Texas and Virginia.

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

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**Reporting Period:** The results reported here are from work conducted July 2019 to June 2021.

### ABSTRACT

This project identifies resistant germplasm and develops molecular tools to support the companion project “Breeding Pierce’s disease resistant winegrapes”. Over 400 accessions collected from Mexico and southwest USA were tested for Pierce’s disease (PD) resistance. Genetic maps and quantitative analysis were completed for a total of 15 accessions including b40-14, b41-13 and T03-16. The first two collected from Mexico and the latter collected from Texas. The QTL analysis identified PD resistance on the chromosome 14 that explained greater than 50% variation in three accessions. So far 13 accessions have been identified with strong PD resistance on chromosome (Ch) 14. The accession, b42-26, has small effect QTLs on four chromosomes, 8, 10, 14 and 17 with additive effect. Limited mapping strategy with phenotypic and genotypic data of Ch14 indicated that ANU67 is the only accession so far with PD resistance not linked with chr14. RNA sequencing was completed for a panel of six lines within the b40-14 genetic background. A pilot study to compare the stem anatomy between different grape species was initiated to gain a better understanding of the difference among species and PD resistant and susceptible accessions to determine if there is any correlation. 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 RGA14 (resistance gene analog) and 18 were sequenced verified and plants were transformed with each gene as well both together in the same line. Promising results were obtained with one RGA14 line with a better cane maturation index and lower ELISA readings. This finding agrees with results from sequencing of cDNA from b43-17, the original source of resistance, inoculated with *X. fastidiosa*, showing the amplification of fragments that comprise sequences identical to RGA14 but different from RGA18. Two St. George RGA18 lines did not show 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 2021. We are also using a reverse genetic approach using CRISPR-Cas9 systems on b43-17 and U0505-01 to knock out candidate genes to expedite the process of identifying resistant candidate genes.

## LAYPERSON SUMMARY

This project provides molecular genetic support to the PD resistant winegrape breeding program by providing mapping and marker development. It also identifies new sources of PD resistance, and studies the genetic diversity of the southwestern US and Mexican grape species and how they resist PD.

## INTRODUCTION

This project provided molecular support to the grape breeding project – “Breeding Pierce’s disease resistant winegrapes”. Previously, we identified a dominant form of PD resistance termed *PdRI* in the *V. arizonica/candicans* accession b43-17, which we mapped to chromosome 14 (Riaz et al. 2006; Riaz et al. 2008). Markers linked to *PdRI* were used to breed PD resistant grapes (Riaz et al. 2009). We have surveyed over 400 accessions of *Vitis* species growing in the southern US 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 (Riaz et al. 2020). 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 geographically and genetically diverse 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 (Ch) 14 in conjunction with greenhouse screen data to determine if the PD resistance in these 14 accessions is different from the previously identified *PdRI* locus (Riaz et al. 2018). Genetic mapping and QTL analysis for three accessions (b40-14, T03-16 and b41-13) with larger populations was completed and they had a major locus on chromosome 14. We initiated a project to identify potential differences in stem anatomy among the different grape species and their relationship with resistance / tolerance to the *Xylella fastidiosa* (the bacterial causal agent of PD). Previous studies have shown that xylem anatomy might 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 genes and promoters from that same species (Holmes et al. 2013). We are also utilizing a reverse genetic approach via the 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 *PdRIb* were verified and constructs were developed to test their function. Transformation experiments with the *PdRI* 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 a climatically controlled growth chamber environment and RNA was sequencing. Embryogenic callus cultures of *V. vinifera* cvs. Chardonnay and Thompson Seedless and *V. rupestris* St. George are being maintained to test the function of these 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, carry out genetic mapping of two new highly resistant lines b41-13 and T03-16 for use in stacking PD resistance genes.
2. Complete a physical map of the *PdR2* region from the b42-26 background 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, carry out genetic mapping of two new highly resistant lines b41-13 and T03-16 for use in stacking PD resistance genes**

We evaluated the genetic diversity of PD resistance in grape germplasm collected from the southwestern US and Mexico. A total of 326 accessions were genotyped and 266 were screened for disease resistance. In addition, 12 PD resistant hybrid cultivars developed from southeastern US grape species, were evaluated for PD resistance. We observed an east-west divide within the germplasm that was reflected in their PD resistance. Results of this study were published in PLOS One “Genetic analysis reveals an east-west divide within North American *Vitis* species that mirrors their resistance to Pierce’s disease”.

Earlier work published in 2018 identified to three accessions that needed detailed mapping work. Genetic mapping and QTL analysis was completed for b41-13, and T03-16. T03-16 collected from the Big Bend region of Texas, and b41-13 from Tamaulipas state in Mexico were good candidates for expanded use and analysis based on their strong resistance and simple inheritance of PD resistance.

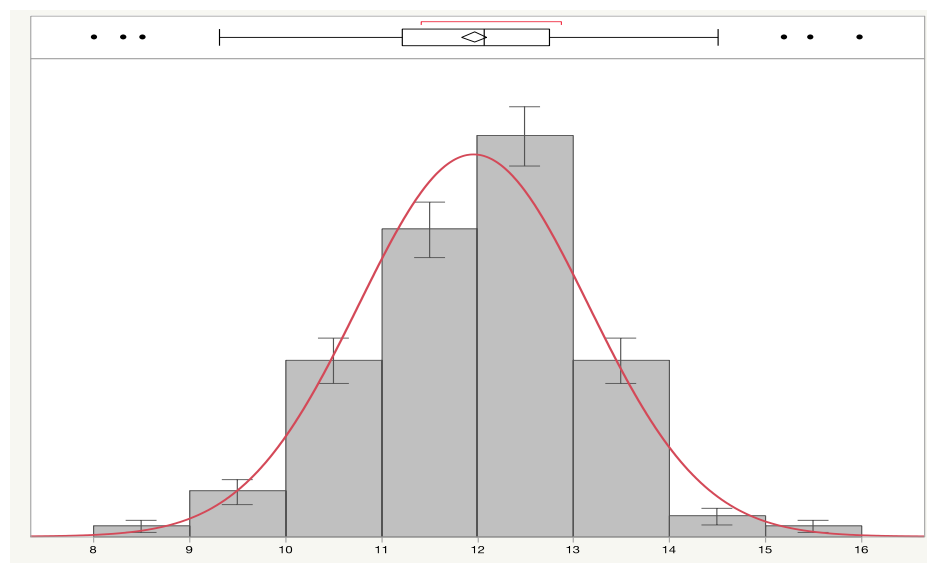
A total of 295 seedling plants from the b41-13 F1 population and 285 seedling plants from the T03-16 F1 population were established in the field. Table 1 provides information on population sizes, number of markers tested for polymorphism for b41-13 and T03-16, and number of markers that were completed on larger populations to develop genetic maps. A manuscript “Genetic mapping of Pierce’s disease resistance in germplasm collected from the Southwestern US and Mexico” is ready for submission in the American journal of Enology and Viticulture. 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 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 US and Northern Mexico indicates 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 US and Mexico.

**Table 1.** List of accessions used to characterize additional sources of PD resistance and their data. 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



**Figure 1.** Distribution of ELISA values for inoculated genotypes of the F1 05347 population with resistant accession b42-26 as the male parent.

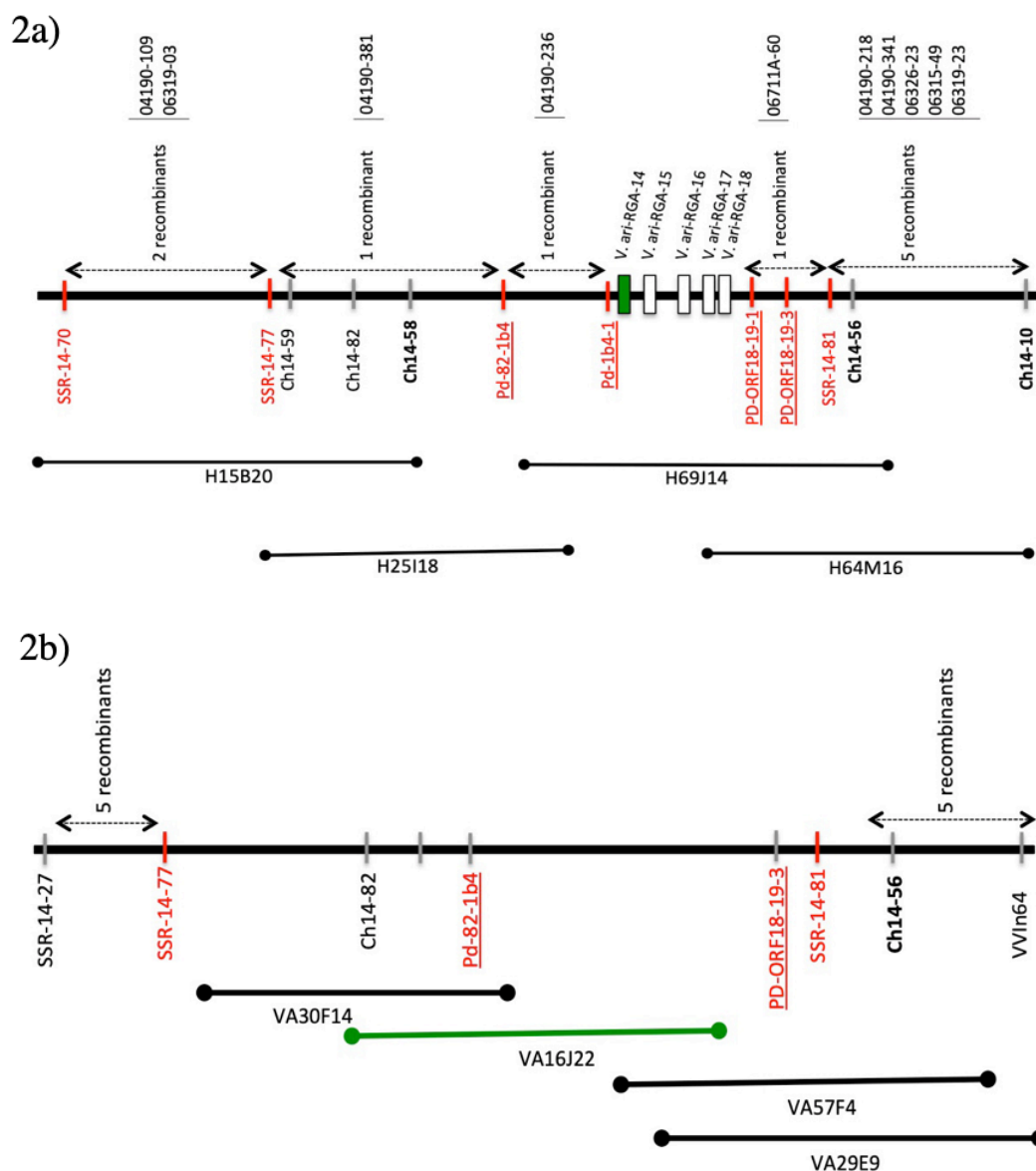
We also completed genetic mapping and QTL analysis of b42-26, which was collected from Loreto, Baja California. A genetic map was completed with 189 SSR markers covering 825 cM representing 18 grape chromosomes; no polymorphic markers were identified for chromosome 19. Results showed multiple small effect QTLs on chromosome 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/ml). Similar genomic regions were identified for the cane maturation index. Figure 1 shows the distribution of CFU/ml values for the tested 323 genotypes from the F1 population (see more details in previous reports). A manuscript titled "Identification of Pierce's disease resistance in a *V. girdiana* hybrid from Baja California Sur, Mexico" is ready for submission.

Previously we tested a small population with 27 F1 plants that have PD resistant accession, ANU67 in their background. Resistance was not identified on chromosome 14 with targeted mapping. Crosses were made to develop the 16361 population and 55 additional plants were tested. The greenhouse screen was completed, and analysis was carried out. The distribution of ELISA values showed that almost all plants are in the intermediate range (6 plants (10.80-12.0); 58 plants (12.1-14.0); 13 plants (>14.1). QTL analysis did not identify any affinity to Ch 14 indicating that potentially minor loci are present on other chromosomes. However, our goal was to identify major loci to accelerate breeding.

We initiated a small pilot study to compare the shoot anatomy of different grape species. Previous studies have shown that susceptible *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 included accessions that are known to have PD resistance on chromosome 14 to study these anatomical traits. Cuttings were made to produce plants for March-April 2020 inoculations – resistance evaluations are expected in December 2020. We were planning to use high-resolution computed tomography (HRCT), 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. However, due to the COVID19 pandemic, all microscopy and HRCT facilities are open only for the essential work with limited hours. Therefore, this pilot study was postponed until after Summer 2021. In Spring 2020, we extracted DNA and marker tested 320 seedling plants from different crosses for *PdR1* and to verify cross purity.

**Objective 2. Complete a physical map of the PdR2 region from the b42-26 background and carry out comparative sequence analysis with b43-17 (*PdR1a* and *b*) and b40-14 (*PdR1c*).**

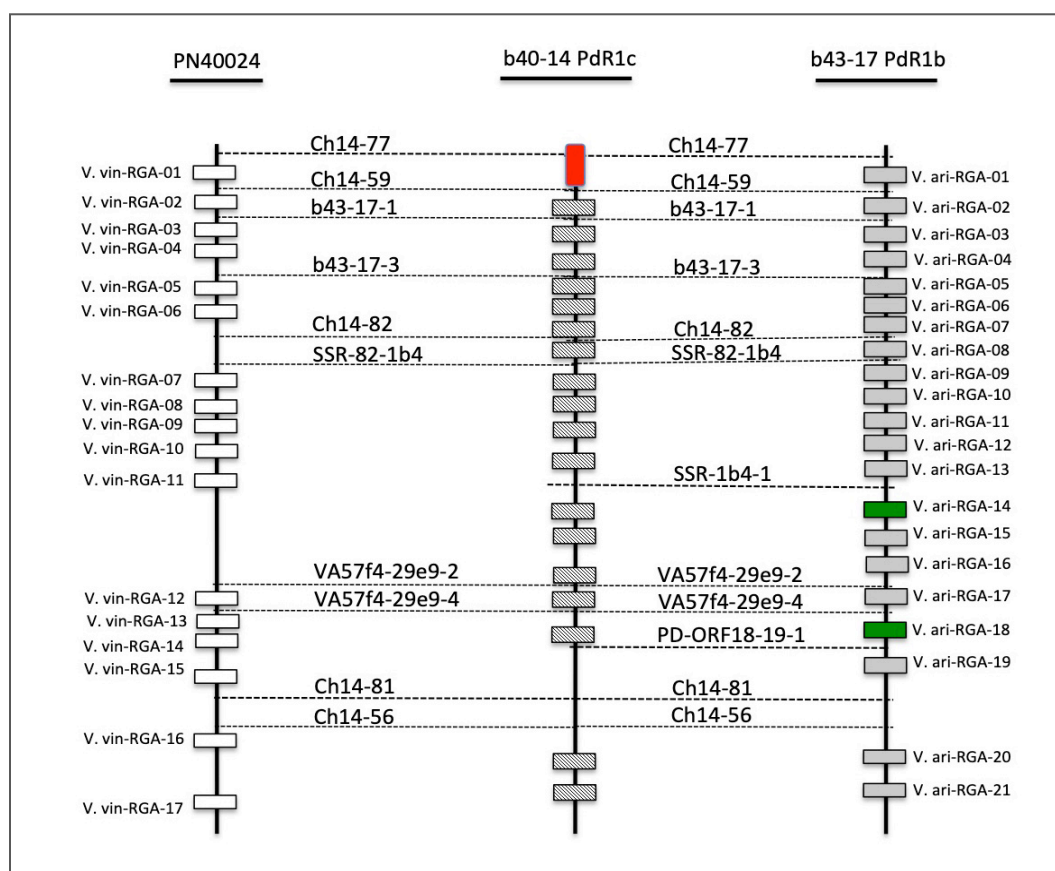
We completed the physical maps of the *PdR1a*, *PdR1b* and *PdR1c* loci from the b40-14 and b43-17 backgrounds. The physical map of *PdR1b* spans 604 Kb and includes the flanking markers Ch14-77 and Ch14-81 used for marker-assisted screening. The physical map of b40-14 (*PdR1c*) covers 426 Kb and consists of four overlapping BAC clones. BAC clone H43I23 (206 Kb) that contains *PdR1a* was also sequenced and it showed complete homology to the sequence of the *PdR1b* haplotype, indicating that the parents of b43-17 must be closely related. Figure 2 presents the physical maps of *PdR1b* and *PdR1c*.



**Figure 2.** Physical map of PD resistant accessions. 2a) b43-17 map, markers in bold were used as probes to screen the library, marker in red are SSR markers. Four underlined markers were developed from the b43-17 sequence, 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. The genetic window of the *PdR1b* locus is limited to 82 Kb between markers SSR-1b4-1 and PD-Orf18-19-1 – five ORFs in that region were associated with disease resistance (Fig. 3). A total of 21 ORFs were identified in the 604 Kb sequence of *PdR1b* in comparison to the 18 ORFs in the *PdR1c* sequence.

The PN40024 sequence was 230 Kb with many gaps, implying that some ORFs were unaccounted for. The Cabernet Sauvignon (CS) reference sequence within the flanking markers was 527 Kb long. All three sequences had an abundance of transposable elements dispersed within the resistance gene analogs (RGA). Genome sequence comparisons show sequence divergence in the region of the RGA and the sequences had greater than 90% homology in the genomic region flanking *PdR1*. 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. In collaboration with Dario Cantu, whole genome sequencing and assembly is completed for b40-14. The accessions b42-26, b43-46 and b43-17 are being sequenced. The results of sequence comparison in coming years will provide more comprehensive understanding of evolution of PD resistance.



**Figure 3.** Comparison of open reading frames (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 Ch1459 and Ch14-77 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. We are finalizing the manuscript documenting these physical maps.

**Objective 3. Employ RNA-seq to understand genome-wide transcriptional changes of the pathways regulated by defense-related genes in b40-14.**

We completed a time course experiment to monitor the bacterial level in control and inoculated resistant and susceptible plants. For this purpose, we used three resistant and three susceptible plants from the 07744 population with PD resistance from b40-14 (*PdR1c*). Plants were propagated and the experiment was carried out in growth chambers with temperature and humidity control to reduce the variance. The complete experiment was carried out twice to obtain four biological replicates for each genotype per time point (Table 2). 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 30cm above point of inoculation for ELISA screening. RNA from multi point extractions were pooled, and 120 libraries were developed. RNAseq libraries were prepared using the Illumina TruSeq RNA sample preparation kit v.2 (Illumina, CA, USA), following Illumina's protocol (Low-throughput protocol) and barcoded individually. Finally, libraries were evaluated for quantity and quality with the High Sensitivity chip in a Bioanalyzer 2100 (Agilent Technologies, CA) and Qubit (Invitrogen, CA). Libraries were sequenced at single-end 100-bp mode on an Illumina HiSeq400 sequencer (DNA technologies Core Facility, University of California).

**Table 2.** Lay out of the experimental plan in the controlled environment growth chambers

	Genotypes	Inoculated Replicates	Time Point	Uninoculated Replicates	Time Point	Number Plants
Experiment 1	6 (3R, 3S)	2	4	2 (3R, 3S)	1	60
Experiment 2	6 (3R, 3S)	2	4	2 (3R, 3S)	1	60
Total		4				120

Data analysis is divided into three steps: Quality Control and preprocessing, Mapping, and differential expression analysis. We have completed the steps of quality control and preprocessing (reads quality check by FASTQC, trimming and quality check). On average, each line had 15 million untrimmed reads, and after quality check and trimming, 12 million reads were available for the next stage of mapping to different genomes.

For the second step, we have the opportunity to map the transcriptomes to multiple reference whole genome sequences. The whole genome sequence of susceptible Cabernet Sauvignon (CS) and PN40024 are available and more recently b40-14 (resistance background of genotypes used for experiments) whole genome sequence was completed in the Cantu lab (UCD). The PN40024 was a mostly homozygous accession and represents one haplotype, but CS and b40-14 represent four haplotypes or genomes of their parents and mapping of the transcriptome to each haplotype is required to calculate number of mapped reads used for differential expression (DE) analysis of genes. In order to evaluate the quality of experiments and the level of variability among different replicates of the same line, we mapped the transcriptome data to the whole genomes of CS and b40-14. Over all 76-78% of the reads mapped to the b40-14 genome and 73-75% reads mapped to the CS genome.

Multidimensional scaling plot of transcriptomic data that mapped to the CS genome had a tight cluster of sequence data for each resistant and susceptible full sib genotype indicating that the experiments had minimum amount of variation and each resistant and susceptible genotype is unique in its transcriptome.

In the next phase, we are mapping the reads to fully assembled individual haplotypes of CS and b40-14 and differential gene expressions analysis will be carried out. We have also completed the physical map of *PdR1c* (reported in Objective 2), and the reads will be mapped to that sequence to identify potential candidate genes that are turned on upon infection.

#### **Objective 4. Cloning PD resistance 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 3 sequences, V.ari-RGA15, 16 and 17, are shorter and contain a large number of transposable elements (TE). 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 Final Report for CDFA Agreement Number 14-0137-SA and 17-0427-000-SA for details).

#### **Objective 5. Comparing the PD resistance of plants transformed with native vs. heterologous promoters.**

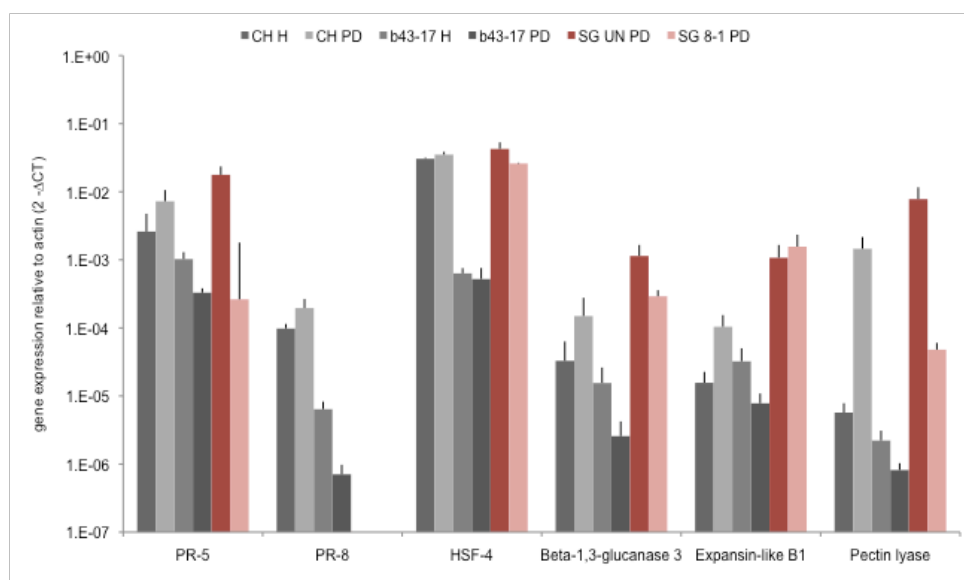
We have established *Agrobacterium* mediated transformation systems followed by regeneration of plants from embryogenic callus and meristematic bulks (Agüero et al. 2006, Xie et al. 2016). *Agrobacterium tumefaciens* strain EHA 105 pC32 was chemically transformed with pCLB2301NK-14 or pCLB2301NK-18 and subsequently used to transform embryogenic calli of *V. vinifera* cvs. Chardonnay (CH), Thompson Seedless (TS) and the rootstock *V. rupestris* St. George (SG). The evaluation of 44 transgenic lines of CH and TS (10-11 lines per genotype x 2 constructs) showed that all transgenic lines displayed disease symptoms, although with different degrees of intensity – TS being considerably more susceptible than CH. Although some lines exhibited reduced symptoms or lower bacteria concentrations, none reached the levels of the resistant biocontrols (See Final Report for CDFA Agreement Number 14-0137-SA and 17-0427-000-SA for details).

Plant regeneration from transgenic SG has been more challenging, however promising results were obtained with one RGA14 line. Cane Maturation Index Means of untransformed and transgenic were 4.9 and 1.7, respectively, while Leaf Scorching Index Means were 4.5 and 3. Shoot regrowth of the transgenics were cut back for sampling 12 wks. after inoculation. None of the untransformed resumed growth, all of the transgenics did. ELISA tests also produced significant differences between untransformed (410,000 cfu/ml) vs. transgenic (120,000 cfu/ml). This finding agrees with results from sequencing of cDNA from b43-17, the original source of resistance, inoculated with *X. fastidiosa*, showing the amplification of fragments that comprise sequences identical to RGA14, but different from RGA18. Expression analysis through qPCR confirmed RGA14 expression in transgenic SG. Furthermore, the expression of 6 genes known to be upregulated in infected *V. vinifera* Thompson Seedless (Zaini et al, 1918) was analyzed in

b43-17, Chardonnay and transgenic St George. We confirmed that the expression of these genes increases in infected *V. vinifera* while 5 out of 6 decreased in infected b43-17. The lower expression levels of thaumatin, beta-1-3 glucanase 3 and pectin lyase in SG 8-1 could be linked to the presence of the transgene (Figure 4).

Three RGA18 lines, inoculated in January, July and December 2019, did not display any tolerance. The rest of the SG-RGA14/18 lines were dwarf and grew very slowly, consequently more transformations were initiated in 2019. Currently, we have several new lines growing *in vitro*, 7 of which have been acclimated to the greenhouse after PCR testing for transgene detection. In addition, co-transformations with both pCLB2301NK-14 and pCLB2301NK-18 have produced lines of TS, CH and SG that are also in the micro plant step (Table 3), however one line, which has been positive for both genes, was acclimated for further testing.

Transformation of meristematic bulks of susceptible genotypes selected from the 04-191 population, which are 50% *vinifera*, 25% b43-17 and 25% *V. rupestris* A. de Serres are also being pursued. One of these genotypes, designated 29-07, produced one PCR positive line that did not display tolerance after *Xylella* inoculation this January (Table 3). Evaluating these lines plus additional transgenic SG could help to clarify the role of genetic background in *PdR1b* resistance.



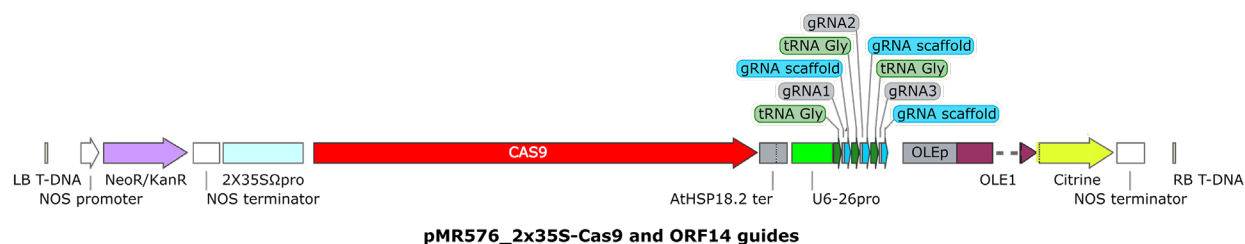
**Figure 4.** Gene expression of Thaumatin (PR-5 VIT\_18s0001g11930), EPC3-3 chitinase (PR-8 VIT\_05s0094g00200); heat shock factor 4 (HSF-4 VIT\_07s0031g00670); Beta1,3-glucanase 3 (VIT\_06s0061g00100); expansin-like B1 (VIT\_00s0309g00050) and pectin lyase (VIT\_14s0066g01060) in healthy (H) and Xf infected (PD) CH, b43-17, untransformed SG (SG UN) and transgenic SG 8-1. Expression of PR-8 in SG was undetermined. Bars represent SE.



**Table 3.** Transgenic lines obtained in 2019 and 2020

Genotype	No. lines Total	No. lines PCR tested*	No lines PCR positive	Lines tested against <i>Xf</i>
<b>pCLB2301NK-18</b>				
St. George	6	4	4	3
29-42	1	1	0	
<b>pCLB2301NK-14</b>				
St. George	8	8	8 (7**)	1
29-07	9	9	1	1
47-26	1	0		
47-50	1	0		
<b>Co-transformations (pCLB2301NK-18 + pCLB2301NK-14)</b>				
T. Seedless	5	4	1**	
Chardonnay	2	2	0	
St. George	2	2	0	

\* Lines not tested will be analyzed when they reach the adequate sample size  
 \*\* acclimated to greenhouse conditions and multiplied to be tested in the next cycle

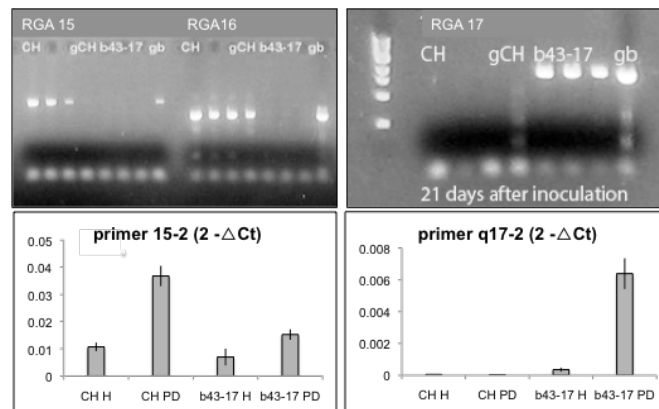
**Figure 5.** Schematic representation of the construct used to knockout RGA14.

In addition, a reverse genetic approach, CRISPR-Cas, is being used on b43-17 and U0505-01. b43-17 is the *PdR1b* source of resistance and U0505-01 is 87.5% *vinifera*, 6.25% *rupestris* and 6.25% b43-17. U0505-01 is also heterozygous for the *PdR1b* molecular markers and highly resistant to PD. We have produced meristematic bulks (MB) of b43-17 and embryogenic cultures (EC) of U0505-01 and completed the construction of a gRNA expression vector targeting RGA14 in collaboration with Dr. M. Ron, from Anne Britt's lab (Figure 5). Calli are currently growing in selection and/or germination medium; however, a first plant has regenerated from a U0505-01 callus. In this plant, DNA editing was verified by sequencing, which proves the efficacy of our gRNA.

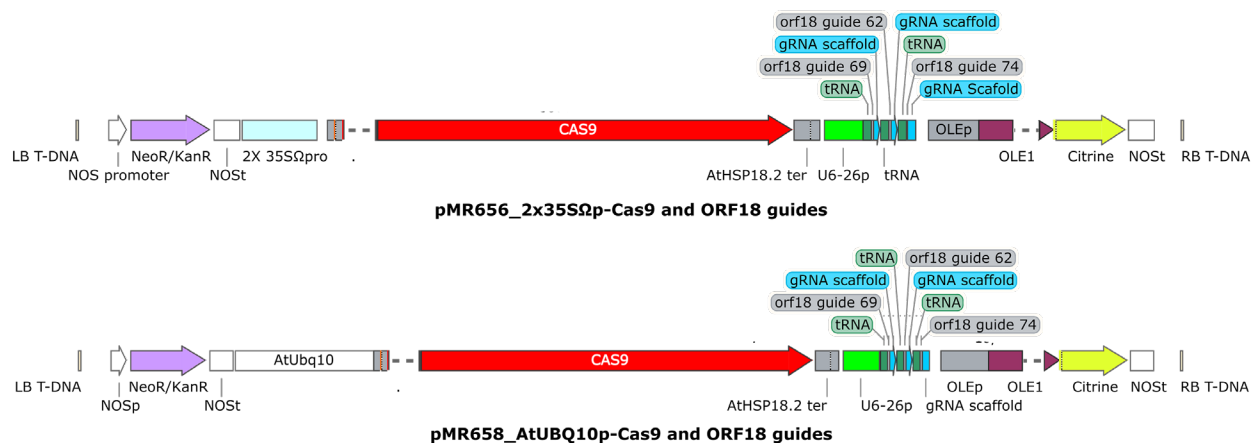
The 82 kb region flanked by the markers linked to the *PdR1b* locus was analyzed manually through repeat identification (<https://www.girinst.org/censor/index.php>), alignment (<https://www.ebi.ac.uk/Tools/msa/clustalo/>; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and blast against Pinot noir PN4002412 ([https://plants.ensembl.org/Vitis\\_vinifera/Info/Index](https://plants.ensembl.org/Vitis_vinifera/Info/Index)), Cabernet Sauvignon

(<https://cantulab.github.io/data.html>) and b40-14 (source of *PdR1c*). Results revealed that the RGA14 homolog is found in b40-14 but is disrupted in PN and CS; RGA15 homologs are almost identical in the other 3 genomes; RGA16 and 17 have longer versions in the other 3 genomes; and the promoter of RGA18 is disrupted in b43-17 and b40-14. These results confirm that RGA14 and RGA18 are the strongest *PdR1b* candidates, therefore, to be the target for knockout.

We have also tested b43-17 cDNA to analyze RGA15, 16 and 17 expression 21 days after inoculation. RGA15 and 16 did not amplify in b43-17, while RGA17 not only amplified in infected b43-17 but also had increased level of expression in comparison with healthy b43-17 and CH (Figure 6). Based on these results, we incorporated RGA17 in the knockout experiments. gRNA expression vectors targeting RGA17 and RGA18 were completed by Dr. Ron this June and transformation experiments started the first week of July. These experiments will compare two different promoters for CAS (Figure 7).



**Figure 6.** PCR amplification of RGA15, 16 and 17 (top) and RGA15 and 17 expression level of RGA15 and 17 relative to actin (bottom). gCH and gb mean genomic CH and b43-17, respectively.



**Figure 7.** Schematic representation of the constructs used to knock out RGA18. The same vectors are used in RGA17 constructs.

## CONCLUSIONS

We completed greenhouse screening, marker testing and QTL analysis of breeding populations from 13 new resistance sources. Genetic mapping and QTL analysis was completed for the b41-13 and T03-16 accessions. Results show that PD resistance resides on chromosome 14 at the same genomic position of *PdR1*. This brings the number of accessions with the *PdR1* locus to 13. 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 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 level of resistant and tolerant control plants. Promising results have been obtained with one line of SG RGA14. Testing of RGA14 and 18 in SG 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 this purpose, we have initiated CRISPR-Cas9 knock outs in resistant genotypes.

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**ISOLATION AND WHOLE GENOME SEQUENCING OF *CURTOBACTERIUM* SP.  
STRAIN TXMA1 FROM A GRAPEVINE SHOWING PD-LIKE SYMPTOMS IN  
TEXAS, U.S.A.**

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**Reporting period:** The results reported here are from work conducted from October 2020 to  
September 2021.

**ABSTRACT**

In September 2019, samples of grapevines (cultivar Blanc du Bois) with leaf marginal necrosis symptoms resembling those of Pierce's disease (PD) were collected in an experimental plot in Monte Alto, Texas. An initial investigation attempted to detect the presence of *Xylella fastidiosa*, the pathogen of PD. PCR detection of *X. fastidiosa* following a previously described procedure (Chen et al., 2015) was not successful. In vitro culture experiments using PW medium (Davis et al., 1981) found no *X. fastidiosa* colonies during the 30-day incubation period at 28 C. In contrast, yellow-pigmented colonies (YPCs) were observed within seven days. The YPCs were selected and triple-cloned. DNA fragments of 16S rRNA gene were amplified by PCR using an universal primer set (Weisburg et al., 1991) and sequenced by Sangers' method. BLASTn search using the 16S rRNA gene sequences against GenBank database showed sequence similarity (>96%) to multiple strains of *Curtobacterium* sp. The YPC strain was designated as *Curtobacterium* sp. strain TXMA1. DNA of strain TXMA1 was extracted and sequenced on a MinION device (Oxford Nanopore Technologies, UK). Basecalling was performed using Guppy v.5.0.11. Nanopore sequence reads were assembled using Flye v2.9 (Kolmogorov et al., 2019). The TXMA1 genome consists of one circular contig of 3,454,876 bp with GC content of 71.74% and ~110x coverage, 3,213 open reading frames (ORFs), 47 tRNA genes, four complete 16S-23S-5S rRNA operons, and three additional noncoding RNA genes. The whole-genome sequence has been deposited in GenBank under accession number CP083910. No plasmids were identified. Average nucleotide identity (ANI) to genome assemblies of 138 *Curtobacterium* strains deposited in GenBank was determined using fastANI v1.1 (Jain et al., 2018). TXMA1 has >98% ANI to two *C. oceanosedimentum* strains (NS263 and NS2359), and >95% ANI to *C. sp.* strain SGAir0471. The association of strain TXMA1 to PD-like symptoms of grapevine remains unclear. The availability of in vitro culture and whole genome sequence will facilitate future research in the bacterial characterization that would benefit grape disease management.

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## ISOLATION AND CHARACTERIZATION OF GRAPEVINE *TRICHODERMA* SPP. FOR BIOLOGICAL CONTROL OF VINEYARD DISEASES

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**Reporting period:** The results reported here are from work conducted from May 2020 to October 2021.

### ABSTRACT

Grapevine trunk diseases, including Pierce's disease, Bot canker, Eutypa dieback, and Esca, can substantially reduce vineyard yields over time. Pesticide applications are the current major control method, but these cannot be viewed as long-term solutions due to the build-up of resistance and environmental concerns. Thus, an alternative or complimentary strategy to pesticides for trunk disease control is needed. One such strategy is the use of biological control agents, which commonly includes the use of various fungi, such as *Trichoderma* spp.. Efforts have been made to isolate novel strains of *Trichoderma* spp. in Californian vineyards in order to identify and obtain those that are most adapted for survival in the unique growing regions of the state, ranging from Southern California, to the Central Coast, and throughout the Central Valley. These strains underwent or will undergo screening consisting of competitive co-plating with pathogens, testing spent media for toxin production, and *in planta* testing to observe capacity to reduce pathogen growth and possible mechanisms. Furthermore, select strains will have complete draft genomes obtained to discover molecular markers related to biocontrol functions. One strain has already been sequenced, with its mitogenome analyzed and published (Wallis et al. 2021) and assembly of a complete draft genome underway. Completion of this work should yield novel biocontrol *Trichoderma* spp. strains for reducing grapevine trunk diseases, potentially prolonging vineyard lifespans.

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Wallis, C.M., Chen, J., Perez de Leon, B. 2021. Mitochondrial genome resource of a grapevine strain of *Trichoderma harzianum*, a potential biological control agent for fungal canker diseases. PhytoFrontiers, <https://doi.org/10.1094/PHYTOFR-08-21-0052-A>

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### ACKNOWLEDGEMENTS

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# **Section 2:**

# **Glassy-winged Sharpshooter**

## CRISPR-MEDIATED GENOME MODIFICATION OF *HOMALODISCA VITRIPENNIS* FOR THE GENETIC CONTROL OF PIERCE'S DISEASE

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**Reporting Period:** The results reported here are from work conducted July 2020 to October 2021.

### ABSTRACT

This project will establish genome editing of Hemiptera pests as a viable and cost-effective strategy for their sustainable, environmentally-friendly insect control. We focus on the glassy-winged sharpshooter (GWSS, *Homalodisca vitripennis*). GWSS is the vector of the bacterial pathogen *Xylella fastidiosa* (XF), the pathological agent of Pierce's disease (PD) of grapes and other fruit, nut and ornamental crop species in California. The impetus for this project comes from four sources: (1) The ongoing need to develop new feasible, fast-acting strategies for the control of GWSS in California; (2) The revolutionary impact that the implementation of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) endonucleases has had on applied genetics in the past five years<sup>1-3</sup>; (3) Our in-house whole genome and transcriptome GWSS next-generation sequencing (NGS) and nanopore sequencing platforms; and (4) We have achieved high frequency CRISPR/Cas9-mediated genome editing in GWSS using insect embryo microinjection techniques developed in our laboratories and have achieved high rates of mutagenesis targeting two genes involved in eye pigmentation, *white* and *cinnabar*. During this funding period, we demonstrated the nature of the mutations in *white* and *cinnabar* and have built on our foundational studies and established stable genetic lines of both mutant strains, now extending to the 4<sup>th</sup> and 5<sup>th</sup> generations. We used reciprocal pair matings to establish that both genes are located on the autosomes rather than the X (sex) chromosome. We then targeted the *cinnabar* gene using two approaches to achieve knock-in mutagenesis (e.g., gene insertion into the GWSS genome). Using CRISPaint technology, we were able to direct integration of a genetic marker gene sequence into the *cinnabar* locus. Using homology-directed repair and oligonucleotides containing the wild-type *white* and *cinnabar* sequences, we were able to achieve reversion at both loci at relatively high frequencies, marking the first time knock-in mutagenesis has been achieved in Hemiptera. In addition, we have leveraged our new whole genome and transcriptome sequencing of GWSS to identify additional candidate genes for CRISPR-mediated genome editing to facilitate our future gene insertion strategies. With our technical breakthrough of high-frequency editing of GWSS, we are setting the foundations to generate and test, for the first time, genetic control strategies in this important pest insect species of Californian agriculture.

## LAYPERSON SUMMARY

Genetic-based control strategies for pest insects have been severely constrained by the ability to alter an insect's genome at precise locations. This hurdle has been overcome by the development of CRISPR-based technology when there is a physical means for delivering this technology into an insect's genome. We have solved this issue for the glassy-winged sharpshooter (GWSS). We will use a CRISPR-based technology in a proof-of-concept strategy to generate strains of sharpshooter that break the transmission cycle of Pierce's Disease, thereby reducing, or even eliminating, the financial impact of this pest to Californian agriculture. Our ability to develop genome-edited GWSS strains is an indication of the impact that CRISPR-based technology has on modern genetics and novel genetic control mechanisms for insect pests, such as GWSS. We have characterized *white* eye-color mutants and found *white* mutants display mosaic white and orange eyes and these mutations are inherited for a minimum of five generations. We identified a second eye-color gene *cinnabar* and these mutants have brilliant orange eyes or eyes with orange striations. We have shown that these mutations are inherited for multiple generations. Using crosses with wild-type GWSS, we have shown that the *white* and *cinnabar* genes do not reside on the X (sex) chromosome. Finally, we have shown that it is possible to insert a gene into the GWSS genome using two distinct CRISPR-based technologies. With our technical breakthrough of high-frequency editing of GWSS, we are setting the foundations to generate and test, for the first time, genetic control strategies in this important pest insect species of Californian agriculture.

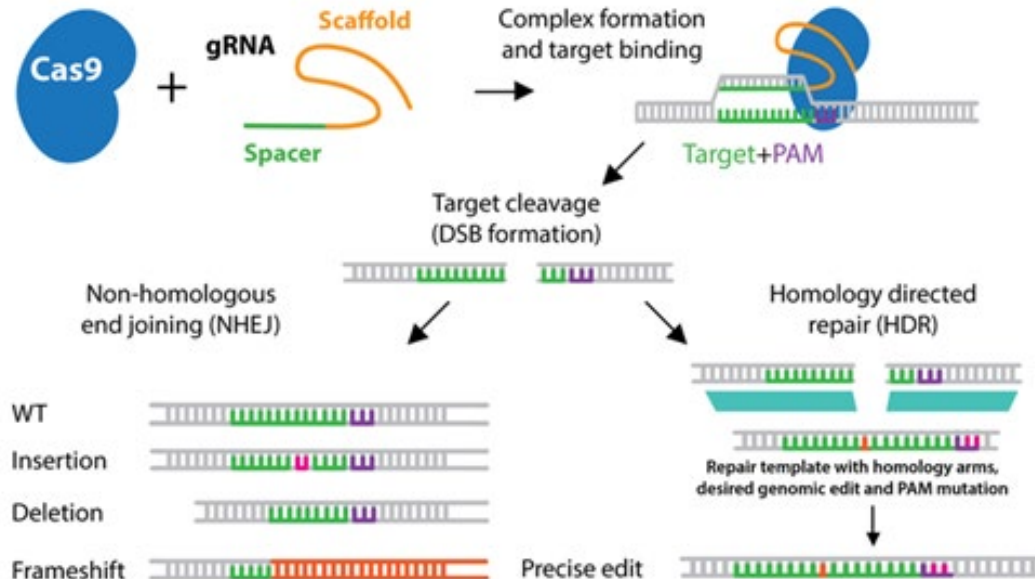
## INTRODUCTION

Our objective is to develop and deploy CRISPR-based technologies in GWSS (Figure 1). As a proof-of-concept, we propose to generate genetic strains of GWSS that express a fluorescent protein at a non-lethal target locus (a "knock-in" mutation). The development of efficient methods to create knock-in GWSS lines will provide the genome insertion site to express genes that have the potential to break the *Xylella fastidiosa* transmission cycle. *X. fastidiosa* (XF) binding to the GWSS foregut is mediated by the bacterium's afimbrial adhesins<sup>4</sup> and by XF's lipopolysaccharide O antigen<sup>5</sup>. In our proof-of-concept experiments, we will express segments of two chitin-binding proteins in GWSS. These peptides should be direct binding competitors with XF. The expression of an afimbrial adhesin and other chitin-binding proteins should saturate XF-binding sites and prevent XF binding to the foregut. XF's residence in the foregut is essential for multiplication of this pathogen, its persistence and transmission to its plant host. By interfering with the ability of XF to reside within the foregut, we should be able to break the XF transmission cycle.

To accomplish these goals, we are using CRISPR/Cas9 technology to generate genome-edited GWSS to develop GWSS strains that cease to transmit the causal agent of PD. We are able to make eye-color mutations at high frequencies at two target loci using CRISPR/Cas9 technologies. A short introduction to these technologies is provided in Figure 1. Two different types of mutations (knock-out vs. knock-in) arise from the how the double-stranded DNA break (DSB), which is generated by the Cas9 nuclease near a PAM (Protospacer Adjacent Motif) site, is repaired within GWSS cells (Figure 1). Knock-out mutations (or loss-of-function mutations) are generated by the more frequent mechanism of repair, which is called the non-homologous end-joining pathway (NHEJ). This pathway generates an array of small and large deletions,

additions and base-pair changes centered around the site of the original DSB (immediately upstream from the PAM site adjacent to the gRNA).

Knock-in mutations will allow us to insert a novel gene construct into GWSS to test for its ability to control the transmission of XF. Knock-in mutants can be created with the less frequent mechanism of DSB repair called homology-directed repair (HDR) (Figure 1). HDR also uses sgRNAs and Cas9 and results in the insertion of new DNA sequences in a target gene at the DSB. While this can result in loss of function to the target gene, the insertion of new sequences by HDR also creates gain-of-function mutations. An alternative approach, called CRISPaint, uses non-homologous-end-joining DSB repair to insert gain-of-function gene sequences into target sites and can do so at a higher frequency than HDR-mediated repair.



**Figure 1. Overview of CRISPR/Cas technologies for making knock-out and knock-in mutants.** To make knock-out mutants, we microinject Cas9 and gene-specific sgRNA. Cas9 endonuclease is directed to specific sites (PAM sites) in the GWSS genome by the sequence-specific guide RNAs (sgRNAs). Cas9 makes a double-stranded DNA break (DSB) near the PAM site. If the break is repaired by the NHEJ system, mutations occur near the PAM site (left). To make a knock-in mutation, we inject Cas9, gene-specific gRNAs and the gene of interest that has “arms” with sequence identity to the genome insertion site. The less efficient HDR system repairs the DSB and the gene is inserted into the GWSS genome (right). (Figure from Addgene)

## OBJECTIVES

### **Objective 1: Generation of Gain-of-Function Mutants and Target Site Identification for Disruption of *Xylella* Transmission.**

Given our exceptionally high frequency for the *white* and *cinnabar* knock-out mutations, we expected rapid success for generating knock-in mutants. In order to generate knock-in mutations in GWSS at optimal target sites, we first set the foundations for the project with the following four goals:

**Goal 1:** Demonstration of inheritance of the *white* knock-out mutants.

**Goal 2:** Identification of the target sites for high frequency creation of knock-in mutants

**Goal 3:** Identification of constitutive promoters and fluorescent marker genes for the GWSS knock-in technologies

**Goal 4:** Creation of GWSS knock-in mutants in the *white* gene (Proof-of-concept).

### **Objective 2: Generation of genetic strains of GWSS expressing XF chitin-binding proteins in their foregut to break the XF transmission cycle.**

We propose that by engineering GWSS to secrete XF afimbrial adhesins or PD1764, we can substantially reduce the XF population within GWSS. Lower levels of XF will decrease the probability of its persistence in the foregut and transmission during sharpshooter feeding on its host plants. To generate these genetic strains that block transmission of XF, we have three goals in this objective:

**Goal 1:** Identification of small chitin-binding peptides that block XF binding to chitin, foregut extracts and transmission to host plants.

**Goal 2:** Identification and utilization of gene promoters that are active in the GWSS foregut epidermis to direct expression of proteins in the GWSS cuticle

**Goal 3:** Testing of the ability of chitin-binding proteins expressed from the GWSS foregut to block XF transmission.

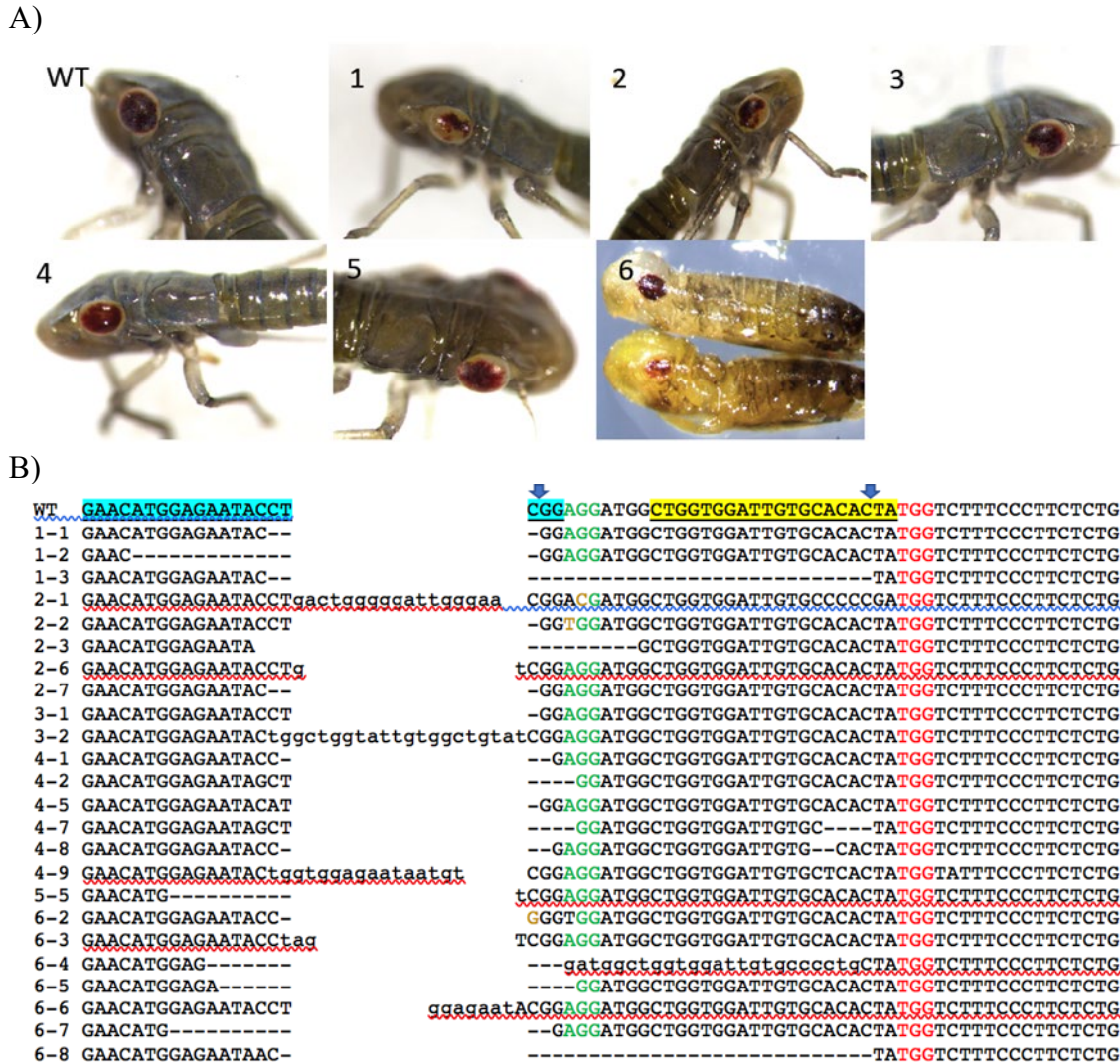
In late Fall quarter of 2020, we recruited a Laboratory technician to the project. In the Winter/Spring quarters of 2021, we appointed one postdoctoral scholar (70% time) to assist with the sequencing and bioinformatics of the project and then our full-time postdoctoral scholar to assist with the molecular genetics and biochemistry of the project.

We have made very substantial progress on Objective 1 and have completed three of the four goals in year 1 and have made substantial progress on the fourth goal. Our molecular/biochemistry postdoctoral scholar, who is essential for all aspects of this program, arrived in spring of 2021 (due to delays in visa approvals and COVID quarantine requirements). When they arrived they focused entirely on Objective 1. As Objective 2 is dependent on Objective 1, we have not made progress on Objective 2's goals.

## RESULTS AND DISCUSSION

### **Objective 1: Generation of gain-of-function mutants and target site identification for disruption of *Xylella* transmission**

To aide with this objective, we first established GWSS rearing conditions to avoid the winter reproductive diapause. This provides a continuous pipeline of GWSS embryos for our experiments. We also demonstrated the ability to perform pair and pool matings throughout the year.



**Figure 2. CRISPR/Cas9 mutagenesis of the GWSS *white* gene. Panel A:** Phenotypes of a wild-type (WT) and six CRISPR/Cas9 *white* mutants of GWSS (#1-6) with mosaic eye color. **Panel B:** Sequence of the *white* gene in WT GWSS and the six GWSS mutants (#1 to #6). The region surrounding the two gRNAs is shown. With the exception of insect #5, multiple mutations were recovered from each nymph. The first number of each sequence denotes the nymph number (Panel A) that the sequence was obtained from. PAM sites are colored green and red. The two 20-nt gRNA sites are shaded in blue and yellow with the Cas9 cleavage sites within them indicated by arrows. Deletions are denoted by dashes, additions by lower case letters, and base-pair changes by brown uppercase letters.

**Goal 1. Demonstration of inheritance of the *white* knock-out mutants.**

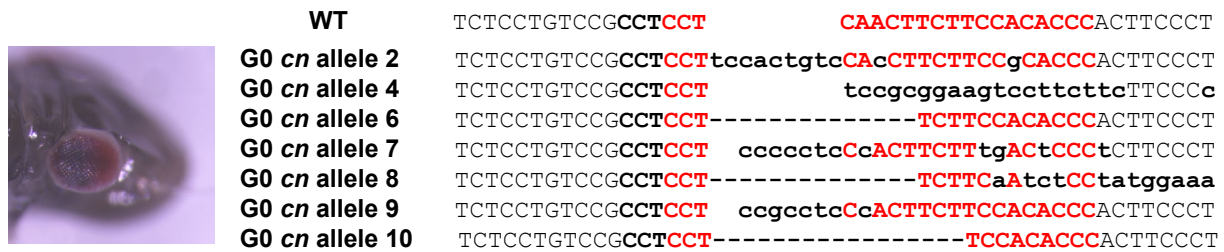
Prior to the funding of this grant, we had preliminary evidence that we could make *white* (*w*) knock-out mutants. This goal was expanded to include knock-out mutagenesis of the *cinnabar* (*cn*) gene since we observed a measure of unviability of some *w* mutants. We established that based on eye-color phenotypes in the G0 generation (the insects that emerged from injected embryos), CRISPR-mediated editing occurred at high frequencies in GWSS with mutagenesis at the *w* locus occurring between 61-80% (Figure 2) and mutagenesis at *cn* occurring at 59% (Figures 3 and 4). We then:

1. Created four *w* lines that inherited the CRISPR-generated mutations to the G1 generation.
2. Maintained one *w* line (Line WhA) for the next five generations, thereby showing mutant line stability.
3. Demonstrated that pair- and pooled-matings are productive in GWSS.
4. Used amplicon DNA sequencing to identify all mutated *w* sequences in four original lines and used conventional DNA sequencing to follow the inheritance of three *w* alleles through the subsequent three generations (G1-G3). This line is now in the G5 generation.
5. Created a *cn* mutant line (CnA) in which two *cn* alleles were segregating with this line. This line is now in the fourth generation (G4).
6. Used reciprocal pair mating to map the *white* and *cinnabar* loci to the autosomes and not the X (sex) chromosome.
7. Discovered that the red color or sections and veins of the GWSS forewing are due to the presence of the pteridine pigment.

**Goal 2. Identification of the target sites for creation of knock-in mutants (gain-of-function mutants).**

Our observations of our *w* and *cn* genetic lines through five and four generations, respectively, of breeding anecdotally suggested that the *w* genotype may have significant behavioral changes, some of which result in some detrimental phenotypes. We are investigating the pleiotropy of these *w* mutations to determine their full effect but our observations are consistent with other studies that show that mutations in the *w* gene can be deleterious<sup>6</sup>. Therefore, while *w* has proven to be an excellent genetic marker for our proof-of-principle experiments, we cannot use the *w* gene for generating CRISPR/Cas9-mediated gain-of-function (“knock-in”) mutations, as we originally proposed. Therefore, we have focused our current knock-in strategies on the *cn* gene. However, using our recently published new GWSS genome annotation<sup>7</sup>, we performed bioinformatic analyses to identify 12 other genes that could serve as suitable genetic markers for knock-in and knock-out mutagenesis in GWSS (Table 1). This is a resource we may explore in year 2 of the grant if needed.





**Figure 3.** Sequence of the cinnabar target region in a G0 cinnabar mutant. One of the first cinnabar mutant (Male G0 #4) with its subtle mosaic phenotype is shown. Its seven mutant alleles are aligned with the wild-type cinnabar region. The gDNA region is shown in red bold font. Deletions are indicated as bold dashes. The PAM site is indicated in black bold font. Insertions are indicated as bold lower-case letters. Spaces were used to align the mutant and wild-type sequences.

G0 *cinnabar* mutant male  
Mosaic-eye phenotype



G1 *cinnabar* mutant male  
orange-eye phenotype



**Figure 4. Phenotypes of G0 and G1 *cinnabar* mutants.** Examples of the mosaic eye and orange eye phenotypes seen in the G0 and G1 generations.

**Table 1.** Candidate genes for use in knock-in (gain-of-function) experiments<sup>7</sup>

Gene name	Gene ID	Category	Scaffold	Start	Stop	Strand
<i>scarlet</i>	J6590_063422	Eye color marker	scaffold_912	460005	478693	-
<i>brown</i>	J6590_023567	Eye color marker	scaffold_152	394070	408336	+
<i>white</i>	J6590_025764	Eye color marker	scaffold_175	597341	620522	-
<i>punch</i>	J6590_079319	Eye color marker	scaffold_1776	27869	36915	-
<i>purple</i>	J6590_010106	Eye color marker	scaffold_46	401764	405463	+
<i>cinnabar</i>	J6590_030756	Eye color marker	scaffold_237	304451	312309	+
<i>rosy</i>	J6590_021669	Eye color marker	scaffold_136	1442727	1477619	-
<i>sepia</i>	J6590_059208	Eye color marker	scaffold_778	21807	32946	+
<i>vermillion</i>	J6590_086284	Eye color marker	scaffold_2636	59559	69160	+
<i>ebony</i>	J6590_055645	Body color marker	scaffold_679	520340	534402	-
<i>curly</i>	J6590_045190	Wing shape marker	scaffold_458	853125	882297	+
<i>miniature</i>	J6590_040001	Wing shape marker	scaffold_363	1027789	1033916	+
<i>vestigial</i>	J6590_019057	Wing shape marker	scaffold_113	220253	229632	+
<i>bar</i>	J6590_017333	Eye shape marker	scaffold_97	1592445	1593704	-

For a Pierce's Disease genetic control strategy to work effectively, the gain-of-function mutants produced in this project should: (1) not impact GWSS fitness and (2) be easily identified. Therefore, the genes targeted with the CRISPR-mutagenesis strategies must be chosen carefully. Several potential target sites in the GWSS genome are needed for the placement of the knock-in gene cassette; as efficiencies of CRISPR-mutagenesis may vary depending on the target gene and certain phenotypes will be easier to identify. However, all evidence to date suggests that *cinnabar* meets these demands.

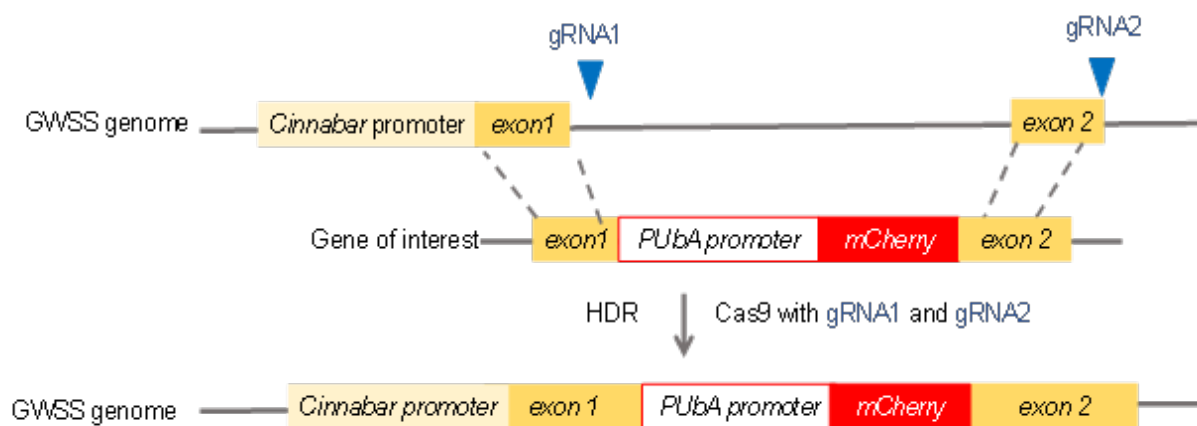
### **Goal 3. Identification of constitutive promoters and fluorescent marker genes for the GWSS knock-in technologies.**

Knock-in mutations are generated by homology-directed repair (Figure 5) or a new technology called CRISPaint (Figure 6)<sup>8,9</sup>. Based on frequencies of knock-ins in other organisms and the

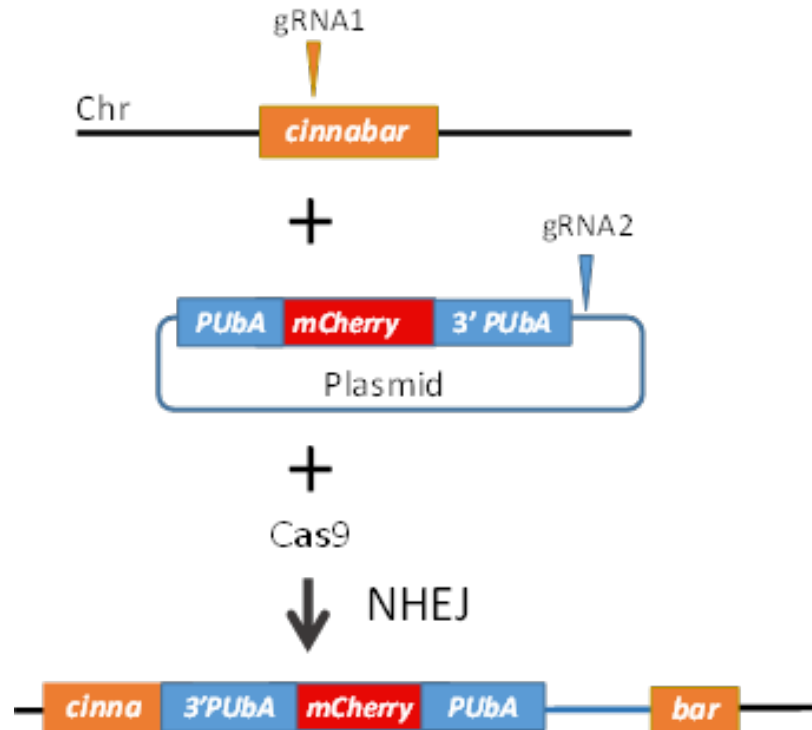
high frequency of knock-out mutant generation in GWSS, we expect that we will get one knock-in mutant in every 30 embryo injections. As we can easily inject 100s of GWSS embryos in a single sitting, we should easily identify these GWSS mutants.

To easily identify knock-in mutants, we will make chimeric genes that express a protein that fluoresces when the GWSS is exposed to a certain wavelength of light. The structure of a typical chimeric gene is shown in Figures 5 and 6. We determined if GWSS embryos, nymphs and adults naturally fluoresce. We see little red fluorescence and low levels of green and blue fluorescence. Therefore, we expect that proteins that fluoresce in the red spectrum offer the best opportunities for detection. However, it is possible that highly visible blue and green fluorescent proteins will be useful in nymphs and adults.

To make chimeric genes, we need gene regulatory sequences (promoters and 3'untranslated sequences) to drive the expression of the fluorescent proteins. We have successfully amplified PCR fragments for the GWSS *actin1A*, *actinB*, *actinC*, and *polyubiquitin (PUBA)* promoters and 3'-untranslated sequences. Sequence verification is currently underway. We have also identified other germ-line and constitutive promoter and regulatory sequences from GWSS. These are currently being sequence verified and chimeric genes will be assembled soon.



**Figure 5. Chimeric genes for making GWSS knock-out mutants.** We will make a *cinnabar* gene knock-out mutant by inserting a gene into *cinnabar* (orange-yellow), Cas9, two gRNAs, and the gene-of-interest is injected into GWSS embryos. Cas9 makes double-stranded breaks at PAM sites (blue arrows) and HDR recombines the gene-of-interest into the GWSS genome using the *cinnabar* gene arms (orange-yellow). The chimeric genes to be inserted fuses the polyubiquitin (*PUBA*) promoter (white box) with the *mCherry* reporter sequence (red box). If we are successful, we will have red fluorescent GWSS.



**Figure 6. A CRISPaint strategy to knock out *cinnabar*.** Cas9, gRNA1 for *cinnabar*, and gRNA2 for CRISPaint donor vector, the mCherry plasmid will be injected into 2.5-hr embryos. NHEJ should integrate the *PUbA*:*mcherry* cassette into *cinnabar*. Red fluorescence is evidence of integration.

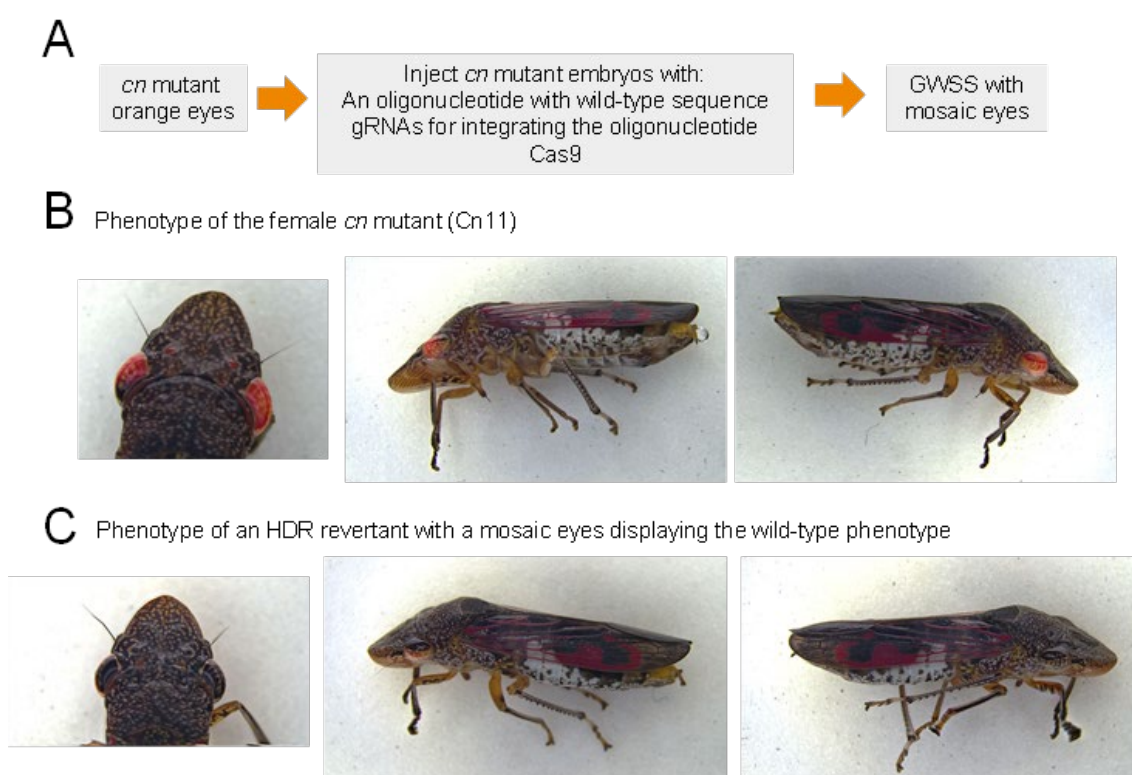
From a different independently funded project focusing on whitefly, we have chimeric genes with regulatory sequences from the whitefly (*Bemisia tabaci*). We will also determine if the whitefly *actin 1*, *actin 2* and *PUbA* are active in GWSS. If one or more are active, we can use the whitefly promoters for the GWSS experiments. This could save us time by eliminating the need to make gene constructs with the GWSS regulatory sequences, as the whitefly genes are ready to deploy.

To rapidly screen chimeric genes for activity in GWSS, we will directly inject these genes (as plasmids) into GWSS embryos. We will examine the embryos at 2, 4, 6, 8, 16, and 24 hr post-injection with fluorescence microscopy. Genes that are actively expressed in GWSS will emit light of a specific color. For example, we will identify red (dsRed or mCherry), blue (CFP), green (GFP), or orange (mOrange) GWSS if a promoter is active. We have performed preliminary experiments with two whitefly gene constructs to date.

#### Goal 4: Creation of GWSS knock-in mutants in the *white* gene (Proof-of-concept).

We proposed to use both HDR (Figure 5) and CRISPaint (Figure 6) to determine if we can create knock-in mutants (gain-of-function) in GWSS. We have preliminary data suggesting that both strategies will be successful.

We have begun to test whitefly chimeric genes for function in GWSS; these data are preliminary but tantalizing. We used the CRISPaint strategy (Figure 6) to introduce CRISPaint plasmids that have a whitefly *PUBA promoter:mCherry reporter* or *PUBA promoter:GreenLantern reporter*. In these experiments the chimeric genes were integrated into the *cinnabar* gene using gRNAs and Cas9.



**Figure 7. A putative HDR gain-of-function (knock-in) mutant. Panel A.** The scheme for the proof-of-concept knock-in experiment. A female *cn* mutant is mated with a *cn* males within the *cn* mutant colony. Her egg masses are injected with an oligonucleotide (ODN2), two *cn* gRNAs, and Cas9. Eggs hatch and nymphs with mosaic eyes (predominantly wild-type with patches of orange), which are putative revertants, are allowed to develop to adulthood. **Panel B.** The phenotype of the Cn11 mutant female adult, whose eggs were injected. Notice the orange eyes with orange striations. The left eye (middle panel) has a small patch of brown in the upper right corner. **Panel C:** The phenotype of a representative revertant with predominantly wild-type eye color. The right eye (right panel) has the wild-type phenotype, while the left eye (middle panel) is wild-type on the top half of the eye and a *cn* phenotype on the lower half of the eye.

We have recovered putative gain-of-function *cinnabar* mutants from these injections and are analyzing them for evidence of integration of the plasmid into the *cinnabar* locus. Based on our preliminary data assessing the frequency of *cn* phenotypes from a small number of mutants, CRISPaint-mediated gene integration occurs at relatively high frequencies.

We also have initiated experiments to integrate genes *via* HDR-mediated repair. We changed our initial proposed strategy. In these experiments we have used *w* and *cn* mutants that we generated in **Goal 1**. We decided to integrate a wild-type gene *via* HDR into the eye-color mutants to restore the wild-type phenotype. To this end, we designed oligonucleotide sequences that would restore the wild-type genotype at each locus. We should produce G0 late embryos, nymphs and adults with eye colors that were mosaic for the mutant and wild-type phenotypes. Preliminary results are very encouraging with these expected phenotypes recovered from both experiments with frequencies between 5.3-13.8% (Figure 7).

Our initial data were presented as a poster at the on-line annual meeting of the Entomology Society of America in November 2020 by Dr. Redak. A manuscript describing the improved genome sequence of GWSS was submitted and published in July 2021 in the journal G3, which is a publication of the Genetics Society of America.

Ettinger CL, Byrne FJ, Collin MA, Carter-House D, Walling LL, Atkinson, PW. Redak RA, Stajich JE (2021) Improved draft reference genome for the Glassy-winged Sharpshooter (*Homalodisca vitripennis*), a vector for Pierce's disease.

G3. <https://doi.org/10.1093/g3journal/jkab255> Published July 20, 2021.

Our current data are being presented as a talk (live, in person) at the annual meeting of the Entomology Society of America in November 2021 by Dr. Pacheco. The first manuscript on GWSS gene editing at the *white* and *cinnabar* loci will be submitted for review for publication in November, 2021:

De Souza Pacheco I, Doss A-LA, Vindiola BG, Brown D, Ettinger CE, Stajich JE, Redak RA, Walling LL, Atkinson PW Efficient CRISPR/Cas9-mediated genome modification of the glass-winged sharpshooter *Homalodisca vitripennis* (Germar). In preparation.

## CONCLUSIONS

In order to develop and establish genetic control strategies for GWSS, we first needed to extend CRISPR/Cas9 technologies into this hemipteran pest to establish a reliable, robust genetic platform. During year one, we have improved GWSS rearing in the lab (breaking diapause), have assembled and improved its genome along with its transcriptome<sup>7</sup>, have developed a method for introducing genetic material into it, have made transgenic GWSS with mutations that are inherited for multiple generations, have knocked-down and knocked-out genes (De Souza Pacheco et al 2021, in preparation), and have phenotypic evidence for precise knock-in mutagenesis. With these achievements, we have propelled GWSS from being an orphan pest with no genetic tools to a near-model genetic organism. We have already fulfilled seven of the ten steps that were recently suggested as being necessary to establish an organism as a genetic model<sup>10</sup>. While our gene-insertion data are new and preliminary, we are close to embarking on Goals 2 and 3 of our project that test the ability of adhesion proteins to block GWSS transmission of *X. fastidiosa*. Our research these past 16 months has established a genetic

platform in GWSS which may well become a model insect for the Hemiptera with a direct benefit to California agriculture.

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**GWSS PERFORM FEWER XF-INOCULATING BEHAVIORS ON RESISTANT *VITIS CHAMPINII* GRAPEVINES THAN ON SUSCEPTIBLE *VITIS VINIFERA* CV. 'CHARDONNAY'**

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**Reporting Period:** The results reported here are from work conducted from October 2020 to September 2021.

**ABSTRACT**

Since 2001, an effort has been made by the University of California-Davis, in cooperation with the USDA Agricultural Research Service, to breed Pierce's disease (PD)-resistant grapevines. *V. arizonica* line b43-17 was found to have strong resistance to *Xylella fastidiosa* (*Xf*) infection. Line b43-17 also has *V. candicans* and *V. rupestris* in its genetic background. We used AC-DC electropenetrography (EPG) to measure and compare the stylet probing behaviors of *Homalodisca vitripennis* (Germar), glassy-winged sharpshooter (GWSS), between *V. champinii*, a *V. candicans/V. rupestris* natural hybrid with moderate density of trichomes, and *V. vinifera* cv. 'Chardonnay,' which lacks trichomes. Results found that GWSS performed more frequent and longer events of XNC (representing salivation-egestion behaviors that eject *Xf*) on Chardonnay than on *V. champinii*, with or without trichomes. Also, probing was delayed on *V. champinii* with trichomes, while there was no delay when trichomes were removed. Thus, EPG can detect a novel type of grapevine resistance to *Xf* – to the vector's inoculation of bacteria – in addition to resistance to bacterial infection that is the basis for most resistance breeding. Future research will develop the means to use EPG to screen grapevines for this novel type of resistance.

**LAYPERSON SUMMARY**

Since 2001, a major effort has been made to breed Pierce's disease (PD)-resistant grapevines. The wild grape *V. arizonica* line b43-17 was found to have strong resistance to *Xylella fastidiosa* (*Xf*) infection (bacterial multiplication and spread in the plant), which has now been backcrossed into elite lines of cultivated grapevine. Line b43-17 also has *V. candicans* and *V. rupestris* in its genetic background. We used AC-DC electropenetrography (EPG) to measure and compare glassy-winged sharpshooter (GWSS) feeding behaviors between *V. champinii*, a *V. candicans/V. rupestris* natural hybrid with a moderate amount of hairs on its leaves and stems, and *V. vinifera* 'Chardonnay,' which lacks such hairs. Results found that GWSS performed more frequent and longer *Xf* inoculation behaviors (the XNC waveform) on Chardonnay than on *V. champinii*. XNC represents salivation then uptake and ejection of a saliva-xylem fluid mixture, which can eject *Xf* when it is present in the functional foregut. Thus, EPG can detect a novel type of grapevine

resistance to *X. fastidiosa* – to the vector's feeding and inoculation of bacteria – in addition to resistance to bacterial infection that is the basis for most resistance breeding today. Future research will develop the means to use EPG to screen grapevines for this novel type of resistance.

## INTRODUCTION

(This Report is derived from portions of a 2021 publication by Backus et al. (Backus et al. 2021).)

***Xylella*, its vectors, and the PD breeding program.** Pierce's disease (PD) is caused by the xylem-dwelling bacterium *Xylella fastidiosa* (*Xf*), which is transmitted (acquired, retained, then inoculated) to grapevines by sharpshooter leafhoppers and spittlebugs (Redak et al. 2004, Backus 2016, Ranieri et al. 2020a). Once acquired, *Xf* colonizes the functional foregut (precibarium and cibarium) of the vector, forming transient biofilm that can be inoculated directly into grapevine xylem during stylet probing (Backus and Morgan 2011).

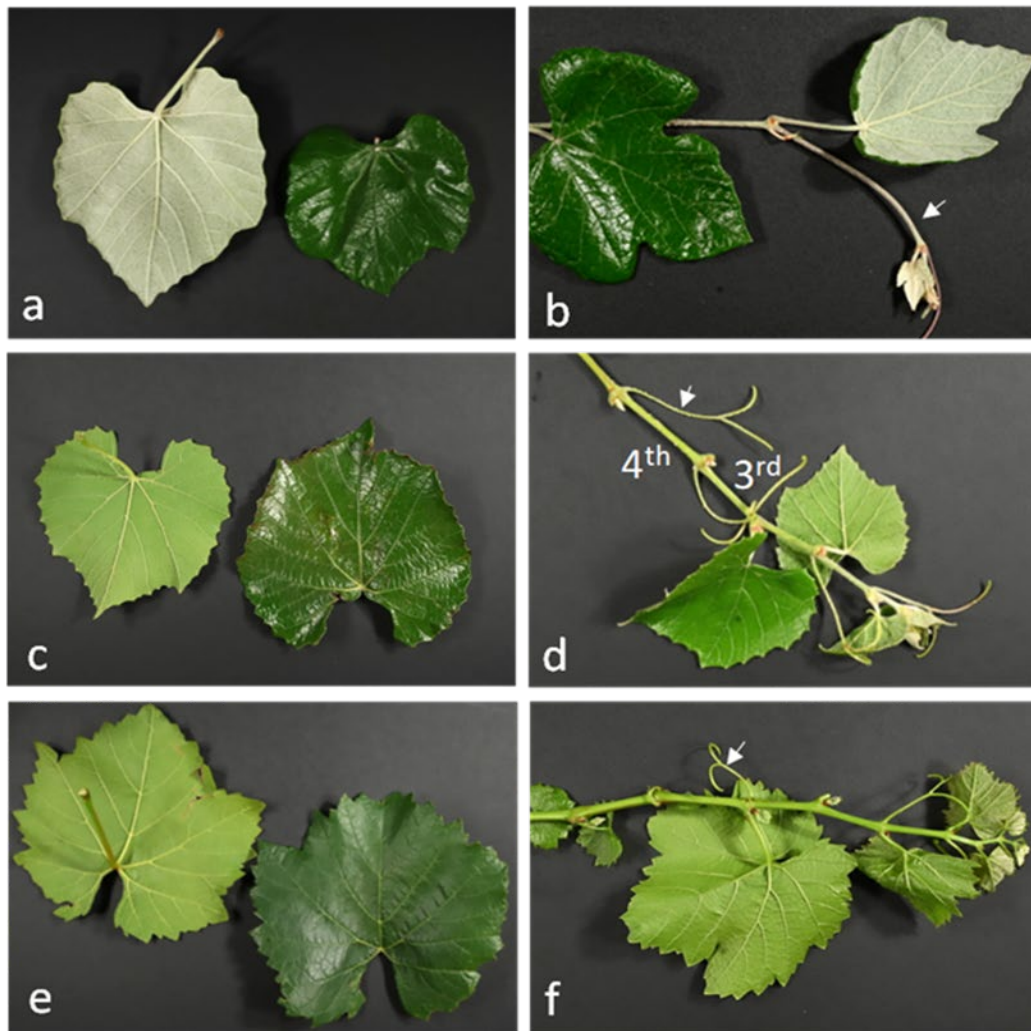
Since 2001, a major effort has been made to develop PD-resistant grapevines through classical breeding of wild grapevines with the many cultivars of *Vitis vinifera*. The project is centered in the Walker program at the University of California, Davis for wine grape breeding, in collaboration with the Ramming/Ledbetter program at USDA ARS in Parlier, CA, for table grape/raisin breeding. They discovered that *Vitis arizonica* Engel., line b43-17, collected near Monterrey, Mexico, has strong resistance to *Xf* infection (Ramming et al. 2009, Riaz et al. 2009). The resistance is inherited as a single dominant gene, named *PdR1*, that has been genetically and physically mapped (Krivanek et al. 2005).

The Mustang Grape (*Vitis candicans* Engel., recently re-named *Vitis mustangensis* Buckley but referred to herein as *V. candicans*) is very common in central Texas from north of Dallas to south of San Antonio, where it is usually found on fertile moist soils. The shoots and leaves of *V. candicans* are covered with dense, relatively short trichomes (Fig. 1 a, b, arrowhead) hence the species name, which means 'bright white.' Its range overlaps with several other wild *Vitis* species and it forms intergrading hybrids with *Vitis acerifolia* Raf. or *Vitis rupestris* Du Lot (the latter hybrid is termed *Vitis champinii* Planch) (Walker et al. 2019). The present study used *V. champinii* cv. 'Ramsey,' which is also covered with trichomes, but less densely and of longer length (Fig. 1c, d, arrowhead); it was originally collected near Boerne, Texas. In contrast, there are no trichomes on the cultivated grapevine, *V. vinifera* cv. 'Chardonnay' (hereafter termed Chardonnay) (Fig. 1e, f, arrowhead). *Vitis candicans* has been researched in the Walker PD breeding program, but it has poor fruit characteristics and only moderate resistance to *Xf*. Although *V. arizonica* accession b43-17 also appears to have some *V. candicans* and *V. rupestris* in its background, neither *V. candicans* nor *V. champinii* possesses the *PdR1* gene.

Mechanisms of grapevine resistance to the post-inoculation infection process (multiplification and systemic spread) of *Xf* are not completely understood. *PdR1* is clearly not the only resistance gene, because bacterial titers in stem samples are lower in several wild grapevine species that lack *PdR1*, including *V. candicans* and *V. champinii*, than the relatively higher titers that develop in *V. vinifera* (Fritschi et al. 2007). There are at least two studied mechanisms. First, cell-to-cell movement of *Xf* is impaired in resistant grapevines because their cell walls lack the

polysaccharide substrates for the bacterial cell wall-degrading enzyme of *Xf* (Sun et al. 2011). Second, some resistant *Vitis* spp. possess xylem sap phytochemistry that is not conducive to development of mature biofilm by *Xf*. Xylem sap of *V. champinii* was shown to inhibit *Xf* colony growth more severely than did sap from susceptible Chardonnay or any other resistant species tested including *V. candicans* (Hao et al. 2016). In contrast, xylem sap of Chardonnay fosters *Xf* biofilm growth (Shi et al. 2012). Thus, despite lacking *PdR1*, *V. champinii* possesses at least two likely vector- or *Xf*-affecting resistance mechanisms: trichomes and xylem chemistry.

Our study is the first to investigate the effects of these putative resistance mechanisms on vector stylet probing.



**Figure 1.** Appearance of grapevine genotypes discussed in this paper. **a**, leaves, **b**, shoots and tips of *Vitis candicans*. **c**, leaves, **d**, shoots and tips of *Vitis champinii* cv. 'Ramsey.' **e**, leaves, **f**, shoots and tips of *Vitis vinifera* cv. 'Chardonnay.' **b**, **d**, **f**, white arrowheads point to tendrils or stems where trichomes are best visible. **d**, internodes on *V. champinii* are designated by '3<sup>rd</sup>' (third internode) and '4<sup>th</sup>' (fourth internode). All leaf images are proportional in size, same magnification (1x) as original photograph. All shoot tips are magnified 1.3x, compared with leaf images.

**Electropenetrography and the *Xf* inoculation mechanism.** Choosing grapevines for a PD resistance breeding program would be enhanced by detecting plants that can resist vector probing behaviors that control *Xf* inoculation. Detecting such novel traits requires electropenetrography (EPG), to understand exactly when during stylet probing the *Xf* is inoculated into xylem on resistant plants. EPG has been used to study *Xf* vector feeding for over 25 years (Backus and Shih 2020). A series of studies has identified the mechanism of *Xf* inoculation into xylem cells during vector probing, as well as the EPG waveforms that represent these inoculation behaviors (Backus 2016, Backus et al. 2019a, Cornara et al. 2020).

In brief, a vector performs specific testing and tasting behaviors when its stylets first contact a xylem cell, as part of a finding-and-accepting behavioral process that is crucial for the insect's host plant and xylem cell selection. Represented by the first parts of the EPG X wave (XN and XC1, combined in this study and called XNC; XN is also called Xe (Cornara et al. 2020)), this tasting/ testing process first secretes a combination of gelling and watery saliva into the xylem cell (Backus et al. 2012), near-simultaneously bringing xylem sap mixed with watery saliva up the stylets to the precibarium, which is lined with chemosensory organs (Backus 1988, Ranieri et al. 2020a). The fluid is rapidly swished around in the precibarium (Ruschioni et al. 2019), then egested (ejected outward from the precibarium and stylet tips) (Backus and Morgan 2011) back into the xylem cell (Backus 2016). Due to the highly turbulent fluid dynamics of egestion in the complicated architecture of the precibarium (Marcus et al. , Ranieri et al. 2020b), and possibly also enzymatic action of the saliva (Backus and Morgan 2011, Backus et al. 2012), *Xf* biofilm is scrubbed off the precibarial cuticle and swept out the stylet tips.

The second part of the EPG X wave (XC2) represents a few cibarial pumps (trial ingestion), probably to test the strength of the stylet connection to the xylem cell, formed by its sturdy gelling-saliva sheath (Backus et al. 2012). If the connection is not strong enough, or the cell contents don't taste acceptable, the insect repeats the salivate-uptake-swish-egest process (XNC) to thicken the salivary sheath and taste the fluid contents. If the connection is found to be strong and cell contents taste acceptable during XNC, the insect directly commences a longer, sustained ingestion event (C2). Thus, after these repeated XNC-XC2 alternations (X waves), the insect either accepts the xylem cell leading to many hours of sustained ingestion, or it rejects and abandons the cell to start the process of finding and accepting a xylem cell all over again (Backus et al. 2009).

From the sharpshooter's point-of-view, the xylem-cell finding and accepting process represented by the X wave, which also inoculates *Xf*, is part of a stepwise series of critical feeding decisions performed after arrival on a plant. All hemipteran probing behaviors are progressive and occur in a consecutive, temporal order, with the performance of each main step contingent upon completion of the previous step. Ultimately, if successful, the probing process consummates in sustained ingestion to obtain nutrition (Backus 1988, Ranieri et al. 2020a).

We propose that the most favorable probing process for a sharpshooter would consummate in (temporally) long durations of sustained ingestion (C2) of a highly nutritional blend of xylem cell contents. EPG cannot measure phytochemistry and nutrition, but it can measure how the insect manages its time in each of the steps of the probing process. It is logical that the insect can achieve long, sustained ingestion by minimizing time from initial plant encounter to first

sustained xylem ingestion. In a favorably managed probing process, insects would perform short non-probing (standing, walking) and test-probing (sensory testing external to the plant) activities before beginning the first deep, exploratory probe. During a deep probe, it would perform short pathway activities with few xylem cells tested/tasted along the way. The insect would final-test/taste/establish connection to a xylem cell as quickly as possible, therefore produce few X waves to accept a mature xylem cell. Thereafter, cibarial pumping (ingestion, C2) would progress as rapidly as physically possible, with as few interruptions for salivation (wave-form N) as necessary.

Given the above feeding model, we propose that it is possible to also determine which behaviors would be less favorable, both for the insect and for *Xf* inoculation. In this way, we can predict the type and quality of the probing process recorded on any grapevine genotype. The long-term goal of our work is to aid breeding of grapevines resistant to PD by adding the novel trait of resistance to the vector's behaviors that control *Xf* inoculation. The present project was a proof-of-principle step towards that goal, using GWSS, *Homalodisca vitripennis* (Germar).

## OBJECTIVES

1. To use EPG to compare the stylet probing process of GWSS on Chardonnay (a cultivated grapevine that is susceptible to PD) versus *V. champinii* (a wild grapevine that is resistant to the post-inoculation infection and symptom development process of *Xf*).
2. To determine whether trichomes (removed by gentle scraping or not) would affect sharpshooter feeding.

Thus, the question we asked for this study was, how do these grapevines, and/or their trichomes, impact achievement of a favorable probing process by GWSS?

## RESULTS AND DISCUSSION

**Experimental design.** We used a 2x2 factorial design. The first factor was genotype, *V. champinii* or Chardonnay. The second factor was plant treatment, that is, external stem surfaces were gently scraped on plants from each genotype to remove trichomes (*V. champinii*) or to simulate removal (Chardonnay), or the surface was not scraped. See (Backus et al. 2021) for scraping technique. Scraped plants were returned to the greenhouse for a 5 – 14 day healing period, which resulted in all scraped stems turning a pale tan color. Otherwise, all scraped plants were healthy and normal in appearance.

**Electropenetrography (EPG).** Details of EPG methods can be found in (Backus et al. 2021). Recordings were performed using a randomized complete block design, with two blocks per day. Four grapevine plants (two Chardonnays - one scraped and one not scraped, and two *V. champinii* - one scraped and one not scraped) were placed into each of two Faraday cages (each with four head stage amplifiers, one per insect) on the same day. Therefore, eight insects were recorded on eight plants per day for 20 h per day for 10 days, organized as 5 contiguous days per wk for 2 wks. A total of 80, field-collected GWSS were recorded, with 20 insects recorded for each of the four treatments. Each wired insect was placed on the scraped or unscraped internode (usually third, sometimes fourth; Fig. 1d) of a young stem, with the plant remaining upright.

**Waveform names used and their meanings.** The Backus naming convention for sharpshooter waveforms was used for all measurement of recordings (Backus et al. 2019b, Backus and Shih 2020). Four waveform phases, 12 families/types, and six categories for measurement are

described in the full article (Backus et al. 2021). Only results from three phases and seven families are described herein (Table 1).

**Table 1.** EPG waveform nomenclature used in this report.

<b><u>Phase/ Waveform</u></b>	<b><u>Description</u></b>	<b><u>Biological Meaning</u></b>
<b>Non-probing/test-probing phase:</b>		
Z	Standing and/or walking	Electrical contact only made through the feet, low voltage
T	Test probing	1-2 sec, very shallow stylet insertions, single or in contiguous clusters
<b>Pathway phase:</b>		
A	Initial puncture of plant surface	Formation of salivary sheath trunk; combination of A1 and A2
B1	Stylet progression	Extension, formation of salivary sheath branch(es), initial contact/tasting/testing of xylem cells
B2	Stylet chiseling	Brief stylet retraction or protraction followed by stylet chiseling through the sheath wall to form a sheath branch or extension, respectively, done after rejection and abandonment of a tested xylem cell
<b>X wave phase:</b>		
XNC	Tasting/testing in xylem	Rapidly sequential sheath/watery salivation, fluid uptake into the precibarium, egestion/outward flow of fluid from stylet tips. Performed to taste and test suitability of recently contacted xylem cell. Combination of previous XN and XC1.
XC2	Trial ingestion	Contiguous, repeating voltage plateaus (cibarial pumping), <299 sec event duration, to mechanically test the strength of the salivary sheath connection into a xylem cell.

**Data preparation and statistical analysis.** Dataset compilation and error correction were performed using the Ebert 1.0 SAS programs; statistical analysis was performed using the Backus 2.0 SAS program (SAS Institute, 2008; Backus et al. 2007, Ebert et al. 2015). Only the first 10 h of each recording were measured and analyzed, because previous work (Krugner and Backus 2014) showed that insects adapted to plants and/or achieved sustained ingestion in the second 10 h, leading to no significant differences among genotype x treatment groups. Variables were analyzed using mixed ANOVA via the GLIMMIX procedure in SAS v. 9.4. Data were transformed using either log (for durations) or square root (for counts), as necessary to correct for heterogeneity. In tables herein, all *F* and *P* values from main effects and interactions are presented, but only means from significant main effects (indicated by different uppercase letters) are shown. If comparisons among all four genotype x treatment groups were warranted,

protected Fisher's least significant difference (LSD) tests were used for subsequent pairwise comparisons. Results were considered significantly different at  $\alpha = 0.05$ , except in one case when we used  $\alpha = 0.06$ . Only the most important findings are described herein; see Backus et al. (2021) for all results.

**Findings and their interpretations.** Waveform data demonstrated that our field-collected GWSS were highly plastic in their stylet probing processes, with each insect reacting individually to the spectrum of stimuli (both external [trichomes] and internal [cells along the stylet pathway and xylem cells]) presented by each genotype x treatment group of plants. Although this plasticity caused a high degree of variability in our ANOVA results, nonetheless, key behaviors rose above the variability to be significantly different in both counts and durations, especially for grapevine genotype.

**1. Cohort-level effects.** Table 2 (next page) shows that there were no significant differences between genotypes for (overall) probing duration per insect (PDI, [mean] probing duration per insect). Nonetheless, sharpshooters made significantly fewer (about half as many) stylet probes ([mean] number of probes per insect, NPI) on *V. champinii* as on Chardonnay. In addition, (mean) number of events (all waveforms combined) per insect (NEI) were significantly different for genotype, with 50% more events made on Chardonnay as on *V. champinii* (Table 2).

**2a. Waveform-level effects: Non-probing/test probing.** Data for waveforms Z and T are shown in Backus et al. (2021). In summary, treatment (scraping or not scraping) strongly influenced the earliest stage of the probing process on *V. champinii*. On not-scraped plants, sharpshooters delayed initiation of the first probe, culminating in sustained ingestion being shorter on plants with intact trichomes, compared with plants whose trichomes had been removed (scraped). Significantly fewer, but much longer, non-probing (walking, standing) events were performed because fewer test probes were made, resulting in numerically more than twice as much non-probing. Therefore, trichomes impeded initiation of deep probing; this part of the process was considered less favorable on not-scraped plants but favorable on scraped plants. Perhaps because of the large body size (~12 mm) of GWSS, the insects eventually overcame the relatively mild physical impediment of the trichomes on not-scraped plants. Fewer test probes presumably were made because they detected that the plant's surface interior was acceptable, allowing deeper, exploratory stylet probing to commence quickly.

**Table 2.** Cohort-level, non-sequential variables describing GWSS feeding. Durations in sec.

Waveform/ Genotype	Probing Duration per Insect (PDI)			Number of Probes per Insect (NPI)			Number of Events per Insect (NEI)		
Chardonnay	33,912.66	± 744.73	A	9.42	± 2.13	A	98.63	± 9.79	A
<i>V. champinii</i>	31,091.37	± 1,224.72	A	4.78	± 0.82	B	74.90	± 8.86	B
	P values	F <sub>1, 74</sub>		P values	F <sub>1, 74</sub>		P values	F <sub>1, 74</sub>	
genotype	0.1023	2.74		<b>0.0498</b>	*	3.98	<b>0.0075</b>	*	7.55
treatment	0.6225	0.24		0.2575	1.30		0.8288	0.05	
interaction	0.1708	1.91		0.5646	0.33		0.8392	0.04	



In contrast, on Chardonnay, as soon as the stylets shallowly punctured the plant surface, stimuli located just inside the plant were at first not acceptable, causing stylet withdrawal and significantly more test probes to be performed, regardless of treatment. Over time, probably as insects became hungrier, they overcame their reluctance to probe and eventually commenced deeper, exploratory probing.

**2b. Waveform-level effects: Pathway.** Significant differences between geno-types occurred in Pathway phase, which is the search for a xylem cell (Table 3, next page). Interestingly, one of the few significant differences in the treatment main effect occurred for overall duration per insect (WDI; [mean] waveform duration per insect) for waveform A. This first probing waveform lasted over twice as long *overall* for not-scraped plants as for scraped plants. Although strictly numerical, slight increases in both number of A events (NWEI; [mean] number of waveform events per insect) and their per-event durations ([mean] waveform duration per event per insect; WDEI) could have contributed to their per-insect overall significance. Interestingly, this difference probably was not directly caused by trichomes on *V. champinii*. Genotype x treatment data showed that overall durations (WDI) of A on not-scraped Chardonnay, although not significant, were three-to-five times longer than on the other three genotype x treatment groups (data not shown). Thus, some feature of not-scraped Chardonnay epidermal cells, perhaps thicker cuticle, seemed to be more difficult for the stylets to penetrate than on the other genotype x treatment plants.

**Table 3.** Waveform-level, non-sequential variables describing Pathway Phase. Durations in sec.

Waveform/ Genotype	Number of Waveform Events per Insect (NWEI)			Waveform Duration per Event per Insect (WDEI)			Waveform Duration per Insect (WDI)					
<u>A (Initial puncture and sheath base formation [mixed A1 and A2])</u>												
Not Scraped	3.44	±	0.39	A	33.41	±	13.47	A	113.02	±	53.95	A
Scraped	2.97	±	0.63	A	20.52	±	3.04	A	47.80	±	8.95	B
	P values		F <sub>1, 66</sub>		P values		F <sub>1, 66</sub>		P values		F <sub>1, 66</sub>	
genotype	0.5675		0.33		0.1230		0.33		0.1036		2.73	
treatment	0.1993		1.68		0.4830		1.68		<b>0.0455</b>	*	4.16	
interaction	0.8521		0.04		0.3129		0.04		0.6108		0.26	
<u>B2 (Chiseling through sheath wall to make a new branch)</u>												
Chardonnay	7.46	±	1.54	A	8.26	±	0.70	A	28.88	±	4.95	A
<i>V. champinii</i>	3.93	±	0.63	B	7.26	±	0.46	A	8.26	±	6.16	B
	P values		F <sub>1, 52</sub>		P values		F <sub>1, 52</sub>		P values		F <sub>1, 52</sub>	
genotype	<b>0.0314</b>	*	4.89		0.3072		1.06		<b>0.0194</b>	*	5.82	
treatment	0.7155		0.13		0.6757		0.18		0.6967		0.15	
interaction	0.8760		0.02		0.6309		0.23		0.8223		0.05	

All variables for B1 (searching and salivary sheath formation) were not significantly different for any factor, therefore they are not shown. The lack of significance means that the internal physical and chemical stimuli along the pathway were apparently found acceptable to the insects, so that the stylets could quickly follow a stimulatory sensory gradient (Backus 1988, Ranieri et al. 2020a) to mature xylem cells on all genotype x treatment groups.

In contrast, significant differences for genotype were found for B2 (formation of sheath branches or extensions) (Table 3). Significantly twice as many B2 events per insect, on average (NWEI), were made on Chardonnay (range: 1 – 13) as on *V. champinii* (range: 1 – 34); B2 was nonetheless not significantly different in per-event durations. Thus, larger numbers of same-duration B2 events ultimately led to significantly 3.5 times as long an overall duration (WDI) of B2 on Chardonnay as on *V. champinii*. On *V. champinii*, only a few xylem cells needed to be rapidly tested for preliminary acceptance during the short Pathway phase.

Finding and preliminarily accepting a xylem cell proved more challenging for GWSS on Chardonnay, requiring many B2 sheath-branching events. We infer that most xylem cells that were sequentially encountered were found sensorially inhibitory, therefore rejected and ultimately abandoned. This is because, in a previous study, a closely related sharpshooter species, *Homalodisca liturata* Ball, rejected 74% of tasted/tested xylem cells on Chardonnay, chiefly small, immature xylem cells (Backus et al. 2009); a B2 event to cut a new sheath branch followed each abandonment. In the present study, more frequent branching led to longer durations of Pathway phase (data not shown), more salivation and sheath formation as stylets moved from xylem cell to cell. Interestingly, every insect performed at least one B2 event; thus, no insect on Chardonnay or *V. champinii* found an acceptable xylem cell on its first attempt.

**2c. Waveform-level effects: X wave phase.** The largest number of significant differences between genotypes occurred with X waves. The first part of the X wave, XNC, was significantly more frequent (NWEI) and overall longer in duration (WDI) on Chardonnay than on *V. champinii* (Table 4, next page). Thus, a larger number of XNC events of stereotypically similar per-event duration were performed so much more frequently on Chardonnay that the overall XNC duration was longer. We infer that, on *V. champinii*, tasting/testing/ejecting of chemical compounds in xylem fluid during XNC required less time, with fewer, same-duration XNC events and overall shorter duration of XNC, than on Chardonnay. XC2, trial ingestion, was also significantly different on *V. champinii* than on Chardonnay. However, for this X wave behavior, number of events (NWEI) was shorter on *V. champinii*, while per-event durations (WDEI) were longer, compensating for one another so that the overall XC2 durations were not significantly different (Table 4).

As each xylem cell was sequentially contacted, GWSS performed more XNC events to taste/test the chemical stimuli in the cell on Chardonnay than on *V. champinii*. Also, more XC2 trial ingestion events were performed to mechanically test the strength of the salivary sheath, for briefer durations on Chardonnay. Over time, more xylem tasting/testing was performed on Chardonnay than on *V. champinii*. Eventually, a xylem cell was found acceptable for sustained ingestion on Chardonnay, again perhaps due to increasing hunger and debilitation. Consequently, the process of finding and accepting xylem cells for sustained ingestion was considered favorable on *V. champinii* but not favorable on Chardonnay.

**Table 4.** Waveform-level, non-sequential variables describing X wave phase. Durations in sec.

Waveform/ Genotype	Number of Waveform Events per Insect (NWEI)			Waveform Duration per Event per Insect (WDEI)			Waveform Duration per Insect (WDI)					
<u>XNC (X wave salivation, fluid uptake, and egestion [mixed XN and XC1])</u>												
Chardonnay	18.42	±	1.76	A	20.72	±	1.99	A	376.73	±	44.88	A
<i>V. champinii</i>	12.95	±	1.44	B	19.92	±	1.24	A	290.47	±	56.72	B
	P values		F <sub>1, 74</sub>		P values		F <sub>1, 74</sub>		P values		F <sub>1, 74</sub>	
genotype	<b>0.0094</b>	*	7.12		0.8980		0.02		<b>0.0248</b>	*	5.25	
treatment	0.7279		0.12		0.2602		1.29		0.3849		0.76	
interaction	0.9422		0.01		0.6816		0.17		0.6403		0.22	
<u>XC2 (X wave trial ingestion [XC2 only])</u>												
Chardonnay	16.95	±	1.77	A	72.91	±	7.94	A	1,141.85	±	151.07	A
<i>V. champinii</i>	11.87	±	1.38	B	90.78	±	6.53	B	938.02	±	89.54	A
	P values		F <sub>1, 72</sub>		P values		F <sub>1, 72</sub>		P values		F <sub>1, 72</sub>	
genotype	<b>0.0162</b>	*	6.06		<b>0.0274</b>	*	5.07		0.3424		0.91	
treatment	0.6966		0.15		0.1422		2.20		0.6442		0.22	
interaction	0.5047		0.45		0.6285		0.24		0.8318		0.05	

**2d. Waveform-level effects: Sustained ingestion phase.** The variable motivation to overcome negative, inhibitory cues (described above) may explain part of the extreme variability in overall duration of sustained ingestion among insects. While sustained ingestion trended 13% longer on Chardonnay than on *V. champinii*, it was not significantly different between genotypes or treatments. This is probably because GWSS is strongly motivated to consummate the stylet probing process and engage in sustained ingestion for many hours, or risk dehydration and death. See the original article for more details (Backus et al. 2021).

**Favorable or not favorable?** Fig. 3 in Backus et al. (2021) summarizes all of our findings about fundamental differences in how the GWSS probing processes were modulated by Chardonnay versus *V. champinii*. In brief, neither grapevine genotype was completely favorable nor not favorable. Instead, their behaviors comprised a spectrum of activities depending on phase of probing. The most important differences between genotypes were finding and accepting a mature xylem cell for sustained ingestion. For that phase of the probing process, *V. champinii* elicited far more favorable probing than did Chardonnay.

**Implications for inoculation of *Xylella fastidiosa* by vectors.** Our work conclusively demonstrates that grapevine genotype can significantly affect the performance of stylet probing behaviors chiefly responsible for *Xf* inoculation into grapevine xylem cells (Backus 2016, Backus et al. 2019b, Cornara et al. 2020). Most importantly, both B2 events (sheath branching to contact multiple xylem cells) and XNC events (tasting/ testing xylem cell contents, then egesting fluids perhaps containing *Xf* cells) were significantly higher in frequency and overall duration on Chardonnay than on *V. champinii*. This result occurred regardless of scraping or not-scraping the

grapevine stems. Increased *Xf* inoculation behaviors on Chardonnay ultimately means that more bacteria could be inoculated into more xylem cells, if bacteria were present in the functional foregut prior to onset of stylet probing, thus priming a more lethal infection. Therefore, less favorable probing by a vector can lead to greater susceptibility to *Xf* infection for the grapevine.

Until the present work, grapevine resistance by wild *Vitis* spp. like *V. champinii* or susceptibility by *V. vinifera* Chardonnay were considered to be solely due to post-inoculative, anti-microbial or symptom-development traits of the plant. Our EPG findings strongly support that other traits of the plant can trigger more favorable or less favorable parts of the probing process for the vector. When performance of the xylem-accepting behaviors that control bacterial inoculation is reduced, vector behavior can play an important role in grapevine resistance to *X. fastidiosa*. Chemical/mechanical cues recognized by the vector can therefore comprise all-new plant traits for plant resistance to *Xf* in grapevine, which can be recognized via EPG.

## CONCLUSIONS

Our work supports that the stylet probing process of GWSS, probably other sharp-shooters, and also spittlebugs (Backus and Shih 2020), is highly plastic and responsive to the external and internal sensory cues of grapevines during a step-by-step, sequential decision-making process (Backus 1985, 1988, Ranieri et al. 2020a). Stylet probing can be significantly altered from favorable to less favorable in response to different genotypes of grapevines; grapevines susceptible to *Xf* stimulate less favorable probing to be performed. Consequently, a major result of this work is that EPG can detect a novel category of grapevine resistance to *X. fastidiosa* – to the vector's probing process and inoculation of bacteria – in addition to the bacterial infection process that occurs after inoculation. Future research could develop EPG protocols for use in screening grapevine genotypes for resistance to vector inoculation of *Xf*.

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**RIVERSIDE COUNTY GLASSY-WINGED SHARPSHOOTER PROGRAM:  
TEMECULA VALLEY & COACHELLA VALLEY**

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**Reporting Period:** The results reported here are from work conducted November 2020 to  
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**ABSTRACT**

For approximately 20 years portions of Riverside County have been part of an area-wide program for the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS). The goal is to limit Pierce's disease spread by suppressing vector populations in commercial citrus, an important reproductive host for this insect, before they move into vineyards. The area-wide program originally consisted of insecticide applications to citrus groves along with monitoring of GWSS populations – to guide grapegrower treatment decisions. The treatment element of the program was halted in 2013 for Temecula Valley, and both monitoring and treatment were halted several years prior for Coachella Valley. Monitoring of GWSS populations continues to occur in Temecula, with approximately 160 yellow sticky traps placed throughout citrus groves and select vineyards being inspected on a biweekly basis. Starting two seasons ago approximately 150 traps were also deployed at the interface of vineyards and citrus groves throughout Coachella Valley. In Temecula, seasonal patterns were fairly typical but the magnitude of GWSS total catch was modest compared to historic averages. In Coachella, GWSS continues to appear to be rare if not absent in the area. Collectively these results show differences in GWSS activity between the two regions, neither of which is indicative of the resurgence of GWSS populations that has been observed in recent years 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 a pathogen that causes Pierce's disease. In Riverside County, area-wide control programs played an important role in reducing the impact of this invasive vector and disease following severe disease outbreaks 20 years ago. Monitoring continues to occur for GWSS to guide grapegrower 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 pretty typical patterns of GWSS activity, with overall fairly low numbers of insects. Monitoring near Coachella Valley table and raisin vineyards indicates that GWSS is rare if not absent from the area.



## **INTRODUCTION**

Economic analyses of the impact of Pierce's disease 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 Temecula of "100s" of 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 Temecula were lost to PD over the course of a few years.

In response to PD epidemics occurring in Temecula 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 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 Temecula approximately a decade after the initiation of the area-wide control program estimated that GWSS abundance had decreased 2000-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. Monitoring programs are important for identifying where and when vector populations might be rebounding (e.g., Haviland and Stone-Smith 2016) or, given substantial interannual variability in activity, to capture and prepare for anomalous years (Daugherty and Soto 2017).

In Riverside County there are two major grape production areas, both of which also have significant citrus production. In Temecula Valley and surrounding areas there are approximately 4500 ac of citrus (~1/3 of which is grown in Temecula Valley itself) and 2500 ac of wine grapes. In the Coachella Valley, there are approximately 7000 ac of table grapes and 8500 ac 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 Temecula Valley
2. Monitor GWSS in select Temecula vineyards adjacent to identified GWSS hotspots in citrus
3. Monitor GWSS populations in select citrus groves throughout Coachella Valley
4. Disseminate newsletters for stakeholders on sharpshooter seasonal abundance in Temecula and Coachella

Double-sided yellow-sticky cards (14x22 cm; Seabright Laboratories, Emeryville, CA) are being used to monitor for adult sharpshooters in citrus. Approximately 155 such sticky traps were deployed in citrus groves and select vineyards throughout the Temecula Valley. An additional 150 traps were deployed throughout Coachella Valley, primarily at the interface between vineyards and citrus groves.

All traps were labeled, then georeferenced with a handheld GPS monitor. Traps were attached with large binder clips to wooden stakes around the perimeter of the grove. For large groves traps are also placed in the interior. The total number of traps depends on the size of the block. The traps were inspected and replaced approximately every 2 weeks during the Summer and Fall (May through October) and monthly the rest of the year. At each inspection the number of adult GWSS and smoketree sharpshooters (*Homalodisca liturata*; STSS) were recorded.

To supplement trapping in Coachella Valley, this summer and fall we also conducted tap sampling to survey for GWSS. Tap sampling of citrus trees occurred at more than a dozen locations on approximately a biweekly basis between late June and mid-October.

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 (<http://temeculagwss.ucr.edu/>).

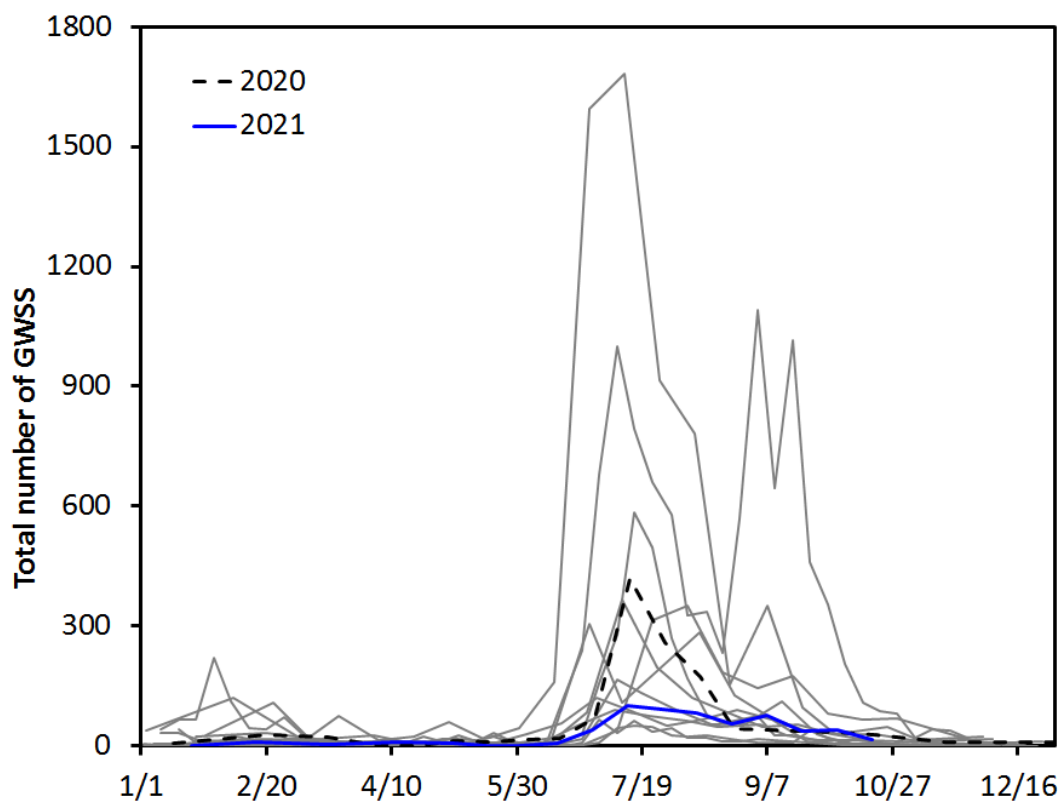
## RESULTS AND DISCUSSION

Monitoring results from Temecula Valley showed fairly typical patterns of GWSS activity over the year in 2021 (Figure 1). Specifically, peak GWSS catch occurred in mid-July, and a secondary peak in early September that occurs in some years. Overall, GWSS catch was fairly modest – lower than 2020 and far lower than in 2008 and 2017, but also higher than especially low years,

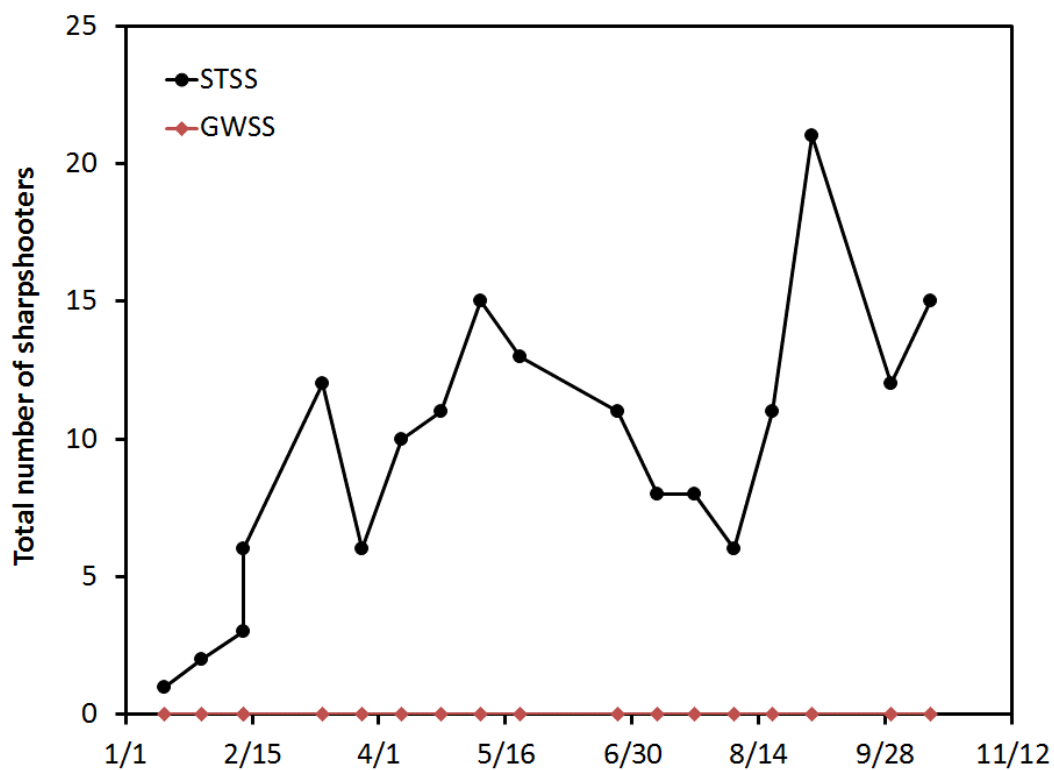
In Coachella Valley, no traps captured GWSS adults over the 17 censuses conducted thus far in 2021 (Figure 2). Over this same period, the native STSS was captured consistently, albeit in fairly low numbers (Figure 2). For the tap sampling, over the more than 100 censuses conducted between June and October, no GWSS were collected.

## CONCLUSIONS

Glassy-winged sharpshooter in Temecula Valley continues to exemplify an insect whose dynamics show substantial interannual variability, with overall trap activity this year that was modest relative to past years. In light of such variability, Temecula grapegrowers are encouraged remain vigilant with respect to the monitoring and management activities for GWSS and PD in their vineyards. Conversely, in Coachella Valley, GWSS appears to be rare if not absent entirely in 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 was reported in previous years in other areas of California.



**Figure 1.** Seasonal total GWSS catch in 2020 and 2021 compared to prior years.



**Figure 2.** GWSS and STSS trap catch in Coachella Valley in 2021.

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# **Section 3:**

## **Other Pests and Diseases of Winegrapes**

## MONITORING GRAPEVINE RED BLOTCH VIRUS AT RUSSELL RANCH FOUNDATION VINEYARD

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**Reporting Period:** The results reported here are from work conducted July 2020 to November 2021.

### ABSTRACT

Foundation Plant Services (FPS) works closely with the California Department of Food and Agriculture (CDFA) to register and certify grapevines and is the source of foundation planting material for California nurseries. FPS established a new foundation vineyard, Russell Ranch vineyard (RRV), in 2010 and by 2019, it included 4,761 vines. In 2013, immediately following the identification of grapevine red blotch virus (GRBV), FPS tested all grapevines in RRV for GRBV using a qPCR assay; no infected vines were detected. In 2014-2016, no GRBV infected vines were detected during maintenance testing but in 2017, five GRBV infected vines were detected, representing the first incidence of GRBV in RRV. Infected vines were removed to minimize spread. That same year, increased numbers of the three-cornered alfalfa hopper (*S. FESTINUS*), *Spissistilus festinus*, the only confirmed GRBV insect vector, were observed in RRV as well as associated feeding damage. In 2018 and 2019, GRBV infection rates increased by 0.5% and 7.1%, respectively. A survey of 80 free-living vines in areas surrounding RRV identified 12 GRBV-infected vines. Spatial-temporal analyses indicated that GRBV was introduced from outside sources and then spread within the vineyard. This newly funded project will monitor the GRBV infection rate in RRV and monitor vineyard insect populations over the next year.

### LAYPERSON SUMMARY

Red blotch disease is an economically significant grapevine disease that is widely distributed in US vineyards. Its widespread distribution suggests that grapevine red blotch virus (GRBV), the causal agent of the disease, was probably introduced into vineyards primarily via infected planting stock. However, epidemiological studies have shown that secondary spread does occur within vineyards in patterns consistent with transmission by a flying insect. The three-cornered alfalfa hopper, *Spissistilus festinus*, has been shown to transmit GRBV in greenhouse studies (Bahder et al. 2016; Flasco et al. 2021) but other insect vectors have also tested positive for GRBV, even though none have been shown to transmit the virus (Cieniewicz et al. 2018,

Cieniewicz et al. 2019). Current GRBV management practices recommend using certified planting material to establish new vineyards or to replace GRBV positive vines in infected vineyards. Due to the importance of eliminating GRBV from certified material, Foundation Plant Services (FPS), the source of foundation planting material for California nurseries, tested all the grapevines in its two foundation vineyards in 2013, shortly after GRBV was identified. No GRBV infected vines were detected in RRV, consistent with the fact that all RRV vines had been through micro-shoot tip culture for virus elimination. However, in 2017, five GRBV positive vines were detected. Despite vine removal to reduce virus spread and implementing a vector control program, GRBV infections increased by 0.5% and 7.1% in 2018 and 2019, respectively. A survey of 80 free-living vines in areas surrounding RRV identified 12 GRBV infected vines. Conversely, GRBV infections decreased in the Classic Foundation vineyard over the same six years from a high of nine infected vines in 2013 to one in 2019. We propose that GRBV was introduced into RRV from outside sources and then spread within via an insect vector. Despite similar management practices and similar geographic locations, GRBV spread did not occur in the Classic Foundation vineyard. Controlling secondary spread in vineyards, including RRV, will be a key component of minimizing grapevine red blotch disease.

## INTRODUCTION

Many details of the epidemiology of GRBV are not well understood. In particular, the identity of a primary vector and its role in spread of the virus under field conditions remain largely unresolved, although several studies have been done that indicate secondary spread does occur (Dalton *et al.* 2019; Cieniewicz *et al.* 2018; Cieniewicz *et al.* 2019). This lack of knowledge about the primary mechanism of spread of the virus limits the capacity to mount effective disease management responses since it is unclear which tactics should be used. While the outbreak of GRBV in RRV is a serious blow to the clean plant approach for wine grapes in California and the voluntary certification program, it also offers a unique research opportunity to characterize the statistical properties of a GRBV outbreak and thereby gather invaluable information about mechanisms of disease spread.

## OBJECTIVES

Objective 1: Determine annual GRBV infection rates.

Objective 2: Spatiotemporal analysis of GRBV infected vines.

Objective 3: Remove GRBV infected vines and replace with healthy virus-tested vines.

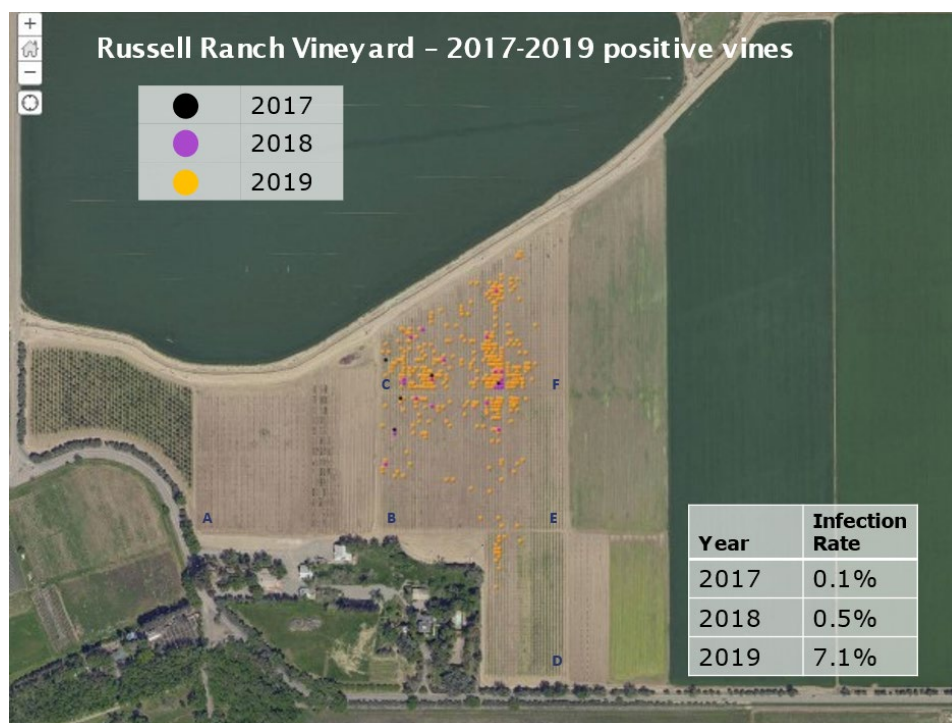
Objective 4: Monitor insect populations while maintaining current vector control measures.

Objective 5: Disseminate research progress and results.

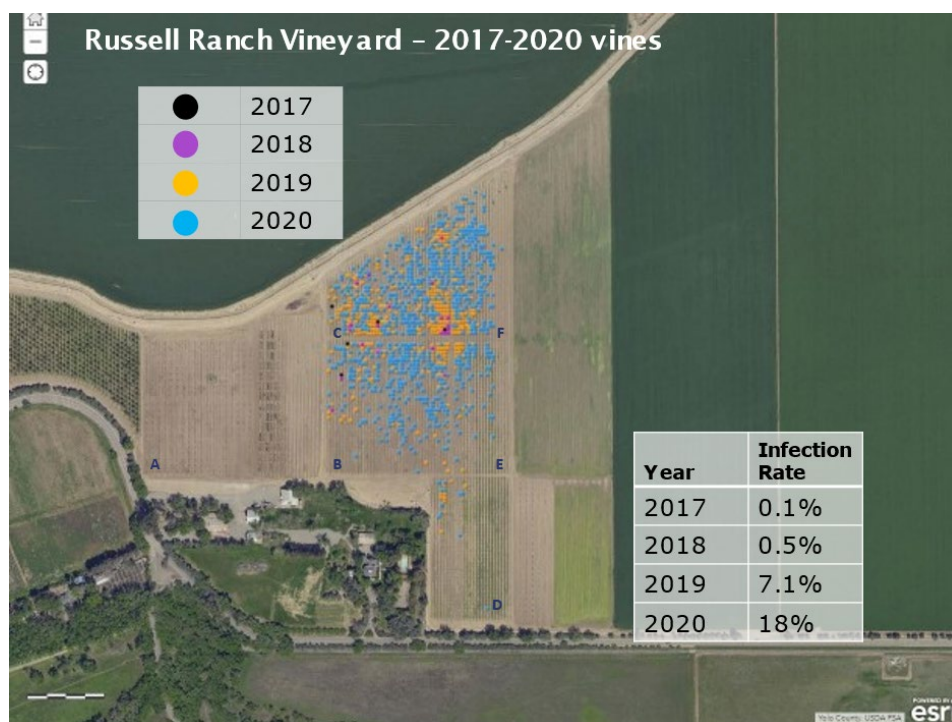
## RESULTS AND DISCUSSION

**Objective 1. Determine GRBV infection rates.** We began sampling and testing the 4,367 vines in RRV in early July 2020, focusing on areas with past aggregated spread (Figure 1).

Sampling and testing were completed in August 2020; 788 newly infected vines were detected, representing an annual infection rate of 18% (Figure 2).



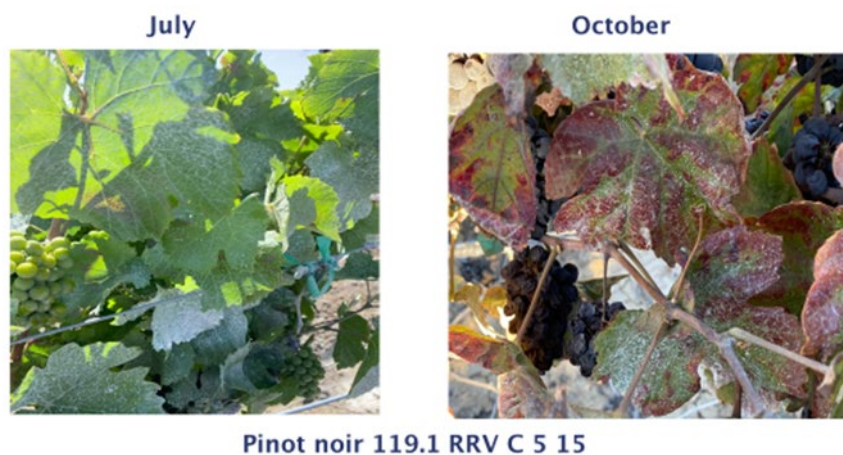
**Figure 1.** Distribution of GRBV infected vines in Russell Ranch vineyard. Colors indicate the year that infected vines were detected. Black = 2017; Purple = 2018; Yellow = 2019



**Figure 2.** Distribution of GRBV infected vines in Russell Ranch vineyard. Colors indicate the year that infected vines were detected. Black = 2017; Purple = 2018; Yellow = 2019; Blue=2020.



a)



b)



c)



**Figure 3.** a) A Pinot noir 119.1 vine that was positive for GRBV by qPCR in July but was asymptomatic until October; b) GRBV positive sister vines of Calmeria 04.1 showing different symptom severity between vines; c) an asymptomatic GRBV positive proprietary rootstock selection photographed in October.

Vines were monitored for GRBV symptoms on a bi-weekly basis from July through October. In general, GRBV-positive vines did not show symptoms in July and August (Figure 3a). Even when they did start showing symptoms, they were highly variable both among and within GRBV infected vines (Figure 3b). Some varieties still have not shown symptoms in October (Figure 3c).

We estimated GRBV incidence in 2021 using randomized quadrat sampling rather than sampling every vine. Petioles from 400 vines were sampled in August, processed and tested; this data is currently being analyzed.

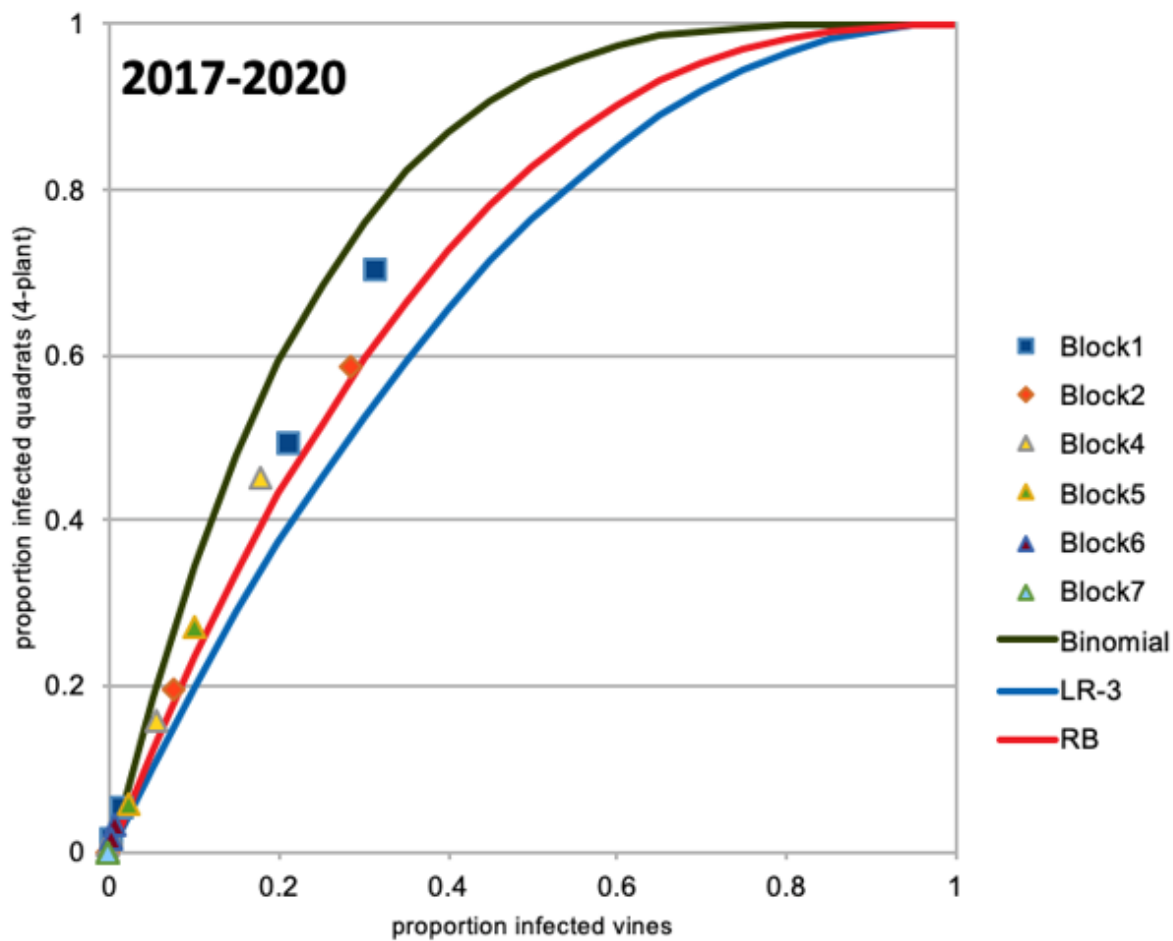
**Objective 2: Spatiotemporal analysis of GRBV infected vines.** We analyzed the 2018 - 2020 GRBV incidence by dividing RRV vineyard blocks into contiguous grids of quadrats of vines, with each quadrat containing the same number of vines. This allowed us to characterize the spatial pattern of infected vines as the frequency distribution of the number of infected vines per quadrat. If the disease has a random pattern across the vineyard, the frequency distribution will follow a binomial distribution with the binomial probability parameter equal to the incidence of vines in the whole vineyard. A random pattern would be expected if disease arrived in RRV from external sources and landed at random locations. Aggregated or patchy disease patterns arise when secondary disease spread occurs from initial points of infection and hence the extent to which the pattern of disease deviates from a random pattern towards a patchy pattern provides an index of the importance of secondary infection. This data is shown in Figure 4.

The displacement of the 2017-2020 GRBV incidence data below the binomial curve indicates that there is more patchiness in the pattern of infected vines than what would be expected by chance. The blue line is the relationship for Grapevine leafroll-associated virus 3 (GLRaV-3; Arnold et al. 2017). The GRBV data are between the GLRaV-3 line and the binomial, indicating that there is patchiness but not as much as for the GLRaV-3 data. This indicates that the dispersal of GRBV is being driven by a process that is contagious but has a longer mean dispersal distance than GLRaV-3. This supports the idea that GRBV is being spread by a flying insect, or one that moves more on average than mealybugs, the vector for GLRaV-3. We are in the process of analyzing the 2021 spatial distribution.

**Objective 3: Remove GRBV infected vines and replace with healthy GRBV-tested vines.** In July 2020, we planted 394 Cabernet franc vines in locations near the GRBV-infected vines that were removed in 2017-2019 (Figure 5). All 394 vines were tested for GRBV and were negative.

All sentinel vines were collected and tested for GRBV in March, June, and August 2021; no GRBV positive vines were detected. Sentinel vines will be tested again using lignified canes in October-November 2021.

Although we had planned to remove newly infected vines, it is not feasible to remove and replace the 788 vines that were detected in 2020. Instead, these vines are being left in place to preserve valuable germplasm for future virus-elimination therapy and to monitor the within-vine distribution of GRBV over time.



**Figure 4.** Graph showing the frequency distribution for grapevine leafroll-associated virus 3 (blue), grapevine red blotch virus (red) and a binomial distribution (black).





**Figure 5.** Distribution of GRBV-tested sentinel vines (green) in RRV blocks.



**Figure 6.** Distribution of yellow sticky cards in RRV blocks.

**Objective 4: Monitor insect populations while maintaining current vector control measures.**

In 2017, *S. festinus* and associated feeding damage was seen frequently in RRV with less evidence in 2018 - 2021, despite increased GRBV incidence. We placed yellow sticky cards in RRV blocks beginning in August 2019 to monitor insect populations (Figure 6). We did not test insects for GRBV.

Traps were collected monthly, and insects identified and counted (Table 1). While *S. festinus* is the only confirmed vector of GRBV to date, other candidates that have consistently tested positive for GRBV include *Scaphytopius graneticus* (*S. graneticus*), *Melanoliarius sp.*, *Colladonus reductus*, *Calladonus coquillettii*, and *Osbornellus borealis* (Cieniewicz et al. 2018, Cieniewicz et al. 2019). *Erythroneura elegantula* (Western grape leafhopper), *Erythroneura ziczac* (Virginia creeper leafhopper), and *Erythroneura variabilis* (Variegated leafhopper) are not currently suspected GRBV vectors but were included because they are common grape pests and occurred in relatively high numbers.

**Table 1.** Total and average vector insects per collection period by year (2019-2021).

Insect	Common Name	RRV Total	2019 RRV (2 collection periods)	2020 RRV (8 collection periods)	2021 RRV (8 collection periods)
<i>Melanoliarius sp</i>		211	0.5	12.125	14.125
<i>Spissistilus festinus</i>	Three-cornered alfalfa hopper (TCAH)	120	7.5	12.5	0.625
<i>Scaphytopius graneticus</i>	Scaphy	38	1	4.25	0.25
<i>Colladonus reductus</i>		15	0	0.223	0.0381
<i>Caladonus coquillettii</i>		2	0	0.0037	0.0036
<i>Osbornellus borealis</i>		1	0	0	0.004
<i>Erythroneura variabilis</i>	Variegated leafhopper (VLH)	756	1	61.75	32.5
<i>Erythroneura elegantula</i>	Western grape leafhopper (WGLH)	225	0.5	21.75	6.25
<i>Erythroneura ziczac</i>	Virginia creeper leafhopper (VCLH)	14	0.5	0.875	0.75

Over the 18 collection periods, 1,382 insects were collected and identified on yellow sticky cards. These were dominated by *E. variabilis* and *E. elegantula*, insects commonly found in grapevine canopies (Table 1). Of the suspected vector insects, *Melanoliarus sp.* was the most abundant and comprised 15.3% of all specimens on sticky cards, followed by *S. festinus* at 8.6% and *S. graneticus* at 2.7%. *C. reductus*, *C. coquilletti* and *O. borealis* were the least abundant and together comprised 1.3% of the total.

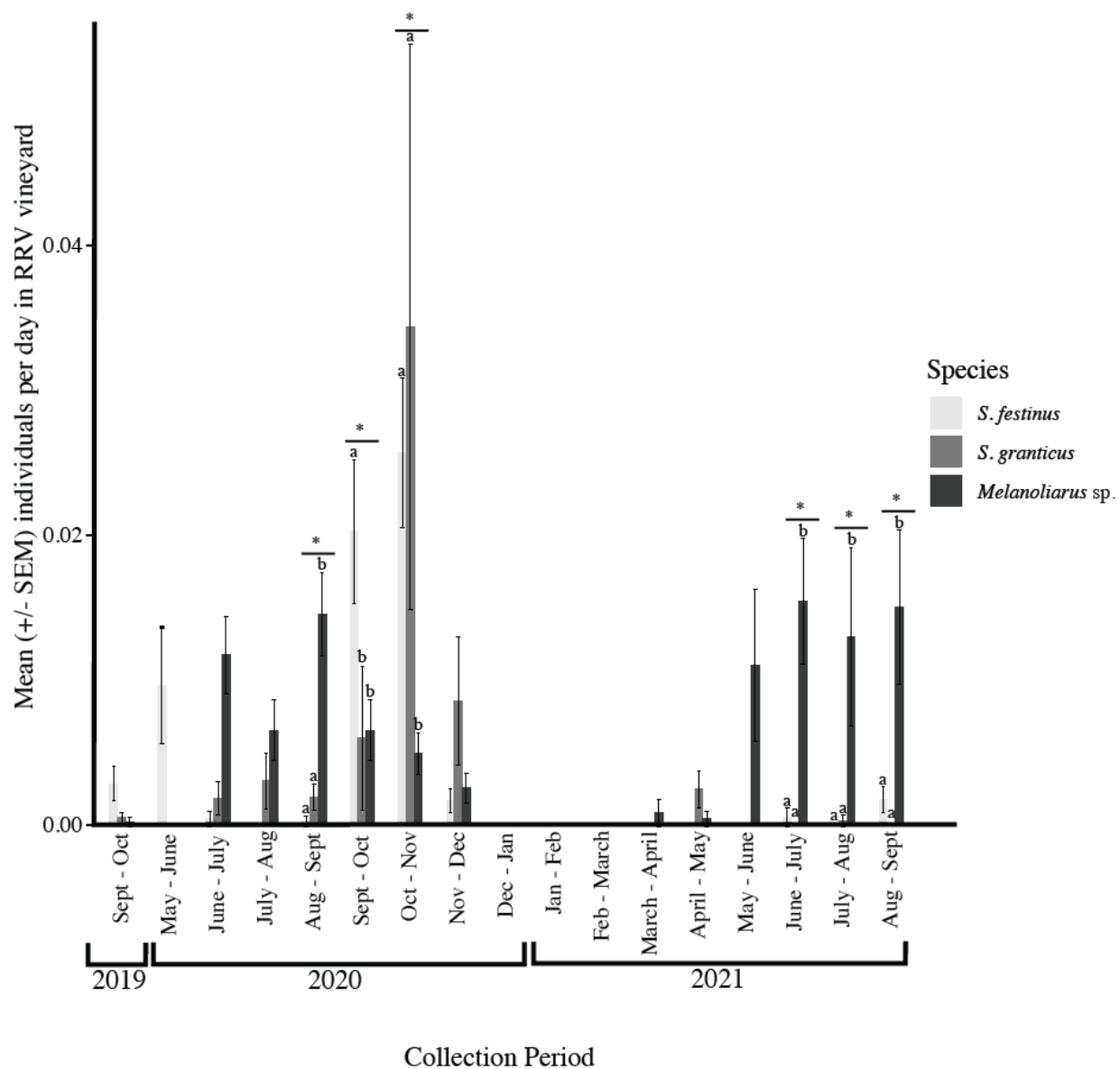
To further analyze possible differences in the abundance of suspected vector insects at RRV, insect count per day for each sampling period was calculated by dividing all insect counts by the number of days that sticky cards were left in the field. *C. reductus*, *C. coquilletti* and *O. borealis* incidence was too low for any meaningful analysis. A comparison of insect counts per day while controlling for collection period showed that *Melanoliarus sp.* were more common than *S. festinus* and *S. graneticus* in June-September for both 2020 and 2021, with the difference being significant in August-September 2020 and June-September 2021 (Figure 7). *S. festinus* was more common than *S. graneticus* and *Melanoliarus sp.* in September-October 2021 and both *S. festinus* and *S. graneticus* were more common than *Melanoliarus sp.* in October-November 2020. We don't have insect counts yet for September-November 2021 to know if is also true this year.

We also compared insects per day across the different RRV blocks. While there were no significant differences among blocks for *S. festinus*, there were some significant differences among blocks for *S. graneticus* (Figure 8) and *Melanoliarus sp.* (Figure 9).

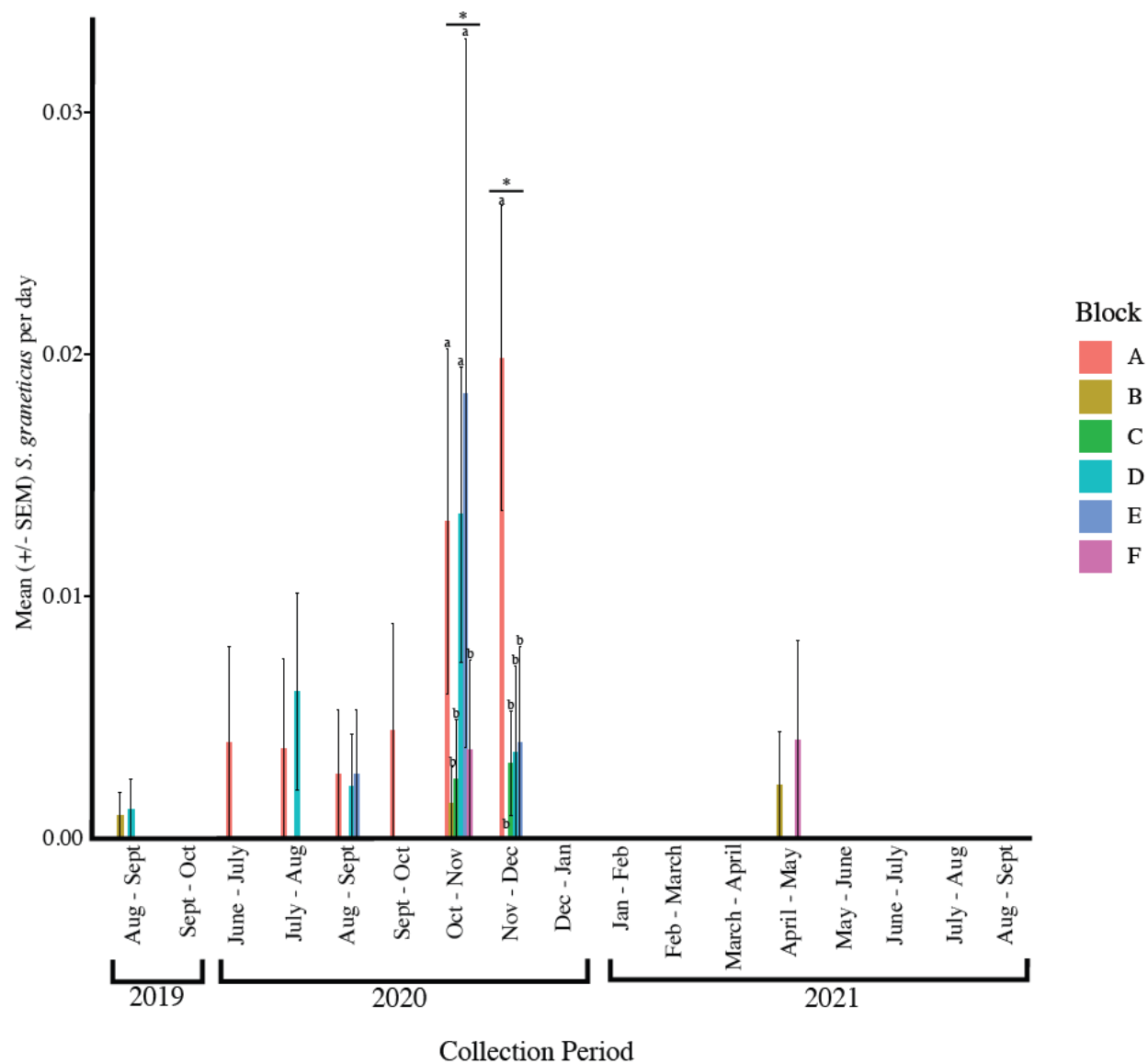
## CONCLUSIONS

Establishing new vineyards with virus-tested, certified planting material will continue to be an essential component of GRBV control. However, the introduction of GRBV at Russell Ranch from outside sources demonstrates that starting with clean planting material may not be enough to stop GRBV from entering vineyards at some point. Once GRBV has been introduced into vineyards, our work indicates that spread can be rapid with annual rates up to 18%. It is unclear what contributed to the high annual rates in 2019 - 2020 at RRV following the initial infections in 2017. Our sampling strategy has always consisted of multiple petioles from mature basal leaves to account for possible uneven virus distribution within vines due to vine growth stage (Setiono et al., 2018). However, we do have recent evidence that GRBV distribution within relatively newly infected vines is highly uneven, making it difficult to detect (unpublished data). These vines almost certainly contribute to false negative test results in any given year's testing and could serve as inoculum sources for transmission that same year if a vector is present. How soon these infections can be reliably detected requires field transmission experiments with a known vector. Until this information is available, optimal testing strategies cannot be determined.

We hope to have a more accurate estimate of annual spread rates in areas of highly aggregated GRBV infections as we continue to test the sentinel vines. To date we have not detected GRBV in these vines, but during sampling in October-November of this year, we did notice girdled petioles, which indicates that TCAH is present and feeding on these vines.

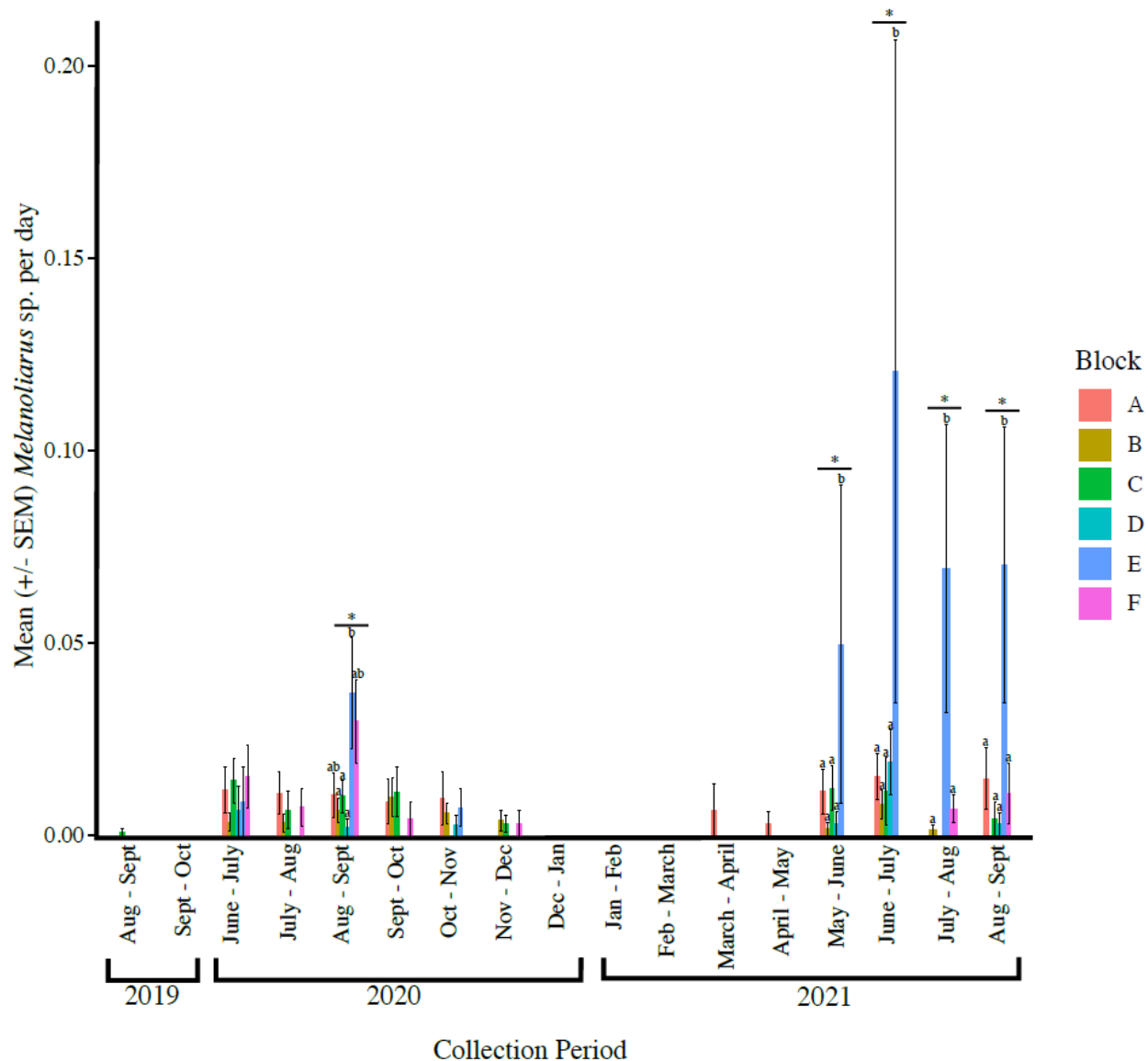


**Figure 7.** Mean  $\pm$  SEM of individuals per day on Russell Ranch vineyard yellow sticky cards by month and year. Statistical significance between insects within a collection period is shown by letters and asterisks.



**Figure 8.** Mean  $\pm$  SEM of *S. graneticus* per day on Russell Ranch vineyard yellow sticky cards by month and year. Statistical significance between insects within a collection period is shown by letters and asterisks.





**Figure 9.** Mean  $\pm$  SEM of *Melanoliarius* sp. per day on Russell Ranch vineyard yellow sticky cards by month and year. Statistical significance between insects within a collection period is shown by letters and asterisks.

While it's clear that GRBV has continued to spread at RRV, we did not gain any specific insights on a vector from this work. Spatial-temporal analyses for 2017-2020 indicates that spread is occurring via a vector that is more mobile than mealy bugs. The insect data from yellow sticky cards indicates that all the current suspected vector insects are present at RRV, although *C. reductus*, *C. coquilletti* and *O. borealis* were present in very low numbers. It is interesting that *S. festinus* peaked in September-November 2020, instead of mid-summer, which was the peak time reported in another CA vineyard (Cieniewicz et al. 2018). This peak in 2020 coincides with the

girdling that we observed on sentinel vines in October-November of this year. It will be interesting to see if this observation is supported by an increase in *S. festinus* counts on yellow sticky cards when they're collected and counted. If *S. festinus* is an important field vector at RRV, this later peak could have important implications for the timing of controls measures and sampling for testing.

Finally, we were especially interested in determining if there were differences in insect numbers between block A and the remaining RRV blocks since no GRBV positive vines have been detected in block A (Figure 2). However, the only significant difference between block A and the other blocks was a higher count for *S. graneticus* in November-December 2020. This would represent a negative correlation between the absence of GRBV infected vines and the presence of a suspected vector insect, although we did not test insects for GRBV, so the significance of this observation is unclear.

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## **GENOMICS RESOURCES FOR IDENTIFICATION, TRACKING, SURVEILLANCE, AND PEST MANAGEMENT OF VINE MEALYBUG IN VINEYARDS**

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**Reporting Period:** The results reported here are from work conducted July 2020 to November 2021.

### **ABSTRACT**

Vine mealybug is an important insect pest of wine and table grapes in California. Currently there is a lack of genomic resources for this pest and having such resources will facilitate research on pest biology and novel management strategies. This project aims to produce a high-quality reference genome and annotation for vine mealybug as well as whole genome-based assessment of the genetic variance present among vine mealybug populations in California. These genomic resources will enable future research on many aspects of pest biology such as development of insecticide resistance. Development of molecular markers based on genome information will also help to improve tracking of vine mealybug population spread across different grape-growing regions.

### **LAYPERSON SUMMARY**

Continued use of chemical control for insect pests such as mealybugs is likely to lead to development of insecticide resistance. It is necessary to explore alternative control strategies based on a detailed understanding of pest biology. DNA sequence information for vine mealybug will enable development of new pest control technologies. This project will expand DNA sequence information for vine mealybug representative of pest populations across California. This information will be used to track pest populations, evaluate prevalence of insecticide resistance, and develop new pest control technologies based on novel genetic targets.

### **INTRODUCTION**

Vine mealybug, an insect pest in most grape-growing regions of California, causes damage to clusters and spreads viruses. There is no tolerance for vine mealybug in table grape, and damage to clusters needs to be kept at very low levels for wine grapes. Treatment still relies heavily on insecticides to keep populations low, and growers spend an estimated \$123 to \$500/acre annually to manage mealybugs. Concerns over insecticide resistance development have prompted further study of vine mealybug biology. However, genomic resources are lacking for this species. Development of a high-quality reference genome for this pest will facilitate ongoing research on all aspects of vine mealybug biology.

Vine mealybug is invasive to the United States, and is believed to have entered California through plant material imported from Israel based on the sequences of two housekeeping genes (6). While these two genes were sufficient to track the movement of highly divergent populations of mealybugs, it is unlikely that these two markers will be useful in tracking CA populations. The recent (1990s) introduction of vine mealybugs into California created a genetic “bottleneck,” limiting the genetic diversity that is needed to track insect movements in the state. Current resources for vine mealybug are insufficient for creating basic molecular tools for assessing local populations. New and robust molecular markers are needed to track populations of vine mealybug in California and monitor differences among populations related to traits such as virus-favorability and insecticide resistance that have been shown to vary among populations in other piercing sucking insects (7).

## OBJECTIVES

Objective 1. Develop genomic resources for vine mealybug

Objective 2. Develop genetic markers to track mealybug populations in California.

## RESULTS AND DISCUSSION

**Objective 1. Develop genomic resources for vine mealybug.** For genome sequencing, vine mealybugs were reared individually on grape leaves (cv Chardonnay) suspending in petri dishes containing water agar (1% agar). Once insects reached the adult stage and males and females could be distinguished, each individual insect was placed in a 1.5 ml microfuge tube and flash frozen with liquid nitrogen. Samples were stored at -80C until DNA extraction. DNA extraction protocols were first validated for use on individual insect samples (both male and female) to ensure sufficient quantity and quality of high molecular weight DNA could be obtained from a single insect. Using validated protocols for insect sampling and DNA extraction, sequencing was performed on a single adult female insect using PacBio HiFi sequencing. A high-quality genome assembly has been obtained using this protocol (Table 2). RNA sequencing (RNAseq) was performed on pooled insects (3-4 males and females were pooled in 3 independent replicates) and results are being used to annotate the genome. Mapping statistics for RNAseq are in Table 3.

**Table 1.** Vine mealybug collections

<b>Year Collected</b>	<b>County</b>	<b>Number of locations</b>
2020	Kern	6
2020	Fresno	3
2020	Monterrey	1
2020	San Luis Obispo	1
2020	San Joaquin	2
2021	Kern	3
2021	Fresno	1
2021	San Joaquin	2
2021	El Dorado	1*

\* Potentially grape mealybug

**Table 2.** Vine mealybug reference genome assembly statistics

	<i>Primary Scaffolds</i>
<i>Cumulative length</i>	369,955,584
<i>Cumulative gap length</i>	1,378,171
<i>Number of gaps</i>	397
<i>Number of sequences</i>	1,323
<i>Average sequence length</i>	279,634
<i>Median sequence length</i>	187,185
<i>Maximum sequence length</i>	2,677,772
<i>N50 Length</i>	485,632
<i>N90 Length</i>	144,606
<i>N50 Index</i>	239
<i>N90 Index</i>	783
<i>BUSCO % - Eukaryota (255 BUSCOs)</i>	
<i>Complete BUSCOs</i>	92.9
<i>Complete and single-copy BUSCOs</i>	84.3
<i>Complete and duplicated BUSCOs</i>	8.6
<i>Fragmented BUSCOs</i>	2.0
<i>Missing BUSCOs</i>	5.1
<i>BUSCO % - Insecta (1367 BUSCOs)</i>	
<i>Complete BUSCOs</i>	89.9
<i>Complete and single-copy BUSCOs</i>	82.4
<i>Complete and duplicated BUSCOs</i>	7.5
<i>Fragmented BUSCOs</i>	2.3
<i>Missing BUSCOs</i>	7.8

**Table 3.** Mapping statistics for RNAseq on male and female vine mealybugs.

ID	Raw reads			Filtered reads					Mapping reads on diploid assembly			
	Fragment count	Cumulative length	Average length	Fragment count	Cumulative length	Average length	Passing reads %	Passing bases %	Mapping reads	Mapping bases	Mapping reads rate %	Mapping bases rate %
F1	50,240,191	5,074,259,291	101	49,478,308	4,881,411,009	98.66	98.48	96.20	43,037,509	4,248,927,533	86.98	87.04
F2	67,319,147	6,799,233,847	101	67,112,152	6,691,798,423	99.71	99.69	98.42	59,867,355	5,973,449,452	89.20	89.27
F3	62,588,108	6,321,398,908	101	62,406,481	6,231,141,996	99.85	99.71	98.57	55,220,822	5,518,230,292	88.49	88.56
M1	42,101,320	4,252,233,320	101	41,802,565	4,149,244,252	99.26	99.29	97.58	34,641,083	3,457,002,717	82.87	83.32
M2	77,737,719	7,851,509,619	101	77,395,351	7,740,212,689	100.01	99.56	98.58	69,093,048	6,923,145,796	89.27	89.44
M3	80,090,402	8,089,130,602	101	79,824,727	8,006,814,110	100.30	99.67	98.98	71,533,557	7,184,128,172	89.61	89.73

**Objective 2. Develop genetic markers to track mealybug populations in California.** From July to October of 2020, vine mealybug samples were collected from vineyards in Fresno, Kern, San Joaquin, San Luis Obispo, and Monterey counties (Table 1). All populations were confirmed by PCR (8) to be *Planococcus ficus*. PCR primers for vine mealybug microsatellite markers were developed based on the fragmented publicly available *Planococcus ficus* partial genome using Primer 3 and MISA software (9). Approximately 700 primers were designed associated with microsatellites of various length and bases. A subset of 40 primers was selected for testing diversity within vine mealybug using an M13-tailed tag assay to determine base pair differences. DNA was extracted from a set of 10 mealybugs collected on location from the greenhouse at the San Joaquin Valley Agricultural Sciences Center (SJVASC). None of the primers showed any variability in sequence length among the mealybugs tested. Using the new high-quality reference genome, primer design will be re-evaluated and tested on a wider range of vine mealybug populations. In 2021, six additional vine mealybug populations were collected from Fresno, Kern, and San Joaquin counties. Insects from these populations will be reared in the lab and used for additional whole genome sequencing.

## CONCLUSIONS

A high-quality vine mealybug reference genome was created from single insect DNA extraction. Work is ongoing to annotate the reference genome using RNAseq data from male and female vine mealybugs. Although little genetic diversity was observed initially based on microsatellite markers, new genomic references will be used to screen for additional marker candidates.

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

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### **ABSTRACT**

Successful extension programs support the uptake of data-driven solutions to management challenges. This project seeks to improve extension outcomes by exploring factors influencing the adoption of best management practices, using grapevine leafroll and red blotch diseases as model systems. Using quantitative surveys and qualitative interviews, the project team identified economic, technical knowledge, and social-behavioral factors that are important to decision-makers in the wine grape industry. The quantitative survey demonstrated that the cost of practices, salability of fruit from diseased vines, and knowledge of disease ecology are related to degree of adoption and perceived cost of practices. The qualitative interviews delineated specifically when and how the cost of practices and product salability act as barriers and drivers and identified production demands as another economic factor. Additional factors related to technical knowledge and social-behavioral aspects include decision-maker knowledge of the problem, current scientific understanding of disease ecology, outreach activities, intra-company teamwork, regional industry cooperation, and public policy instruments. We also explored the importance decision-makers place on various educational resources, the ways in which they access these resources, and how they use those resources within their professional networks to make informed decisions on virus management.

### **LAYPERSON SUMMARY**

Grapevine leafroll (GLD) and red blotch (RBD) diseases are consequential viral diseases of grapevine that are actively managed by wine grape industry professionals in the western United States. Management guidelines for GLD encourage the planting of virus-screened material, the local eradication (through vine removal) of diseased vines, and management of vector populations. Since RBD was more recently identified, specific guidelines are under development, but will likely include similar practices. Uptake and implementation of these practices varies among growers and across regions. To understand why, we conducted a survey and interviews with growers in California and Washington and identified numerous economic, knowledge, and social-behavioral factors that affect adoption. We also collected feedback from decision-makers



on educational resources. We are using these data to formulate recommendations for extension programs that maximize effective dissemination of knowledge and promote the uptake of management practices to create positive outcomes for affected growers. For example, public and private investments that support research and outreach programs to address knowledge gaps, increase economic efficiency, and improve our understanding of disease ecology. Overall knowledge of disease ecology and uptake of practices was high among survey respondents, suggesting that research and outreach programs to date have largely been successful. Addressing remaining knowledge gaps around transmission biology of RBD can further improve adoption. Further analysis of the interview data will provide additional insight into peer-learning among agriculturists, the role of regional disease management programs, government and industry standards, and the economic costs of viral 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 (RBD) 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 RBD 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.

Global adoption of management practices for grapevine viruses has been noted as low or suboptimal in their implementation, with few studies on why this is the case (Fuchs 2020). 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. The more extensive literature on adoption for other farming practices, such as Integrated Pest Management (IPM), has identified numerous barriers and drivers for adoption that can be broadly characterized as economic, technical knowledge, or social-behavioral factors (Lefebvre et al. 2015; Peshin et al. 2009).

Economic factors influencing adoption of virus management practices could be the expense of removing diseased vines or blocks (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).

Technical knowledge factors may include misperceptions about scientific conclusions and personal capability to control disease. An added challenge is the lag between the emergence of the pest or disease and the availability of evidence-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 practical obstacles to overcome in implementing technical knowledge, such as the availability and reliability of monitoring tools for vectors, assays for pathogens, and the training of staff to identify insects and diseased vines.

Agricultural knowledge is created and disseminated collaboratively through a network of industry professionals including extension agents, scientists, industry organizations, and growers themselves (Phelps et al. 2012; Lubell et al. 2014). Social-behavioral factors relate to how this network act as individuals, in collaboration, and in relation to public policy instruments targeting behavior change (e.g., standards, regulations, government support). Such factors may include individual differences in willingness to try new practices, effective knowledge dissemination by outreach and extension, ability for growers to collaborate regionally, and the impact of plant material certification programs. Additionally, outreach events and materials must successfully communicate with the target audience to support adoption of beneficial practices. 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. This project is extending Hoffman's work to the specific of issue of GLD and RBD to (1) quantify the usefulness of various resources; (2) assess differences in resource preference by position within the company; and (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 using GLD and RBD as model systems.

**OBJECTIVE 1:** Use quantitative and qualitative tools to identify factors influencing adoption of GLD and RBD management practices in California and Washington.

**OBJECTIVE 2:** Develop recommendations for improving outreach programs that support greater adoption of best management practices for GLD and RBD, 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 identify factors influencing adoption of GLD and RBD management practices.**

***Quantitative Questionnaire (Survey):*** We deployed a 43-question quantitative tool to capture demographic characteristics and measure (1) adoption rates of disease management practices, (2) perceptions regarding usefulness of educational resources, and (3) drivers and barriers to adoption of management practices. From April 2019 to December 2020, a total of 154 responses were collected at regional seminars or using an online survey tool. Participants from the California and Washington wine grape industry included vineyard directors (10%), managers and owners (25%), viticulturists (28%), consultants or pest control advisors (16%), and other employees involved in grape production (21%). Respondents from California (CA) worked in 13 grape pricing districts, with the largest groupings from District 4 (Napa; 47.8%), District 3 (Sonoma/Marin; 7.5%), District 7 (Monterey/San Benito; 6.7%), District 5 (Solano; 3%), and 12.7% working across multiple districts. Most respondents from Washington worked in multiple districts; Yakima Valley (68%) and Horse Heaven Hills (47%) were the most represented.

Although adoption of individual management practices varied (29.7% to 75.3%), most respondents had adopted at least one practice for GLD (89.6%) and RBD (88.4%), and most respondents had adopted multiple practices for each disease. The most widely adopted practices for GLD were removal of diseased vines, virus assays, and vector management. For RBD, virus assays of planted blocks and nursery vines were the most widely adopted, followed by removal of diseased vines.

Economic factors related to the cost of practices and salability of product were observed to influence adoption. Costs associated with identification, removal and replanting diseased vines were a common barrier for both diseases, while the costs of vector control were a barrier specifically for GLD. The cost of GLRaV-3 vector management was a greater barrier in California, where the invasive vine mealybug is present, compared with Washington. Growers able to sell grapes from GLRaV-3 or GRBV-infected vines were less likely to adopt management strategies or perceive them as cost-effective. This salability factor had a regional component: respondents in California were less able to sell infected product compared to Washington. Consequently, California growers were willing to invest more in control programs than Washington growers.

Acquisition of technical knowledge of disease ecology by respondents was also a factor in adoption. Respondents with technical knowledge of disease ecology were more likely to adopt management practices and to perceive them as cost-effective. In contrast, with a lack of knowledge of GLD ecology, notably that mealybugs are vectors, adoption was reduced, and practices were perceived as less economical. Factors influencing adoption were broadly generalizable across diseases, but knowledge of disease ecology was more closely associated with adoption for GLD than for RBD, likely reflecting the existing knowledge gaps for RBD.

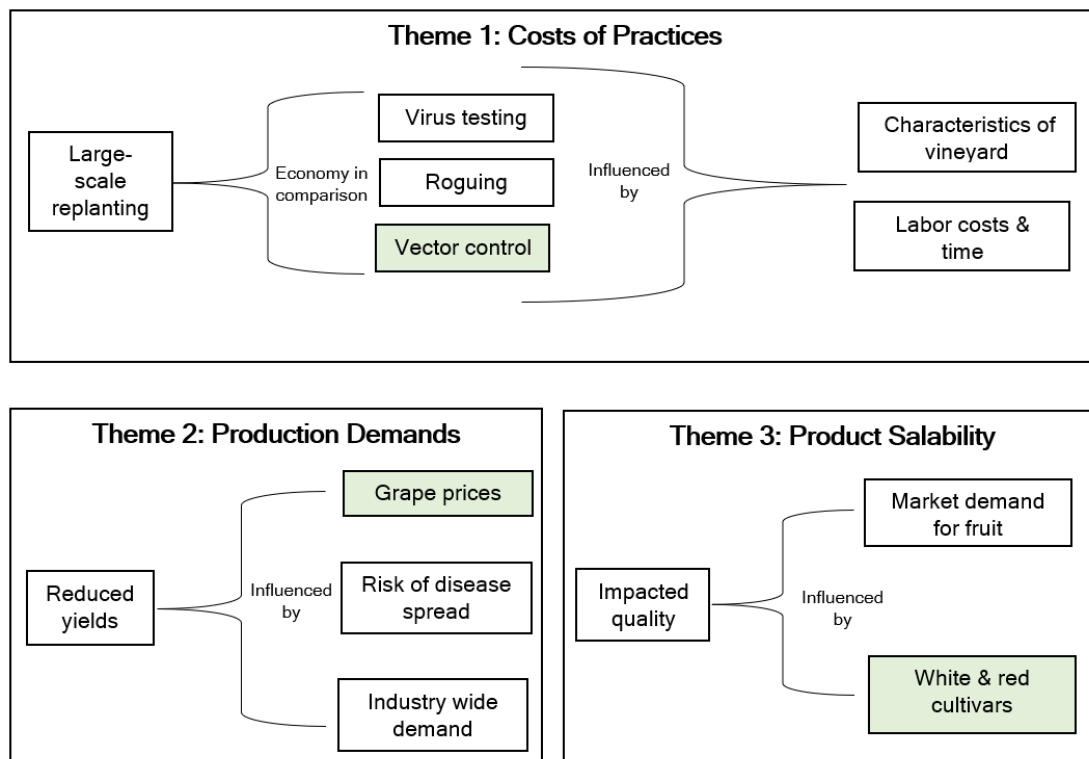
***Qualitative Questions (Interviews):*** Semi-structured interviews were conducted with a subset of survey respondents from Napa County (n=24), Lodi and Central Coast (n=8), and Washington (n=10). Between November 2020 and March 2021, interviews were conducted either in-person or over Zoom. Each interviewee responded to 11 open-ended questions, exploring topics such as knowledge gaps, regional approaches to disease management, government and industry standards, and the economic impact of viral diseases. Follow-up questions were asked as necessary. Participants were also asked to share their views on educational resources for virus management. Interviewing in this manner allowed interviewees to freely generate and explain information, was less restricted by the researchers' preconceptions, and permitted broad and detailed exploration of the reasons behind adoption. Following each interview, audio recordings were transcribed, and thematic analysis conducted using R program "RQDA". This technique organizes and describes interview data in rich detail and makes sense of shared meanings and experiences by identifying commonalities about the way virus management was discussed. In addition to the thematic analysis, we are using RQDA algorithms, such as Fruchterman-Reingold and Kamada-Kawai, to explore relationships between people and resources in the grape industry. Economic themes that emerged from this analysis are described in Fig. 1, and include the cost of practices, product salability and production demands. Description of themes in other categories is ongoing and will be reported subsequently.

In summary, the 'costs of practices' sub-themes revealed that the cost of large-scale replanting (block or whole vineyard) was a universal barrier to removing infected vines with the economy of virus testing, roguing, vector control, and associated practices (scouting; mapping) measured against their ability to avoid large-scale replants. The degree to which practice costs were a barrier to adoption is influenced by the surrounding economic considerations. These include the characteristics of individual vineyards such as existing financial resources, site specific aspects (e.g., organic vs. conventional), and the long-term strategy for specific vineyard blocks (e.g., young vs. mature or heritage blocks). Labor costs and time required to implement practices were highlighted as very significant barriers to adoption. Interviewees provided many examples of occasions when practices could not be implemented because there was a shortage of staff for scouting, roguing and replanting, tending to replacement vines, or because staff time was diverted to other pressing tasks.

The 'production demands' sub themes revealed that reduced yields from GLD and RBD are a common driver for the adoption of management practices to maintain economically viable production. However, the capacity of reduced yields to drive adoption are determined by grape price, risk of disease spread, and wider industry production demands. High grape or wine prices tend to drive greater adoption of practices. However, where prices were low, interviewee's adoption was often related to whether red or white cultivars were grown. High regional demand

for grapes were perceived to incentivize growers to take shortcuts to fast track production, such as by planting non-certified vines which were more likely to be virus-infected. Lastly, risk of spread of the pathogens and potential to reduce future yields was an important regional component. For example, interviewees from the California Central Coast viewed RBD as an existential threat that required vigorous application of management practices, because of rapid spread and the devastating impact on yields. In contrast, Washington interviewees did not observe RBD spread in their region and would not necessarily actively manage the disease.

Consideration of the 'product salability' sub themes revealed that reduced quality drove efforts for vine removal and replacement. However, when fruit quality met requirements, decision-makers were reluctant to remove infected vines, even if viticultural staff had concerns about disease spread. When quality was a barrier to adoption, demand for fruit was a determining factor. Specifically, when demand for fruit is high, it was considered easy to sell infected product of lower quality and there was less incentive to adopt management practices. When there is an oversupply of fruit, wineries demand higher quality product, leading to greater adoption of management practices. For GLD, a clear distinction emerged between white and red cultivars, with many interviewees less likely to adopt practices for white cultivars because quality was easier to achieve.



**Figure 1.** Themes and sub-themes for economic factors influencing adoption of management practices for grapevine leafroll and red blotch disease.

**Objective 2. Develop recommendations for improving outreach programs that support greater adoption of best management practices for GLD and RBD, and guidelines for adapting these to improve current and future responses to grapevine pests and diseases.**

The importance of economic factors suggest that research and extension efforts can be most effective by focusing on measures that reduce the costs to individual growers and incentivize the removal of diseased vines. This may include assessments that are collected across a region and used collectively to subsidize practices. One example is the Napa County Wine Grape Pest and Disease Control District, where assessment funds are used in part to support monitoring and biological control of pests, such as the invasive vine mealybug. Replication of this program in other wine grape growing regions could improve regional efforts to reduce the spread of pests and diseases. Development of labor-saving technologies could be particularly effective in reducing cost and increasing adoption. For grapevine viruses, this may include decision-support or automated tools that increase the efficiency and reproducibility of visual symptom assessments and diseased-vine mapping.

Acquisition of technical knowledge of disease ecology by respondents was a key factor driving adoption. This emphasizes the importance of research and extension programs to develop an evidence-based understanding of pathogens, vectors, conditions for spread, and economic impact. Research programs that address critical knowledge gaps in disease ecology and management, and outreach programs that make evidence-based information accessible to a diverse audience should therefore be prioritized for funding support.

Recommendations on outreach programs for viral diseases of grapevine will continue to be refined. For example, educational videos, when they are of high quality, originate from a reputable source and are translated into Spanish (or other languages), can be an effective method for disseminating information. They may be particularly useful for internal efforts to train incoming or less experienced staff. Educational videos are falling short of their potential as a valuable resource, as evidenced by their low reported usage in our survey. Efforts to increase their adoption should raise awareness of existing videos as well as the production of new video resources. Likewise, factsheets and newsletters originating from a reputable source are a key resource for multiple levels of the industry. They are used to train staff (crew; interns; field scouts; technicians) and to pass on information to upper-level managers. To be most effective they must be short, straightforward, available in multiple languages, and include copious photos. Social media is likely to be a poor resource to disseminate information on viruses. Individuals in the industry are unlikely to seek out virus related information on social media platforms and many do not use social media for professional purposes. Reluctance to engage on a public platform relates mainly to marketing and perceptions that may result public postings.

## **CONCLUSIONS**

Economic, technical, and social-behavioral factors influence the adoption of management practices for grapevine leafroll and red blotch diseases. The cost of practices—in time, labor, and outlay—are important considerations, as are production demands and salability of product. Specifically, yield, quality and grape pricing were contributing factors. Therefore, individual or collective practices that reduce the economic burden of adoption can improve regional disease management outcomes. The most influential technical factor is the availability and acceptance of

evidence-based management practices. Prioritizing research and outreach programs that develop and disseminate an evidence-based understanding of disease ecology and management can reduce the detrimental effects of viral diseases. Lastly, programs that improve regional camaraderie and collaboration, as well as supportive learning environments within individual organizations are social factors that can increase adoption of management practices.

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## **IMPROVED DECISION-MAKING FOR GRAPEVINE LEAFROLL AND RED BLOTCH DISEASES USING RAPID IDENTIFICATION TOOLS AND A REGIONAL APPROACH TO MONITORING AND MANAGEMENT**

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**Reporting Period:** The results reported here are from work conducted July 2021 to October 2021.

### **ABSTRACT**

Grapevine leafroll (GLD) and red blotch (RBD) are consequential viral diseases of grapevine. Fundamental knowledge of disease ecology is critical to successful mitigation programs, which incorporate tactics such as provision of virus-screened plant material, removal of inoculum reservoirs, either as individual or groups of vines, and reduction of vector population by cultural or chemical practices. Removal of inoculum reservoirs is contingent on rapid, accurate identification of infected vines, either by visual symptoms or molecular assay. We are developing an artificial intelligence tool to improve vision-based assessments of symptomatic vines. And are adopting the use of a rapid assay to detect infected vines. Combined, these tools can improve users' ability to identify and remove infected vines to reduce viral inoculum in commercial vineyards. For red blotch specifically, our more limited understanding of disease ecology has complicated the development and uptake of management practices. To address knowledge gaps in disease ecology and uncertainties in management, UCCE-Napa scientists and growers are monitoring 23 unique, commercial vineyard blocks over the 3-year project period. Data on insect populations, disease incidence, vine removal and vineyard floor management will be collected. Spatial and temporal trends in the measured variables will be explored to improve our understanding of RBD epidemiology and inform the development and implementation of management guidelines. Educational activities will include networking (focus group) meetings, seminars, field days and workshops, as well as the development of handouts and instructional videos.

### **LAYPERSON SUMMARY**

Grapevine leafroll (GLD) and red blotch (RBD) are among the most consequential grapevine diseases (GLD). Because these diseases are incurable, mitigation efforts to reduce spread include

(a) sourcing virus-screened plant material; (b) removing diseased vines individually (roguing) or redeveloping high-incidence blocks; and (c) reducing vector populations. Successful roguing requires accurate identification of diseased vines, which can be challenging when symptoms are confusing, asynchronous, or absent (such as in white-berried cultivars). This project seeks to increase the accuracy of visual assessments and improve vine removal efforts using artificial intelligence (AI) and an “in-house” assay. We will also address fundamental questions of RBD ecology by harnessing the power of grower-collected data. Regional monitoring of the disease and vector, combined with network-based learning, will address uncertainties in RBD epidemiology and management. Educational opportunities will include field days, workshops, seminars, and networking groups. Educational products will include handouts and instructional videos. This project will advance the use of emerging technologies to identify diseased vines and support grower-coordinated efforts to reduce the economic and environmental impacts of GLD and RBD for the grape industry.

## 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), it occurs in every major grape-growing region, infecting wine, juice and table grape cultivars and 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 (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) result in significant economic losses from GLD (Atallah et al. 2012; Ricketts et al. 2015). Currently, eleven mealybug (Pseudococcidae) and eight soft scale (Coccidae) species are recognized vectors of GLRaV-3 (Naidu et al. 2014; Herrbach et al. 2017). The deleterious effects to the vine and fruit, combined with rapid spread under ideal conditions, have elevated GLRaV-3 management efforts worldwide (Almeida et al. 2013, Pietersen et al. 2013, Naidu et al. 2014, Poojari et al. 2017).

Red blotch disease (RBD) has emerged in the last decade as an important viral disease of grapevine. The causal agent, *Grapevine red blotch virus* (GRBV; Yepes et al. 2018), has been found in *Vitis vinifera* plant material in major viticulture regions throughout North America (Cieniewicz et al. 2017; Krenz et al. 2014; Sudarshana et al. 2015). This disease has significant economic impacts, resulting from altered sugar, acid and phenolic accumulation (Blanco-Ulate et al. 2017; Girardello et al. 2019). The resultant wines may have lower ethanol concentration, higher acidity and less color (Girardello et al. 2019). Economic losses may reach up to \$170,000 per acre over the lifespan of a vineyard, including cost penalties for suboptimal fruit and detraction from negotiating power for price points (Ricketts et al. 2017).

Because GLD and RBD are incurable, preventative practices include the use of virus-tested plant material to reduce initial infections (Arnold et al. 2019). Strategies aimed at reducing vector populations may also be implemented, depending on the pathogen and vector species. Identification and removal of leafroll-diseased vines (roguing) reduces vine-to-vine spread (Almeida et al. 2013; Pietersen et al. 2013; Bell et al. 2017, 2018; Arnold et al. 2019). To accomplish this, symptoms are visually assessed in the fall and infected vines marked for removal. One of the primary challenges to roguing is the correct assessment of visual symptoms, since they can resemble those caused by a variety of biotic and abiotic ailments, such as spider

mite feeding damage, girdling, mineral deficiency or toxicity, and root or cambium damage (Sudarshana et al 2015). Furthermore, there is a 1-year latent period for GLRaV-3, meaning that infected vines can be present in the vineyard without visual symptoms (Blaisdell et al. 2016). A latent period for GRBV has not been established experimentally (Cieniewicz et al 2017). Although the development of RBD symptoms can be characteristic, the severity and timing of symptom onset varies across seasons (Sudarshana et al. 2015). In some situations, premature leaf fall can affect growers' ability to identify symptomatic vines. Successful roguing therefore requires an investment in training of field scouts and in diagnostic assays, both of which can be time and cost intensive (Bell et al. 2017). Visual assessments of symptoms may over or underestimate total disease incidence. In small plot research, a high level of confidence in visual symptom assessment can be accomplished by assaying a large number of vines and correlating symptom expression to results (Bell et al. 2017; Cieniewicz et al. 2017). However, growers have more limited resources to conduct extensive sampling, creating uncertainty in the face of critical and costly management decisions. We aim to address this by developing a pair of tools to improve the rapid identification of diseased vines.

Artificial intelligence (AI) is the theory and practice of developing computer systems that perform tasks that are generally expected to require human intelligence. These systems use algorithms to turn data inputs, such as a photograph, into data outputs, such as the identification of a disease. In modern AI development, practitioners give the computer system numerous examples of the data inputs (i.e., photographs) with corresponding examples of the data outputs (i.e., disease diagnoses) and the computer system uses the examples to write the instructions for the algorithm. In effect, the computer learns how to write the algorithm based on the examples, hence these types of algorithms are called machine-learning algorithms. A neural network, also referred to as deep learning, is a type of machine-learning algorithm. Computer vision is a field within the broader AI discipline. Computer vision has progressed to the point where it can identify anything that a person can visually identify. For example, computer vision models can classify skin lesions as cancerous with the same level of accuracy as dermatologists (Esteva et al. 2017). Computer vision can also identify abiotic and biotic soybean stressors from leaf symptoms with 94% accuracy (Ghosal et al. 2018). Computer vision performance has improved because the machine-learning algorithms have improved. In the current state of computer vision, the limiting factor is not the machine-learning algorithm, but rather the size and diversity of training dataset (Sun et al. 2017). Given a sufficient number of examples of photographs of a plant disease and given sufficient representation of the diversity of the symptoms among the photographs, machine-learning-based computer vision models can learn to accurately identify any plant disease with visual symptoms.

The LAMP (loop mediated isothermal amplification) method is a point-of-use, DNA-based assay that has been developed for detection of GRBV (Romero Romero et al. 2019). The assay does not require special facilities, expensive equipment or highly trained laboratory personnel. It offers a unique, "in-house" testing alternative to the current industry practice of sampling and sending plant material to commercial testing labs for virus analysis using polymerase chain reaction (PCR) assays. The LAMP assay is 10,000 times more sensitive than PCR, requires an initial investment of \$4000, and subsequent costs are \$3-\$5 per sample. Preliminary data indicate a strong consistency between LAMP and PCR results. The benefit of this assay is that it would enable growers to assay large numbers of samples and quickly generate results. There is a

learning curve to proficiency. The assay is highly sensitive, so users must implement strict protocols to avoid potential contamination. Our efforts are complementary, but not duplicative, to those of the Cornell University team. As described previously, visual assessments for RBD are challenging given that symptoms can be inconsistent, variable and asynchronous. Furthermore, an unclear latent period and asymptomatic, white-berried cultivars can complicate efforts to remove pathogen reservoirs. We will therefore explore the LAMP assay as a complement to visual symptom assessments to improve growers' ability to identify and remove GRBV-infected vines in commercial vineyards.

We will use network-based learning to increase uptake of these technologies and improve disease management outcomes. This builds on previous efforts for GLD management by decision-support networks in Oakville and Rutherford, California (2012-2016). Group members monitored male mealybug flights and tracked GLD incidence across a regional area of ca. 2000 acres. They shared data and coordinated removal and replant strategies among neighbors. These types of extended decision networks, that involve a community of extension educators, crop advisers and growers, influence the adoption of new practices by fostering key relationships that catalyze social learning and group problem-solving (Gent et al. 2013; Hoffman et al. 2015). Analysis of the grower GLD dataset suggests that individual actions combined with community efforts resulted in successful disease management (MacDonald et al., submitted). Given this, we will adopt a similar strategy for monitoring RBD vector(s) and disease incidence. In summary, this project advances the use of emerging technologies and coordinates regional networking groups to reduce the economic and environmental impacts of grapevine leafroll and red blotch diseases.

## **OBJECTIVES**

The goal of this project is to develop and apply emerging technologies to identify diseased vines and catalyze grower-driven data collection efforts to improve decision-making and viral disease mitigation efforts by the grape industry.

### **OBJECTIVE 1: Build the grape industry's capability to rapidly identify diseased vines**

using (a) artificial intelligence to assess vines based on visual symptoms; (b) applications of the LAMP assay, an "in-house" DNA-based diagnostic tool for grapevine red blotch virus.

### **OBJECTIVE 2: Improve red blotch disease management through network-based learning.**

We will catalyze grower-driven, regional monitoring of vector(s) and disease and use spatiotemporal analyses to address fundamental questions of RBD ecology and management.

## **RESULTS AND DISCUSSION**

### **Objective 1. Build the grape industry's capability to rapidly identified diseased vines**

#### **1a. Artificial intelligence: Compile database of photographs of diseased & healthy vines**

With the goal of developing artificial intelligence (AI) for the rapid vision-based identification of diseased vines, we are compiling a robust collection of validated photographs of diseased and healthy vines. We are focused principally on photographs depicting vines with visual symptoms associated with confirmed GLRaV-3 and GRBV infections, as those are the most commonly occurring viral diseases of grapevine in California and are the focus of this study. We are also collecting photographs of other common canopy symptoms such as potassium deficiency, measles (Esca), spider mite feeding damage and girdling, as well as healthy, asymptomatic vines. Both the research team and growers are contributing photographs, with associated diagnostic

assay results to confirm the infection status of the photographed vine. We have also reached out to commercial laboratories and industry organizations (Napa Valley Grapegrowers) to support data collection efforts through their members and clients. To date, we have curated over 6,000 photographs toward our goal of 10,000 images. During the next two project periods (Winter 2021-22 and Spring 2022) we will build a preliminary model that will be tested against a library of collected images with the goal of releasing a beta version of the application to industry users in various grape growing regions of California by fall of 2022. The beta version will be refined over time as we identify areas where the model is performing well and where it needs improvement, while remaining transparent with end users regarding its limitations in the preliminary stages.

**1b. Artificial intelligence: Explore partnerships to deploy AI tools to identify diseased vines**

Tule Technologies and The New Zealand Plant & Food Research Institute are key partners supporting the development of AI technology and its adoption by end-users in the grape industry. Action on this sub-objective is contingent on the development of an AI application, as described previously and is scheduled to commence in late summer of 2022.

**1c. Applications of LAMP assay for detection of GRBV to complement visual assessments and improve vine removal programs.**

Vine-by-vine removal (roguing) of visually symptomatic vines is a proven strategy for GLD management but has not been assessed experimentally for RBD. Roguing efforts are compounded by the difficulty in identifying RBD visual symptoms, variability in the degree of symptom expression, asynchronous symptom development and lack of knowledge about the latent period. We are exploring applications of the LAMP assay to complement visual assessments and improve detection of GRBV-infected vines for subsequent removal. Towards this goal, from September 17- October 20, 2021, we assayed 632 vines from 9 unique vineyard blocks that have been anonymized based on their location within the Napa Valley AVA (Oak Knoll, Oakville River East, Oakville West, Rutherford Central, Rutherford West, St Helena, St Helena Central, Wooden Valley and Yountville). Additional vines will be assayed subsequently, from an additional two blocks (Oakville River West, Rutherford West2). Our experiences this season have demonstrated multiple applications of the LAMP assay:

(1) The LAMP assay can be used to confirm the accuracy of visual assessors. For example, 10 samples were collected from “St Helena” prior to visual mapping assessments. LAMP assays confirmed that visual symptom assessments matched the vines’ infection status, increasing our confidence in visual assessments.

(2) The LAMP assay can be used to confirm the infection status of vines with confusing symptoms. For example, “Oakville River West” had 18 vines that had poor growth and were sufficiently weakened to make it difficult to assess visually. Of these, six returned a positive result in the assay and 12 returned a negative result. Their infection status would not have been determined without the assay, and this uncertainty could have led to infected vines being retained or uninfected vines being removed, at increased expense to the grower.

(3) The LAMP assay can be used confirm the infection status of vines with symptoms resulting from mixed virus infections. For example, “St Helena Central” and “Rutherford West” (both *V. vinifera* cv. Cabernet Sauvignon clone 337) predominantly express symptoms of GLRaV-2,

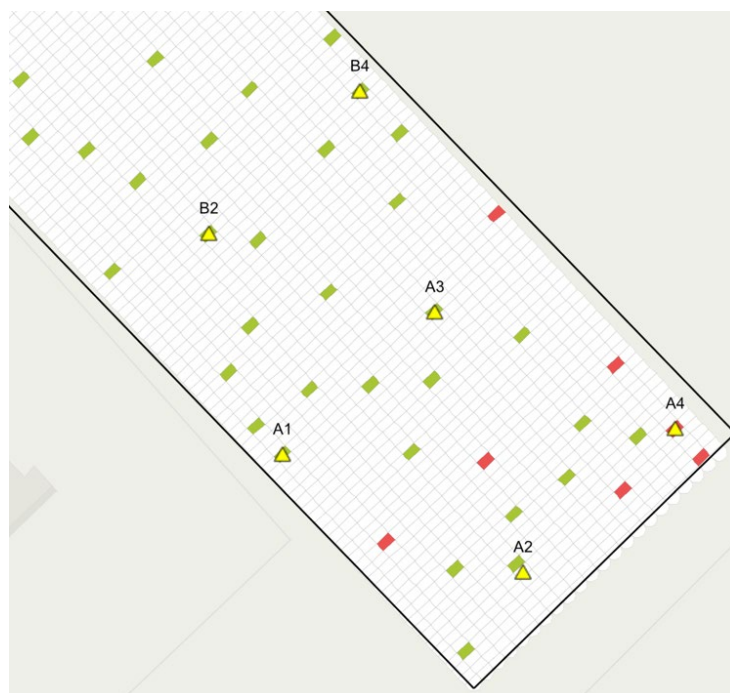
making it difficult to visually assess for symptoms of RBD. We tested approximately 50 vines in each block, finding two GRBV-infected vines in “Rutherford West” and nine GRBV-infected vines in “St Helena Central”, corresponding to relatively low rates of infection. However, the pattern of distribution at “St Helena Central” (depicted in figure 1) suggests a hotspot in the southern portion of the block that could be further monitored to assess incidence and potential for spread.

(4) The LAMP assay can be used to confirm the infection status of vines with light or newly developing symptoms. We observed an array of symptom severity at our field sites. Often, vines expressed subtle symptoms that were limited in distribution within the vine to one shoot or a few lightly symptomatic leaves. For example, at “Oakville West” we recorded three vines with subtle, limited symptoms (Figure 2); all three vines were positive for GRBV in LAMP assays.

(5) The LAMP assay can be used to identify vines that were infected, but not symptomatic at the time that visual assessments were conducted. We observed red blotch symptoms developing over an extended period, starting in early July, and continuing through October. We observed variability between sites as to when symptoms first appear, and variability within a site as to when vines develop symptoms (Figure 3). We refer to this as asynchronous symptom development. This asynchronicity complicates our ability to identify an optimal date for visual symptom assessments, suggests that multiple mapping passes may be needed to identify all symptomatic vines, and challenges growers or pest control advisers who have to balance the need to assess multiple sites before leaf fall.

(6) The LAMP assay can be used to assess infection status of asymptomatic vines. Although the potential impacts of GRBV on white cultivars has not been explored, one concern is that the asymptomatic blocks may be a reservoir of GRBV inoculum. To assess that potential, 81 and 80 asymptomatic *V. vinifera* cv. Sauvignon blanc vines at “Rutherford Central” and “Oak Knoll”, respectively, were assayed. Of these, 8 (“Rutherford Central”) and 11 (“Oak Knoll”) tested positive for GRBV. Additionally, two *S. festinus* specimens collected in July 2021 at “Oak Knoll” tested positive by qPCR analysis, as part of a collaboration with Cornell University (Marc Fuchs, Principal Investigator).

(7) The LAMP assay is rapid, reliable, and efficient alternative to traditional diagnostic assays. To conduct the LAMP assay, users must have a clean space, training, and general proficiency in laboratory techniques. However, the wine grape industry is uniquely positioned to adopt in-house assays, as wineries have laboratory spaces where fruit, juice and wine samples are assessed using standard practices. Staff of these enology laboratories are familiar with standard operating procedures and practices to ensure cleanliness and reproducibility. In roughly 4 weeks, our team collected and assayed more than 600 samples, and produced assay results for 50-75 samples within 48 hours of sample collection. The volume of samples as well as the speed with which we returned results meant that we could immediately apply the results to improve visual assessments of GRBV-infected blocks. In summary, the removal of symptomatic vines relies on accurate and timely visual assessments. However, there are limitations to visual assessments, and the LAMP assay can be used to complement or replace these assessments. Ultimately, the LAMP assay can be used to complement or replace visual assessments and improve our ability to reduce GRBV inoculum and reduce spread.



**Figure 1.** At “St Helena Central”, 48 vines were assayed for GRBV, of which 9 were positive and 39 were negative. Infected vines were clustered in the southern and eastern portions of the block. Rectangles represent individual vines within the block. Those that are colored green had a negative result and those colored red had a positive result.



**Figure 2.** A Cabernet Sauvignon vine at “Oakville West” with very limited and difficult to distinguish symptoms of red blotch disease. Because the symptoms are subtle in coloring and limited to a single shoot and few leaves, this vine was not be marked for removal, although a LAMP assay returned a positive result for GRBV.





**Figure 3.** Cabernet Sauvignon vines at “Oakville West” have symptoms of red blotch disease (red blotches on blade). The vine on the right has been marked for removal with red flagging tape, but the one on the left has not been marked. Due to asynchronous symptom development, the vine on the left did not have symptoms when the vines were assessed

**OBJECTIVE 2: Improve red blotch disease management through network-based learning**

We are catalyzing existing grower networking groups to implement regional monitoring to improve our understanding of red blotch ecology and management, focusing in three areas:

**2a. Coordination and training: recruitment and support for grower-driven data collection.**

We recruited 11 growers to monitor insect populations and incidence of symptomatic vines in 16 unique vineyard blocks. Additionally, the project team is monitoring 10 blocks, concurrent with objective 1c, described previously. Insect monitoring was conducted at weekly intervals from June through September, using an array of 6 yellow panel traps in the lower canopy in each block. Incidence of RBD symptomatic vines will be recorded annually in October for each block. To support these efforts, the UCCE team developed educational resources, including handouts that are accessible on a project-based webpage (<https://ucceviticulturenapa.wixsite.com/uccevitnapa/red-blotch>) and held 4 educational sessions to train participants to identify target insect(s), distinguish disease symptoms and share data.

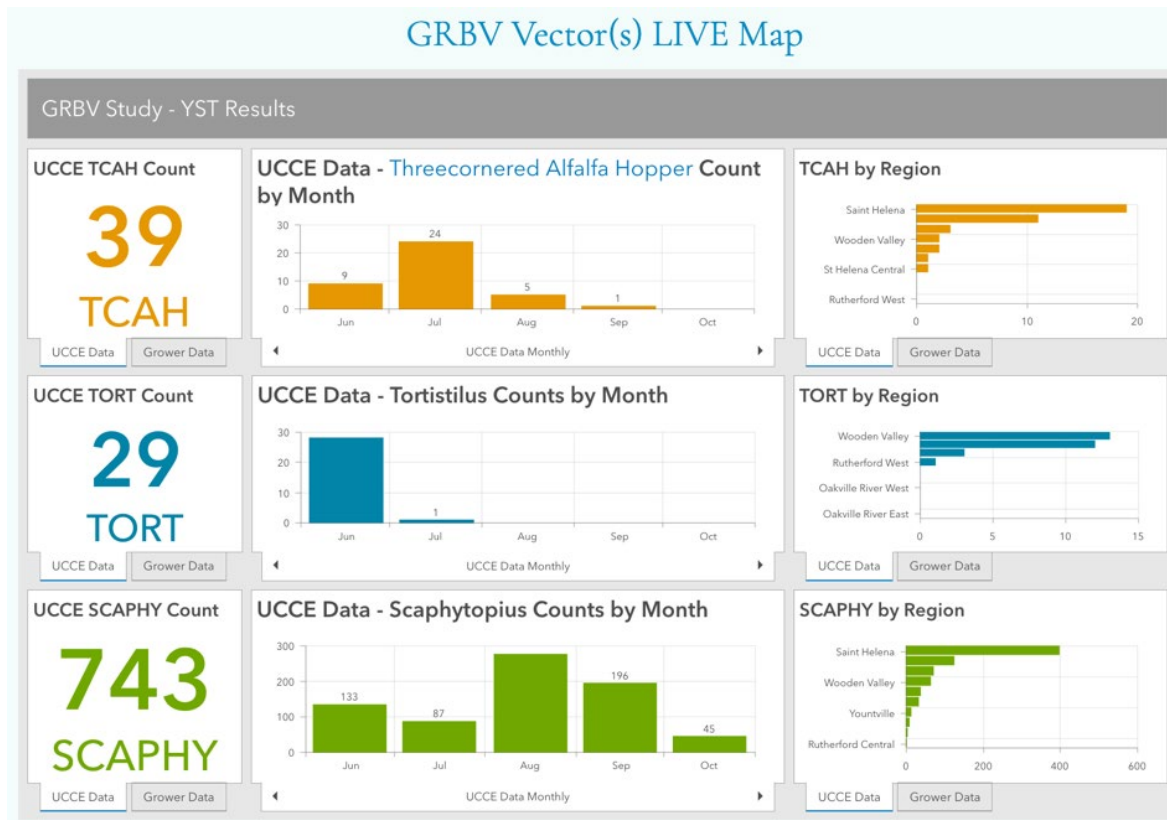
**2b. Data sharing: increase growers’ access to actionable information.**

Technological advances have improved data-sharing capabilities, increased accessibility to actionable information, and fostered greater engagement among participants. We are utilizing online platforms (Google Sheets and ArcGIS Dashboards) to record and share data in real-time. Specifically, the UCCE team created an online, shared data sheet (Google Sheet). Participants enter data using their anonymized code. This includes descriptive data (one time, at start of the study), vector data (weekly), red blotch disease incidence (annually) and management data

(annually). The data sheet is linked to an ArcGIS online dashboard (<https://ucceviticulturenapa.wixsite.com/uccevitnapa/data-dashboards>) that updates automatically and is viewable in real-time. Data are sorted by various parameters, and both the community (grower) and project team (UCCE) data are visualized and summarized.

## 2c. Data analysis: exploring trends in vector populations and disease spread.

Activities associated with this sub-objective will not commence until the final project year, when sufficient data are collected to ensure a robust analysis.



**Figure 4.** Community (grower) and project team (UCCE) data are visualized using ArcGIS Dashboard (Esri, Redlands, CA). Total trap captures for 2021 include 39 *S. festinus*, 29 *T. albidosparsus*, and 743 *Scaphytopius graneticus* on UCCE-monitored traps at 10 unique vineyard sites in Napa County.

## CONCLUSIONS

This is an incipient project that has made significant progress on its objectives and laid the foundational groundwork for successful outcomes. We are actively sourcing and taking photographs of diseased vines, having collected 2/3 of the photographs that will be used to build a vision-based application to assess symptoms of red blotch and leafroll disease in the vineyard. We are on-track to release a pilot version of this application in time for the next virus mapping season (September and October 2022). This decision-support tool will create efficiencies for

growers' marking and removing diseased vines. We have identified 6 scenarios where the LAMP assay for GRBV complements vision-based assessments of disease symptoms. We demonstrated that the LAMP assay for GRBV is a rapid, efficient, and reliable alternative to conventional laboratory-based diagnostic assays. We developed an educational video demonstrates the LAMP assay for GRBV, and complementary educational materials that detail supply needs and standard processes, as well as a webpage (<https://ucceviticulturenapa.wixsite.com/uccevitnapa/red-blotch>) that is a repository for all project-based educational materials. We recruited 11 participating growers who are monitoring 16 vineyard blocks. The project team monitored an additional 10 vineyard blocks. All data are viewable in real-time on the above webpage, increasing participants' engagement and increasing access to actionable information. We have several outreach activities planned for the winter project period to disseminate preliminary results. Taken together, our activities are developing decision-support tools, increasing access to actionable information, improving the efficiency of efforts to reduce virus inoculum in commercial vineyards, and overall reducing knowledge gaps for red blotch disease management and engaging the grape industry to address the intractable problem of these viral diseases.

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## SPOTTED LANTERNFLY OUTREACH AND MAPPING THE RISK-PRONE AREAS IN CALIFORNIA

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**Reporting Period:** The results reported here are from work conducted between January 2021 to October 2021.

### ABSTRACT

The spotted lanternfly (SLF), *Lycorma delicatula*, is an invasive pest in the United States and several California crops are at high risk if it ever arrives in the state. To continue outreach efforts in California that were initiated in 2014 when the pest was first detected in the United States, i) a webpage was created with various resources, ii) masks were produced with a large SLF image and distributed to growers, pest control advisors, researchers, and private citizens, iii) presentations were made at multiple extension meetings, iv) information was presented in a radio talk show, and v) information about SLF resources and efforts were emailed to more than 1700 stakeholders. To help understand the SLF risk to various counties based on cultivated and wild hosts several maps were created based on the acreage, value, and distribution of hosts to identify low to high-risk areas.

### LAYPERSON SUMMARY

SLF is an invasive pest that is rapidly spreading to several states in the eastern United States and moving westward. Apples, grapes, cherries, walnuts, and other high-value commodities and several landscape species in urban and residential areas are at risk if this pest invades California. Researchers are working on developing biological control solutions and other strategies to manage this pest. However, the best strategy is to prevent or at least delay its invasion to new areas. To increase the awareness of this pest, various outreach efforts have been made by developing outreach material and giving presentations. Based on the distribution of 22 cultivated and 70 wild hosts of SLF in California, various maps were generated. These efforts will help Californians to identify, report, and manage this pest if it is accidentally introduced into the state.

### INTRODUCTION

The spotted lanternfly (SLF) [*Lycorma delicatula* (Hemiptera: Fulgoridae)] is an invasive planthopper, which causes a significant damage to apples, grapes, stone fruit, trees used for timber, and other hosts (Dara et al. 2015). Native to China, SLF was first reported in 2014 in Pennsylvania and has been rapidly spreading in the eastern United States and moving westward. California has 22 cultivated and about 70 wild hosts of SLF and include several high value crops such as apples, cherries, grapes, and plums. The tree-of-heaven, an invasive species, is a favorite host of SLF and is widely distributed in California. SLF is also a nuisance pest with 100s or 1000s of individuals infesting landscape trees and hosts in residential areas. This pest deposits eggs on inanimate objects such as vehicles, furniture, stones, and packages and thus spread to other areas through the movement of these objects. Awareness of the pest and its damage potential, ability of Californians to recognize and report the pest if found, and the knowledge of

control practices will help prevent accidental transportation of eggs or other life stages from the infested areas to California and prepare the citizens to take appropriate actions. Outreach efforts have been made in California since 2014 through extension articles, presentations at extension meetings, videos, social media posts, and personal communication (Dara, 2014).

Wakie et al. (2020) modeled the establishment risk of SLF in the United States and around the world and indicated that many coastal regions and the Central Valley of California are among the high-risk areas. Considering the risk to several high-value commodities and the presence of several wild hosts that are distributed all over California, mapping the risk-prone areas based on the cultivated hosts, their acreage and value in different counties, and the distribution of wild hosts will help both growers and other Californians to prepare for potential invasion of SLF.

## **OBJECTIVES**

1. Continue existing outreach efforts to increase the awareness of SLF risk in the Central Coast area and other parts of California.
2. Map areas prone to the SLF risk in California based on cultivated and wild hosts, and acreage and value of cultivated hosts.

## **RESULTS AND DISCUSSION**

### **Objective 1. Spotted lanternfly outreach**

California, with a wide range of cultivated and wild plant species, is at risk of SLF infestations. Movement of inanimate objects with SLF life stages and travel from the SLF-infested areas could potentially bring this pest to California. SLF deposits eggs on vehicles, shipping packages, and other objects and covers with a protective coating, which can look like a splash of mud and can go unnoticed. Previous outreach efforts included extension articles in UCANR eJournal Pest News and trade journals, media interviews for podcasts and ag media, webinars, talks at various extension meetings, short and detailed outreach videos, surveys, and social media posts. As a part of this project, the following outreach efforts have been made.

A webpage (<https://ucanr.edu/spottedlanternfly>) was created to provide a brief overview of SLF with links to YouTube videos, scientific and extension articles, and other resources.

Two hundred facemasks with a large picture of SLF were ordered and distributed to growers, pest control advisors, researchers, members of Farm Bureaus or commodity boards, Agricultural Commissioners, and private citizens since August 2021. An article was written by Mike Hodgson in Lompoc Record about the masks ([https://lompocrecord.com/business/agriculture/ag-adviser-using-masks-printed-with-insect-pest-to-raise-public-awareness/article\\_79d41cc3-4a07-5cc2-9869-f21510a7590c.html](https://lompocrecord.com/business/agriculture/ag-adviser-using-masks-printed-with-insect-pest-to-raise-public-awareness/article_79d41cc3-4a07-5cc2-9869-f21510a7590c.html)).

A presentation titled “Spotted lanternfly: risk-prone areas in California and current management options” was made at the annual meetings of the California Association of Pest Control Advisors in October, 2021. Another presentation will also be made in November 2021 at the annual meetings of the Association of Applied IPM Ecologists.

SLF information was provided in the KVEC 920 Hometown Radio talks show with the guest host Dan Shadwell ([https://www.ivoox.com/en/hometown-radio-10-11-21-3p-guest-host-dan-shadwell-audios-mp3\\_rf\\_76662245\\_1.html](https://www.ivoox.com/en/hometown-radio-10-11-21-3p-guest-host-dan-shadwell-audios-mp3_rf_76662245_1.html)).



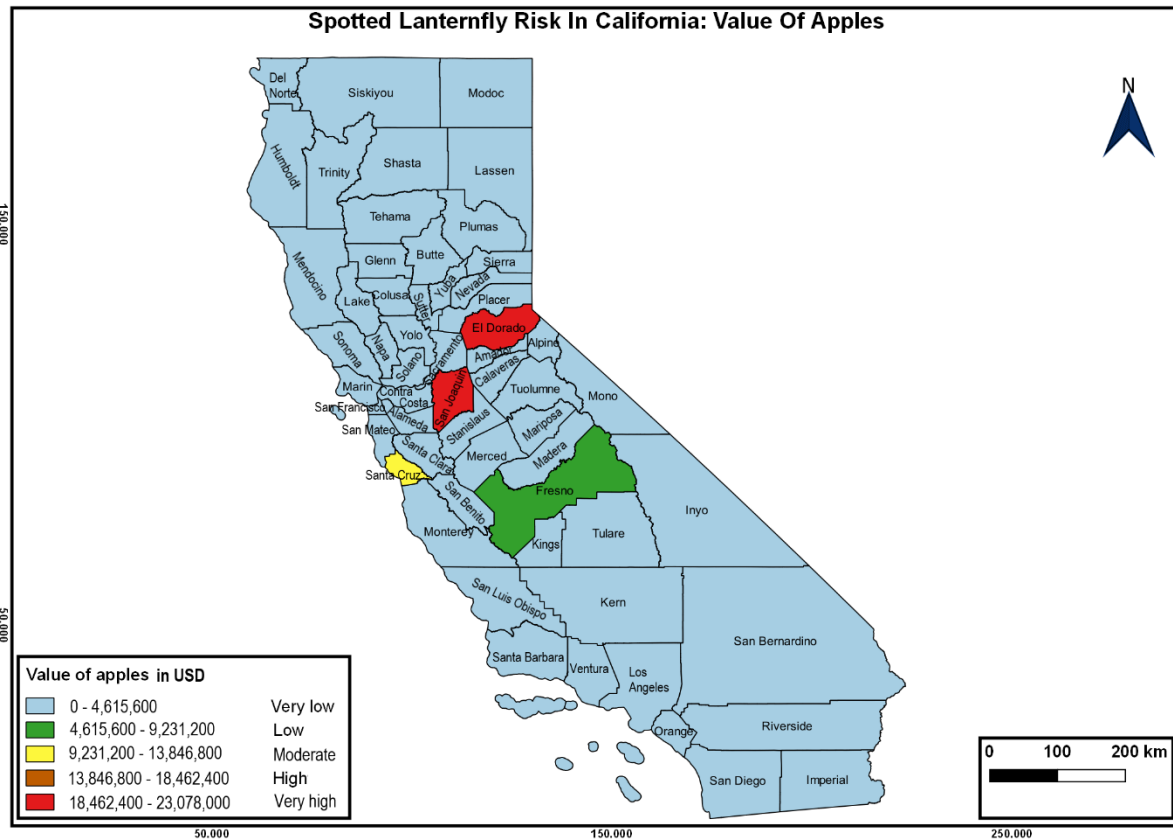
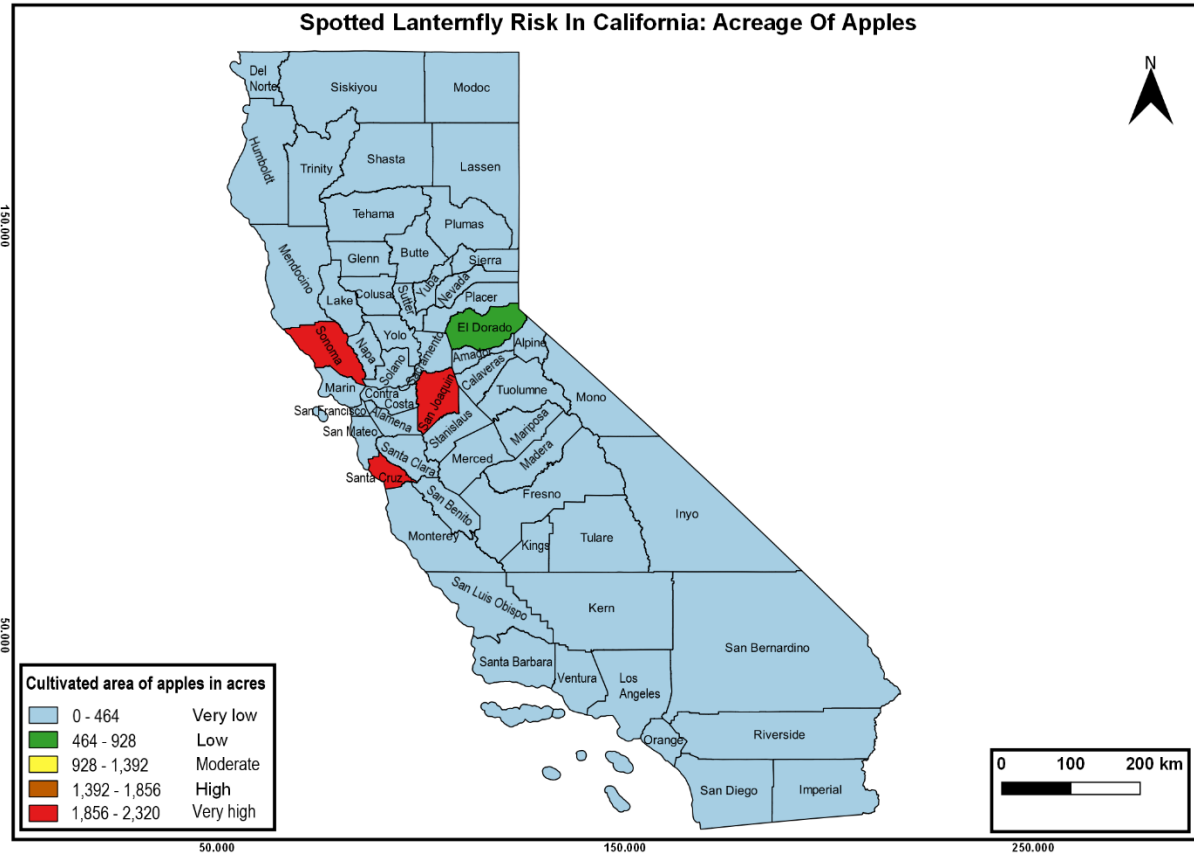
Emails were sent to a list of more than 1700 stakeholders in and outside California on 23 February and 16 September 2021 with information about SLF resources and outreach efforts.

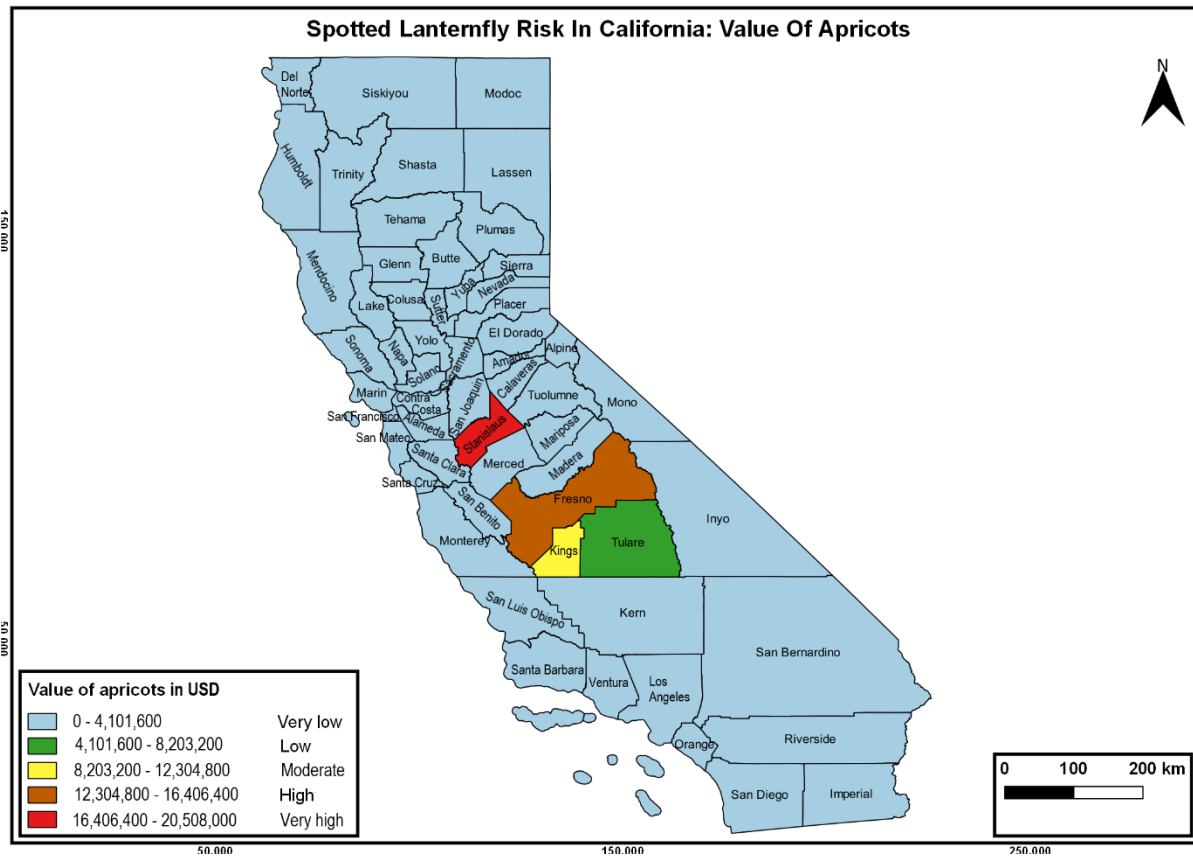
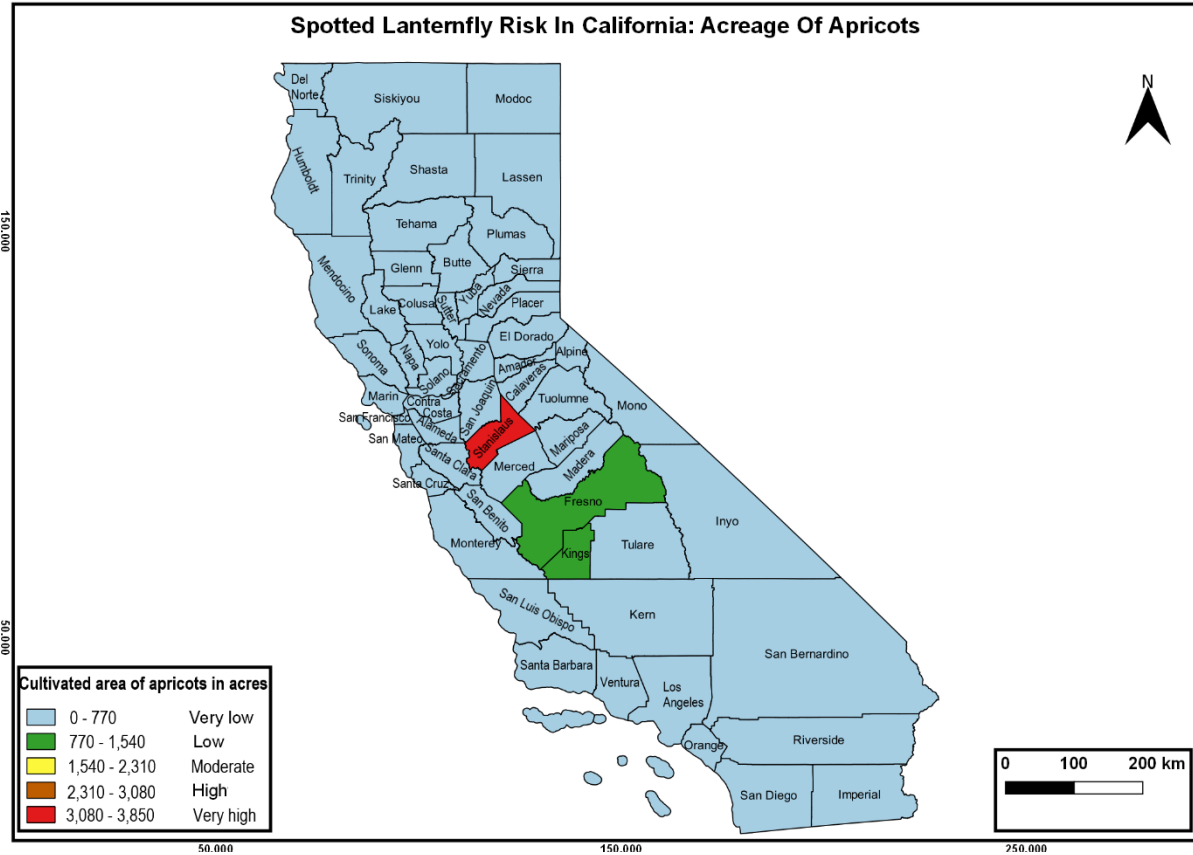
## **Objective 2. Mapping spotted lanternfly risk-prone areas in California**

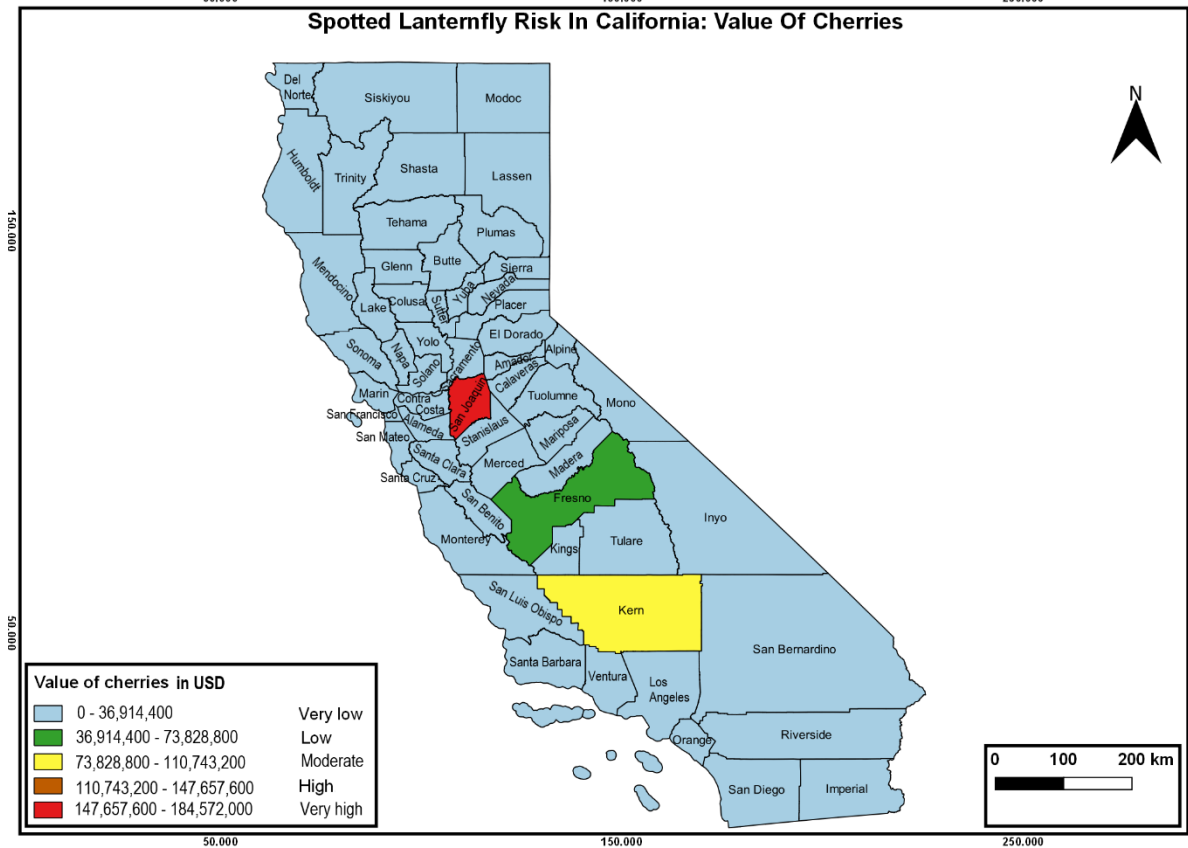
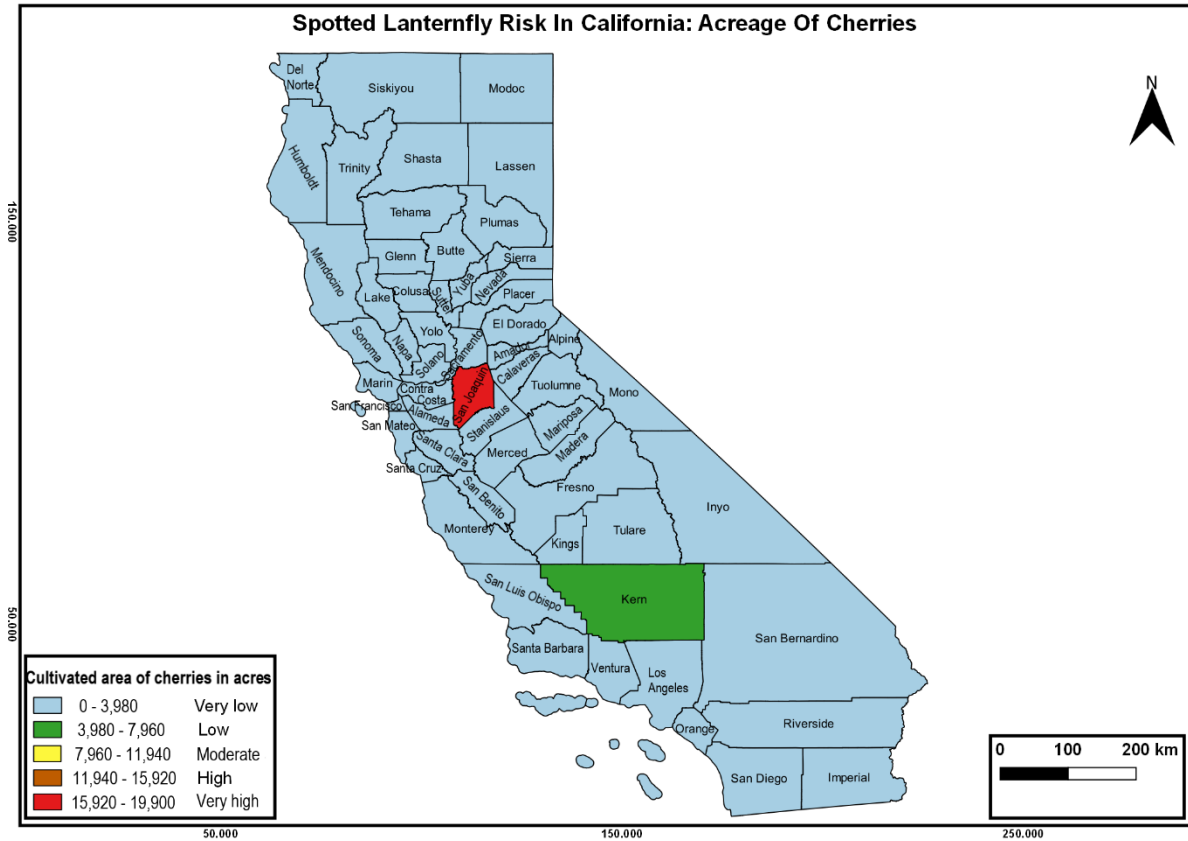
The list of SLF hosts is continuously evolving with host specificity studies in various places. Based on two published resources (Dara et al. 2015; Barringer and Ciafré 2020) 22 cultivated and 70 wild hosts appear to be present in California. Plant species that support some of the feeding life stages or all life stages were included in preparing these lists. The cultivated hosts include apples, apricots, basil, blueberries, butternuts, cherries, cotton, grapes, hibiscus, hops, mock orange, nectarine, peaches, pears, persimmon, plums, pomegranates, roses, soybean, sponge gourd, tea, and walnuts; and the wild hosts include *Acacia* sp., American hazelnut, Amur corktree, American linden, American sycamore, arborvitae, Argentine cedar, Asian white birch, bee balm, big-toothed aspen, black gum, black hawk, black locust, black walnut, Bladder senna, boxelder, chestnut oak, chinaberry tree, Chinese boxwood, Chinese juniper, Chinese parasol tree, Chinese wingnut, dogwood, Eastern white pine, edible fig, false spiraea, fireweed, five-stamen tamarisk, flowering dogwood, Forsythia, Glossy privet, greater burdock, grey alder, hemp, hollyhocks, honeysuckle, hornbeam, Japanese angelica, Japanese boxwood, Japanese maple, Japanese snowball, Japanese zelkova, jujubes, *Kobus magnolia*, Northern spicebush, Norway maple, *Osmanthus* sp. (Devilwoods), *Oxicodendron vernicifluum* (Lacquer tree), perennial salvia, Persian silk tree, plane tree, Poinsettia, poplars, princess tree, red maple, sapphire dragon tree, sassafras, sawtooth, serviceberry, silver maple, slippery elm, snowbell, staghorn sumac, sugar maple, tree-of-heaven, tulip tree, Virginia creeper, white ash, wild grape, and willows.

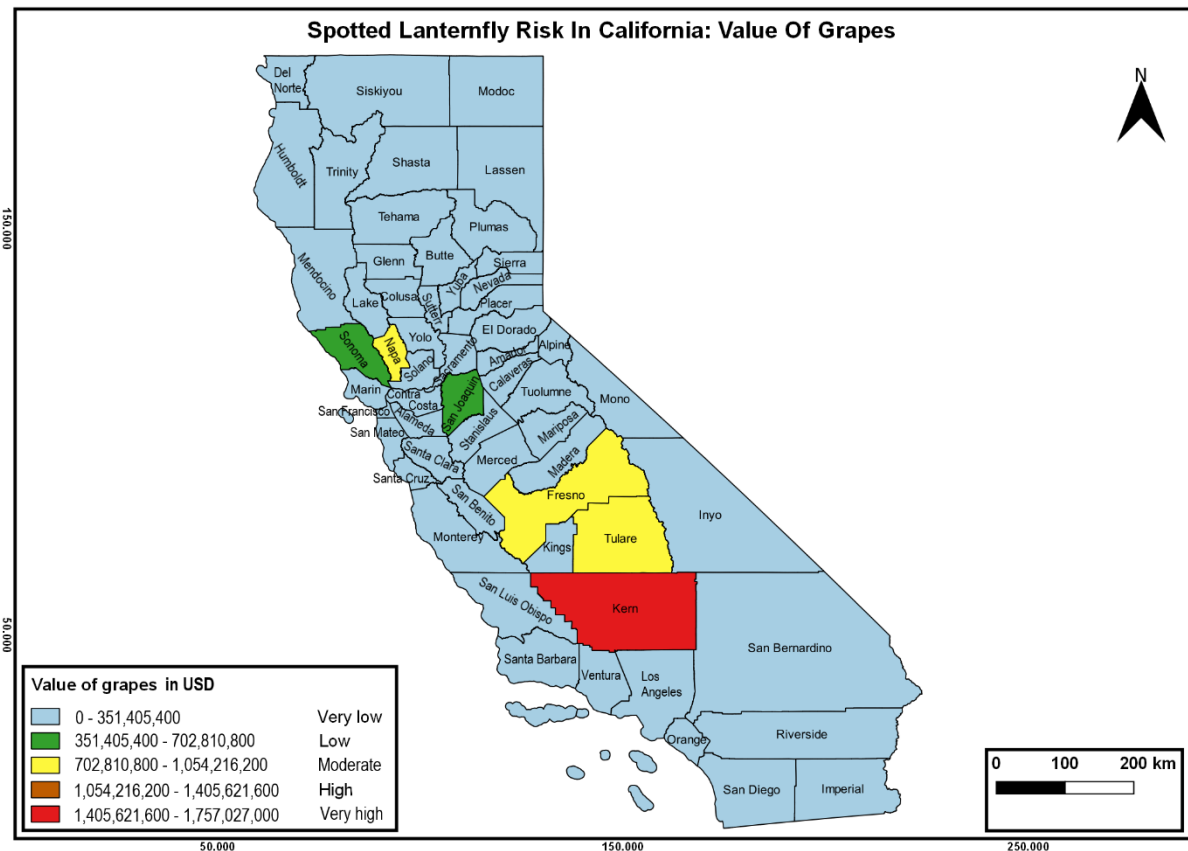
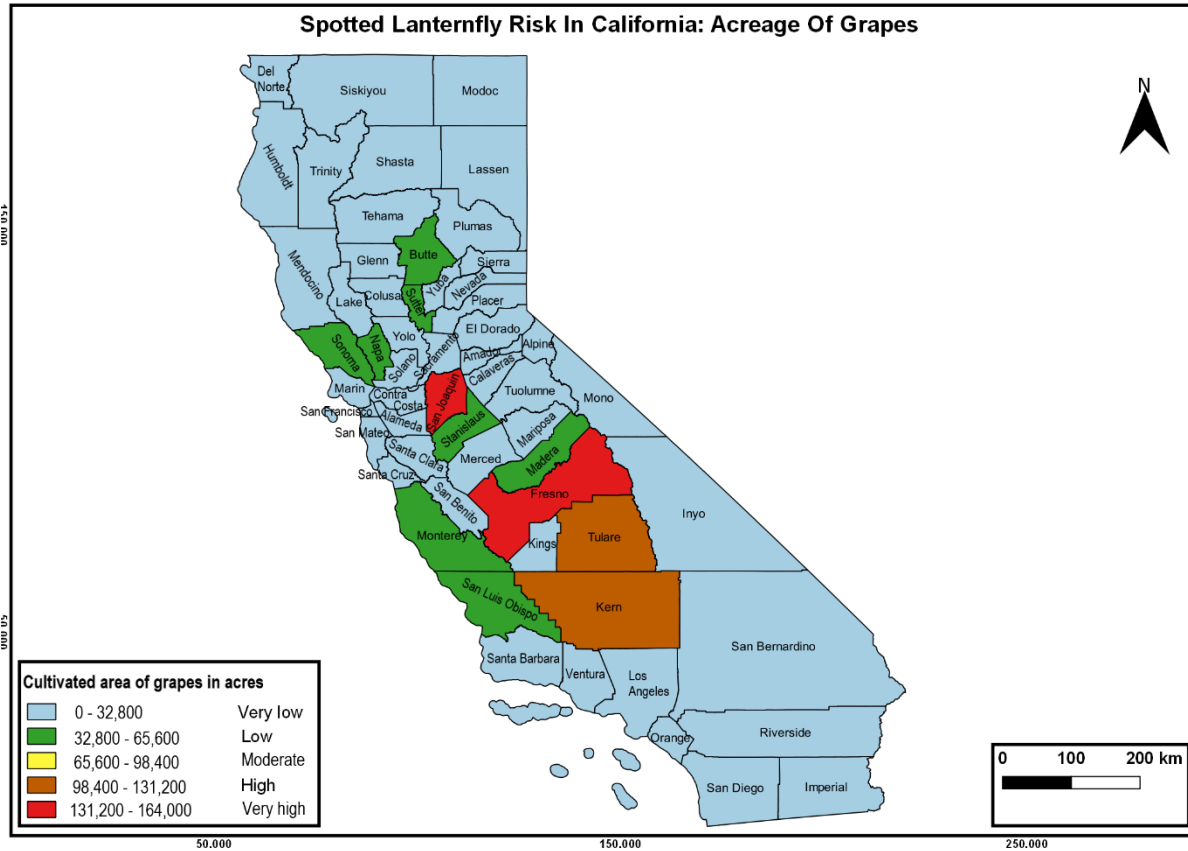
The summary of country crop reports from the California Department of Food and Agriculture (CDFA 2018) was used to determine the value and acreages of the cultivated hosts. To determine the distribution of wild hosts various online resources were used. SLF risk levels were determined as very low, low, moderate, high, and very high for the number of hosts, acreage and value of each cultivated host, and other such parameters within each county. The highest risk value within each parameter was used to determine 'very high' category and 4/5, 3/5, 2/5, and 1/5 were used for high, moderate, low, and very low categories, respectively. In other words, 0-20% risk was considered very low, 21-40% as low, 41-60% as moderate, 61-80% as high, and 81-100% as very high for each measured parameter. Data were entered into a spreadsheet and maps were generated using QGIS open-source cross-platform geographic information system application.

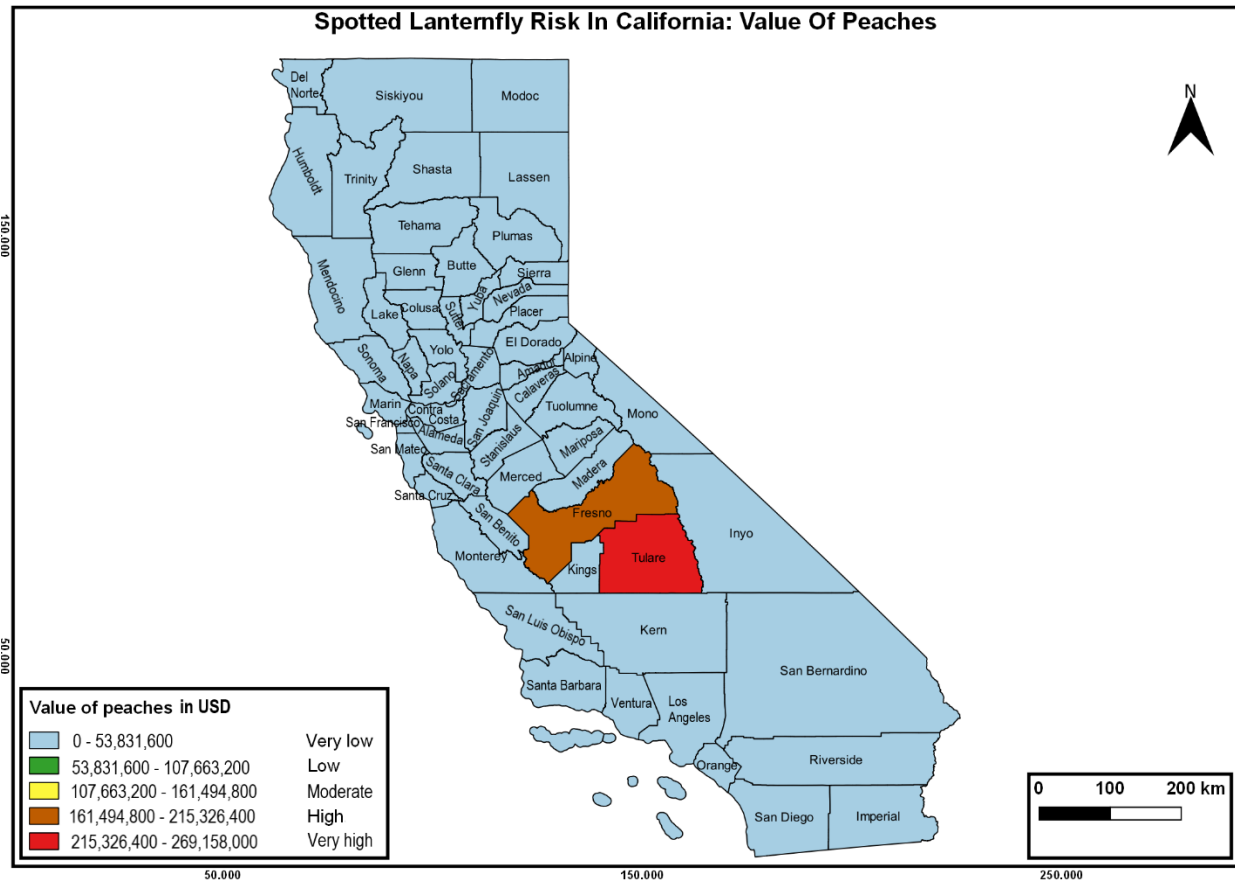
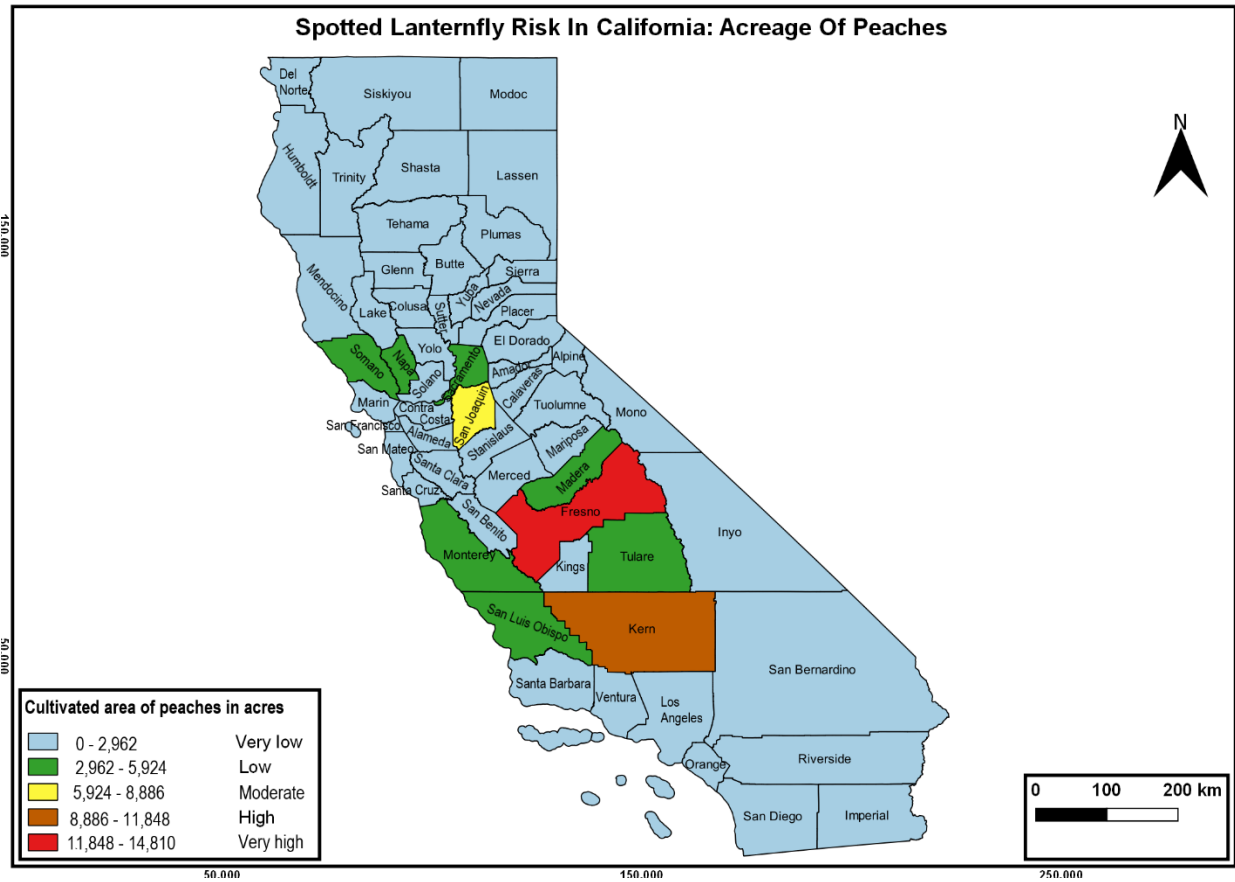
A few examples of maps based on the crop value and acreage of some individual crops or top five cultivated crops in California along with the distribution of 22 cultivated and 70 wild hosts.

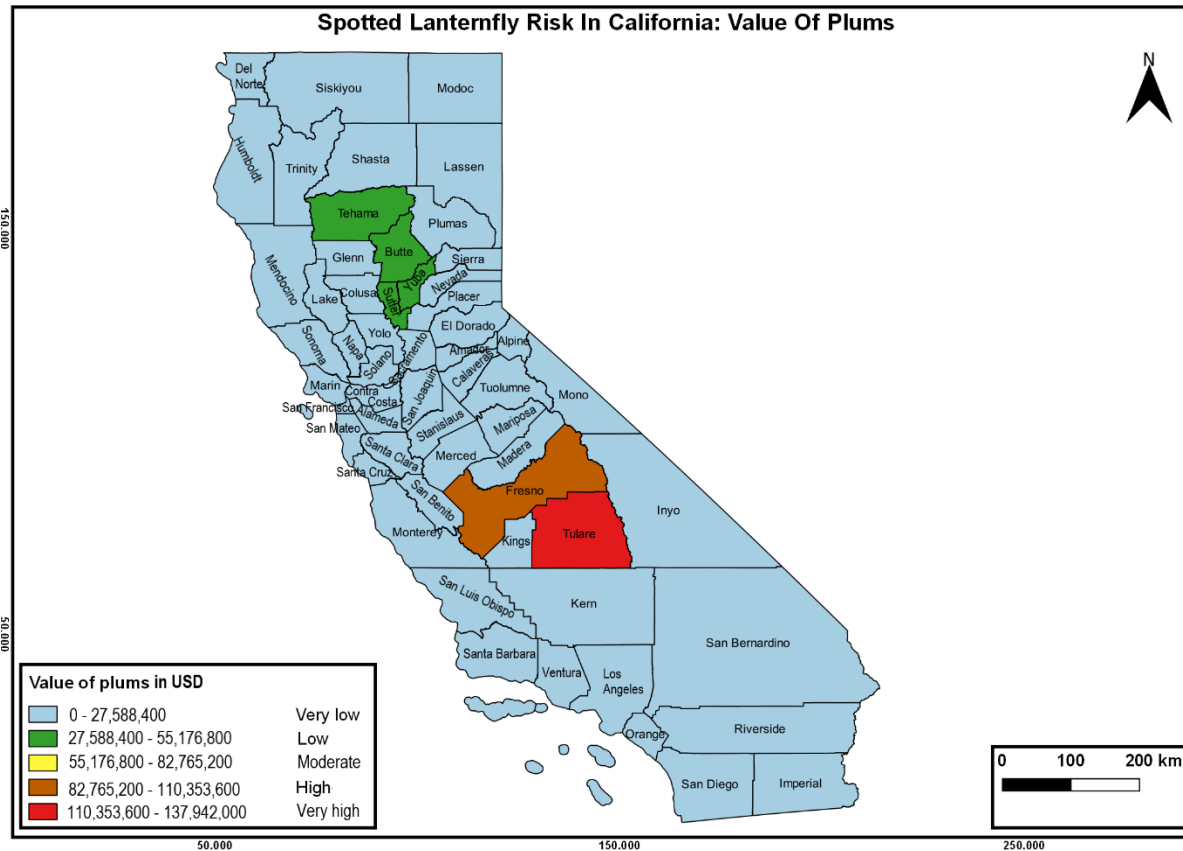
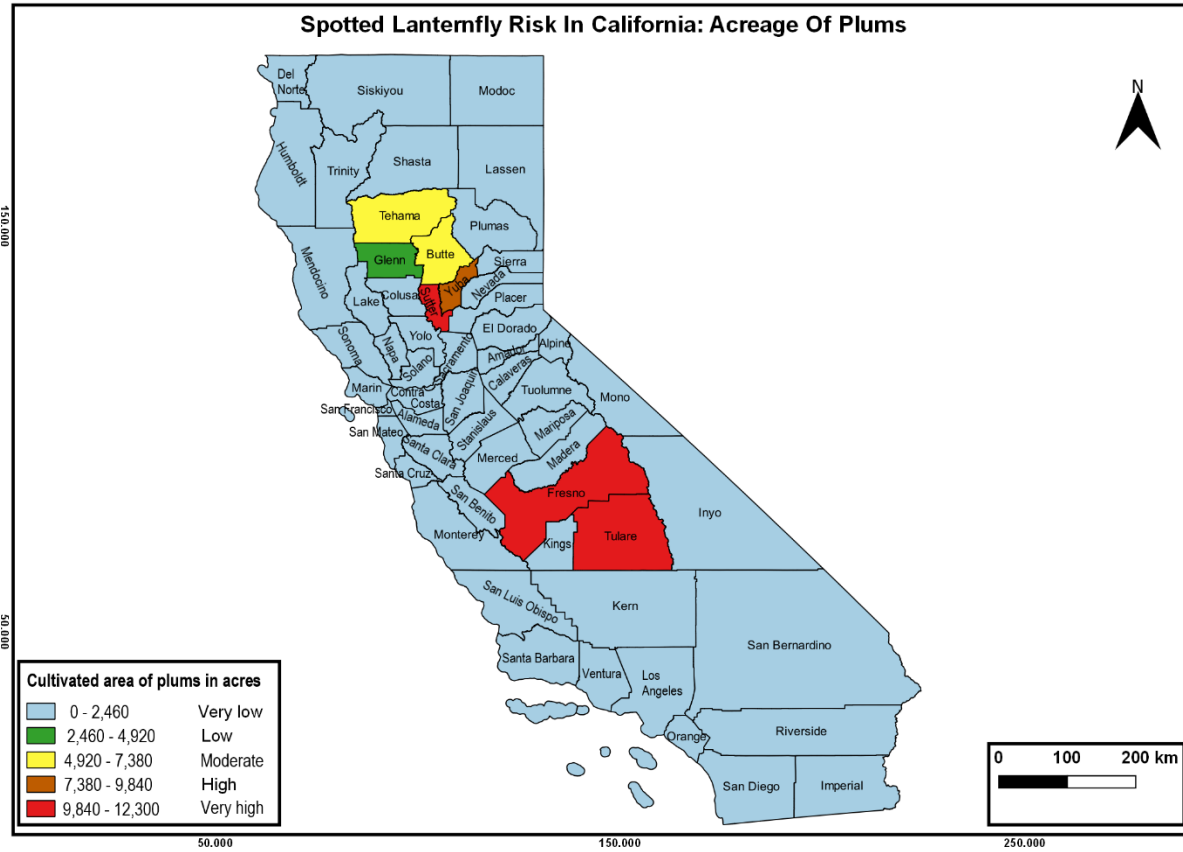




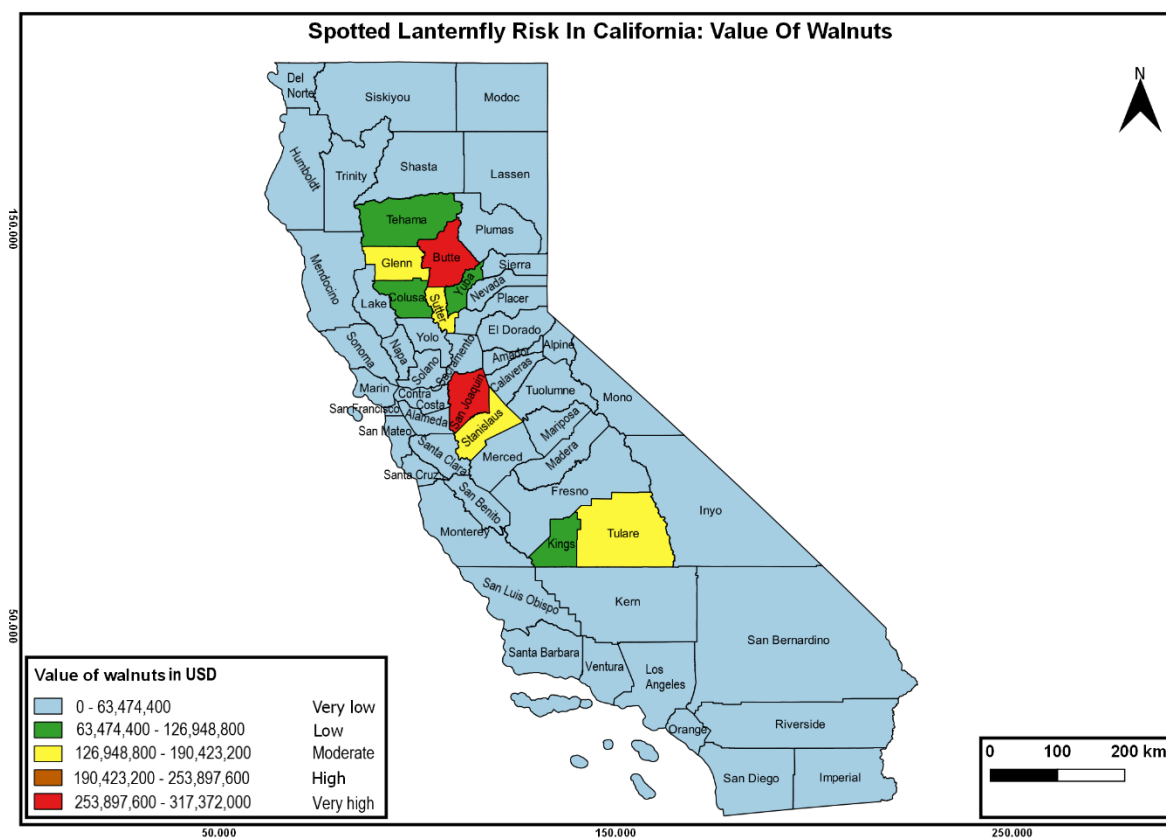
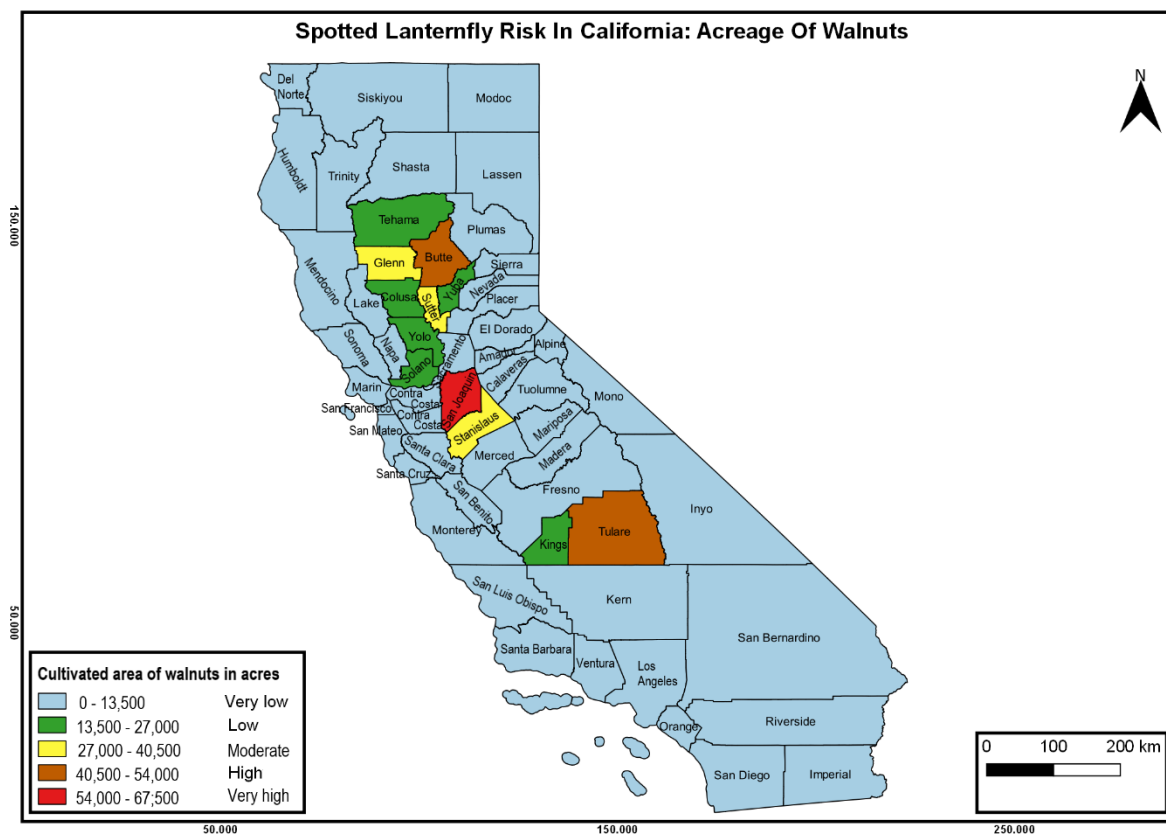




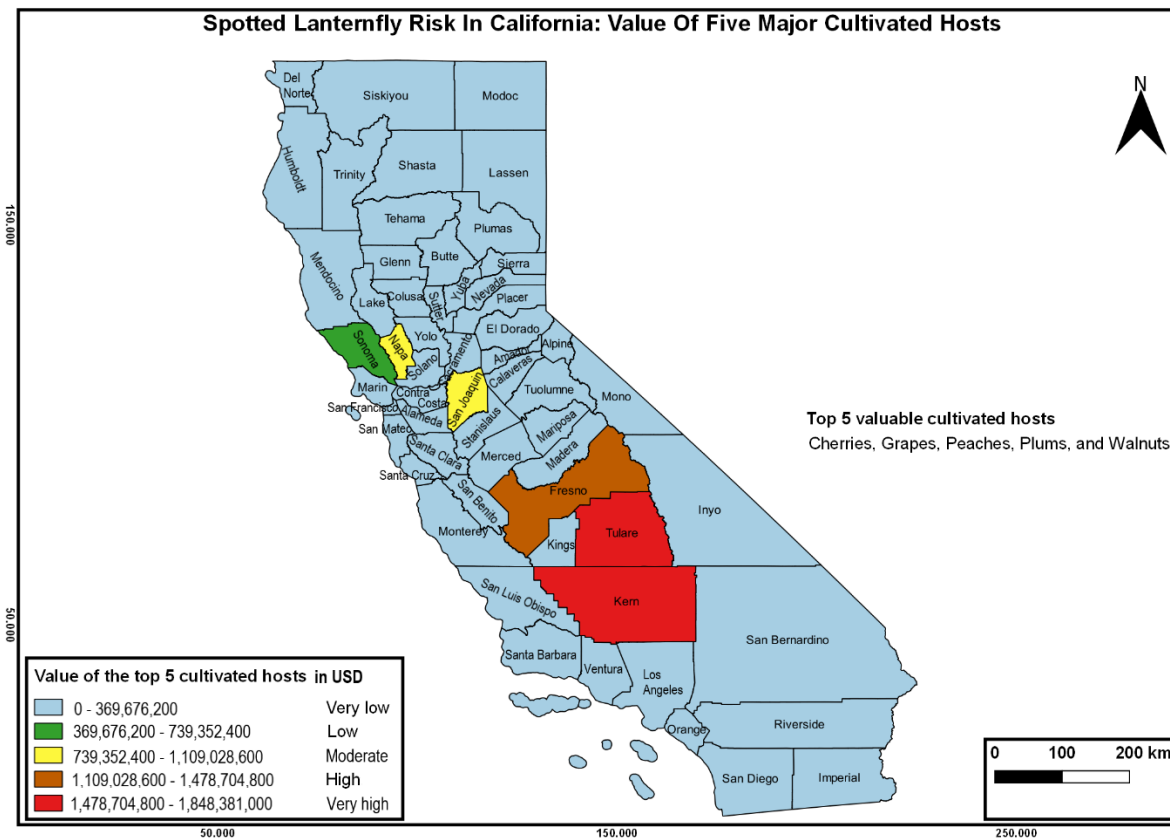
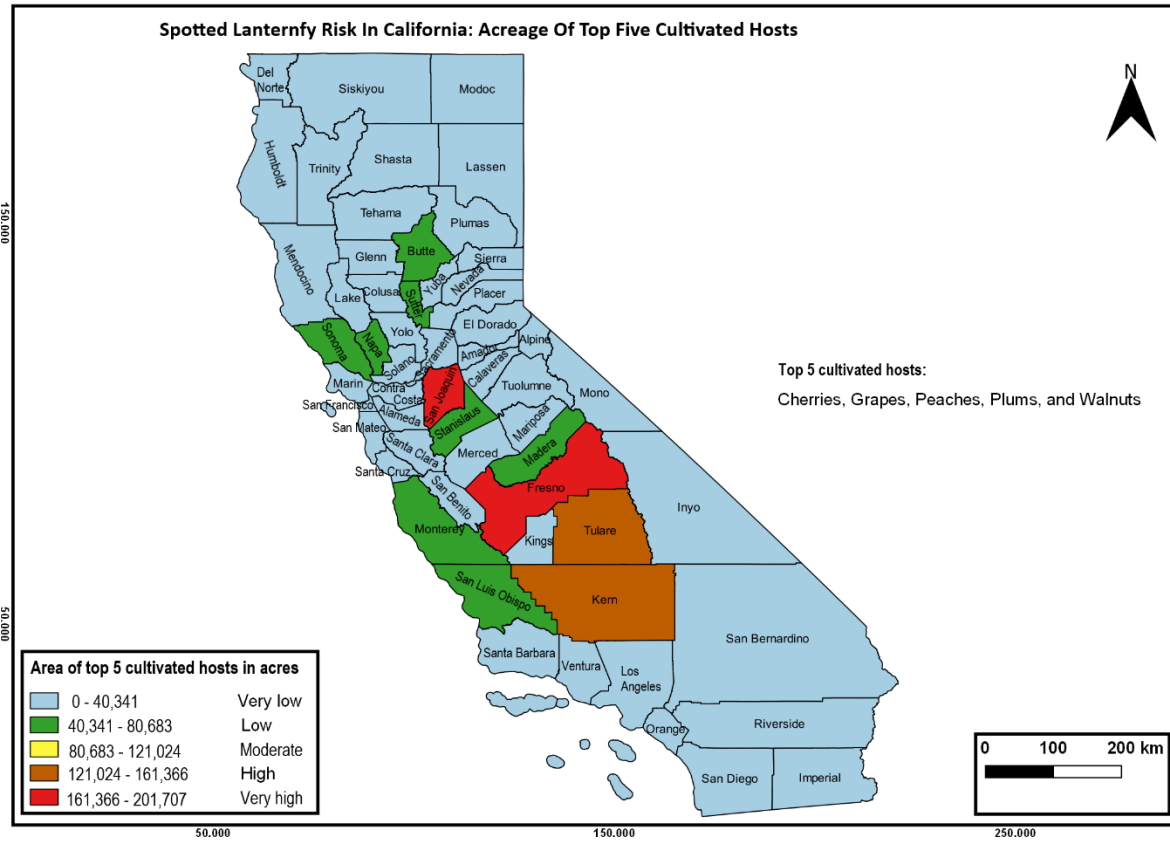


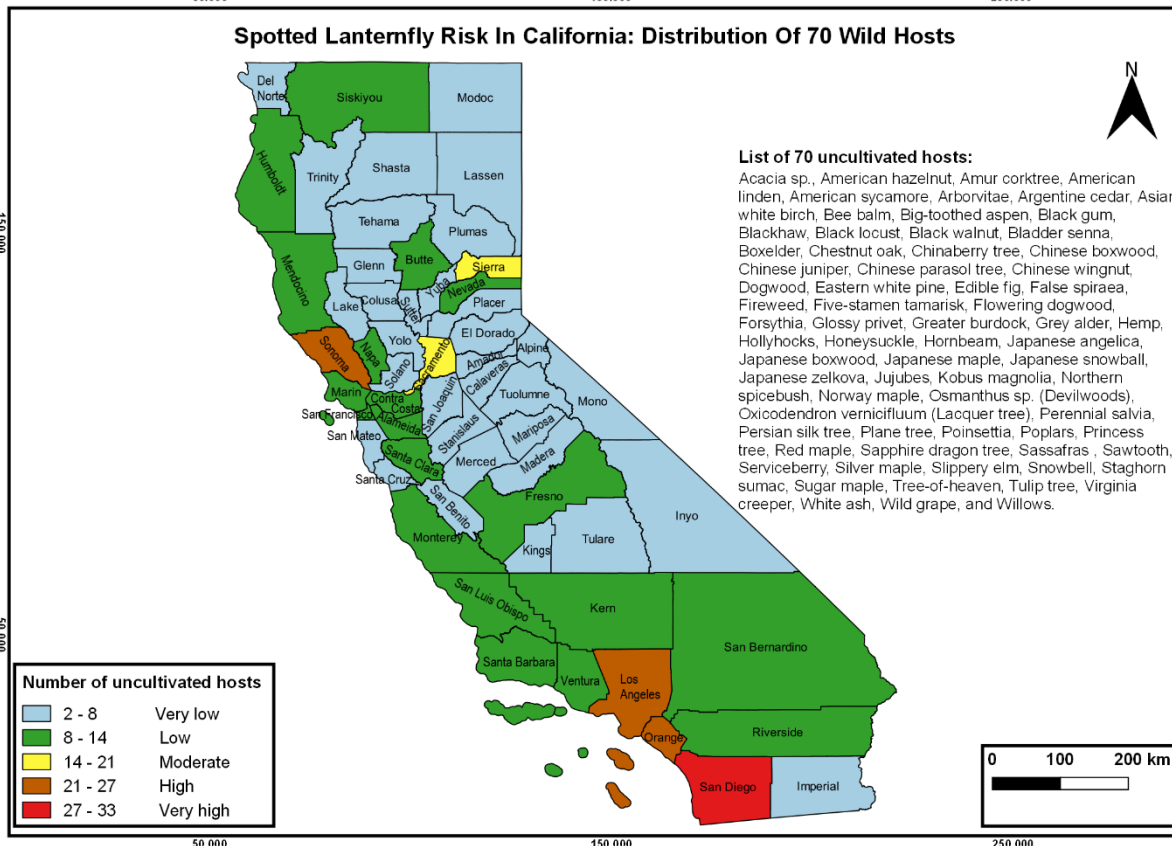
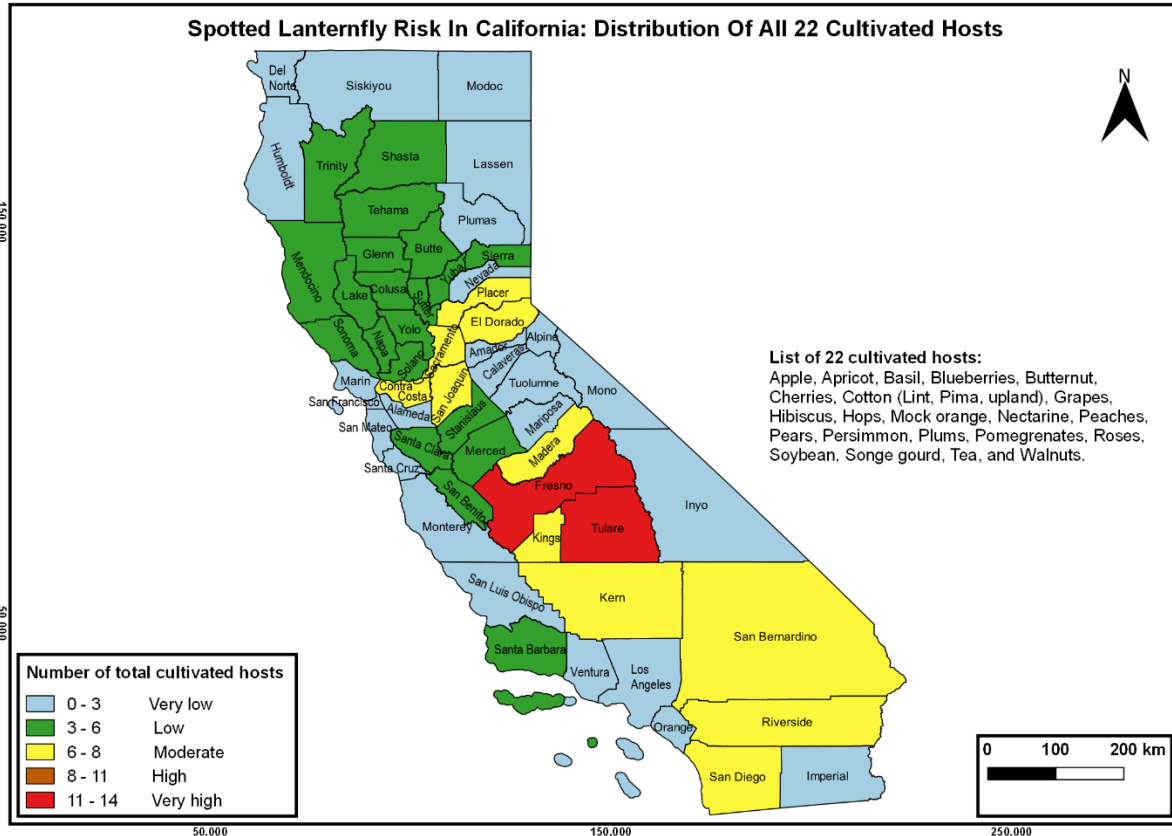












Based on these maps, the entire state of California is at some level of risk. In addition to the commercially produced crops, several backyard or landscape plant species such as roses, grapes, peaches, plums, and others are present throughout the state and can harbor SLF. Such host plants in residential and urban landscapes can serve as SLF sources for commercial crops. The tree-of-heaven is present throughout California and several such uncultivated hosts can serve as sources of undetected infestations. While researchers are working on appropriate biocontrol solutions such as releasing natural enemies, other control options such as synthetic and microbial pesticide applications, sticky traps, removal of egg masses and wild hosts, and other strategies can help manage SLF. In the meantime, Californians will benefit by knowing about this pest and its potential risk to the state. The ability to identify, destroy or capture, and report the pest to county and state departments or University of California Cooperative Extension offices will help prevent or delay SLF invasion and spread in California.

## CONCLUSIONS

California is at the risk of SLF invasion and spread. Depending on the number of cultivated crops, their acreage, value, and the distribution of wild hosts, the risk level varies in various counties throughout the state. Outreach efforts are helping to alert Californians about SLF and its damage to cultivated crops and nuisance in urban and residential areas.

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## FUNDING AGENCY

Funding for this project was provided by the California Department of Food and Agriculture.

## **DEVELOPING A GMO-FREE RNA INTERFERENCE APPROACH TO MITIGATE RED BLOTCH NEGATIVE IMPACTS ON GRAPE BERRY RIPENING**

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**Reporting Period:** The results reported here are from work conducted July 2020 to November 2021.

### **ABSTRACT**

RNA interference (RNAi) is a defense mechanism against many plant pathogens, including viruses. The concept relies on producing 20-24 nucleotides, small RNA fragments originating from plants or pathogens to enable its degradation. RNA interference has been extensively used in crop protection platforms. Currently, most approaches are based on the conventional use of transgenic plants expressing double-stranded RNAs (dsRNAs) against selected targets (endogenous plant genes, virus, fungi, bacteria). However, the use of Genetically Modified Organisms (GMOs) has raised scientific and public concerns. Approaches alternative to the production of Genetically Engineered material involves the direct exogenous application of RNA molecules to trigger the RNAi mechanism in the plant, which has the potential to address public and industry concerns (Dalakouras et al., 2020). Our project proposes developing a dsRNAs application tool to mitigate Grape Red Blotch Virus (GRBV) on grape berry composition. As the first step of a long-term project, we propose identifying the Red Blotch Virus's genomic regions targeted by the grapevine RNA interference mechanism during the early stages of viral infection. Two main sets of data are expected to be generated, namely Small RNAome and methylome. Both are parts of the Transcriptional Gene Silencing (TGS – [methylome]) and Post-Transcriptional Gene silencing ([Small RNAome]) that are activated during the early phases of viral infection. The generation of these data will serve as a foundational basis for identifying "hot spots small RNA molecules" on the viral genome targeted by the grapevine. The long-term application of this information will serve to implement the experimental exogenous application of dsRNA molecules to trigger RNAi against the virus to mitigate the adverse effects of GRBV on grape berry composition. Our first milestone in the current project was to identify the earliest phases of the GRBV activity in leaves of in vitro plants following an Agrobacterium infection using the infectious clone (NY358 – JQ901105.2), kindly donated by the CoPI-Perry. Our preliminary experiments indicate that we can detect the presence and the activity of the GRBV virus as early as 18 days post-infection (dpi) and still detect it after six weeks. An additional Agrobacterium infection experiment is currently conducted with a collection of infected plantlets sampled every three days from the infection time. From this experiment, we expect to identify a

most refined window of the early infection. Once validated, this experiment will be repeated to generate the genome-wide datasets.

### **LAYPERSON SUMMARY**

RNA interference (RNAi) is a conserved biological response across living organisms (animal or plant cells) initiated by the presence of double-stranded RNA molecules from various pathogens, including viruses. The RNA interference mechanism initiated in the plants will lead to a cascade of molecular events that are meant to repress the activity of the virus and its propagation within the plant. Once infected, the plants will recognize and produce specific nucleic regions of the viral genome to activate the RNA silencing machinery. These regions are named "hot spot" regions. The main goal of this project is to identify these "hot spots" of the Red Blotch Virus. In the long-term, this knowledge could help develop innovative technology tools like ectopic RNA molecules application in vineyards to mimic the virus's presence and make the plants immune or "primed" to further infections like a vaccine will do. This might limit the propagation of the Red Blotch Virus from plant to plant and could potentially mitigate its negative effect on grape berry ripening of already infected plants. The current project will use Next Generation Sequencing technologies to identify these "hot spots" during the early stages of Red Blotch Virus infection in tissue culture material. Once the nucleic regions are identified, the continuum of the project will involve trials for systemic application, through a spray, of these RNA molecules to either infected plants to mitigate the adverse effects of Red Blotch on ripening, or to non-infected plants, to trigger their immune responses to viral infection. If confirmed in a greenhouse setting, the next step will be to assess the ds-RNA formulation in field trials.

### **INTRODUCTION**

GRBV is a geminivirus of the geminiviridae family (<https://viralzone.expasy.org/>) responsible for Red Blotch symptoms on the grapevine, a widespread disease in most grape-growing areas of North America (Krenz et al., 2014). Its impact on fruit quality ranges from reducing sugar content to significant impairment of the ripening process, dramatically reducing fruit quality. Impaired allocation of carbohydrates from the leaves to the fruit appears to be the primary cause in slowing the ripening progress of infected berries (Blanco-Ulate et al., 2017). More recently, water deficit practices promoting ripening were not found to compensate for the ripening delay in GRBV infected grape berries (Levin and Kc, 2020). While long-term effects of the virus become more documented, the early steps of the interaction between this virus and grapevine are still poorly understood. Among various defense mechanisms a plant can develop against a DNA-based virus-like GRBV, Post-Transcriptional Gene Silencing (PTGS) and Transcriptional Gene Silencing (TGS) are seen as two central regulatory mechanisms during the famous "arms race" between viruses and plants (Prasad et al., 2019). In both pathways, the generation of several classes of viral small interference RNAs (vsiRNAs) with different lengths (21/22/24 nucleotides long) is responsible for triggering TGS and PTGS to neutralize the DNA replication of the virus and the transcription of the viral genes (Figure 1). Characterizing via Next Generation Sequencing technologies viral small RNA populations that are processed by the plant cell machinery and confirming the effects on the biology of the virus (viral transcript and methylation analyses) is the main objective of the currently funded project to identify the "hotspots" of the virus that could be exploited for the use of dsRNA ectopic application in the field to mitigate the effects of the virus. Since the acceptance of the project in June 2020, the PI has recruited a graduate student from Italy who has started working on the project on October 1st. Through

cooperation with Dr. Perry, the PL Deluc has been issued by the USDA-APHIS services a permit to import two bitmer infectious clones containing two variant strains (NY175 and NY358) of the Red Blotch virus (USDA -APHIS Permit #: 20-176-103m). These infectious clones will be essential to study the early phases of grapevine GRBV infection through two primary methodologies described below.

## OBJECTIVES

The overarching goal of the proposed research is to build the foundational knowledge for the future development of a molecular field-based tool, the Spray Induced Gene Silencing (SIGS), aiming at reducing the negative impacts of Grapevine Red Blotch Virus (GRBV) on the ripening process of the grape berry. It will provide the growers with a new practice to potentially control the spread of the disease within a vineyard and mitigate Red Blotch symptoms in infected plants.

**Objective 1.** Identify the viral genomic regions that are targeted by the RNAi machinery through an integrated OMICs approach.

**Objective 2.** Validate the silencing effect of the identified dsRNAs, and evaluate the uptake, their processing into siRNAs, and their systemic effects.

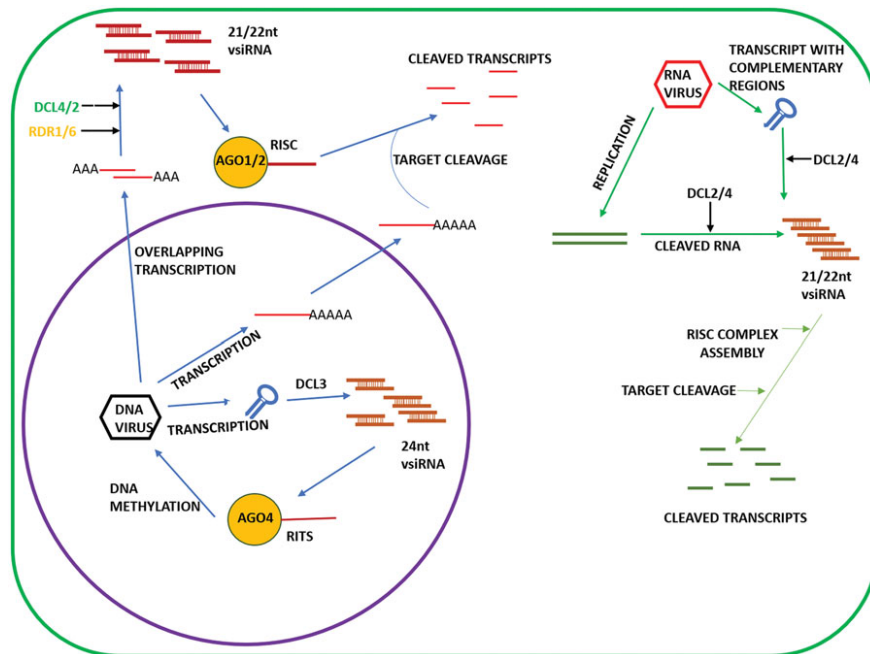
**Objective 3.** Determine the efficacy of Layered Double-Hydroxide (LDH) nano clay particles to stabilize and extend the lifespan of the dsRNAs sprayed onto the grapevine leaves.

The CDFA has currently funded objective 1 of the long-term research project. For Objective 1, we have started to conduct a series of experiments designed to precisely monitor the dynamics of the early infection of grapevine plants via several methods of viral inoculations. This first phase of the project is *i)* to have an experimental system in place to detect the virus in its early phase of infection, *ii)* to delineate the dynamics of the viral titer during the early phase of infection following a vacuum-assisted Agroinoculation of the virus, and *iii)* the methodology for inoculation of healthy material in tissue culture condition, and *iv)* to evaluate the viral spread of healthy material through grafting.

However, there is a need to design good experiments aiming to delineate the early steps of the viral infection in the grapevine. This progress report will describe the Project Leader's strategies and contingency plans to properly characterize the early phases of GRBV infection on in vitro grapevine material. Knowing this information will be essential to adequately capture the PTGS and TGS events that we planned to characterize through High Throughput sequencing. To do so, we will develop in parallel two main complementary approaches with the final objective to determine which methods will satisfy our aim to capture the earliest detection of the virus in the newly infected grapevine material.

The use of *Agrobacterium tumefaciens* as a means to deliver *in planta* viral-based vectors designed to trigger the gene silencing is one essential component of the methodology. The *Agrobacterium* containing the viral-based vectors can be delivered through leaf infiltration and inoculation through a vacuum (Muruganantham et al., 2009) (Muruganatham et al., 2009), colony inoculation procedure or Agropriking (Koeda et al., 2017), and Agrodrenching (Ryu et al., 2004; Yepes et al., 2018). The agrodrenching that can be vacuum-assisted is adequate to deliver different viral-based vectors to the grapevine. Yepes et al. (2018) demonstrated the Agrodrenching technique was vital to validate the causative leaf reddening symptoms to the presence of the virus and to satisfy part of Koch's postulates. The concept of *Agrobacterium-*

*mediated Inoculation* through drenching is to expose the plant rhizosphere to a liquid culture of *Agrobacterium tumefaciens* containing a viral-based binary vector, which will penetrate the through a series of timed vacuum cycles separated by vacuum releases. This method can be optimized with the use of various repetitive processes of vacuum under specific conditions. The team will use the Agroinoculation method assisted with vacuum as a means to deliver the GRBV genome. Once applied, the team will identify the number of days required to infect the upper part of the plants and the dynamics of viral accumulation.



**Figure 1.** Plant-virus interaction mediated by sRNAs from Prasad et al., 2019. In RNA viruses, transcripts with self-complementary regions are processed by endogenous DCL4/2 of plants to generate 21/22 nt *virus small interfering* RNA generation. In DNA viruses, overlapping transcription and transcripts with self-complementary regions are sources of dsRNA which can act as substrates DCLs. DCL4 generates 21 nt vsiRNAs that can associate with AGO2 to cleave viral transcripts. DCL3 produces 24 nt vsiRNAs which associate with AGO4 to form functional RITS complex to methylate viral DNA. DCL: Dicer-like protein; vsiRNA: virus-derived siRNA; RDR: RNA dependent RNA polymerase; AGO: Argonaute.

A second methodology proposed will be the viral transmission via grafting. Symptoms of the Grape-Leafroll virus, which is also transmitted through grafting, can be detected as early as two to three weeks after micrografting (Pathirana and McKenzie, 2005). By analogy with other geminiviruses, the GRBV is anticipated to restrict the phloem (Sudarshana et al., 2015). The team will use a grapevine material (infected by Or1a isolate) generously offered by the cooperator Martin. This material will serve as a mother plant to infect healthy grapevine material through green grafting in a tissue culture environment (see video: <https://www.dropbox.com/s/xethmqff374vrjxj/Grafting%20protocol.mp4?dl=0>).

The overall proposed research aims to build the foundational knowledge for the future application of a molecular field-based tool, the **Spray Induced Gene Silencing (SIGS)**, to reduce the negative impacts of Grapevine Red Blotch Virus (GRBV) on the ripening process of the grape berry. The overarching research project consists of three main objectives. In Objective 1\*, we will identify the viral genomic regions targeted by the RNAi machinery by conducting a genome-wide study including High-throughput sequencing of coding and non-coding genes and methylome analyses from infected grapevine materials. From Objective 2, we will validate the silencing effects of identified dsRNAs, and we will evaluate their uptake, processing into siRNAs, and their systemic effects on the whole plant level. Finally, in Objective 3, we will determine the efficacy of Layered Double-Hydroxide (LDH) nano clay particles to stabilize and extend the dsRNAs' lifespan sprayed onto the grapevine leaves.

The CDFA has currently funded objective 1 of the long-term research project, which includes as a first step the characterization of the early accumulation of GRBV in newly infected grapevine material. Unlike most reports, the current research aims to study the early immune responses of grapevine without waiting for the disease symptoms to manifest several months later. Following two main procedures, Agroinoculation and green grafting using infected grapevine material (Pinot noir infected with Or1a isolates) as a means to contaminate healthy grapevine material, the team will further evaluate the best methods for detecting the early dynamics of viral accumulation by either gentle pricking of newly infected materials followed by LAMP-PCR assays (Romero Romero et al., 2019) or by standard DNA and RNA extractions of newly infected plants followed by conventional and quantitative PCR-based assays.

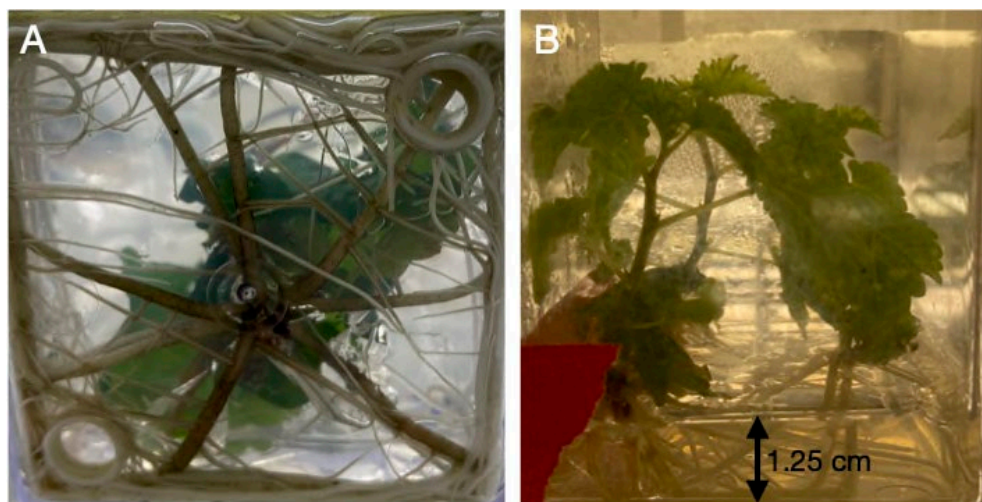
## **RESULTS AND DISCUSSION**

### **Conducting an Agroinoculation approach to detect the earliest accumulation of GRBV in the infected material. \***

The Project Leader Deluc received, from the cooperator Perry, two infectious bitmer clones (Permit issued in July 2020) of the GRBV in the *Agrobacterium tumefaciens* GV3101 strain. This strain contains 1.5X GRBV genome cloned into a pBin-19 binary vector from two variants (NY175 [GB accession #: KF147916] and NY358 [GB accession #: JQ901105]) of the Red Blotch Virus. We follow the methodology optimized by Yepes et al. (2018) with slight modifications due to the used plant material. In short, plantlets will be transferred in a sterilized vacuum chamber equipped with a remote vacuum gauge/bleed valve to control the intensity of the vacuum ranging from 650 to 720 mm Hg. The authors in Yepes et al. (2018) have tested several vacuum cycles. They have demonstrated the best results for *Agrobacterium* inoculation with two cycles of 3.5 minutes at 720 mmHg separated by a vacuum release between the two cycles. The efficacy of the Agroinoculation-mediated delivery of the genetic information depends on the cultivars and the stage of the plantlets. For the current project, the team will be using microvine plant material extensively used in the lab for genetic engineering (Gouthu and Deluc, 2018). This model has several advantages compared to traditional cultivars. It grows fast, and the plant regeneration through somatic embryogenesis is streamlined, giving the option to produce hundreds of individual plants within two to three months (Boss and Thomas, 2002; Chaïb et al., 2010). For the past five years, we have consistently observed a significantly higher root-to-shoot fresh biomass ratio (Figure 2a) with the microvine plants compared to traditional genotypes. This will be an essential factor to optimize the surface contact between the plant material and the *Agrobacterium* solution during the Agroinoculation experiment. Usually, the subculture of two-node plantlets leads to rooted plantlets after 10 to 15 days and 5 to 7 cm height



plants within a month (Figure 2b). To increase the number of plantlets, we are in the process of generating at least 250 individual plantlets, which will be used for the Agroinoculation and green grafting experiments. In addition, we are in the final stage of the genome sequencing of the microvine from another funded project. This will be essential for the downstream analyses of the current research proposal (plant and viral transcriptomes, small RNAomes, methylome).



**Figure 2.** Microvine plants in tissue culture **A)** Root growth from 8-week-old plant and **B)** Height of 5-week-old microvine plant.

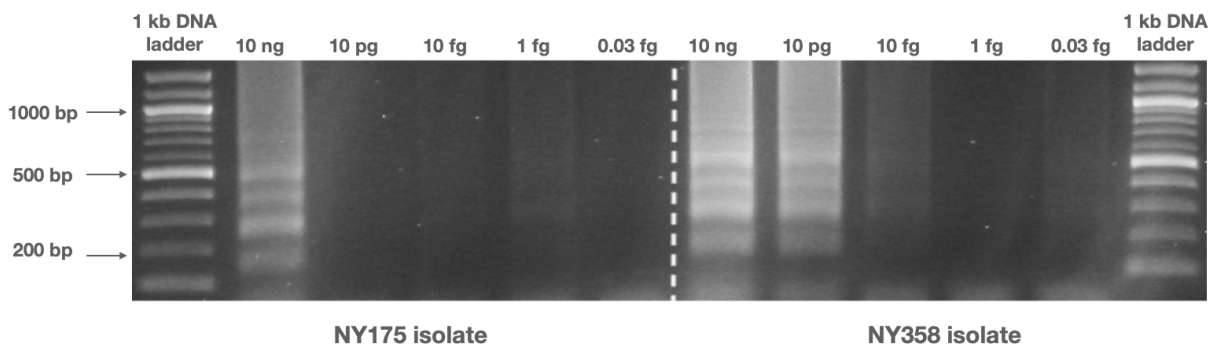
### **Developing a green grafting experiment to examine the dynamics of early accumulation of the GRBV through grafting.**

While Yepes et al. (2018) has demonstrated the transmission of the virus to the upper part of plants through AgroInoculation assisted with vacuum, the efficiency is relatively low and strongly relies on the genotype. As a contingency plan, we propose to explore the infection of GRBV through green grafting. In our lab, we developed a streamlined procedure for green-grafting that can be visualized at this URL (<https://www.dropbox.com/s/xethmqff374vrj/Grafting%20protocol.mp4?dl=0>), for which we have confirmed the transmission of GLRaV3 no less than three weeks after the grafting procedure as previously described (Pathirana and McKenzie, 2005). As mentioned above, GRBV is likely to be transported through the phloem, but there is not so much scientific report validating this assumption. We propose to prove it by transmitting the GRBV virus to healthy microvine material through a micro-grafting procedure using Pinot noir plant materials infected with the Or1a isolate (Dr. Martin donation). We are in the process of regenerating around 200 individual plants from embryogenic cells through a standard protocol for grapevine regeneration (Gouthu and Deluc, 2019). Our survival rate for green grafting and scion growth ranges from 65% to 85%, respectively. Most variations are usually based on the genetic background of the grapevine material used as rootstocks or scions.

### Evaluating conventional and sensitive PCR-based methods to detect early accumulation of GRBV from Agroinoculated and graft-transmitted tissue cultured plants.

There are several methods to detect the virus in plant samples. A rapid and sensitive test based on a Loop-mediated isothermal AMPlification reaction was found to detect up to 1 femtogram of virus in infected samples from mature plants (Romero Romero et al., 2019). We have begun to test the LAMP PCR system with the two infection clones (NY358 and NY175) at different dilutions from 10 nanograms to 1 femtogram using LAMP-based primers designed from the genomic sequence of the NY358 isolates. The viral detection was inconclusive beyond ten femtograms of nucleic material as templates (Figure 3). We also began to test the pin-pricking methods proposed by Romero on tissue cultured infected Pinot noir with the Or1a isolate, but we failed to produce the proper amplicons by LAMP-PCR. One potential reason could be a minimal load of virus from tissue culture material that may not be suited to the LAMP-PCR-based assays, usually conducted on mature grapevine plants (Yepes et al., 2018). Another reason could be the nucleic variability among the Or1a and NY358 isolates that could affect the efficiency of the LAMP-PCR reactions. We performed the alignment of several genomes of GRBV to examine the differences among isolates.

On the one hand, we did not find any nucleic mismatches between NY358 and Or1a in the region used to design the LAMP-PCR primers. On the other hand, we observed mismatches between NY175 and 358 precisely at the site of the LAMP-PCR primers, which could explain the lower efficiency of the amplification for the NY175 infectious clone (Figure 3). We are currently extracting total nucleic Acids from grapevine leave samples infected with the Isolate Or1a to conduct conventional and Real-Time PCR reactions to examine the virus's presence and activity.



**Figure 3.** Image of an agarose gel (1.5%) with the different sizes of amplicons usually produced from a LAMP-PCR reaction as described in Romero et al., 2019. The infection (1.5X GRBV cloned to PBin19 expression vector) for NY175 and NY358 isolates were used template. PCR assays using the NY isolates have consistently yielded better results compared to NY175.

## RECENT ACCOMPLISHMENTS

### Agrodrenching experiments

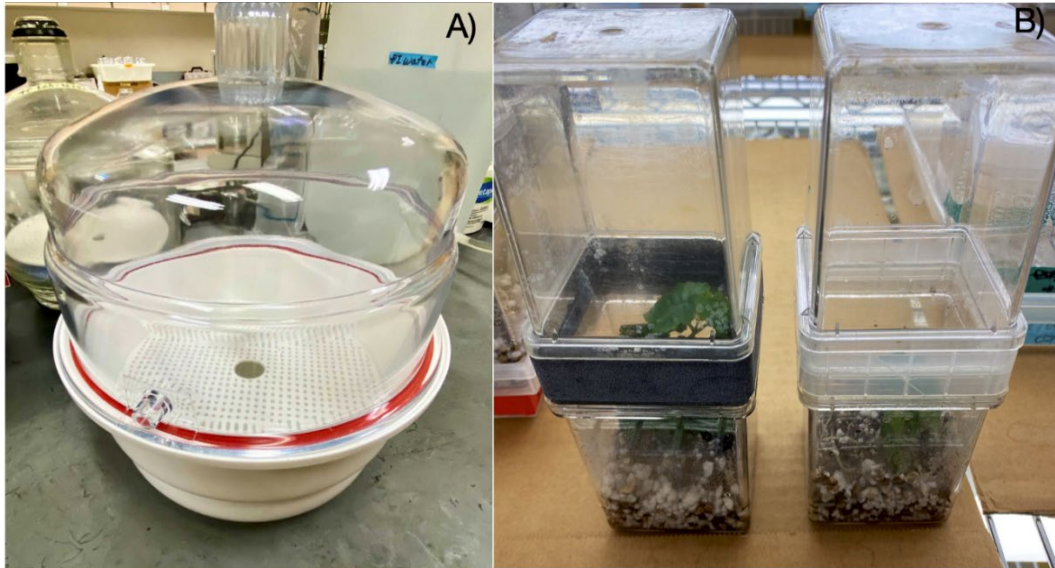
Several adjustments were made from the original protocol described in Yepes et al., 2018. The liquid *Agrobacterium* culture of the infectious clone NY358 was conducted for 30 hours in our current setting to reach an OD<sub>600</sub> of 1.9. The induction and infiltration media were prepared according to Va et al., 20. The Agrodrenching method was conducted with a sterile incubation

chamber (Figure 4A) containing about 750 mL of infiltration medium with an *Agrobacterium* culture at OD<sub>600</sub> equal to 1.0. After a series of vacuum cycles trials, we opted for two cycles of a vacuum of 650 mm/Hg administered for 3.5 min, each separated by a rapid vacuum release. We could accommodate ten healthy microvine plants (6 to 8 weeks old plants after propagation) in the incubation chamber per vacuum treatment. After the Agrodrenching was achieved, the plantlets were transferred into a sterile double-magenta box system (Figure 4B) containing autoclaved vermiculite and 75 ml of growing medium to facilitate vertical growth (Kurth et al., 2012). The growth medium was then replaced three days after the Agrodrenching treatment by a fresh growth medium, ticarcillin and clavulanate (15:1 - Timentin: PlantMedia.com) at 1000 µg/ml final concentration.

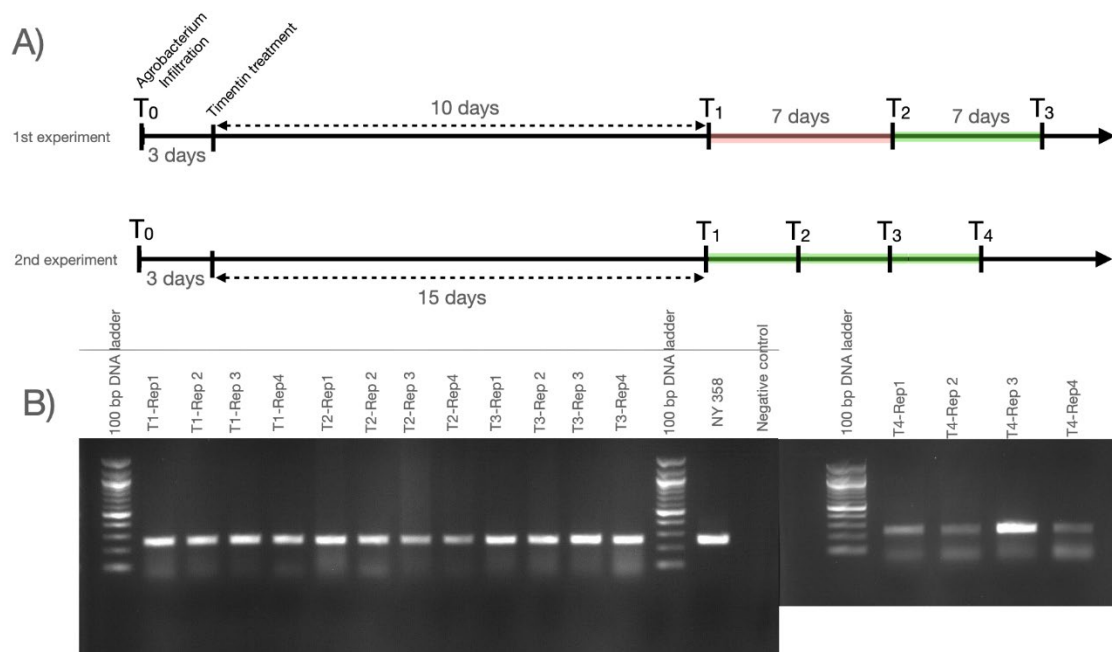
### **Detection method of the viral activity in Agrodrenched plants**

After several assays, we have decided to abandon the LAMP PCR assays to detect the presence of the virus. The lack of reliability of the visual diagnostic component of the kit was part of our decision. We observed too much inconsistency in the visual validation of the resulting amplicons. The conventional PCR-based assays and the Real-Time were found to be more adapted to our purpose. From our first assay, we design the timing of sampling collections to ensure the presence of the virus after a reasonable time (Figure 5A). We could not detect the presence of the virus in the leaves of infected plants before at least 27 dpi. Since our goal is to identify the earliest existence of the virus in the plants, we developed a second infiltration assay with collection times closer to the infection. We could use the virus's coat protein at 22 dpi and after that (Figure 5B). We are currently developing a last AgroDrench experiment with a sampling collection every three days from the infection to encompass the time window for the early phases of viral infection. This will be followed by Real-Time PCR assays targeting the expression of the C1 replicase gene product of the GRBV genome, coat protein (Cp), and viral-induced Dicer-like microvine genes (VitviDCL1/2), part of the RNAi machinery induced during viral infection in plants. If successful, plant materials from this experiment will be used to run the genome-wide analysis.

In September, we conducted a third infiltration experiment with a time collection every three days. At each time collection, we crushed four plants (leaves and stem) to extract total RNA. One extra plant was also collected and stored at -80°C. From this experiment, we expect to improve the timeline for viral presence in the leaves of Agrodrenched plants. This information will be essential to delineate the developmental window representing the exponential phase of the viral increase in the microvine plants before reaching a plateau of accumulation.



**Figure 4.** Agrodrenching experiments **A)** Sterile incubation chamber used for the Agrobacterium-vacuum assisted infection. **B)** Sterile setting for the Agro-infiltrated microvine plants.



**Figure 5.** Agrodrenching experiments **A)** Timeline of the two experiments with successive sampling (T<sub>x</sub>). **B)** Image of an agarose gel stained with Ethidium Bromide. The produced amplicon results from a Reverse-Transcription PCR reaction (35 cycles of amplification) targeting a specific genomic region of the GRBV encoding the coat protein of the NY358 genotype.

### Optimization for extraction of coding (mRNA) and non-coding RNA (small RNAs) for downstream RNA sequencing application.

RNA sequencing technologies require high-quality samples of RNAs. We have tested several extraction protocols and kits (extraction and cleanup kits) designed to enrich and clean coding and non-coding RNA. Differential Alcohol-cationic salt precipitations to enrich the small RNA fraction were also tested.

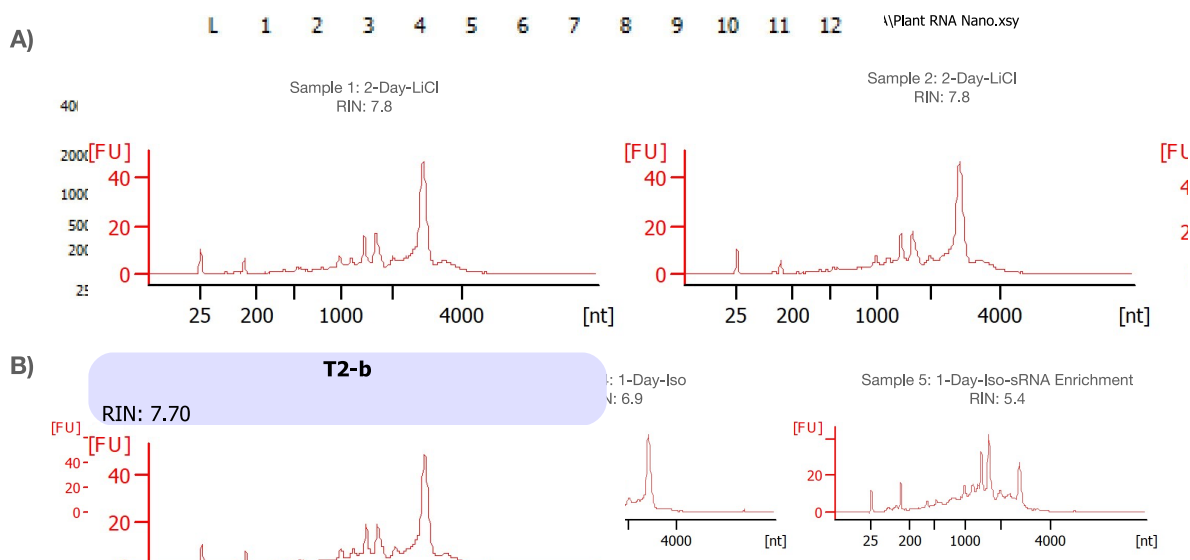
Due to the presence of polysaccharide and phenolic acid matrices in grapevine samples, the extraction of coding RNAs using a Cetyltrimethylammonium bromide (CTAB)-based method has been implemented (Reid et al., 2006). We also tested a user-developed protocol using Plant RNA-Easy mini kit from QIAGEN optimized for plant tissues. We conducted a few modifications to the Reid protocol. We shortened the extraction and precipitation times, reducing the duration of total RNA extraction from two to one day. We also performed two precipitation and procedures (LiCl-based or Alcohol-based) to enrich the Small RNA fraction from the samples as proposed in (Choi et al., 2018; Figueroa-Balderas et al., 2019). We then compared the results for yield and presence of contaminants by spectrophotometry (Nanodrop 2000). We also evaluated the integrity of total RNA molecules for degradation and the likely enrichment for small RNAs within the 25-200 nt range (Agilent BioAnalyzer) (Table 1 and Figure 6).

One additional cleanup step (RNA Cleanup kit) was tested to determine if we could improve the integrity of RNA samples. We found no improvement in the RNA integrity in using the RNA cleanup kit (data not shown). Finally, we tested a user-developed protocol using a modified Plant RNAEasy MiniKit specifically used for polysaccharides enriched tissues adapted from McKenzie et al., 1997. The modified kit (modified Lysis buffer) has the advantage of taking less than one hour to extract total RNA, which will prevent their degradation frequently observed with standard conventional extraction protocols. We tested the kit on a fully developed stem and lignified stem tissues (Table 2 - Figure 7), which poorly yields traditional protocols.

**Table 1:** Yield and quality of total and small RNAs from leave samples collected on Agrodrenched microvine plants post-viral infection

Samples	Protocol	Concentration (ng/μL)	Yield (μg/g FW)	A260/280†	A260/230††	RIN†††
Sample 1 (500 mg)	2-Day-LiCl	156	15.6	2.00	2.40	7.70
Sample 2 (500 mg)	2-Day-LiCl	166	16.6	1.96	2.50	7.80
Sample 3 (500 mg)	1-Day-LiCl	489	48.9	2.04	2.39	8.00
Sample 4 (500 mg)	1-Day-Iso*	264	26	2.14	2.34	6.80
Sample 5 (500 mg)	1-Day-LiCl-sRNA**	419	30	2.29	2.42	5.40
Sample 6 (500 mg)	1-Day-Iso-sRNA**	459	33	2.21	2.31	5.40

\*: Isopropanol + Sodium Acetate Precipitation, \*\*: Small RNA Procedure enrichment according to Choi et al., 2018, †: an absorbance ratio greater than 2 indicates the absence of phenolic and protein contaminants, ††: an absorbance ratio greater than 2 indicates the absence of polysaccharides contaminants, †††: RIN RNA Integrity Number range from 1 to 10. Ranges between 8 to 10 indicate negligible RNA degradation.

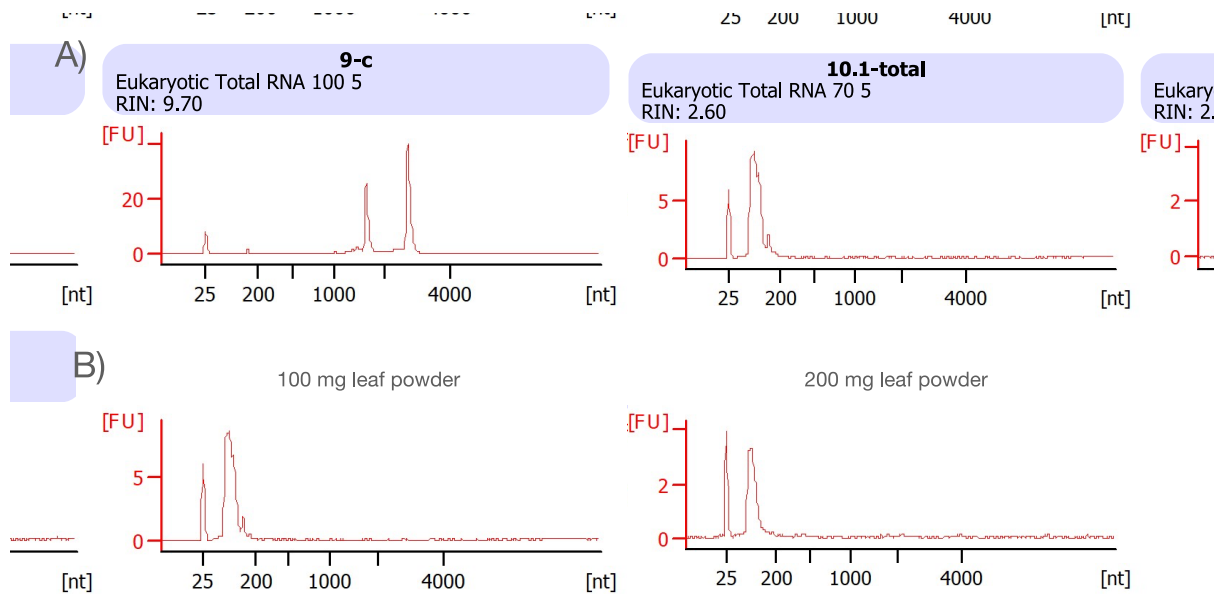


**Figure 6:** RNA Integrity Number from extracted samples using LiCl-RNA and Isopropanol-precipitation for Total and Small RNAs (Reid et al., 2006; Figueroas et al., 2020). A) Electrophoregram plots of total RNAs extracted from two leaves using 2-Day LiCl Protocol. B) Electrophoregram plots of same leaf samples extracted in one day using a LiCl precipitation (Left panel), and Isopropanol precipitation aimed to enrich the small RNA fraction (center panel), and an Isopropanol precipitation along implemented by an additional enrichment of small RNA (right panel) according to Choi et al., 2018.

**Table 2:** Yield, quality, and RIN measures of total RNA on recalcitrant tissues using a modified Plant RNAEasy Minikit (QIAGEN) and small RNAs from leaves tissues Agrodrenched-microvine plants.

Samples	Protocol	Concentration (ng/ $\mu$ L)	Yield ( $\mu$ g/g FW)	A260/280 <sup>†</sup>	A260/230 <sup>††</sup>	RIN <sup>†††</sup>
Sample 7 (young stem, 100 mg)	Modified Plant Mini Kit*	410	123	2.14	2.20	8.10
Sample 8 (lignified stem, 100 mg)	Modified Plant Mini Kit	107	32	2.02	2.06	8.6
Sample 9 (V4 leaves, 100 mg)	mirPremier Kit	70	35	1.94	1.72	-
Sample 10 (V4 leaves, 200 mg)	mirPremier Kit	55	27.5	2.00	1.85	-





**Figure 7:** RNA Integrity using the modified RNAEasy plant mini kit (A) and the MirPremier kit (Sigma-Aldrich) for enrichment of small RNAs (B).

Overall, shortening the LiCl protocol to one day ensured better integrity of RNA compared to the 2-day protocol. The enrichment of small RNAs using isopropanol to precipitate small RNAs was adequate (Figure 7), but over-drying the pellet of RNAs after alcohol precipitation, as recommended by Choi et al., 2019, did not improve the enrichment (Figure 7). The modified plant RNAEasy Mini Kit, which includes a modified Lysis Buffer (4N guanidine Isothiocyanate, 0.2M Sodium Acetate, pH 5.0, 25mM EDTA, 2.5% (w/v) Polyvinylpyrrolidone [MW 40,000], 1%  $\beta$ -mercaptoethanol), has even given the best results in terms of yield, absence of contaminants, and better RNA integrity. Ultimately, mirPremier Kit from Sigma company gave us the best results to enrich small RNA fractions with a decent yield and relatively no contamination of polyphenols and polysaccharides. Interestingly, the amount of enriched small RNAs was found greater using 100 mg of leave powder. However, we could not find a relevant reason for this outcome.

#### **Draft sequencing of the 04C023V0004 genome (Chaib et al., 2010).**

In collaboration with the Center for Quantitative Life Sciences and the Edmund Mach Institute (PI Mickael Malnoy), we are currently sequencing the grape microvine genome (04C023V0004 genotype) using long-read from both PacBio SEQUEL II and Oxford Nanopore technologies. Sequences from both technologies were combined to provide a phased diploid assembly using the PacBio assembler (<https://github.com/PacificBiosciences/FALCON>), Falcon unzip (Chin et al., 2016) (Chin et al., 2016) to separate into haplotype genomes, Quickmerge (Chakraborty et al., 2016), and Minimap2 (Jain et al., 2018) to combine and extend contigs. Phase genomics Hi-C technologies were used to sequence long-range regions within chromosomes. The current genome assembly statistics show for the primary haplotype chromosome assembly 655 contigs for a total length of 497 Mb, with a median length of read exceeding 2.2Mb (N50), and for the secondary haplotype assembly, 1,539 contigs for a total length of 146 Mb and a median length of

reads exceeding 150 Kb. We are currently using other grape genomes to create a Transposable Element Repeats database to mask repetitive sequences. We also are in the process of generating a reference transcriptome using PacBio-Sequel II (Iso-Seq) as evidence-based sequences for de novo gene prediction and functional gene annotation.

#### **Generation and processing of the RNA-Seq data for coding and non-coding RNA.**

mRNAs will be first enriched from total RNA before preparing the library using TruSeq Stranded mRNA Kit (Illumina, USA). Enriched small RNAs will be prepared using TruSeq Small RNA Library Preparation Kits (Illumina, USA). Both coding and non-coding RNA will be sequenced using a NextSeq 2000 instrument at the CQLS center (<https://cqls.oregonstate.edu/core/sequencing/illumina-nextseq-2000>). Reads will be extracted from the Fastq files sequencing data. The quality of the reads will be evaluated using FastQC software, and adaptor sequences will be removed using Cutadapt (<https://github.com/marcelm/cutadapt>)

There is a need to extract structural RNAs (tRNA, rRNA, snRNA) for the read datasets. To do so, reads will be mapped against the Rfam database (Kalvari et al., 2021). The remaining reads will be mapped to the microvine and viral genomes using Bowtie2 (Langmead and Salzberg, 2012). For the viral small interfering RNAs identification, graphical representation of each small RNA class, using MISIS2, will be mapped to the viral genome to infer potential "hotspots" (Seguin et al., 2014). The number of reads aligned to each position of the GRBV genome will be normalized per library to Reads per Million (RPM) to identify "hot spots". Ufold, designed to predict RNA secondary structures on flanking regions of the identified hot spots reads to the viral genome, will be used to demonstrate their functionality as siRNA precursors (Fu et al., 2020). Filtered small RNA reads will also be aligned against the microvine genome using Bowtie2 (Langmead and Salzberg, 2012). Reads that imperfectly aligned, less than 18 nucleotides and greater than 26 nucleotides will be excluded. Reads that mapped to greater than 20 locations will also be excluded. Counts will be generated for known and novel microRNA that passed the previously mentioned filters and all other small RNA sequences. Only reads that have unique sequences will be used for quantitative analyses.

DESeq2 will be used to compare, at each time collection ( $T_{x,y,z}$ ), the abundance of all sRNAs in GRBV with  $T_0$ . miRNA and other sRNAs with an adjusted p-value less than 0.05 will be reported as differentially abundant. Targets of putative and previously annotated miRNAs, as well as other small RNAs of interest, will be predicted using psRNATarget (Dai et al., 2018). Following the DESeq2 analysis, all differentially abundant small RNAs will be compared to the miRVine expression atlas to determine previous identification (Belli Kullán et al., 2015). Total DNA was isolated with the DNeasy Plant Mini Kit (Qiagen). Methylation marks on the viral genome will be inferred by a BS-Seq approach (Li and Tollefsbol, 2011). The reads will be mapped to the GRBV genome using a methylation-aware aligner Bismark (Krueger and Andrews, 2011). Graphical representation of methylation marks to the genome will be performed using an in-house Perl script developed by the Babraham Research Institute.

Filtered reads for total RNAs will be aligned using HISAT2 (Kim et al., 2019) to the 04C023V0004 microvine genome (Chaib et al., 2010). Annotated genes on the microvine genome were based on the V3.3 COST Annotated Reference Pinot Noir genome (Canaguier et



al., 2017). Aligned sequences reads were then assembled into potential transcripts using StringTie (Pertea et al., 2016). Normalized and Variance Stabilizing Transformation counts were generated using DESeq2 (Love et al., 2014). The p-values for statistical significance of the differentially abundant transcripts were adjusted using Benjamini-Hochberg False Discovery Rate to account for multiple statistical tests.

### **Greengrafting**

We have experienced a series of technical hurdles to accomplish this research task, including 1) the incompatibility of young pinot noir GRBV (+) plantlets to properly graft the microvine, 2) a significant variability of the GRBV viral load in infected pinot noir plantlets that were initially propagated from one presumably infected grapevine plant. Given our success with the Agroinfection method, we decided to abandon the green grafting approach to infect the microvine.

### **CONCLUSIONS**

We were successful in infecting the microvine plants *via* Agrobacterium drenching. We are currently delineating the exponential phase by Real-Time PCR using viral and plant RNA Interference-related genes to detect the early stages of viral infection. We have optimized the extraction protocol for total and small RNA. We expect to prepare and sequence the libraries for RNA-Sequencing (mRNA and sRNA) by the end of the year. We will then conduct the bioinformatic analyses early next year. Meanwhile, we will extract the total DNA and perform the Bisulfite-Sequencing too. Overall, we expect to have all the data necessary for their interpretation by the end of Winter, and we expect to have the "hotspots" identified by the end of Spring 2022.

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## ECOLOGY OF GRAPEVINE RED BLOTCH VIRUS

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**Reporting Period:** The results reported here are from work conducted July 2020 to October 2021.

### ABSTRACT

Information on the transmission biology of grapevine red blotch virus (GRBV) by *Spissistilus festinus*, the three-cornered alfalfa hopper (TCAH), is scarce. For example, how GRBV is transmitted by the TCAH is poorly understood and how long it takes for grapevines to exhibit disease symptoms following exposure to viruliferous TCAH is not known. We first determined transmission biology attributes of GRBV by the TCAH and documented an access acquisition period of 10 days when insects are exposed to GRBV-infected grapevines. Then, we revealed transmission of GRBV by the TCAH from and to free-living grapevines. Next, we showed the competence of the TCAH at transmitting GRBV in the vineyard by detecting the virus at three-months post-exposure of healthy grapevines to viruliferous insects in an experimental vineyard. Infected vines are continuously monitored, as they have not shown disease symptoms yet. The recently developed diagnostic loop-mediated isothermal amplification (LAMP) assay for GRBV was validated with cooperating wine grape growers. These growers have received training on the LAMP assay and some of them have adopted it as an on-site diagnostic tool to determine GRBV incidence in vineyards. Information on the ecology of red blotch disease was disseminated to grower communities at conventions, through video conferences, and on-site visits.

### LAYPERSON SUMMARY

Grapevine red blotch virus, the causal agent of red blotch disease, causes substantial losses to the grape and wine industries. This virus is transmitted by grafting and by the three-cornered alfalfa hopper, although this insect is not a pest of grape. Little information is available on how red blotch virus interacts with the three-cornered alfalfa hopper for transmission. We documented 10-days of insect exposure to infected grapevines are necessary for the virus to be transmissible to healthy grapevines. We then revealed transmission of red blotch virus from and to free-living grapevines by the three-cornered alfalfa hopper. Next, the ability of the three-cornered alfalfa hopper at transmitting red blotch virus in the vineyard was shown as early as three-months post-exposure of insects carrying the virus to healthy grapevines in an experimental vineyard. Test vines that became infected via vector-mediated inoculation are continuously monitored, as they have not exhibited disease symptoms yet. Nonetheless, these results support an active role of the three-cornered alfalfa hopper in transmitting red blotch virus in the vineyard. Additionally, an accurate, cheap, and user-friendly red blotch virus diagnostic assay was developed and validated with wine grape growers in Napa and Sonoma Counties. Some of these growers have adopted the assay for on-site diagnosis to determine GRBV incidence in vineyards. Research progress and information on disease ecology was communicated to grower communities.

## INTRODUCTION

Red blotch is one of the most important viral diseases of grapevine in the United States (Cieniewicz et al. 2017a, Sudarshana et al. 2015). It was described for the first time on Cabernet Sauvignon at the UC Oakville Research Field Station in 2008 (Calvi 2011). Red or chlorotic blotches on leaves of black- and white-berried *Vitis vinifera* vines, respectively, delayed fruit ripening, and reduced fruit quality are characteristic symptoms of red blotch disease (Blanco-Ulate et al. 2017, Cieniewicz et al. 2017a, Girardelo et al. 2019, Girardelo et al. 2020, Kurtural et al. 2019, Martinez-Lüscher et al. 2019). The estimated economic impact of red blotch disease ranges from \$5,469 to \$169,385 per acre over a 25-year lifespan of a vineyard (Ricketts et al. 2017).

Grapevine red blotch virus (GRBV) is the causal agent of red blotch disease (Yepes et al. 2018), and the type member of the genus *Grablovirus* in the plant virus family *Geminiviridae* (Varsani et al., 2017). This virus has a single-stranded DNA genome that codes for seven open reading frames (Cieniewicz et al., 2017a, Vargas et al., 2019). Analysis of the genetic diversity among GRBV isolates indicates two phylogenetic groups (Krenz et al. 2014) that are both involved in the etiology of the disease (Yepes et al. 2018).

GRBV is transmissible by grafting and has been reported throughout the United States (Krenz et al., 2014) and Canada (Poojari et al. 2017, Xiao et al., 2015), as well as in Switzerland (Reynard et al. 2018), South Korea (Lim et al. 2016), Mexico (Gasparin-Bulbarela et al. 2018), Argentina (Luna et al. 2019), India (Marwal et al. 2019), and Italy (Bertazzon et al. 2021). GRBV has also been isolated from numerous table grape accessions at the USDA germplasm repository in Davis, CA (Al Rwahnih et al. 2015a), from an herbarium collection at UC-Davis (Al Rwahnih et al. 2015b), and from free-living grapes in Northern California (Badher et al. 2016a, Cieniewicz et al. 2018a, Perry et al. 2016). While long distance dispersal is attributed to the dissemination of infected propagation material, short distance spread within vineyards has thus far only been observed in California (Cieniewicz et al. 2017b, Cieniewicz et al. 2018b, Cieniewicz et al. 2019) and Oregon (Dalton et al. 2019).

GRBV is transmitted by *Spissistilus festinus* [Say, 1830] (Hemiptera: Membracidae), the three-cornered alfalfa hopper (TCAH), from infected to healthy vines under greenhouse conditions (Bahder et al. 2016b). This hopper species is also suspected as a vector of epidemiological relevance in the vineyard (Cieniewicz et al. 2018). Studies in vineyards in Napa Valley showed that populations of *S. festinus* peak from late June to early July (Cieniewicz et al. 2018, Cieniewicz et al. 2019). A differential dynamic of GRBV spread in a Cabernet franc vineyard and an adjacent Cabernet Sauvignon vineyard was associated with the abundance of TCAH populations; higher rates of spread occurred with higher TCAH populations (Cieniewicz et al. 2019). Similar epidemiological work in a Merlot vineyard in New York failed to document spread of GRBV and no TCAH was caught on sticky traps in this vineyard (Cieniewicz et al. 2019).

The transmission mode of GRBV by the TCAH is non-propagative, circulative (Flasco et al. 2021). This means that the virus is acquired by feeding of the TCAH on the phloem of a GRBV-infected plant, circulates up through the food canal, and crosses the midgut and eventually the hindgut of the insect, reaching the hemocoel. Then the virus crosses accessory salivary glands and returns to the plant via the saliva canal of the TCAH (Flasco et al. 2021, Gray et al. 2014).

Interestingly, a recent phylogenetic and morphological analysis of the variability of TCAH populations documented two distinct genotypes with a genetic distance of up to 11% in the mitochondrial cytochrome C oxidase 1 (mt-COI) gene (Cieniewicz et al. 2020). All the specimens from California comprised one phylogenetic clade and all the specimens from the Southeastern United States comprised another clade. Statistically supported subclades were also found among populations from northern and central California (Cieniewicz et al. 2020). No biological significance is assigned yet to these variabilities, but population differences could have important implications in transmission ability or efficiency, as reported for other insect-transmitted geminivirids (Gray et al. 2014). It would be of interest to compare the vector competence and transmission efficiency of GRBV with the two distinct populations of the TCAH.

The TCAH is not considered a direct pest of *Vitis* spp. although it can cause girdling damage of shoots and petioles upon feeding (Badher et al. 2016b). Females oviposit in green grapevine tissue but progeny do not survive to adulthood on grapevines (Preto et al. 2018a). Populations of the TCAH occur at higher populations at vineyard edges near water sources in Napa Valley in California (Cieniewicz et al. 2018, Cieniewicz et al. 2019), and are suspected to primarily reproduce on vineyard middle-row cover crops (Preto et al. 2019). Feeding and reproductive hosts are Spanish clover, dandelion, birdsfoot trefoil, common groundsel, field bindweed, magnus peas, bell beans, blando brome, purple vetch, black medick, subterranean clover, crimson clover, and woollypod vetch. The greatest nymph emergence was observed on plant species belonging to the family Fabaceae (Preto et al. 2018b). Middle-row cover crop legume species were collected in several diseased vineyards in Napa Valley and tested for GRBV in 2014-2018. All samples tested negative for GRBV suggesting a limited role of cover crops in disease epidemiology (Cieniewicz et al. 2019).

Limited information is available on the transmission efficiency of GRBV by the TCAH in the vineyard. Similarly, no information is available on the virus incubation period, the time between initial infection and development of disease symptoms in a vineyard, following inoculation. Badher et al. (2016b) reported three out of 15 plants exposed to TCAH that fed on a GRBV-infected plant were infected with GRBV at five months post-inoculation, and two of these three plants exhibited foliar disease symptoms. Negative control samples for *S. festinus* and plants tested negative for GRBV throughout the study period (Badher et al. 2016b). Will these findings obtained in the greenhouse translate into similar results in a vineyard situation? One of the goals of the proposed research is to study disease progress following TCAH-mediated inoculation of GRBV under commercial vineyards conditions. This work is critical to inform sound disease management practices.

By analogy with other phloem-feeding insects, including vectors of geminivirids, it is anticipated that the TCAH requires primary endosymbionts for supplementing its diet (Gray et al. 2014). In addition, endosymbiotic bacteria within insects have been implicated in the survival of vectors and are suspected to influence the acquisition, retention, and transmission of plant viruses (Kliot et al. 2014). No information is available on the occurrence of endosymbionts in the TCAH. Recent sequencing work has documented the presence of the primary endosymbionts *Candidatus Sulcia muelleri* (*Sulcia*) and *Ca. Nasuia deltocephalinicola* (*Nasuia*), and of the facultative endosymbiont *Ca. Arsenophonus nilaparvataea* (Clarke and Fuchs, unpublished) in TCAH

specimens from California. Characterizing the TCAH endosymbionts is necessary to determine whether they play a role in vector survival or GRBV transmission.

Management of red blotch disease relies upon the accurate detection of GRBV, elimination of 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 commonly involves the use of a polymerase chain reaction (PCR) assay, requiring sophisticated instrumentation and expertise of a testing laboratory. We recently developed loop-mediated isothermal amplification (LAMP) assays to detect GRBV. These assays can be completed in approximately 45 minutes, without the need for significant instrumentation (Romero et al. 2019). Additional needs are a clean workspace, tubes, a pipetting device, and cold storage for the reagents. The LAMP assays are cost-effective and tailored for on-site testing (Romero et al. 2019).

In spite of tremendous progress on the biology and spread of red blotch, research on ecological aspects of the disease is still needed. For example, validating an on-site diagnostic LAMP assay with cooperating vineyard managers is of high 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, and to facilitate the implementation of disease management strategies. A better understanding of how GRBV is transmitted by the TCAH and how the virus interacts with endosymbionts and different genotypes of the TCAH for transmission is also of interest to develop optimal disease management options. Finally, disseminating information on the ecology of GRBV to the industry is essential to communicate research accomplishments and share the latest knowledge on red blotch disease ecology. These are significant components of this continuing project.

## **OBJECTIVES**

Our specific objectives are to:

1. Refine and validate diagnostic GRBV LAMP assays
2. Assess the competence of the TCAH at transmitting grapevine red blotch virus
3. Determine the transmission efficiency of grapevine red blotch virus by the TCAH
4. Characterize tri-trophic virus-vector-endosymbiont interactions for transmission
5. Disseminate information to grape growers, extension educators and service providers

## **RESULTS AND DISCUSSION**

**To address objective #1 and refine and validate diagnostic loop-mediated isothermal amplification (LAMP) assays for GRBV,** LAMP assays for GRBV diagnostics were recently developed (Romero et al. 2019). These assays are very sensitive and false negatives have not been problematic. It is expected that PCR will remain the standard for GRBV testing but LAMP assays offer an alternative testing strategy with limited requirements for instrumentation and potential for implementation by grower or nursery operations. In addition to the reduced cost per sample, the speed in obtaining information is attractive; the primary requirement is the investment of time and labor to perform the assay. Having rapid test results will complement visual assessments of symptoms and can facilitate selecting material for further sampling (or not) and making management decisions for vine replacement. The LAMP assays were demonstrated to growers in Napa and Sonoma Counties in cooperation with farm advisors Monica Cooper and Rhonda Smith, respectively. Follow-up training sessions on how to perform the LAMP assays

have been organized and some vineyard managers have adopted the assays. They are using an optimized version of the assays to estimate the incidence of GRBV in their vineyards and facilitate the adoption of the most appropriate disease management strategy. If adopted at a larger scale, the LAMP assays could facilitate the mitigation of the detrimental impacts of red blotch disease by implementing management tactics tailored to specific vineyards.

**To address objective #2 and determine the transmission efficiency of GRBV by the TCAH,** we characterized the acquisition and inoculation access periods by developing and optimizing a transmission assay in the greenhouse (Flasco et al. 2021). Our findings were consistent with 5-, 8- and 10-day exposure of the TCAH to infected grapevines needed for GRBV to be detectable in dissected guts, hemolymph, and heads with salivary glands, documenting a circulative transmission mode. After a 15-day acquisition on infected grapevines and subsequent transfer on alfalfa, a non-host of GRBV, the virus titer decreased over time in adult insects, as shown by qPCR, illustrating a nonpropagation transmission mode (Flasco et al. 2021). Snap bean proved to be a feeding host of *S. festinus* and a pseudo-systemic host of GRBV following *Agrobacterium tumefaciens*-mediated delivery of an infectious clone (Flasco et al. 2021). The virus was efficiently transmitted by *S. festinus* from infected snap bean plants to excised snap bean trifoliates (90%) or grapevine leaves (100%) but less efficiently from infected grapevine plants to excised grapevine leaves (10%) or snap bean trifoliates (67%) (Flasco et al. 2021). Transmission of GRBV also occurred transstadially but not via seeds. The virus titer was significantly higher in guts and hemolymph relative to heads with salivary glands, and in adults emanating from third compared with first instars that emerged on infected grapevine plants and developed on snap bean trifoliates (Flasco et al. 2021). This study demonstrated circulative, nonpropagative transmission of GRBV by *S. festinus* with an extended acquisition access period, and marked differences in transmission efficiency between grapevine, the natural host, and snap bean, an alternative herbaceous host (Flasco et al. 2021).

More recently, we documented the transmission of GRBV via the TCAH from infected free-living grapevine hybrids (*Vitis californica* x *Vitis vinifera* 'Sauvignon blanc') (Perry et al. 2016) to healthy free-living grapevine hybrids (43%, 6 of 14); from infected free-living grapevine hybrids to healthy wine grapes ('Cabernet franc') (33%, 4 of 12); and from infected wine grapes to healthy free-living grapevine hybrids (100%, 9 of 9) (Hoyle et al. unpublished). As expected, none of the recipient control plants (0%, 0 of 27) exposed to aviruliferous TCAH tested positive for GRBV in PCR. These results illustrated the role of GRBV-infected free-living grapevines in the epidemiology of red blotch disease.

In parallel, experiments were performed in 2020 to investigate the incubation period for symptom development in a vineyard following TCAH-mediated inoculation of GRBV. An experimental vineyard was selected for this study at Cornell AgriTech in Geneva, NY. It was planted in 2008 with Cabernet franc vines grafted onto the rootstock 3309C. The GRBV-free-status of test vines in this experimental vineyard was confirmed by PCR testing of petioles in fall of 2018 and 2019. The vineyard consisted of 6 rows of 24 vine each, including 16 experimental vines surrounded by 128 spacer vines. Treatments included 10 vines exposed to 12-15 viruliferous TCAH and three vines exposed to 12-15 aviruliferous TCAH in June of 2020. Negative controls consisted of three vines not exposed to any TCAH. Insects were released on a single shoot of select vines and were contained in mesh cover sleeve nets (Figure 1).



The TCAH were maintained on vine shoots in sleeve nets for five weeks during which their behavior (development and survival) was regularly monitored (Figure 2). Then the contact insecticide Mustang® Maxx was applied to kill the insects in the sleeve nets. Next leaf petioles from the shoot exposed to the TCAH were collected monthly for GRBV testing by PCR. Similarly, leaf petioles from the shoot next to the one exposed to the TCAH were collected for GRBV testing to determine systemic virus movement within inoculated vines. Results showed that none of the petiole samples from 10 experimental vines exposed to viruliferous TCAH tested positive for GRBV six weeks post-release in mid-August 2020. However, starting at three-months post-release (September 2020, some the experimental vines (33%, 3 of 10) exposed to viruliferous TCAH tested positive for GRBV, as shown by PCR. As expected, the three controls vines exposed to aviruliferous TCAH and the unexposed control vines tested negative for GRBV from July to October 2020. Similarly, petiole samples from shoots adjacent to those exposed to viruliferous and aviruliferous TCAH tested negative for GRBV. These results documented the competence of the TCAH at transmitting GRBV in the vineyard, documenting the role of this insect in red blotch disease epidemiology.

None of the GRBV-infected vines expressed disease symptoms from June to October of 2020 and the virus had not moved away from the inoculated shoot to other shoots of the vines exposed to viruliferous TCAH. Experimental vines were continuously monitored for typical red blotch symptoms in 2021 but none of them had manifested disease symptoms throughout the growing season yet. A lag time between virus presence, as indicated by PCR results, and disease symptom expression is consistent with red blotch disease progress in vineyards in California (Fuchs, unpublished). Similar TCAH release experiments were going to be duplicated in a vineyard in Napa Valley but were not carried out in 2020 due to institutional covid19-related travel restrictions.



**Figure 1.** TCAH contained on a single shoot of a Cabernet franc vine in a sleeve net (center of the photo).



**Figure 2.** TCAH feeding on a ‘Cabernet franc’ leaf in a sleeve net in the vineyard.

Concurrent to the 2020 vineyard studies in New York transmission studies were carried out in the greenhouse. Following an acquisition access period on GRBV-infected plants, cohorts of 10 TCAH were transferred to healthy (GRBV-negative) recipient plants. TCAH were caged on the recipient plants, half of which receiving viruliferous TCAH and the other half serving as a control set and receiving aviruliferous TCAH. An additional group of 10 healthy vines were kept in the greenhouse as untreated controls. Infection by GRBV was monitored over time through petiole testing of recipient plants by PCR. Results showed transmission of GRBV at a low rate (31%, 22 of 72). As expected, vines exposed to aviruliferous TCAH (0%, 0 of 7) and control vines (0%, 0 of 3) tested negative for GRBV. None of the infected recipient vines has shown disease symptom in the greenhouse. These results are consistent with the transmission rate obtained in the vineyard (33%, 3 of 10). Following a dormancy period for two months, plants exposed to viruliferous and aviruliferous TCAH were grown in a greenhouse, and continuously monitored for disease symptom expression. None of these plants exhibited red blotch disease symptoms for 8 months. In 2021, additional transmission assays were repeated with viruliferous or aviruliferous TCAH under greenhouse conditions (Figure 3). Results showed that some plants tested positive for GRBV in PCR (10%, 3 of 30), but none of them showed red blotch disease symptoms 6-months post-exposure to viruliferous TCAH. As expected, none of the plants exposed to aviruliferous TCAH tested positive for GRBV in PCR or manifested disease symptoms. The 2020 and 2021 vines will continue to be rated for disease symptoms.



**Figure 3.** Transmission assays of GRBV with cage TCAH on healthy 'Cabernet franc' vines in a greenhouse.



**Figure 4.** Release of TCAH on select vines indicated by red arrows in a Cabernet Sauvignon vineyard in Napa Valley.



Releases of TCAH were repeated in June of 2021 in the same experimental Cabernet franc vineyard at Cornell Agritech in Geneva, NY. Insects were released on vines other than those selected in 2020. A similar approach was used in a Cabernet Sauvignon vineyard in Napa Valley under USDA-APHIS-PPQ permit P526P-21-02605 (Figure 4). Vines onto which the insects were released in the Cabernet franc and Cabernet Sauvignon vineyards were confirmed negative for GRBV by PCR in 2019 and 2020. Different cohorts of TCAH, i.e., 10, 5 and 2, were used in distinct sized sleeve nets to maximize insect feeding and detectability of GRBV following feeding. In both vineyards in New York and California, the behavior of insects in the sleeves was monitored for feeding and survival. Similarly, vines were monitored for insect feeding damage, as expressed by girdling of petioles and/or shoots and reddening of leaves. Then, following a 4-week exposure, a contact insecticide was applied to kill the TCAH contained in the sleeve nets. Shoots and leaves exposed to viruliferous or aviruliferous TCAH were tested for GRBV and disease symptoms. A few of them tested positive for GRBV in PCR (6%, 2 of 35) but none of them showed disease symptoms even five months post-exposure to the TCAH.

**To address objective #3 and assess the competence of distinct populations of the TCAH at transmitting grapevine red blotch virus,** insect specimens from California and South Carolina were used. Insects from the two colonies acquired virus from GRBV-infected potted vines obtained following agroinoculation with GRBV infectious clones (Yepes et al. 2018) or derived from naturally infected vines. After the acquisition feeding period, 10 insects were transferred to healthy potted vines in small insect cages and allowed to feed. These experiments are under way. Following the inoculation access period, disease symptoms and infection by GRBV will be monitored over time in petioles of recipient plants by PCR. This work will determine if TCAH specimens from California and South Carolina, which are genetically distinct (Cieniewicz et al. 2020), have a differential capacity to transmit GRBV.

In addition, fluorescence *in situ* hybridization experiments have been initiated to illustrate the transmission pathway for GRBV in the TCAH. Specific hybridization probes were designed and used to localize GRBV DNA in different organs of the TCAH that were dissected under an Olympus SZX16 stereoscope. Hybridization results are visualized using fluorescence confocal microscopy on an Olympus FV3000 microscope. Visualization of GRBV in the gut lumen and wall, hemolymph, and salivary glands is expected in viruliferous TCAH by confocal microscopy. This work is performed to illustrate movement of GRBV in the TCAH, and further support a circulative transmission mode.

**To address objective #4 and characterize tri-trophic virus-vector-endosymbiont interactions for transmission,** early Illumina sequencing efforts documented the occurrence of endosymbionts and revealed the presence of three species of bacteria in the bacteriocyte of TCAH specimens from California: *Sulcia*, *Nasuia* and *Arsenophonus* (Clarke et al., unpublished). The annotation of the genomes of these endosymbionts is progressing. Of relevance to the transmission process, populations of the TCAH from various geographic origins and different hosts will be screened for the occurrence of these endosymbionts by PCR using specific primers designed against *Sulcia*, *Nasuia*, and *Arsenophonus* to determine the existence of specimens without secondary endosymbionts. If TCAH populations with distinct endosymbiont compositions are identified, colonies will be established and the behavior of viruliferous and non-viruliferous populations with or without endosymbionts will be tested on

grapevines and alternative hosts such as snap bean; and their transmission capacity of GRBV will be compared.

**To address objective #5 and disseminate research results to farm advisors and the industry,** research findings were communicated to the industry through presentations to growers, industry groups, farm advisors, extension educators, vintners, nurseries, consultants, and service providers. Presentations on the biology and ecology of GRBV were made at the following grower meetings:

Fuchs, M. 2021. Updates on red blotch disease. Lodi district, CA, October 15 (participants = 35).

Fuchs, M. 2021. Updates on red blotch disease. Central Valley, CA, October 14 (participants = 25).

Fuchs, M. 2021. Updates on red blotch virus transmission and disease management in the vineyard. Agri-Analysis LLC and Stamp Associates, March 11 (participants = 120).

Fuchs, M. 2021. Updates on red blotch transmission. National Clean Plant Network for Grapes, February 17 (participants = 41).

Fuchs, M. 2021. Red blotch disease: transmission and management. Webinar of the Canadian grapevine certification network, January 21 (participants = 120).

Fuchs, M. 2021. Leafroll and Red Blotch Viruses: Identification and Management. Meeting of the Maryland Grape Grower Association, January 13 (participants = 55).

Flasco, M., Loeb, G. and Fuchs, M. 2020. Investigating the transmission efficiency of red blotch virus by *Spissistilus festinus* in the vineyard. Cornell Recent Advances in Viticulture and Enology (CRAVE) conference, December 10 (participants = 40).

Hoyle, V. and Fuchs, M. 2020. Vector ecology and virus transmission ability. Cornell Recent Advances in Viticulture and Enology (CRAVE) conference, December 10 (participants = 40).

Fuchs, M. 2020. Viruses: what can we do? Willbur-Ellis Agribusiness, Viticulture meeting, October 28, (participants = 60).

Fuchs, M. 2020. Leafroll and red blotch diseases: what's new? And, what can I do? Sustainable Ag Expo, San Luis Obispo, CA (participants = 250).

We reached out to more than 800 growers, extension educator, integrated pest management specialists, vineyard managers and vintners to discuss research progress on the biology and ecology of GRBV. Information on disease ecology, LAMP diagnostic assays, and disease management recommendations were well received.

## CONCLUSIONS

We documented a circulative, non-propagative transmission mode of GRBV by the TCAH. We provided evidence that the TCAH can transmit GRBV from and to free-living grapevines and inoculate GRBV to wine grapes in the vineyard. Similar low transmission rates (~10-30%) were obtained in the vineyard and greenhouse. Vines infected upon TCAH-mediated GRBV inoculation are monitored in the vineyard and greenhouse for disease symptom development. The recently developed diagnostic LAMP assays for on-site GRBV detection were validated with growers in Napa and Sonoma Counties. Some growers have adopted the optimized version of the assays and use it to assess red blotch disease incidence in vineyards. Information on research progress and ecology of red blotch disease was disseminated to grower communities.

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## RESISTANCE TO GRAPEVINE LEAFROLL VIRUS 3 AND ITS MAJOR MEALYBUG VECTORS

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### ABSTRACT

Leafroll is one of the most devastating and widespread viral diseases of grapevines. We are applying RNA interference (RNAi) to confer resistance in grapevines against grapevine leafroll-associated virus 3 (GLRaV-3), the dominant leafroll virus in diseased vineyards, and to the grape (*Pseudococcus maritimus*) and vine (*Planococcus ficus*) mealybugs, the two most important vectors of GLRaV-3 in California vineyards. Our strategy is to combine RNAi against targets of the virus and the two insect vectors. The target RNAi genes *AQPI<sub>GM</sub>*, *SUCI<sub>GM</sub>* and *NUCI<sub>GM</sub>* from the grape mealybug were identified and characterized. A significantly increased mortality (20%) of grape mealybug nymphs was observed upon feeding on a diet supplemented with dsRNA constructs corresponding to these targets, validating our RNAi approach against the grape mealybug. Efforts to predict the efficacy of dsRNA constructs from the grape mealybug suggested that *AQPI<sub>GM</sub>* and *NUCI<sub>GM</sub>*, but not *SUCI<sub>GM</sub>* should be efficient against the vine mealybug; therefore, we characterized *SUCI<sub>VM</sub>* from the vine mealybug to maximize RNAi efficacy. Bioassays have been initiated to test how *SUCI<sub>VM</sub>* affects the mortality of vine mealybug nymphs upon feeding. Next, *SUCI<sub>VM</sub>* was stacked with *AQPI<sub>GM</sub>*, *SUCI<sub>GM</sub>* and *NUCI<sub>GM</sub>* for optimal RNAi efficacy against the two mealybug species. For GLRaV-3, RNAi constructs were designed in target genes, i.e., *p19.7*, *CP*, *RdRP* and *HSP70h*, and used in grape transformation experiments. RNAi constructs against the grape and vine mealybugs and against GLRaV-3 were pyramided for transformation experiments. Expression of pyramided constructs *in planta* are directed to the phloem by the specific promoter *AtSUC2*. Efforts to produce grape plants stably transformed with RNAi constructs against GLRaV-3 and the two mealybug pests are under way. Research progress and the potential of our approach at conferring resistance to GLRaV-3 and its two major mealybug vectors was communicated to the grape industry.

### LAYPERSON SUMMARY

Leafroll is a widespread viral disease of grapevines that reduces fruit production and quality. There is no cure 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 virus in diseased vineyards, and to the grape mealybug and the vine mealybug, the two most important vectors of GLRaV-3 in California vineyards, using RNA interference (RNAi). RNAi is a potent regulatory mechanism of gene expression that targets the specific degradation of RNA molecules with high sequence identity to a dsRNA inducer. Our strategy is to combine RNAi constructs against targets of the virus and of the two insect vectors.

Three dsRNA targets essential for the virus to complete its infection cycle were selected against GLRaV-3. Two gut RNAi targets critical for successful insect feeding on plant phloem sap were selected against the mealybugs; a third RNAi target was selected for mealybugs to enhance the efficacy of the two gut RNAi targets. Target genes were identified for the grape and the vine mealybugs and characterized. Preliminary results showed that dsRNA constructs against mealybug genes resulted in a significantly increased mortality of grape mealybug nymphs following feeding on an artificial diet supplemented with the dsRNA constructs. Predictive modeling suggested that one of the dsRNA constructs against the grape mealybug is unlikely to provide any cross-reactivity against the vine mealybug. Therefore, we engineered a new dsRNA construct against the counterpart gut gene of the vine mealybug. This new RNAi construct was identified and characterized from dissected gut tissue of vine mealybugs established on winter squash in a growth chamber. The integrity of this new dsRNA construct was validated. It will be used in combination with the three dsRNA constructs from the grape mealybug for optimal RNAi efficacy. For resistance to GLRaV-3, target dsRNA constructs were developed and used in grape transformation experiments for the recovery of stable transformants. Some transgenic rootstock 110R and 101-14 and *Vitis vinifera* cultivars Cabernet franc and Pinot noir were obtained and characterized. Transformation efforts are pursued by using stacked dsRNA constructs against the mealybugs and GLRaV-3, and by directing their expression in the phloem tissue using a tissue specific plant promoter. The production of grape plants stably transformed with dsRNA constructs against GLRaV-3 and the two mealybug pests is advancing.

## INTRODUCTION

Leafroll is one of the most devastating and widespread viral diseases of grapevines. 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, Naidu et al. 2015). 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, e.g., GLRaV-1, -2, -3, -4, -7, and -13, have been identified in diseased vines (Fuchs 2020). Among these six viruses, grapevine leafroll-associated virus 3 (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 one-hour access period of feeding by immature mealybugs (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 are capable to spread 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 (Daane et al. 2012). 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 vines, however, the grape mealybug (*Pseudococcus maritimus*) and the vine mealybug

(*Planococcus ficus*) are the most abundant and widespread 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 of leafroll disease 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 primarily because there is a lack of recognized host resistance (Oliver and Fuchs 2011). Interestingly, some level of resistance to the vine mealybug was recently described in the rootstock genotype RS-3 (Naegele et al. 2020). It will be interesting to see whether this resistance is practical in infested vineyard sites.

Although no practical resistance source is known in *Vitis* sp., resistance can be achieved against GLRaV-3 and its mealybug vectors by applying RNA interference (RNAi). RNAi is a conserved and RNA-dependent gene silencing process that is induced by dsRNA expression to target the specific cleavage of messenger RNA molecules and down regulate their expression. Leveraging RNAi is an elegant approach to develop virus- and insect-resistant plants (Fuchs 2020, Ibrahim and Aragão 2015, Luo et al. 2017, Scott et al. 2013, Tzin et al. 2015). 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 both virus and insect vector.

The goal of our research is to develop a robust RNAi-based strategy against GLRaV-3, the grape mealybug, and the vine mealybug. The basis for our approach is three-fold. First, mealybug survival depends on two genes that are localized to the gut and 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, Arora et al. 2020), with evidence that insect mortality is enhanced by co-targeting via RNAi these two genes with different molecular function but related physiological role (Tzin et al. 2015). The AQP1 product moves water from the anterior of the insect hindgut to the anterior of the midgut through the filter chamber to decrease osmolarity of food. The *SUC1* product breaks down sucrose to glucose and fructose, and the transglucosidase activity of the enzyme synthesizes oligosaccharides from monosaccharides (mainly glucose) to decrease the osmotic pressure. Second, these two gene functions can be targeted by RNAi *in planta*, with evidence from related phloem feeding insects. In addition, RNAi efficacy is enhanced by stacking dsRNA constructs corresponding to *AQP1* and *SUC1* in combination with dsRNA against the gut nuclease (*NUC1*) (Luo et al. 2017). The NUC1 product breaks down nucleic acids and is targeted in our research to increase the stability of dsRNA. Third, RNAi is being successfully applied against viruses of fruit crops such as papaya and plum, among many other crops (Fuchs 2020).

The proposed research is to develop grapevines resistant to GLRaV-3, the grape mealybug, and the vine mealybug using RNAi. Our strategy is to combine RNAi against targets of the virus and of its two most important 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. Our goal is to build on our previous achievements towards effective control of key mealybug pests using RNAi against gut genes that protect the insects

against osmotic collapse and dehydration (Arora et al. 2020). We will continue producing grape plants stably transformed with RNAi constructs against the mealybug pests and GLRaV-3 with the goal of analyzing their level of resistance against the insects, and subsequently against the virus.

Our RNAi strategy has several 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; (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 organisms (grape mealybug, vine mealybug, and GLRaV-3) with minimal impact on non-target organisms; and (iii) prior success of our strategy with other phloem-feeding insects (Luo et al. 2017, Tzin et al. 2015) and fruit crop viruses (Fuchs 2020). 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.

We previously identified the candidate genes coding for the water channel aquaporin AQP1 and the sucrase-transglucosidase SUC1 in the grape mealybug, as well as a gut nonspecific nuclease NUC1, and developed a system to assess the impact of RNAi trigger molecules against these genes on grape mealybug survival. Our findings documented a significantly increased mortality (20%) of grape mealybug feeding on an artificial diet supplemented with dsRNA constructs against *AQP1*, *SUC1* and *NUC1* (Arora et al. 2020). We believe this assay underestimates the RNAi impact since the overall performance on artificial diet is anticipated to be lower compared to a bioassay *in planta*. In addition, the counterpart candidate *AQP1*, *SUC1* and *NUC1* were identified and characterized in vine mealybugs kindly provided to us by Dr. Kent Daane, UC-Berkeley. Predictive modeling recently suggested that the grape mealybug *SUC1* RNAi is unlikely to provide any cross-reactivity against the vine mealybug due to substantial nucleotide sequence divergence and suboptimal processing of the corresponding dsRNA into active siRNA (Arora et al. 2020). Therefore, there is a need to develop a new RNAi against *SUC1* of the vine mealybug. To the contrary, it is predicted that dsRNA constructs against the grape mealybug *AQP1* and *NUC1* RNAi will be effective against the vine mealybug due to very high nucleotide sequence identity and proper processing of the corresponding dsRNA into active siRNA.

For resistance to GLRaV-3, RNAi constructs against p19.7, a viral suppressor of RNAi (Gouveia et al. 2012), 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 activate the anti-viral pathways of RNAi and confer resistance. A few plants of transgenic rootstock genotypes 110R and 101-14 were obtained and characterized. Also, efforts to transform embryogenic calli of *Vitis vinifera* cvs. Cabernet franc and eventually Pinot noir with GLRaV-3 RNAi constructs were initiated and are being actively pursued. To augment the efficacy of our RNAi approach, constructs against mealybugs (*AQP1<sub>GM</sub>*, *SUC1<sub>GM</sub>*, *SUC1<sub>VM</sub>*, and *NUC1<sub>GM</sub>*) and GLRaV-3 (*CP*, *RdRp*, *HSP70h*) are being stacked in various combinations. Their expression *in planta* is driven by the phloem-specific promoter *AtSUC2* (Truernit and Sauer 1995, Zhang and Turgeon 2018).

## OBJECTIVES

The proposed project will address the following objectives:

1. Complete the engineering of optimal dsRNA constructs with predicted efficacy against the grape mealybug and the vine mealybug
2. Continue the development of grape transformants with optimal dsRNA constructs against both mealybug species and grapevine leafroll-associated virus 3
3. Quantify the impact of RNAi-transformed vines on the survival of mealybugs
4. Disseminate information to stakeholders through presentations at conventions

The overarching goal of this proposal is to advance the development of grapevines with resistance to GLRaV-3, the dominant leafroll virus in vineyards, and to both the grape mealybug and the vine mealybug, the two major vectors of GLRaV-3 in California vineyards. We will build on previous accomplishments to complete the engineering of optimal dsRNA constructs against the mealybugs and GLRaV-3 and pursue grape transformation efforts.

## RESULTS AND DISCUSSION

**To address objective 1 and complete the engineering of optimal dsRNA constructs with predicted efficacy against the grape mealybug and the vine mealybug,** we previously developed and characterized *AQPI<sub>GM</sub>*, *SUCI<sub>GM</sub>*, and *NUCI<sub>GM</sub>* from the grape mealybug. Our predictive modelling and theoretical prediction of siRNA efficacy indicated *in silico* a cross-reactivity of *AQPI<sub>GM</sub>*, and *NUCI<sub>GM</sub>* against the vine mealybug counterpart gene transcripts due to high sequence identity and proper processing of dsRNA for siRNA production (Arora et al. 2020). However, *SUCI<sub>GM</sub>* is predicted to have minimal cross-reactivity to corresponding transcripts of the vine mealybug due to weak sequence identity and differential processing of dsRNA with no siRNA target. These results indicated that the *SUCI<sub>GM</sub>* dsRNA will likely not work against the vine mealybug. Therefore, *SUCI<sub>VM</sub>* from the vine mealybug should be used for efficient RNAi-mediated resistance against the vine mealybug.

We engineered a dsRNA construct of *SUCI<sub>VM</sub>* by RT-PCR using total RNA isolated from dissected gut tissue of instars from a colony of the vine mealybug maintained on winter squash in a controlled walk-in chamber at Cornell AgriTech (Figure 1), and specific primers designed from GenBank accession number MT192031. The colony was established with specimens generously provided by Dr. Ken Daane, UC-California at Berkeley, CA under USDA-APHIS-PPQ permit no. P526P-20-4926. The integrity of the amplified *SUCI* fragment was verified by gel electrophoresis and bidirectional sequencing at the Institute of Biotechnology at Cornell University. Sequence analysis showed 100% nucleotide identity of the newly identified *SUCI<sub>VM</sub>* construct with GenBank accession number MT192031 from the vine mealybug. That was previously determined (Arora et al. 2020). In addition, as predicted from earlier work, the sequence identity of the newly identified *SUCI<sub>VM</sub>* construct was lower with that of *SUCI<sub>GM</sub>* from the grape mealybug (76%). Subsequently, *SUCI<sub>VM</sub>* was cloned in a transcription plasmid to produce dsRNA *in vitro*.

Then, the dsRNA to *SUCI<sub>VM</sub>* was engineered into a concatenate dsRNA construct with *AQPI<sub>GM</sub>*, *SUCI<sub>GM</sub>*, and *NUCI<sub>GM</sub>*. This was achieved using specific primer pairs designed to amplify *AQPI<sub>GM</sub>*, *SUCI<sub>GM</sub>* and *NUCI<sub>GM</sub>* from the grape mealybug, and *SUCI<sub>VM</sub>* from the vine mealybug, by PCR, and sequentially cloning each fragment to form a concatenate construct. Specific primer

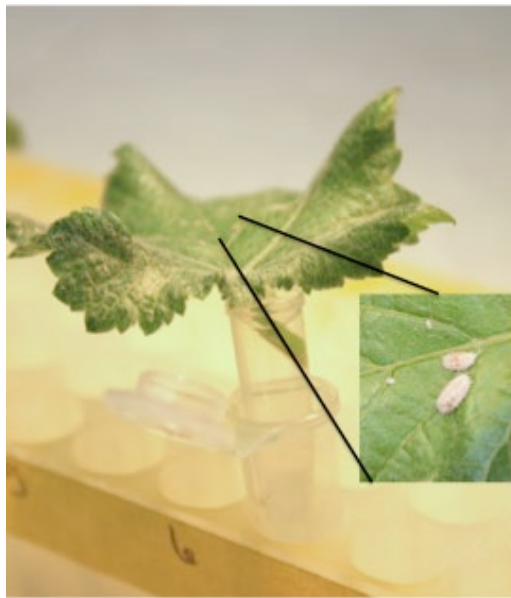
pairs contained additional, unique restriction digestion sites to facilitate the cloning of each fragment into a bacterial plasmid. The concatenate dsRNA construct is almost complete. *In planta* expression of the mealybug dsRNA concatenate construct will be driven by the phloem specific *SUC2* promoter of *Arabidopsis thaliana*, *AtSUC2* (Truernit and Sauer 1995; Zhang and Turgeon 2018). Next, the mealybug dsRNA concatenate construct will be fused to the GLRaV-3 CP, *RdRp* and *HSP70h* concatenate dsRNA construct by restriction digestion and ligation in a bacterial plasmid for expression *in planta*. Alternatively, a pHELLSGATE binary vector will be used to insert the PCR products of interest by directed recombination. The resulting mega concatenate dsRNA construct will be used in grape transformation experiments. It is anticipated to confer effective resistance to GLRaV-3 and its major mealybug vectors.



**Figure 1.** Colony of the vine mealybug on winter squash in a walk-in chamber at Cornell Agritech. The inset shows a single specimen.

Concurrently, we initiated experiments to test the efficacy of *AQPI<sub>GM</sub>*, *SUC1<sub>GM</sub>* and *NUC1<sub>GM</sub>*, and *SUC1<sub>VM</sub>* against the vine mealybug. Experiments are carried out as previously described for the grape mealybug (Arora et al. 2020). Briefly, dsRNA constructs are delivered orally to 2nd–3rd instar vine mealybugs using a sachet of sterile artificial liquid diet sandwiched between two layers of Parafilm<sup>®</sup>. Experiments and treatment are replicated, and data will be analyzed statistically. As controls for non-specific effects of dsRNA, replicated sets of insects are fed with dsRNA of the green fluorescent protein (GFP) gene or dsRNA-free diet. Following a 5-day treatment, the surviving insects are counted, and the guts of individual specimens are dissected for RNA extraction and quantification of gene expression by RT-qPCR using specific primers (Arora et al. 2020).

As an alternative to the use of bioassays based on a sachet, excised grapevine leaves will be explored to test the effect of *AQPI<sub>GM</sub>*, *SUCI<sub>GM</sub>*, *NUC1<sub>GM</sub>*, and *SUCI<sub>VM</sub>* against vine mealybug instars. The use of excised leaves will require vine mealybug to survive and develop for at least a week, and for dsRNA constructs to be successfully administered by capillarity to the leaf blade. Detached leaf bioassay settings were previously investigated to assess grape mealybug survival and development (Figure 2).



**Figure 2.** Close-up the excised a Pixie leaf (Cousins 2007) with its petiole immersed in water and mealybug adults feeding on secondary veins.



**Figure 3.** Environmental chamber devised to test the survival of mealybugs on excised wine grape leaves.

For excised leaf bioassays, the most promising setting identified so far consisted of detached *V. vinifera* leaves in an environmental chamber placed within a walk-in growth chamber (Figure 3). Briefly, excised grapevine leaves were placed in parafilm-covered 2 mL-collection vials containing sterile water with a hole poked through the parafilm to hold upright the petiole submerged in water. Then twenty 2<sup>nd</sup> instar grape mealybug nymphs from the colony maintained on Pixie grapes (Cousins 2007) in the greenhouse were delicately deposited on the underside of detached leaves using a paint brush. Detached leaves in vials were then enclosed in a clear polypropylene 950 mL container (Farbi-Kal, PK32T) sealed with a polypropylene donut lid carrying a nylon screen (BugDorm 5002). The collection vials were regularly replenished with sterile water, as appropriate. Detached leaves stayed turgid, green, and healthy for at least three weeks. In addition, the environmental chamber resulted in active feeding of crawlers primarily at the petiole-leaf junction and midrib of the leaf blade with 70 and 80% of them still alive on the assay leaves after three weeks. Moreover, the mealybugs seemed to develop normally and reached medium size. In a second experiment, we used two potted Chardonnay vines, one treated with a systemic insecticide (imidacloprid), which is toxic to mealybugs, 10 days prior to the assay, while the second vine was not treated. In this trial, we used ten 2<sup>nd</sup> instar nymphs per detached leaf rather than 20. A subset of leaves was destructively sampled weekly, and the number of live, moribund, and dead mealybugs enumerated over a three-week time course. While we observed high survival and normal development on control leaves (70%), we observed low levels of survival (<10%) on leaves treated with insecticide. These results confirmed that a detached assay system should be an efficient approach for evaluating the impact of planned RNAi transformations on the survival of mealybugs.

**To address objective 2 and continue the development of grape transformants with optimal dsRNA constructs against both mealybugs and GLRaV-3**, transformation experiments of embryogenic cultures of rootstock genotypes (101-14 and 110R) and *Vitis vinifera* cvs. Cabernet franc and Pinot noir will continue with GLRaV-3 dsRNA *p19.7*, a viral silencing suppressor (Gouveia et al. 2012), *CP*, *RdRP*, *HSP70h*, and stacked dsRNA constructs of *CP*, *RdRP* and *HSP70h*. These efforts will build on previous transformation experiments with GLRaV-3 dsRNA *p19.7*, *CP*, and *CP+RdRP+HSP70h* constructs (Table 1).

**Table 1.** Summary of independent transgenic grape events with GLRaV-3 dsRNA constructs obtained so far. Elongated embryos are obtained following transformation of embryogenic cultures with *Agrobacterium tumefaciens* carrying binary plasmids with dsRNA constructs of interest that thrive on selective tissue culture and will develop into plantlets.

Genotype	dsRNA	Independent transgenic events
110R	GLRaV-3 <i>p19.7</i>	12
	GLRaV-3 <i>CP</i>	19
	GLRaV-3 <i>CP</i> , <i>RdRP</i> , <i>HSP70h</i>	7
101-14	GLRaV-3 <i>p19.7</i>	8
	GLRaV-3 <i>CP</i>	17
Pinot noir	GLRaV-3 <i>p19.7</i>	3
	GLRaV-3 <i>CP</i>	5
Cabernet franc	GLRaV-3 <i>p19.7</i>	4
	GLRaV-3 <i>CP</i>	elongated embryos



Transformation work will also use stacked dsRNA *AQPI<sub>GM</sub>*, *SUCI<sub>GM</sub>*, *SUCI<sub>VM</sub>*, and *NUC<sub>GM</sub>* constructs and dsRNA *CP*, *RdRP*, *HSP70h* constructs from GLRaV-3. Putative transgenic plants obtained from these transformation experiments will be characterized for transgene insertion by PCR and Southern blot hybridization. Transgene expression will be assessed by RT-PCR and northern blot using specific probes with the aim of identifying independent transformation events. The accumulation of siRNA will be tested using total RNA preparations enriched in small RNAs in select transgenic plants and labeled probes corresponding to RNAi. Extracts from plants transformed with an empty vector or a GFP construct, and nontransgenic plants will be used as controls in RT-PCR, Southern and northern hybridization assays. Independent transgenic grapevine events will be micropropagated in tissue culture and transferred to the greenhouse for bioassays.

**To address objective 3 and quantify the impact of RNAi on the survival of mealybugs,** independent transgenic grapevine events will be separately exposed to grape and vine mealybugs. Transgenic and control plants will be tested for their effect on insect survival using detached leaves and population effects using whole vines. It is anticipated that 10 independent transgenic events per dsRNA construct will be tested.

For population assessments, individual vines will be initially infested with 20-40 crawlers and allowed to develop over four weeks. Small stakes with burlap will be attached to vines to provide habitat for egg-laying. After four weeks the vines will be destructively sampled to determine total mealybug populations as a function of treatment. There will be 10 replicates per treatment combination blocked between two greenhouses (five replicates per greenhouse). Abundance of mealybugs on source and assay leaves or vines will be compared using two factor mixed model ANOVA. We expect mealybugs will have a lower survival and reduced population growth on plants with RNAi constructs against the osmoregulatory genes compared to those on control plants, i.e., plants transformed with RNAi constructs against GFP (with no homology to any mealybug gene) and empty vector controls. Similar results are anticipated with grape and vine mealybugs. Leaf assays will be conducted in controlled environmental chambers, as above described (Figure 3).

These bioassays will inform RNAi efficacy of different transgenic events. The most promising events will be identified based on a consistent low survival rate of the grape mealybug (or the highest mealybug mortality) across replicates. Promising transgenic events will be selected for future experiments under vineyard conditions. It is anticipated that transgenic events expressing *AQPI<sub>GM</sub>*, *NUCI<sub>GM</sub>*, *SUCI<sub>GM</sub>*, and *SUCI<sub>VM</sub>* from both the grape and the vine mealybug without or in combination with the GLRaV-3 dsRNA constructs will perform best against the two mealybug species. Field evaluations of these transgenic events will be addressed in future research proposals.

The level of processing of dsRNA constructs into sRNAs (small RNAs, including siRNA) by the grapevine RNAi machinery will eventually be determined by sRNA-Seq using phloem samples of transgenic and control grapevines. Similarly, the accumulation of sRNA in mealybugs will eventually be tested in pooled specimens exposed to experimental and control transgenic plants. These experiments are essential to demonstrate that the intended transformation is responsible for GLRAV-3 and mealybug suppression. This work will also suggest routes for future

optimization of the transformation design by identifying any processing of the dsRNA constructs that may influence RNAi efficacy. Low recovery and appreciable processing to sRNA molecules will provide a basis for improved design of constructs in future rounds of transformations, especially if intended effects on mealybug performance and GLRaV-3 multiplication are weak.

**To address objective 4 and disseminate research results to farm advisors and the industry,** research progress was communicated to the industry. Presentations were used to disseminate information to growers, industry groups, farm advisors, extension educators, vintners, nurseries, consultants, and service providers at the following grower meetings and on-site visits:

- Fuchs, M. 2021. Updates on leafroll disease management. Lodi district, CA, October 15 (participants = 35).
- Fuchs, M. 2021. Updates on leafroll disease management. Central Valley, CA, October 14 (participants = 25).
- Fuchs, M. 2021. Leafroll and Red Blotch Viruses – Identification and Management. Meeting of the Maryland Grape Grower Association, January 13 (participants = 55).
- Hesler, S., Fuchs, M. and Loeb G. 2020. Management of viruses and mealybug vectors in a commercial leafroll-diseased vineyard. Cornell Recent Advances In Viticulture And Enology (Crave) Conference, December 10, Ithaca, NY (participants = 40).
- Fuchs, M. 2020. Viruses: what can we do? Willbur-Ellis Agribusiness, Viticulture meeting, Oct. 28, webinar, (participants = 60).
- Fuchs, M. 2020. Leafroll and red blotch diseases: what's new? And, what can I do?, Sustainable Ag Expo on Sept. 8, webinar, San Luis Obispo, CA, Invited Speaker (participants = 250).

We reached out to more than 460 growers extension educator, integrated pest management specialists, vineyard managers and vintners to discuss research progress on the application of RNAi against GLRaV-3 and its two major mealybug vectors.

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## **VIRUS-BASED DELIVERY OF INTERFERING RNAS TARGETING GRAPEVINE LEAFROLL-ASSOCIATED VIRUS(ES)**

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**Reporting Period:** The results reported here are from work conducted July 2021 to October 2021.

### **ABSTRACT AND LAYPERSON SUMMARY**

Grapevine leafroll is the most complex viral disease of grapevines worldwide and is economically important in all grape-growing regions (Atallah et al., 2012). At least eleven viruses and several species of mealybugs and scale insects were reported to be associated with the disease complex (Almeida et al., 2013, Naidu et al., 2014). Given the lack of natural resistance in 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 proposed project, we will build upon our successes from the previous funded project and use grapevine virus-based RNA interference (RNAi) approach to target grapevine leafroll-associated viruses. We have successfully developed a vacuum infiltration method to efficiently deliver DNA expression plasmids and viral infectious clones into grapevine plants. This method can be used to deliver expression plasmids and viral vectors directly into greenhouse grown grapevine plants without relying on regeneration from somatic embryos and *in vitro* micropropagation.

We have successfully developed 2 grapevine virus infectious clones: grapevine geminivirus A (GGVA) and grapevine virus A (GVA), that give asymptomatic infections in greenhouse grown grapevines. We will use these 2 viral vectors to deliver and enhance the RNAi efficacy in grapevine rootstocks and scions against GLRaVs. During this report period, we focused on the GGVA viral vectors. We confirmed that our clones of the defective GGVA-76 and GGVA-93 were infectious. Wild type GGVA not only can trigger the production of defective GGVA in various forms, but also facilitate the replication of the defective form of the virus. Those results showed that we can construct RNAi inducers into the defective GGVA and co-infect grapevine plants with the defective GGVA and wild-type GGVA. The wild-type GGVA is expected to enhance the efficacy of RNAi effects based on our results presented in this report. The results obtained during this report period helped us build a plan/protocol moving forward of using this virus a carrier for RNAi delivery. After identifying the best insertion sites in our infectious clones, we will start to construct RNAi inducers targeting grapevine leafroll viruses and test for efficacies. This project will provide new important information and help with development of contemporary strategies and management approaches for the Grapevine leafroll disease and could be directly applicable against other important grapevine viruses such as Grapevine red blotch virus.

## INTRODUCTION

Grapevine leafroll disease (GLD) is one of the economically important diseases of wine grape (*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 is recognized as the most complex viral disease for wine grape production (Almeida et al., 2013, Naidu et al., 2014).

RNA interference (RNAi) is one of the key regulatory processes for gene expression and anti-viral defense in plants (Ding, 2010, Pelechano & Steinmetz, 2013). Since its discovery, RNAi technologies have been used for plant disease control targeting the plant hosts, plant pathogens and their insect vectors (Rosa et al., 2018).

In this funded research, we will our expertise in virology and RNA interference to assess new, effective approach(es) to target GLD. Plant viruses have been used to enhance the RNAi effects targeting a variety of plant pathogens (especially viruses) and insects (Khan et al., 2013, Wuriyangan & Falk, 2013, Tang et al., 2010, 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. We will focus on developing two recombinant viral vectors or plant virus replicons (GGVA- and GVA-based) for RNAi delivery that can be used in rootstocks and scions to target GLRaVs.

## OBJECTIVES

Objective 1: Establish robust grapevine viral vectors for RNAi delivery in grapevine plants

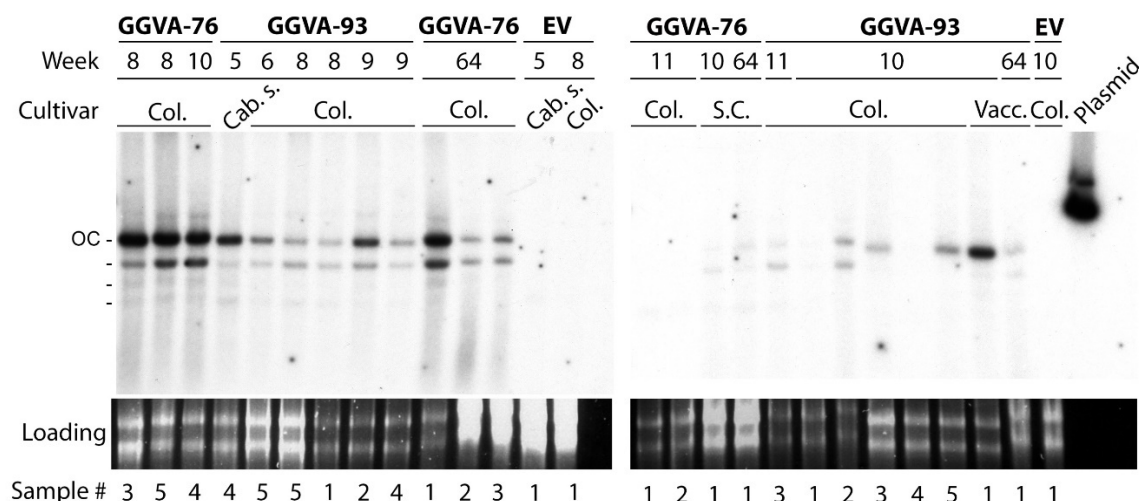
Objective 2: Clone additional RNAi inducers against GLRaVs

## RESULTS AND DISCUSSION

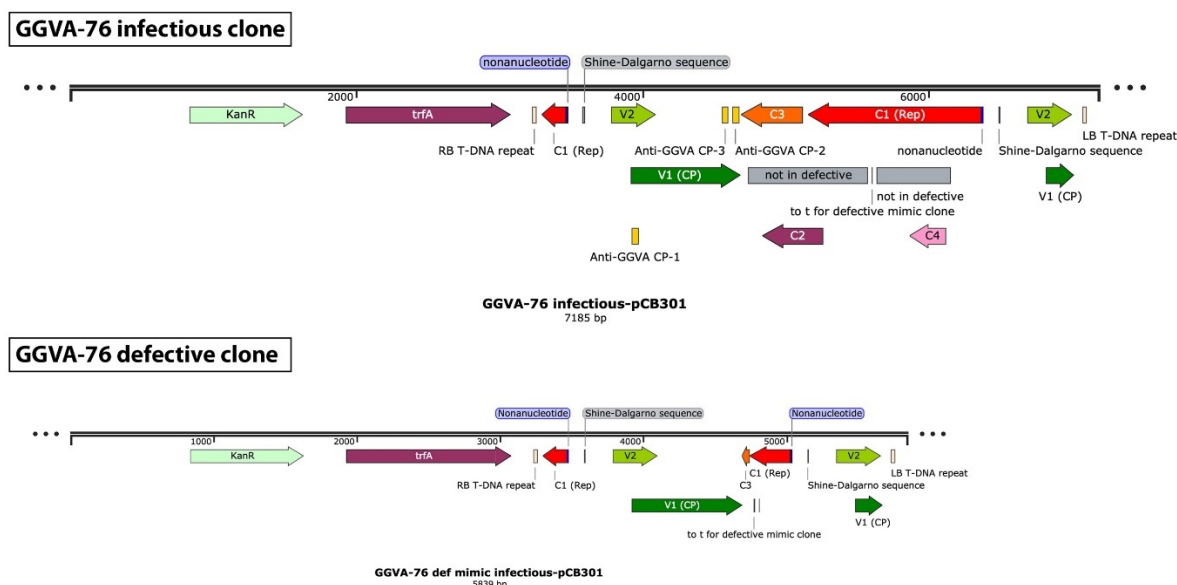
### **Objective 1: Establish robust grapevine viral vectors for RNAi delivery in grapevine plants**

Previously, we established a protocol for delivering viral infectious clones into greenhouse grown grapevine plants. We have successfully delivered viral infectious clones of 2 CA-GGVA isolates and a CA-GVA isolate into 4 different cultivars of full-grown grapevine plants and have detected the viral replication in the tested plants (Figure 1). The tested plants in our pilot assays have tested positive for the CA-GGVA viral replication of the tested clones for over a year, indicating that the infection of the cloned viruses is stable in the grapevine plants.

During our last funded project, we have constructed 2 GGVA multimeric (1.2X) clones of 2 isolates: GGVA-93 [isolate Super Hamburg - Japan (KX570610)] and GGVA-76 [isolate Longyan – China (KX570611)] and the defective GGVA multimeric (1.2X) clone (def-GGVA-93 and def-GGVA-76) (Figure 2). We cloned the multimeric clones of the GGVA-93, GGVA-76, def-GGVA-93, and def-GGVA-76 into pCB301, an *Agrobacterium* compatible binary vector (Figure 2). The clones were then transformed into *Agrobacterium* strain GV3101 for *Agrobacterium* vacuum infiltration. It was reported that the defective GGVA found in Japan, South Korea, and China, has merely ~1.5 k bases in its genome, and is capable of replicating and moving in the infected grapevine plants (Al Rwahnih et al., 2017).



**Figure 1.** Southern blot analyses of GGVA infiltrated grapevine plants. CA-GGVA-76, CA-GGVA-96, and pCB301 (empty vector, EV) were infiltrated into different cultivars of grapevine plants. Each clone was infiltrated into 5 plants of each cultivar. The sample number indicates the plant number of each treatment. The plants were tested at different time points for viral replication/accumulation. The plants tested at week 64 were the plants used in the pilot assays. 0.3 ng of plasmid was used as a control. Col.: colombar, Cab. s.: cabernet sauvignon, S.C.: salt creek, Vacc.: vaccarèse. OC: open circular form (Dry et al., 1997).



**Figure 2.** The constructs of GGVA-76 [isolate Longyan – China (KX570611)] wild-type (WT) and defective (def.) clones. Majority of C1 (Rep) and C3 were truncated in the defective GGVA found in South Korea. The defective clones were designed based on the previous found defected GGVA forms.

### GGVA caused symptoms in *Nicotiana benthamiana*

We tested the defective GGVA clones in both grapevine and *Nicotiana benthamiana* plants. The infiltrated/inoculated *N. benthamiana* plants started to show symptoms for both wild-type (WT) GGVA isolates at about 2 to 3 weeks post infiltration (Figure 3).

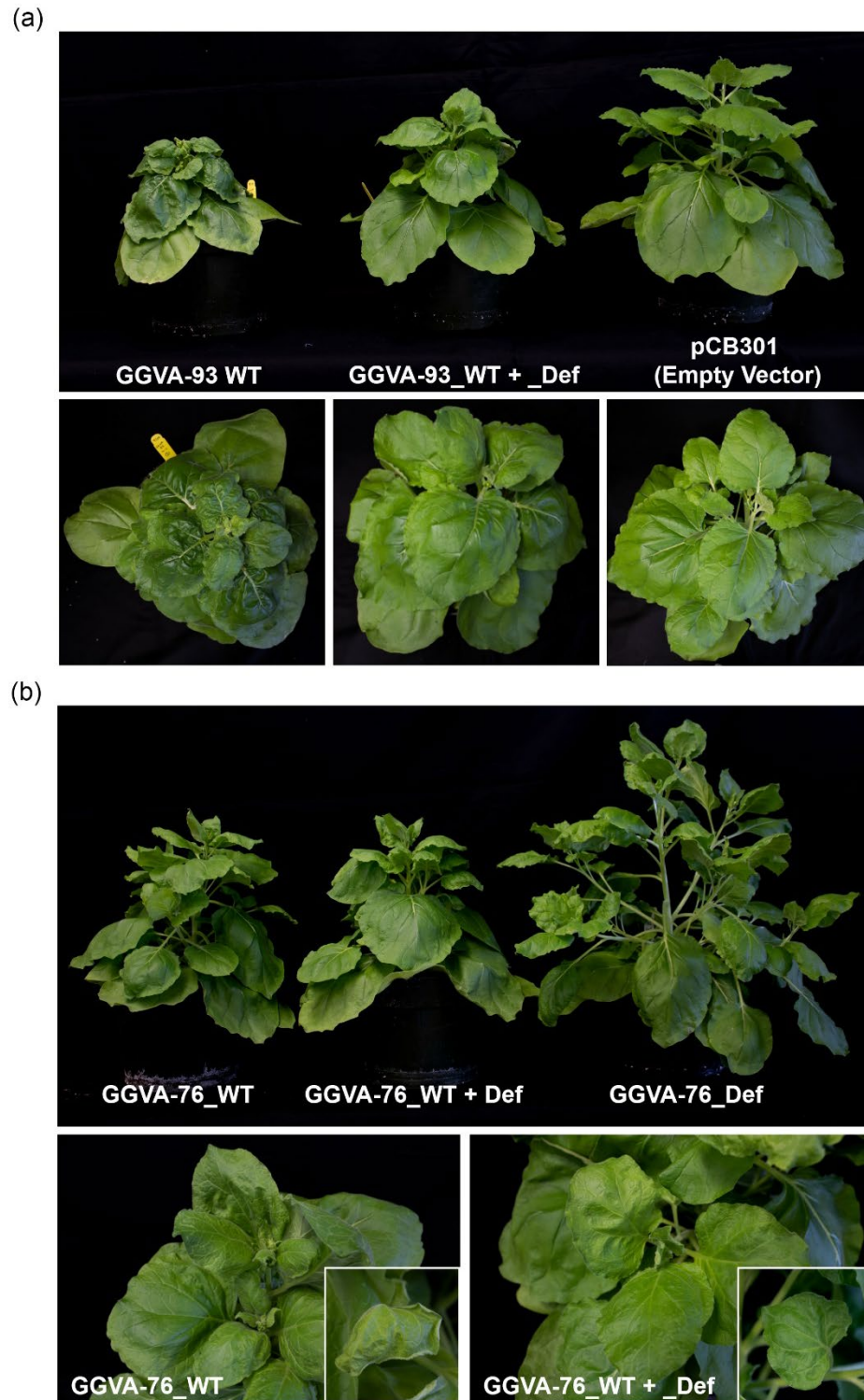
GGVA-93 WT showed much more severe symptoms compared to that of GGVA-76 in *N. benthamiana* plants 3 weeks post infiltration (wpi). Meanwhile, the GGVA-93 and -76 defective clones did not cause obvious symptoms in *N. benthamiana* at 3 wpi.

We then tested mix infection of GGVA WT and defective form and observed if the mix-infection could affect the symptoms in *N. benthamiana* plants. Our results showed that the plants co-infected with WT and defective form of GGVA showed milder symptoms compared to the symptoms caused by WT alone (Figure 4).

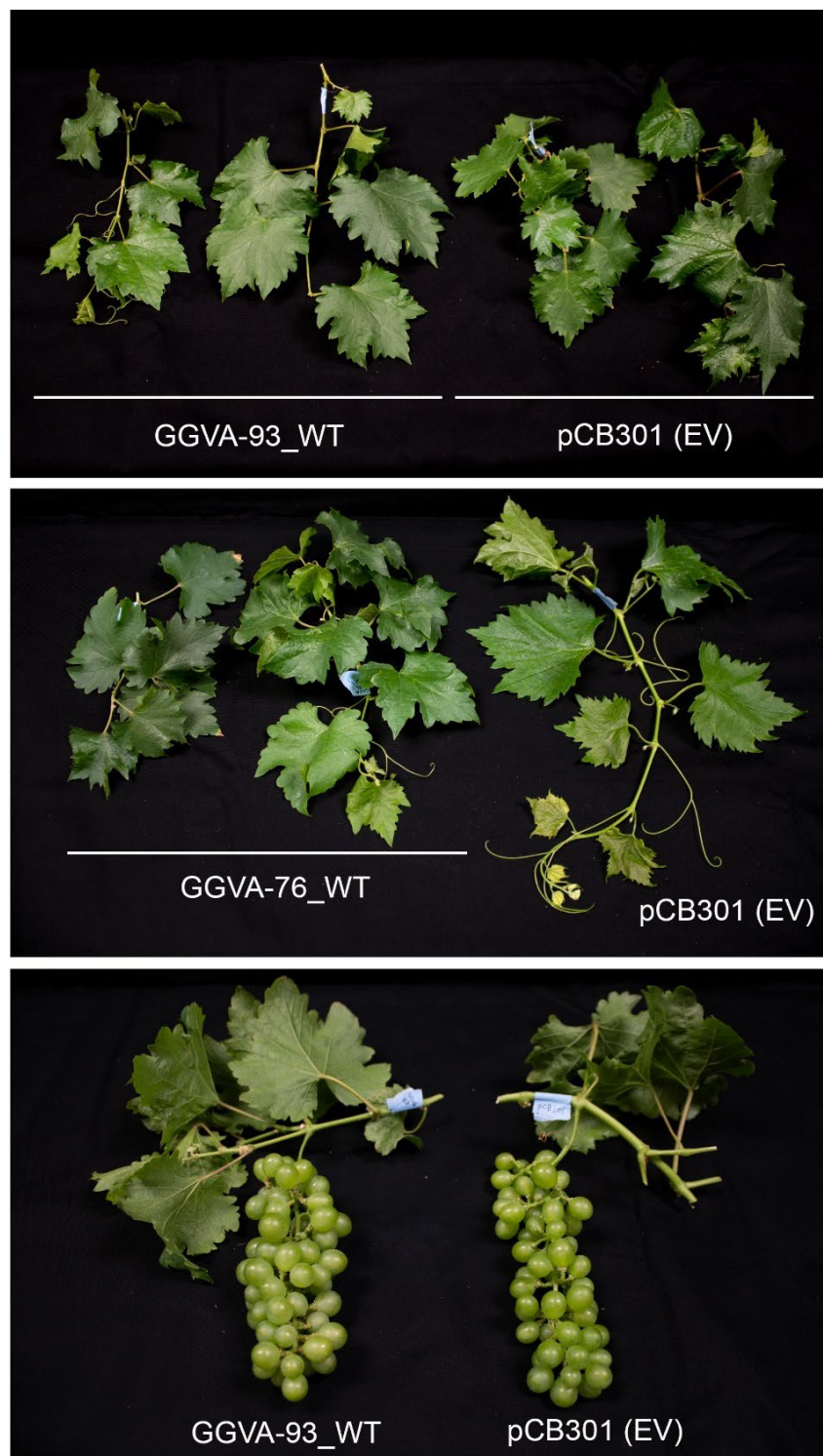


**Figure 3.** Symptoms caused by the 2 isolates of GGVA wild-type and defective form: GGVA-93 [isolate Super Hamburg - Japan (KX570610)] and GGVA-76 [isolate Longyan – China (KX570611)] in *N. benthamiana*. GGVA-93 caused severer symptoms in *N. benthamiana* compared to the GGVA-76. pCB301, a binary vector for *Agrobacterium*, was used as the vector for all the infectious clones. The empty vector (EV) of pCB301 was used as a negative control for the assays.





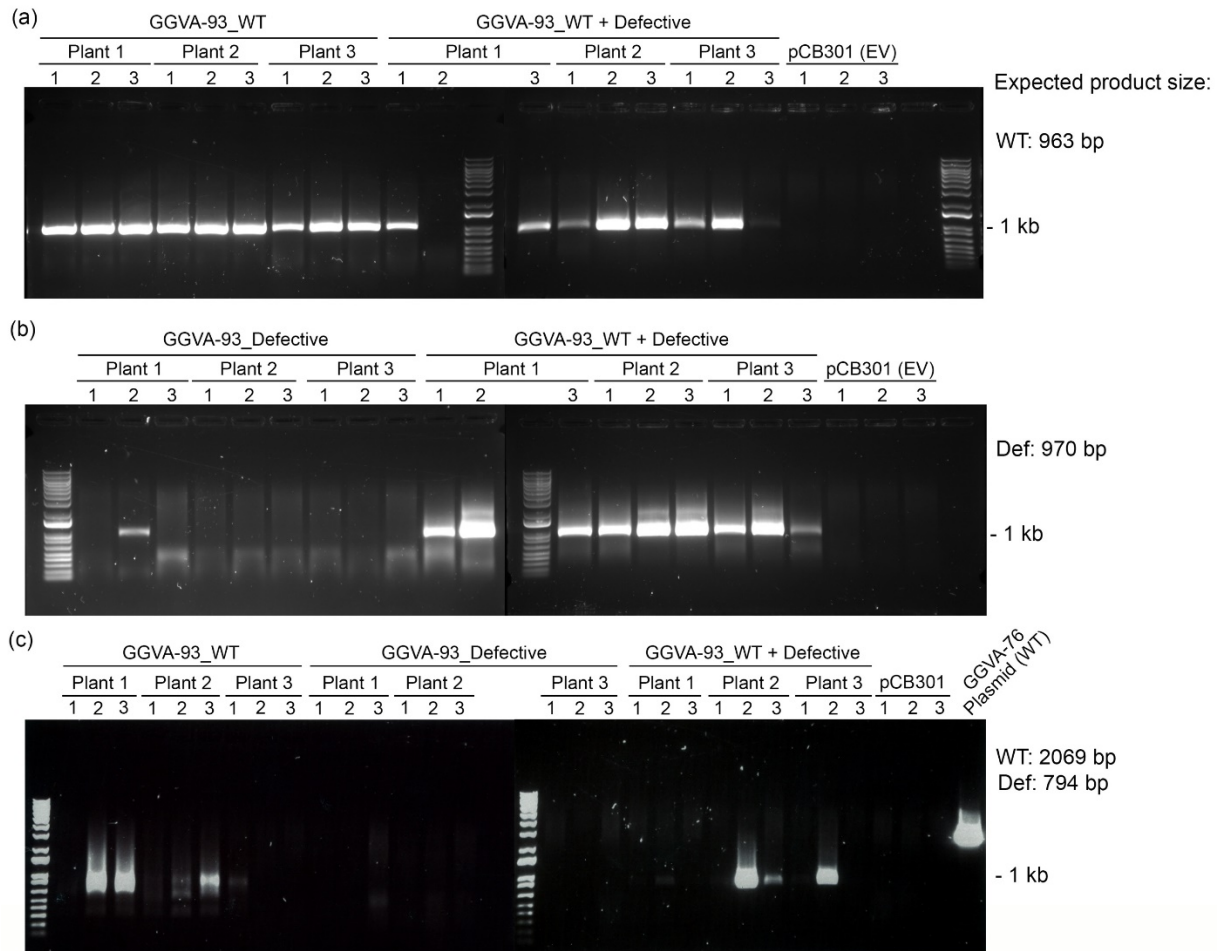
**Figure 4.** The defective form of both GGVA isolates decreased the severity of symptoms caused by the wild-type GGVA in *N. benthamiana* 3 weeks post infiltration.



**Figure 5.** The GGVA infection did not show symptoms in greenhouse-grown grapevine plants. The empty vector (EV) of pCB301 was used as a negative control for the assays.

### GGVA in grapevine plants

There are no obvious symptoms caused by GGVA infection in greenhouse-grown grapevine plants (Figure 5). This makes it an ideal viral vector to deliver RNAi in grapevine plants targeting grapevine leafroll-associated viruses or other diseases, such as grapevine red blotch disease. We also co-infiltrated the GGVA\_WT and GGVA\_Def into grapevine plants. Those grapevine plants will be tested and monitored in the following months.

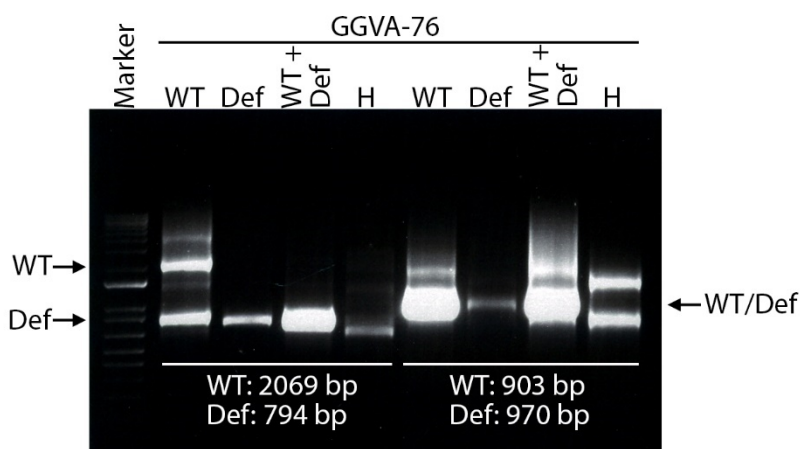


**Figure 6.** Detection of wild-type (WT) and defective (Def) form of GGVA in infiltrated *Nicotiana benthamiana* plants. (a) Primers that only amplify replicating WT GGVA were used for the PCR reactions. The expected product size: 963 bp. (b) Primers that amplify replicating Def GGVA were used for the PCR reactions. The expected product size: 970 bp. (c) Primers that can amplify both WT and Def sequences were used for PCR reaction. The expected product sizes: WT- 2069 bp, Def- 794 bp.

### Molecular analyses for the GGVA\_WT and \_Def in *N. benthamiana* and grapevine plants

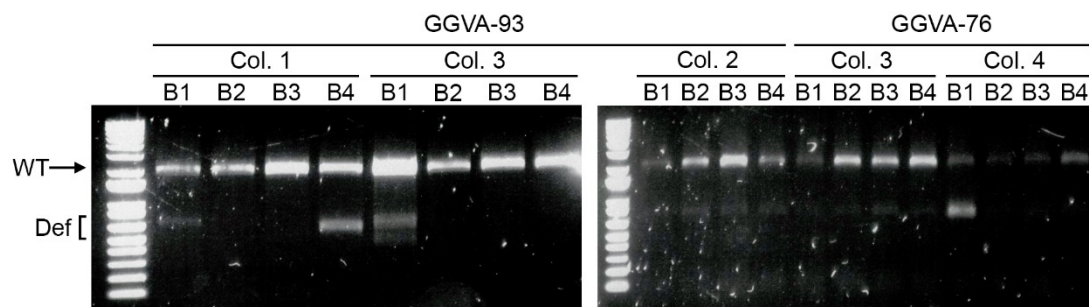
To understand how the GGVA defective form affects the wild-type GGVA, we analyzed the viral replication of WT and Def GGVA of both isolates in plants by using polymerase chain reaction (PCR) and Sanger sequencing (Figure 6). Our results showed that when infected with GGVA\_WT alone, the WT virus produced various defective forms of GGVA in both *N. benthamiana* and grapevine plants (Figure 6c). However, when the *N. benthamiana* plants were co-infected with GGVA\_WT and GGVA\_Def, the cloned defective form dominated and only one form of GGVA\_Def was detected (Figure 6b and c). The results were confirmed by Sanger sequencing.

Only one sample of the *N. benthamiana* plants, that were infiltrated with GGVA\_Def alone, was detected with the GGVA\_Def replication at 3 wpi (Figure 6c). To determine if this was caused by extremely low titer of the GGVA\_Def, we performed a crude viral purification of GGVA, modified based on a geminivirus virion purification protocol (Caciagli et al., 2009). One-third of the partially purified virion was used for total DNA extraction. The extracted DNA was then used for detections of GGVA\_WT and/or GGVA\_Def replication. Our results showed that weak GGVA\_Def replication was detected in the *N. benthamiana* plants that were infiltrated with GGVA\_Def alone, indicating that when plants were infected with GGVA defective form the replication efficiency was very low (Figure 7). When plants were infected with both WT and Def forms of GGVA, the WT virus facilitate the replication of the defective forms of GGVA. It was also noted that after the purification, the smaller variants of defected GGVA were not detected in the PCR reactions, suggesting that the smaller variants of defected GGVA may not be able to be encapsidated into viral particles as efficient as the larger defective forms.

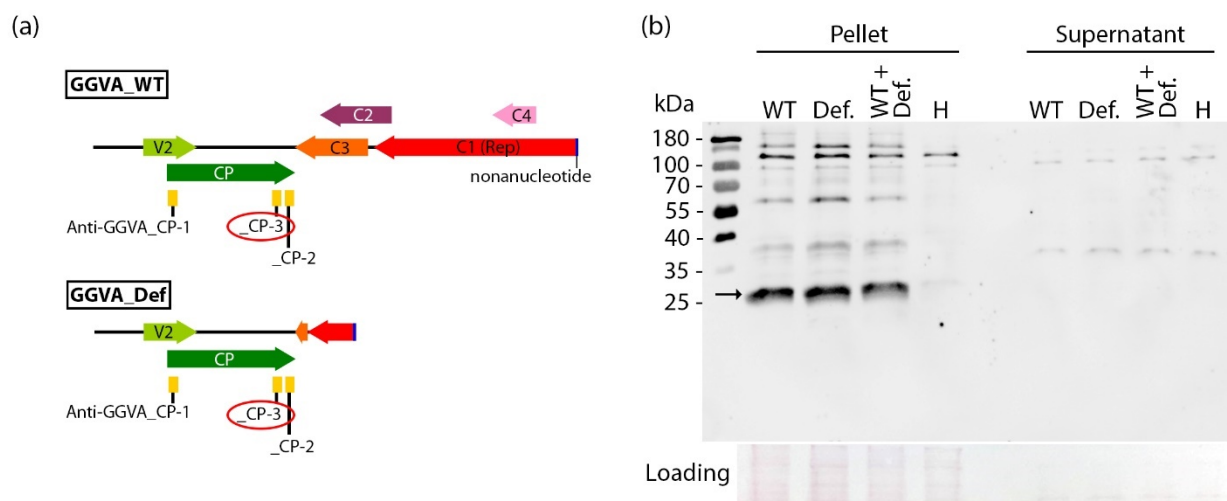


**Figure 7.** Defective GGVA replication was detected in the total DNA extracted from the crude purified viral particles. GGVA-76 infiltrated *Nicotiana benthamiana* plants were used crude purification of the GGVA-76 virions. 2 sets of primers were used to confirm the replication of the defective form of GGVA-76. The expected sizes of PCR products from the first PCR set (left) are – WT:2069 bp and/or Def: 794 bp; from the second set are – WT: 903 bp and/or Def: 970 bp.





**Figure 8.** Both wild type and defective GGVA were detected in grapevine plants infiltrated with wild-type GGVA. Col.: cultivar colombard; B: branch.



**Figure 9.** Antibodies produced with the GGVA coat protein (CP) peptides can be used to detect GGVA CP in the GGVA infected plants with Western blot analyses. (a) Schematic diagram of GGVA\_WT and GGVA\_Def that indicates the peptide regions used to produce GGVA CP antibodies. (b) Western blot analyses. The samples were crude purified for GGVA virions. Both pellets and supernatant fractions from the ultracentrifuge step (last step) were collected for all the samples for analyses. WT: wild type; Def: defective form; WT + Def: co-infiltration of WT and Def; H: healthy control. The membrane was stained with ponceau S for loading controls.

Similar to the results of tests in *N. benthamiana*, grapevine plants infiltrated with the wild-type GGVA of both isolates also showed defective GGVA forms derived from the wild-type virus in some samples (Figure 8).

We also synthesized peptides of different regions of GGVA\_WT coat protein (CP) (Figure 9a) for producing antibodies that can detect GGVA infection in plants. We tested the GGVA infected *N. benthamiana* plants with one of the antibodies (produced with CP-3 peptide, Figure 9a) by Western blot analyses. Due to the low titer of the GGVA infections, we used fractions of the crude viral purification of GGVA for the Western blot analyses (Figure 9b). The results showed that the antibodies were able to detect the GGVA CP at the estimated protein size of ~26-28 kDa in the GGVA\_WT, or GGVA\_Def, or GGVA\_WT and GGVA\_Def (co-infiltrated) infected plants but not in the negative (healthy) controls. The other larger sizes of bands that appeared in the GGVA infected samples but not in the healthy control could be the multimeric forms of CP and/or post-translational modified CP (Figure 9b).

The studies done during this report period showed that we can modify the defective form of GGVA to carry RNAi inducers targeting GLRaVs and co-infect the plants with the wild-type virus for efficient expression without causing serious symptoms in the grapevine plants.

### **Objective 2: Clone additional RNAi inducers against GLRaVs**

The proposed target sequences of GLRaVs in the previous proposal were the POL (RNA dependent RNA polymerase, RdRP) and HSP70h gene sequences (Figure 9, (Maree et al., 2013)). We chose the regions within the 2 genes that are more conserved among different isolates of GLRaVs or different GLRaVs. However, the untranslated regions, such as the 3'-UTR, and other genes could also be used as targets for potential stronger efficacy. The 3'-UTR of the GLRaVs could be a strong target, for instance, because all the subgenomic viral RNAs contain the same 3'-UTR sequence. Additionally, Dr. Maher Al Rwahnih and colleagues reported a small highly conserved region in the 3'-UTR of the virus genome (Diaz-Lara et al., 2018). We will align genome sequences of different GLRaVs to find conserved regions among the viruses in ORFs or non-coding regions and will clone more ideal RNAi inducers to target different regions of the GLRaV sequences to enhance silencing effects and offer stronger protections against the virus infection(s) in the following report periods.

### **CONCLUSIONS**

During this report period, we confirmed that our clones of the defective GGVA-76 and GGVA-93 were infectious. Wild-type GGVA not only can trigger the production of defective GGVA in various forms, but also facilitated the replication of the defective form of the virus. Those results showed that we can construct RNAi inducers into the defective GGVA and co-infect grapevine plants with the defective GGVA and wild-type GGVA so the wild-type GGVA will enhance the efficacy of RNAi effects. The results obtained during this report period helped us build a plan/protocol moving forward of using this virus a carrier for RNAi delivery. We also observed no obvious symptoms that were caused by the GGVA infections in greenhouse grown grapevine plants. This further confirmed that GGVA is an ideal viral vector for RNAi delivery into grapevine plants. We will start cloning markers or reporter genes/tags into our infectious defective GGVA clones and co-infiltrate with the wild-type virus into grapevine plants. We will confirm which insertion sites will have the best expression of the markers or genes. After

identifying the best insertion sites in our infectious clones, we will start to construct RNAi inducers targeting grapevine leafroll viruses and test for efficacies. This project will provide new important information and help with development of contemporary strategies and management approaches for the Grapevine leafroll disease and could be directly applicable against other important grapevine viruses such as grapevine red blotch virus.

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## IDENTIFICATION OF GRAPE CULTIVARS AND ROOTSTOCKS WITH RESISTANCE TO VINE MEALYBUG

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**Reporting Period:** The results reported here are from work conducted July 2020 to June 2021.

### LAYPERSON SUMMARY

Vine mealybug is a major pest to the California grape industry. 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. Select rootstocks and scion cultivars were evaluated under a high mealybug pressure environment to determine how robust the resistance was. 10-17A and IAC572 had higher plant health, but high numbers of mealybugs in the greenhouse suggesting that the resistance will not hold up under extremely high mealybug pressure. Sap quality differences were found between partially resistant rootstocks and other grape vines, which could be a potential target for both management and breeding research. Rootstocks IAC 572, RS-3 and 10-17A have varying levels of resistance under high, medium and low mealybug pressure and can be used for breeding and mapping resistance to vine mealybug in a new CDFA project.

### INTRODUCTION

Mealybugs damage leaves and fruit in grape, and can transmit the economically important Grapevine Leaf Roll Associated Virus (GLRaV). GLR disease control can cost growers \$12,106 to \$91,623 per acre annually in California (Ricketts et al., 2015). Mealybug specific 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.

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 (*Dysmicoccus brevipes* and *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. Our previous work suggested that RS-3 and IAC572 both had partial resistance to vine mealybug under low to moderate mealybug pressure.

## OBJECTIVES

Objective 1: Evaluate grape materials with identified resistance to vine mealybug.

## RESULTS AND DISCUSSION

### Objective 1. Evaluate Grape Materials with Identified Resistance to Vine Mealybug

A previously established vine mealybug colony was used as the source for all insect inoculations. Table grape, wine grape, and rootstocks cultivars were propagated from dormant cuttings for use in a replicated greenhouse trial at UC Kearney (Table 1). RS-3 was not included in the study due to poor rooting.

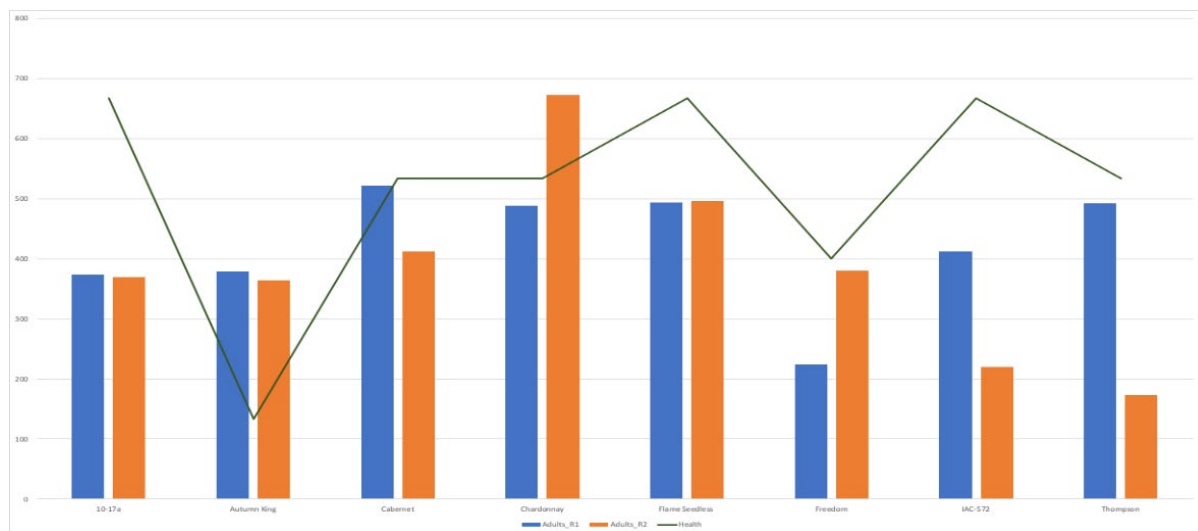
Approximately 20 plants were evaluated for each line. The experiment was repeated once for a total of two times. Vines were evaluated for mealybugs bi-weekly and counted for the total number of juveniles, adult females and egg sacs visible in 1 min. Mealybug density was also related to plant health using a 1 to 5 scale with 5 being healthy and 1 being dead (Fig 1 and 2). Mealybug population growth was calculated using an Area under the insect growth curve (AIGC) based on the AUDPC model by Shaner and Finney (1977). Average AIGC was calculated per line using SAS statistical analysis software.

Moderate mealybug numbers and higher plant health were noted for cultivars 10-17A and IAC572, which were expected to have partial resistance. Because mealybugs were able to move among the vines, it was unclear if the mealybugs on the vines were causing feeding damage. Based on the higher plant health value, despite the presence of mealybugs, it would seem that there was reduced feeding by the insects on these cultivars or they were more tolerant to the insect feeding. Differences in insect severity were observed between the two replicates of the trial suggested vine phenology stage and environment may play a role in the partial resistance. Freedom rootstock, which had high plant health in the first experiment performed poorly in the second in contrast to IAC 572. Mealybug numbers did not vary drastically among the cultivars, but large differences in ovisacs, feeding damage and overall plant health were observed. Mealybug pressure was extremely high in this study, with no sprays or natural predators to reduce insect populations.

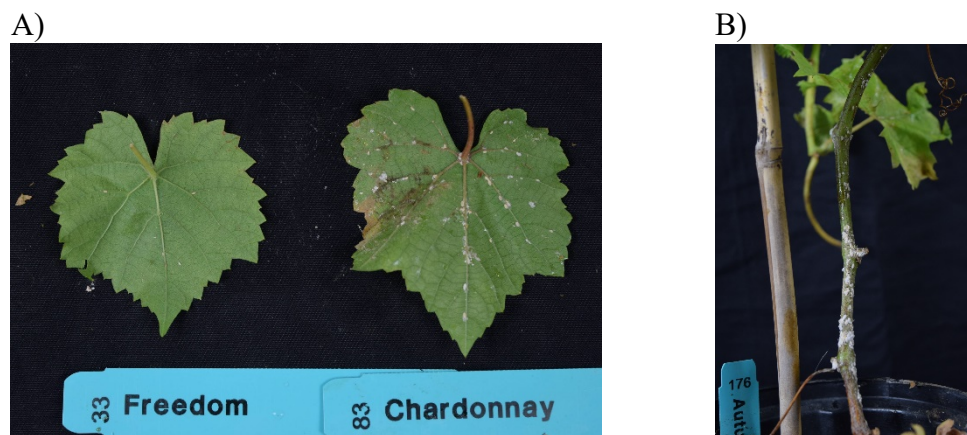
These results differed somewhere from evaluations in the prior CDFA-funded mealybug resistance project where cultivars were evaluated in outdoor pots with exposure to ants and predators, though we tried to minimize predator effects using screen bags to protect mealybugs, and pots were spaced to minimize mealybug spread via walking or wind to nearby pots (Fig 3.)

**Table 1.** Cultivars and species evaluated for mealybug resistance.

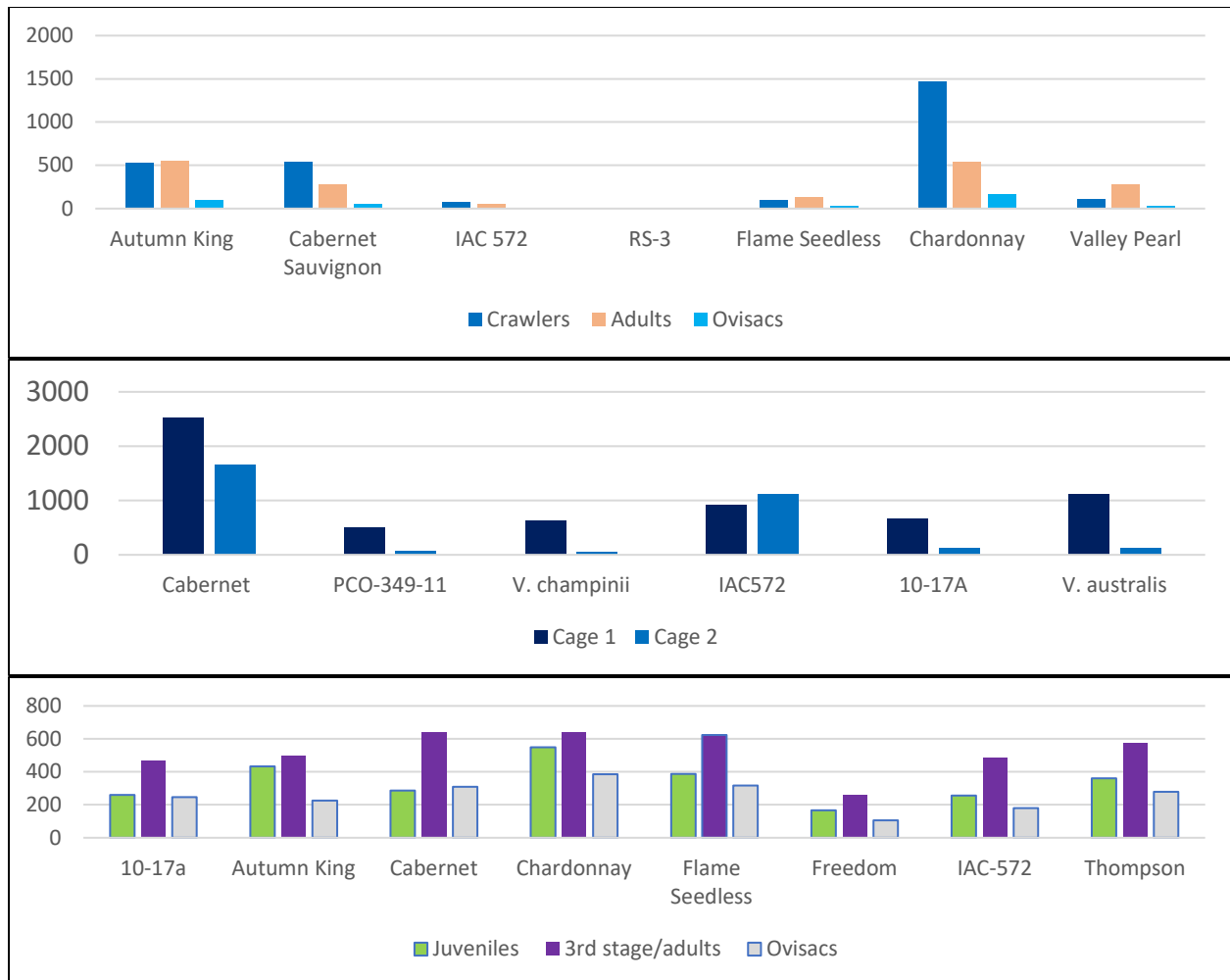
Cultivar	Species	Features
Flame Seedless	<i>V. vinifera</i>	Table grape
Cabernet Sauvignon	<i>V. vinifera</i>	Wine grape
IAC 572	<i>Interspecific hybrid</i>	Rootstock with partial resistance
10-17A	<i>Interspecific hybrid</i>	Rootstock with partial resistance
Autumn King	<i>V. vinifera</i>	Table grape
Chardonnay	<i>V. vinifera</i>	Wine grape
Thompson Seedless	<i>V. vinifera</i>	Table grape
Freedom	<i>Interspecific hybrid</i>	Common rootstock



**Figure 1.** Mealybug density on different grape cultivars in the first (blue) and second (orange) round of the greenhouse experiment. Plant health 1-5 (1 being dead and 5 being healthy) is denoted by the green line and the numbers on the right axis.

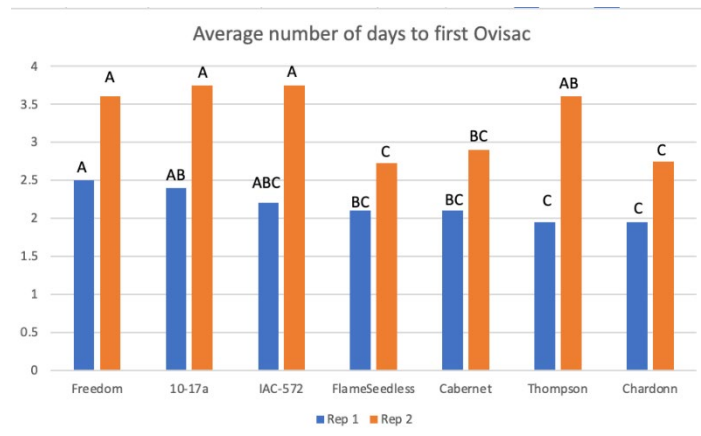


**Figure 2.** A) The underside of two grape cultivars (Freedom and Chardonnay) showing differences in mealybug density and ovisacs. B) Mealybugs and ovisacs along the stem of an Autumn King vine.

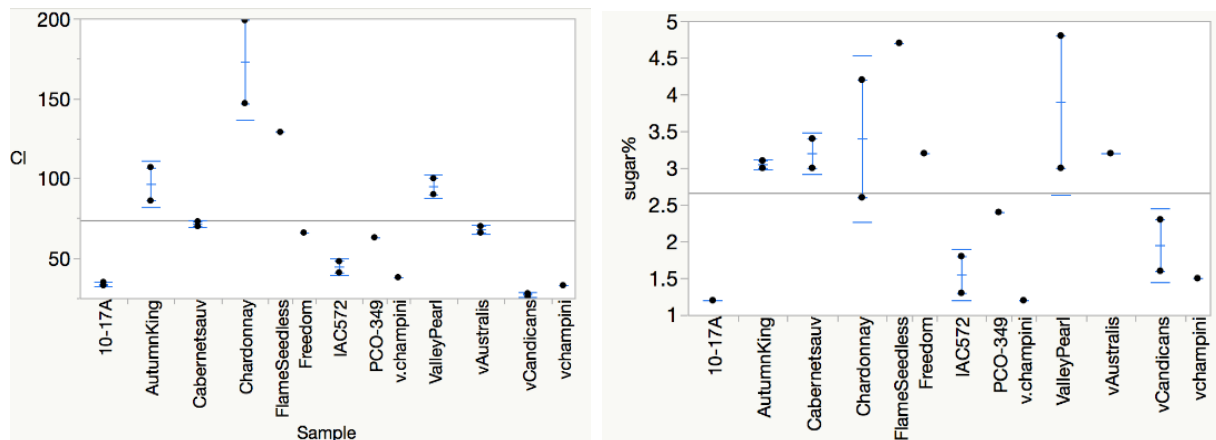


**Figure 3.** Mealybug growth for over time in 3 experiments representing 3 different environments and mealybug densities: Outdoor cages with low density (top box), Outdoor mesh bags with moderate density (middle box) and Greenhouse mesh bags with high population density (bottom box). For the greenhouse mesh screen bag experiment, variability among life stages was also observed.

When speed of mealybug development was compared among lines, differences were observed among cultivars in speed to first ovisac development. Mealybug development (i.e. time to first ovisac) was faster on rootstocks compared to more sensitive cultivars (Fig 4). This was unexpected, as we had initially predicted that mealybugs would develop slower on less favorable vines. It is possible that ovisacs are being developed, but are not viable or have low numbers of offspring, which could partially explain the higher plant health ratings on these rootstocks compared to commercial cultivars like Cabernet, Chardonnay and Flame Seedless.



**Figure 4.** Average number of days to first observed ovisac among select grape vines evaluated in a replicated greenhouse experiment (rep1 and rep 2). Letters that are different across a color indicate significant differences.



**Figure 5.** Chloride content (left) and percent sugar (right) of select grape cultivars from field grown vines based on sap quality from old leaves.

Preliminary assessment of sap quality differences among resistant and susceptible cultivars previously identified were performed. Leaves from unreplicated field grown vines were collected in spring and summer and analyzed for sap quality. Most nutrients observed were within similar ranges between the rootstock and scions evaluated. Only magnesium, potassium and chloride showed consistent variability among known susceptible and resistant cultivars (Fig 5.)

Several presentations were provided to grower groups including the sustainable ag expo (2019) and two San Joaquin Valley Table grape growers meetings (2020). The following publication was also produced:

Naegele R.P., Cousins P., Daane K.D. (2020) Identification of *Vitis* cultivars, rootstocks, and species expressing resistance to a *Planococcus* mealybug. Insects. 11:86

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## FUNDING AGENCIES

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

## EVALUATION OF VITIS ROOTSTOCKS WITH PARTIAL RESISTANCE TO VINE MEALYBUG

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**Cooperator:** Monica L. Cooper | Cooperative Extension | University of California | Napa, CA 94559 | [mlycooper@ucanr.edu](mailto:mlycooper@ucanr.edu)

**Cooperator:** Kent Daane | Department of Environmental Science, Policy, and Management | University of California | Berkeley, CA 94720 | [kdaane@ucanr.edu](mailto:kdaane@ucanr.edu)

**Reporting Period:** The results reported here are from work conducted July 2020 to June 2021.

Significant delays in this project occurred due to COVID-19 restrictions and hiring delays. Objective 1 was halted completely after Dr. Naegele took another position with the USDA ARS in Michigan on sugarbeet and no suitable persons could be identified to finish the project.

This work was designed to prepare for field evaluations and breeding of mealybug resistance. Breeding populations for analyzing genetic components of mealybug resistance developed are planted in the field and ready for continued studies by the new grape breeder, if desired.

### LAYPERSON SUMMARY

Vine mealybug is a major pest to the California grape industry. 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. Understanding how these rootstocks perform in the field under natural and artificial mealybug pressure, and identifying the heritability and genetics behind resistance are the next steps for developing mealybug resistant grapes. This proposal was designed to lay the groundwork for this.

### INTRODUCTION

Mealybugs damage leaves and fruit in grape, and can transmit the economically important Grapevine Leaf Roll Associated Virus (GLRaV). GLR disease control can cost growers \$12,106 to \$91,623 per acre annually in California (Ricketts et al., 2015). Mealybug specific 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.

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 (*Dysmicoccus brevipes* and *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. Our previous work suggested that RS-3 and IAC572 both had partial resistance to vine mealybug under low to moderate mealybug pressure.

## OBJECTIVES

Objective 1: Set up vineyards testing Vitis rootstocks with partial resistance to vine mealybug.

Objective 2: Breed Vitis rootstocks with improved resistance to vine mealybug.

## RESULTS AND DISCUSSION

### **Objective 1: Set up vineyards testing Vitis rootstocks with partial resistance to vine mealybug.**

Grower cooperators for on-site trials were identified and discussions started on logistics for planting. This vineyard was to determine the performance of partially resistant rootstocks in the field under natural conditions and mealybug pressure. To have a high mealybug pressure vineyard, a second vineyard was to be set up at the USDA ARS research station in Parlier, CA. A site was selected and irrigation updated to support vine health, materials were purchased for setting up the field in 2020. This objective was delayed due to Covid and then halted entirely once Dr. Naegle agreed to move to Michigan.

### **Objective 2: Breed Vitis rootstocks with improved resistance to vine mealybug.**

Methods for detached leaf assays were tested in the greenhouse using Cabernet cuttings grown in the lab and field grown vines to test rooting and survival of hydroponically grown leaves. It was determined that field leaves could be used for hydroponic detached leaf assays. Survivability of hydroponic leaves ranged from 50-80% depending on the grape genotype used. It was determined that leaves should be collected earlier in the year to maximize survival, however this was postponed due to COVID restrictions.

Crosses for mealybug resistance were planted in the field and maintained (pruned and managed for disease) on site at the SJVASC. Additional crosses were made for select crosses (RS-3 primarily) that had low seed set the previous year to ensure sufficient numbers for genetic mapping and selection.

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## FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board. Money unspent because the project was not completed was returned to the CDFA.

## INVESTIGATING THE IMPACT OF GRAPEVINE RED BLOTCH VIRUS (GRBV) ON GRAPE SKIN CELL WALL METABOLISM AND SOLUBLE PATHOGENESIS-RELATED PROTEINS IN RELATION TO PHENOLIC EXTRACTABILITY.

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**Reporting Period:** The results reported here are from work conducted July 2021 to October 2021.

### ABSTRACT

Red blotch (RB) disease is a recently identified disease caused by the grapevine red blotch virus (GRBV). Prior to our research little was known about the impact of RB disease on grape and wine composition. Results indicate mainly a significant impact on berry ripening in all varieties studied, with variable impacts on primary and secondary metabolites depending on site and season which had a larger impact than variety. In ripening grapes, factors such as cell wall composition, cell integrity, individual phenolic concentrations, and interactions with each other influence phenolic extractability under winemaking conditions. Pectolytic enzyme degradation of skin cell walls during grape ripening is documented to increase the extractability of anthocyanins. However, there is little known about the impact of GRBV on cell wall composition and structure. Our ongoing work indicates that RB disease increases the amount of pectin (quantified as uronic acid), which is known to bind to tannins during winemaking. Further analysis is needed to understand the degree of methylation or acylation of pectin, potential cross-linkages with potassium and calcium, and pectolytic enzyme activity. We also observed increased levels of soluble proteins in GRBV grape skin cell walls. These proteins may be pathogenesis-related (PR) proteins, which researchers have yet to investigate in GRBV infected grapes. PR proteins are known to accumulate in grapes as a result of bacterial, fungal, and viral infection and lead to the binding of phenolic compounds such as tannins. The current work needs to be expanded to determine how GRBV alters the composition and linkages of the cell wall, PR protein levels, and cell wall modifying enzyme activity. Answers to these questions will increase our understanding of plant-virus interactions and potential mitigation strategies to alleviate the impact of GRBV on grape composition and wine quality. In addition, due to the large seasonal variability that has been observed in RB disease expression in previous studies, investigation of multiple seasons will ensure robust conclusions.

### LAYPERSON SUMMARY

Research in the Oberholster group indicate that grapevine red blotch virus (GRBV) causes a delay in ripening events in grapes, leading to significant decreases in sugar accumulation, color development, and aroma compound accumulation. In addition, our work has indicated that GRBV affects key metabolic pathways that are responsible for the production of compounds

(phenolics) that are important to the color, flavor, and mouthfeel of a final wine. However, there is little known about the impact of GRBV on cell wall composition and structure. It is known that during ripening the grape cell wall changes in composition and integrity, which impacts phenolic extractability during winemaking. In addition, it has been shown that pathogens such as fungi, bacteria, and viruses alter cell wall modifications. Consequently, these changes in the grape cell wall can directly impact the extractability and final concentrations of phenolics in wines. Our current research indicates that grapes infected with GRBV have significantly higher quantities of pectin (acidic heteropolysaccharide groups located in the cell wall) and soluble proteins. Previous studies have shown that higher levels of pectin and soluble pathogenesis-related (PR) proteins result in binding reactions to phenolic compounds such as tannin. Therefore, studying the impact of GRBV on PR proteins, cell wall composition, and cell wall enzymatic processes will aid in understanding plant-virus interactions and potential mitigation strategies to alleviate the impact of GRBV on grape composition and wine quality.

## INTRODUCTION

Grapevine red blotch virus (GRBV), the causative agent of red blotch (RB) disease, has been prevalent in the United States since its identification in 2012<sup>21</sup>. GRBV, a member of the *Geminiviridae* family, is comprised of a circular, single stranded DNA molecule<sup>21</sup>. Reports indicate that the virus is mainly spread through propagation material, but recently *Spissistilus festinus*, a three-cornered alfalfa hopper, was identified as a potential vector<sup>22</sup>. The economic impact of the disease can range from \$2,213/ha to \$68,548/ha in the United States, with rouging or vineyard replacement currently being the most used option by grape growers to control the disease<sup>23</sup>. Studies indicate that GRBV causes a delay in ripening events in grapes, leading to significant decreases in total soluble solids (TSS) levels and anthocyanin concentrations, with higher amounts of titratable acidity (TA)<sup>1,3,4,16</sup>. These alterations are translated into the resulting wines, making wines with less fruit aromas, color, and mouthfeel<sup>6</sup>. Blanco-Ulate et al.<sup>4</sup> demonstrated that GRBV compromises the regulation of ripening in grapes by interfering with transcriptional factors and hormone networks. One key metabolic pathway affected was the phenylpropanoid pathway, which is responsible for flavonoid and anthocyanin biosynthesis. Recent research suggests that the inhibition of translocation of carbon (hexoses) from leaves to the grapes results in the impairment of ripening in GRBV infected grapes, instead of decreases in carbon assimilation<sup>3</sup>.

However, there is little known about the impact of GRBV on cell wall composition and structure. There is limited research on overall plant-virus interactions in regard to fruit skin cell wall metabolism, even though the cell wall plays a crucial role in initiation of virus spread as well as a defense mechanism<sup>19</sup>. It is well known that grape cell walls are made up of cellulose, hemicellulose, lignin, and pectin, that intertwine proteins and polyphenols<sup>17</sup>. One of the important phenomena that occur during grape softening is the degradation and solubilization of the grape cell wall. Research indicates there are significant decreases of type I arabinogalactan, galactose, pectin methylation and acylation, as well as increases in the solubility of galacturonan<sup>17,24</sup>. Abiotic factors alter cell wall modifications by impeding methylesterification of cell wall pectins, increasing cell wall thickening (increasing lignin and cellulose), and increasing cell wall derived proteins, whereas biotic factors have shown to produce enzymes that degrade cell wall polysaccharides<sup>19,20,25</sup>. Consequently, these changes in the grape cell wall can

directly impact the extractability and final concentrations of phenolics in wines. Studying the impact of GRBV on cell wall composition will aid in understanding plant-virus interactions.

To determine the cause of grape cell wall modifications during ripening, Nunan et al.<sup>24</sup> performed a comprehensive study analyzing the expression patterns of main cell wall modifying enzymes during grape ripening. It was observed that the hydrolysis of cell wall galactan may be caused by the upregulation of  $\beta$ -galactosidase. Whereas, the solubilization of pectin polysaccharides may be due to alterations in pectin methylesterase (PME), pectate lyase (PL), and polygalacturonases (PGs)<sup>24</sup>. Through transcriptomic studies involving GRBV infected grapes, Blanco-Ulate et al.<sup>4</sup> found an upregulation of invertase/ (PME) inhibitors at late stages of berry ripening. These enzymes are known to impede the dimethylesterification of cell wall pectins in early berry ripening to control berry enlargement and softening. However, currently no study has investigated the impact of GRBV on cell wall enzyme activities. Further insight on overall cell wall metabolism in GRBV infected grapes is needed to fully understand the impact on phenolic extractability.

A previously research project assessed general differences in skin cell wall composition of grapes that were infected with GRBV and healthy grapes at two harvest points (25 and 27 Brix). The aim was to determine whether RB disease does have an impact on grape cell walls and ascertain the structural groups that need to be investigated further. In depth analysis of the monosaccharides composition of the cell walls was outside of the scope of this project and findings concluded that a deeper examination of the composition and structure of the cell wall was indeed needed to fill gaps in the current knowledge. The current project aims to develop a complete picture of the impact of GRBV on the grape skin cell wall by analyzing specific monosaccharide concentrations and their linkages and then relate this to enzymatic control in the cell wall. In addition, the potential impact of GRBV infection on PR proteins have not been investigated. Both PR proteins and cell wall metabolism play a crucial role in phenolic extractability during winemaking.

For this study, *Vitis vinifera* L. Merlot (clone 12, grafted on 1103P rootstock) will be used from Paso Robles, CA for two seasons (2021 and 2022). Around 30% of grapevines in this block have been tested since 2014, for leafroll virus (GLRaV-1, -3, and -4), rupestris stem pitting-associated virus, and the presence and absence of GRBV. For this study, approximately 60 vines will be tested via qPCR techniques for the presence (RB(+)) or absence (RB(-)) of GRBV. For RB(-) and RB(+), 25 vines each will be selected for grape sampling and analysis of cell wall composition, phenolic content, PR protein content, and enzymatic activity assays. The 25 vines for both RB(-) and RB(+) will be randomly separated into five groups of five vines and treated as biological replicates. Individual berries from each vine will be collected at four phenological stages based on the modified Eichhorn–Lorenz (E-L) system<sup>26</sup>: (i) pre-véraison (E-L 34); (ii) véraison (E-L 35); (iii) postvéraison (E-L 37); and (iv) harvest (E-L 38). All analyses will be performed in triplicate for each of the five biological replicates for each RB(+) and RB(-) treatments (n=15). This results in a total of 120 samples for each analysis in each year analyzed.

## OBJECTIVES

The main objectives of this project are the following:

- 1) Investigate the impact of GRBV on cell wall composition through ripening and at harvest.
- 2) Evaluate the effect of GRBV on cell wall enzyme activity through grape ripening.
- 3) Determine the potential production of pathogenesis-related proteins in grapes infected with GRBV.
- 4) Relate grape cell wall metabolism and pathogenesis-related protein concentrations to GRBV impact on final wine composition through phenolic extractability.
- 5) Develop mitigation guidelines based on findings.

## RESULTS AND DISCUSSION

### **Objective 1. Investigate the impact of GRBV on cell wall composition through ripening and at harvest.**

Funding was only received in October 2021, with the result that a PhD student has not yet been identified to work on this project. Drs Oberholster and Medina Plaza did collect samples for qPCR virus testing from the identified Paso Robles vineyard site (*Vitis vinifera* L. Merlot, clone 12, grafted on 1103P rootstock). Virus testing was performed by Dr. Sudarshana's laboratory. Additionally, grape samples were collected from identified GRBV positive and negative vines at four phenological stages. The next step is to recruit a student to start analysis of the collected samples as soon as possible. This will entail isolation of cell wall material and characterization thereof.

### **Objective 2. Evaluate the effect of GRBV on cell wall enzyme activity through grape ripening.**

Objective 2 will start in 2022.

### **Objective 3. Determine the potential production of pathogenesis-related proteins in grapes infected with GRBV.**

Objective 2 will start in 2022.

### **Objective 4. Relate grape cell wall metabolism and pathogenesis-related protein concentrations to GRBV impact on final wine composition through phenolic extractability.**

Objective 2 will start in 2022.

### **Objective 5. Develop mitigation guidelines based on findings.**

Dissemination of results will mostly take place in 2024 after multivariate statistical analysis of data across both seasons.

## CONCLUSION

As the research project just started there currently are no research conclusions. Next steps are to recruit a PhD student and start method optimization and analysis of collected samples.

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## **EFFECTS OF GRAPEVINE RED BLOTCH DISEASE ON FLAVOR AND FLAVOR PRECURSOR FORMATION IN THE GRAPE AND ON WINE QUALITY**

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**Reporting Period:** The results reported here are from work conducted July 2017 to July 2021.

### **LAYPERSON SUMMARY**

Two irrigation treatment main plots are randomized in two blocks of fields and characterized by varying water application rates (wet and dry) on both red blotch infected (RB+) and non-infected (RB-) Pinot noir grapevines. A wet treatment was irrigated at 100% estimated crop evapotranspiration (ETc) and dry treatment was irrigated at 66% ETc. The impact of red blotch disease on grape and wine quality was studied across three years. Pinot noir grapes were collected during berry ripening till the harvest (one week after harvest in 2019 and 2020) from RB+ and RB- grapevines. Wines were made from Pinot noir grapes with treatments included D+ (dry treatment on RB+ grapevines), D- (dry treatment on RB- grapevines), W+ (wet treatment on RB+ grapevines), and W- (wet treatment on RB- grapevines) conducted through 2018 to 2020. Berry maturity parameters, berry free and bound form C<sub>13</sub>-norisoprenoids, wine anthocyanins, phenolics, and flavor profiles were investigated. The results indicated that red blotch infected grapes had a lower level of total soluble solids at harvest. Wines made from red blotch infected grapes showed lower total phenolic content compared to wine from non-infected grapes. Certain volatile compounds can be affected by both the health status and irrigation treatments of grapevine. Wet treatment may enhance the levels of some volatile compounds in RB+ wines based on the result shown in 2019 and 2020. While the patterns were not consistent throughout the years, which suggested that vintage was also an important factor in the volatile wine profile.

### **INTRODUCTION**

Grapevine red blotch virus (GRBV) is a single-stranded circular DNA virus correlated with grapevine red blotch disease (GRBD) (Krenz et al., 2014). It was first found in Cabernet Sauvignon in California in 2008 and is widespread in North America. The grape species that GRBV can infect 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, disrupting 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 a decrease in 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 also been reported to 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%. At the same time, a total 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 significant decrease in Brix and significantly higher concentrations of tannins and non-tannin phenolics. Anthocyanins were found to be considerably 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. In addition, four mouthfeel and taste sensory attributes were significantly different among the wines (Eridon, 2017). However, the impact of GRBD on grape and wine quality has not been thoroughly investigated, especially from the point of view of grape and wine flavor.

## OBJECTIVES

1. Investigate the effect of GRBD on grape berry development, focusing on the flavor and flavor precursor formation.
2. Investigate the effect of GRBD on wine quality.

## RESULTS AND DISCUSSION

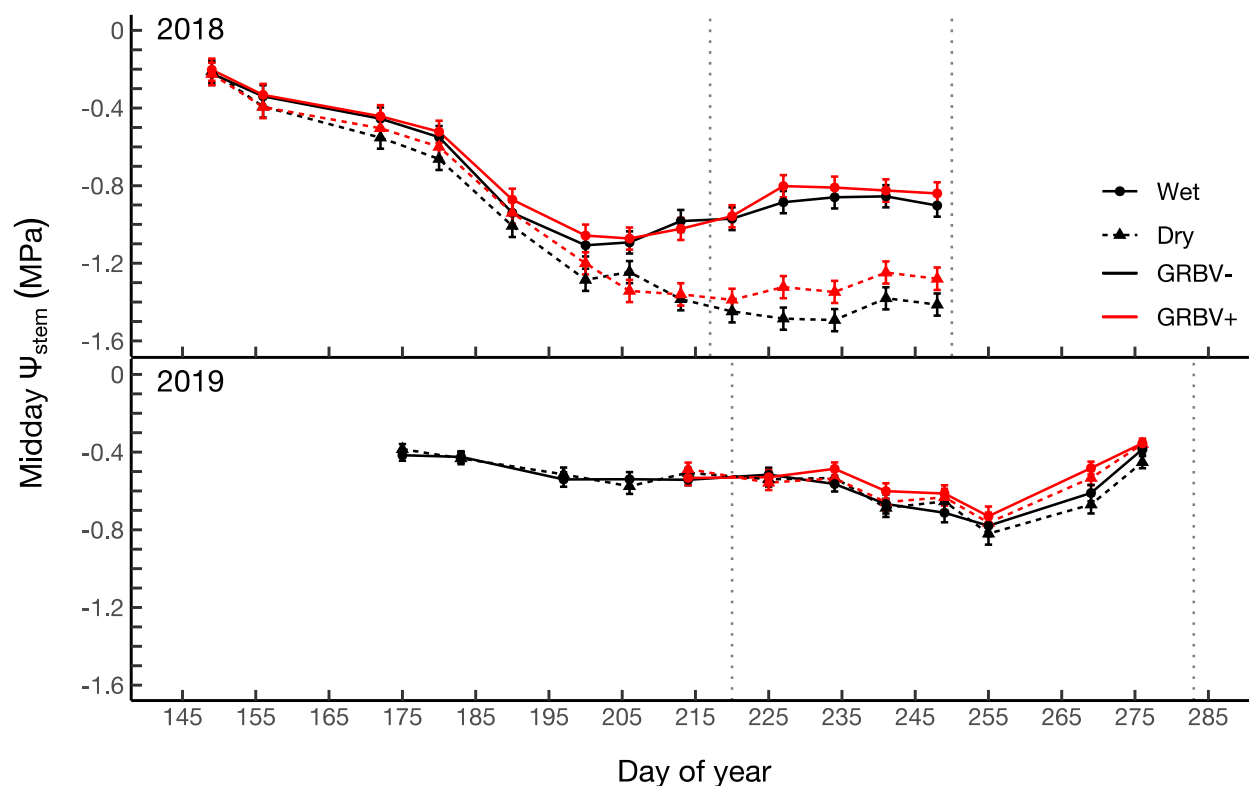
### Vine Water Status

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;  $\Psi_{\text{stem}}$ ) (Table 1). However, irrigation treatments only significantly affected  $\Psi_{\text{stem}}$  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 than healthy vines in both years (Figure 1). Yet, while this difference was observed immediately following veraison in 2018, it was not observed until just before harvest in 2019. Nevertheless, the effects of disease status on  $\Psi_{\text{stem}}$  were only observed post-veraison were consistent between years.

**Table 1.** Analysis of variance (ANOVA) results for the response of midday  $\Psi_{\text{stem}}$  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  $\Psi_{\text{stem}}$  to experimental treatments in 2018 and 2019. Data are means  $\pm$  1 standard error ( $n = 5$  and  $4$  in 2018 and 2019, respectively). Left-most and right-most vertical dotted lines signify the approximate date of veraison and date of harvest, respectively.

**Objective 1. Investigate the effect of GRBD on grape berry development, focusing on the flavor and flavor precursor formation.**

*Grape Berry Development*

Organic acids and total soluble solids (TSS) were measured during the grape ripening process until the harvest (one week after harvest for 2019 and 2020) for RB+ and RB- Pinot noir grapevines from 2018 to 2020. TSS increased in both red blotch positive (RB+) and red blotch negative (RB-) grapes during grape ripening (Figure 2). However, in 2019 and 2020, significantly reduced TSS ( $P < 0.05$ ) can be observed at or after harvest in RB+ grapes than RB- grapes.

Four main organic acid levels were also determined for both RB+ and RB- Pinot noir grapevines from 2018 to 2020. All major organic acids decreased during berry development (Figures 3). In 2019 and 2020, malic acid decreased significantly ( $P < 0.05$ ) after harvest in RB+ grapes. There was no consistent difference in other organic acids concentration between RB+ and RB- grape berries across three years.

Grape phenolic compounds, including catechin, epicatechin, caftaric acid, coumaric acid, vanillic acid, and malvidin-3-o-glucoside, were measured through HPLC across three years. A lower concentration of malvidin-3-o-glucoside was observed during RB+ grape ripening in three years, while the difference was not statistically significant at harvest in 2019 and 2020 (Figure 4). Other phenolic compounds were heavily impacted by year as no consistent results showed across three years.

*Free form and bound form C<sub>13</sub>-norisoprenoids*

For free form C<sub>13</sub>-norisoprenoids, Figure 5-7a indicated the concentrations of  $\beta$ -damascenone and  $\beta$ -ionone were similar during the RB+ and RB- grape berry development across three years, except the  $\beta$ -ionone level of RB+ grapes was higher than RB- grapes at the beginning in 2018 and 2020.

For bound form C<sub>13</sub>-norisoprenoids, RB+ grapes in 2018 showed a higher concentration of bound form  $\beta$ -damascenone during the three weeks (8/17-8/31) before harvest. Minor difference of  $\beta$ -damascenone was found in 2019 RB+/- grapes. The bound form vitispirane level was lower in RB+ grapes than RB- grapes during development in 2018, while the result was inconsistent in 2019. The bound form  $\beta$ -damascenone level was similar between RB+/- grapes except at or after harvest in 2020. Bound form TDN and  $\beta$ -ionone were at similar levels in RB+/- grapes across three years (Figure 5-7b).

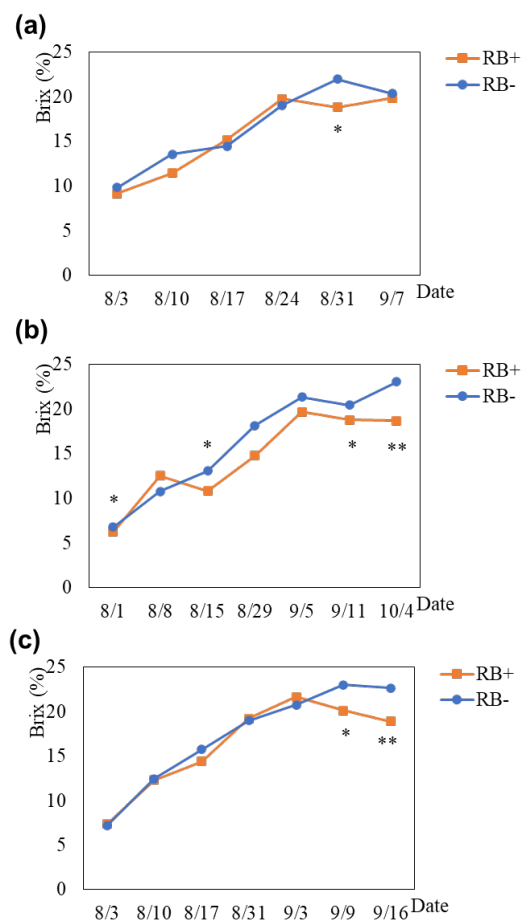
**Objective 2. Investigate the effect of GRBD on wine quality**

*Wine Monomeric Anthocyanin and Total Phenolic Content*

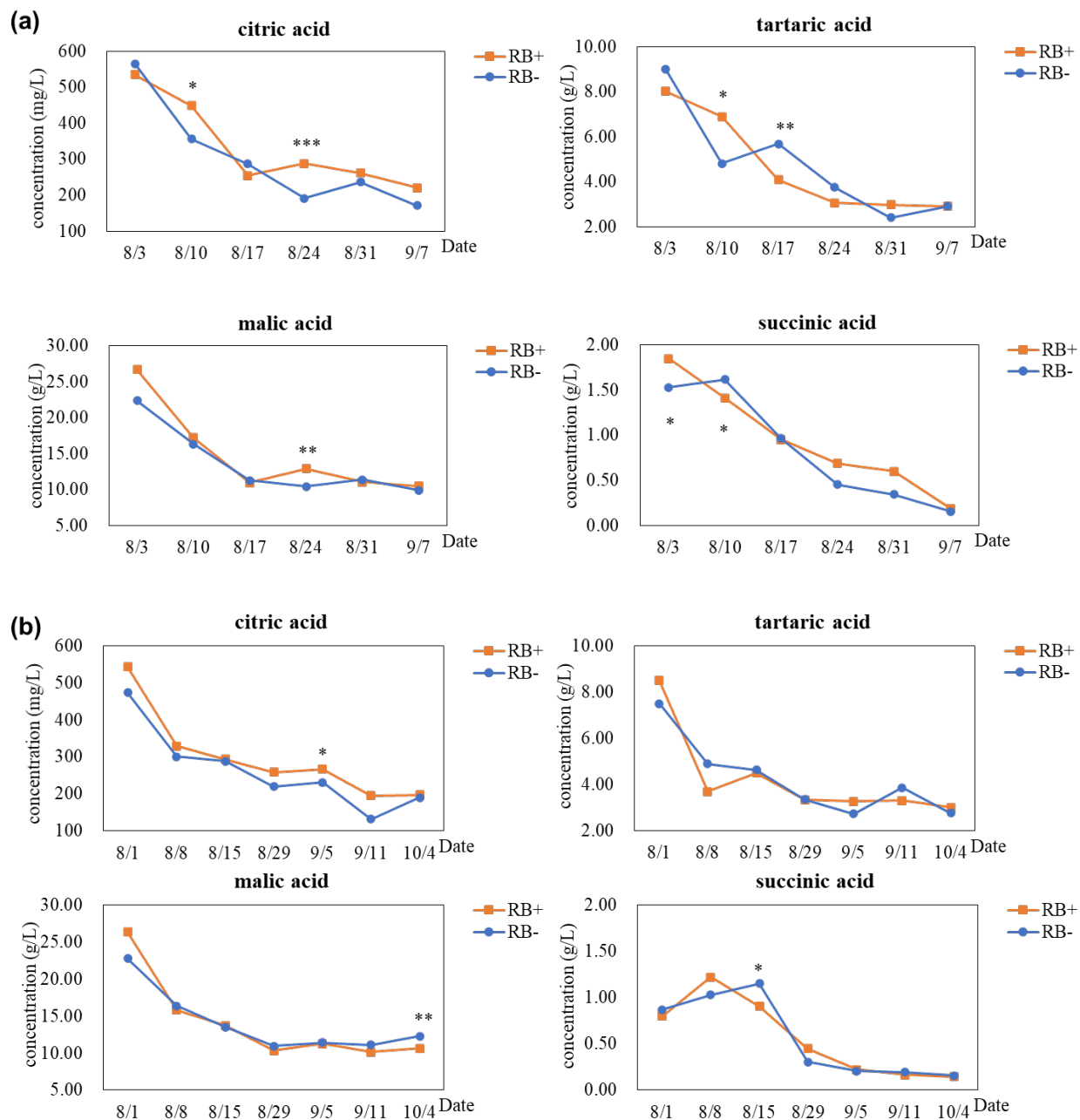
Monomeric anthocyanin and total phenolic content (Table 2) are generally higher for D- and W- wines than D+ and W+ wines. Wine from vines without wet treatment has the highest level of monomeric anthocyanin of the four treatment groups. The difference between red blotch positive and negative wines in 2020 was not significant.

Major phenolic compounds determined by high-performance liquid chromatography are summarized in Table 3-5. Red blotch positive showed lower concentrations of major phenolics

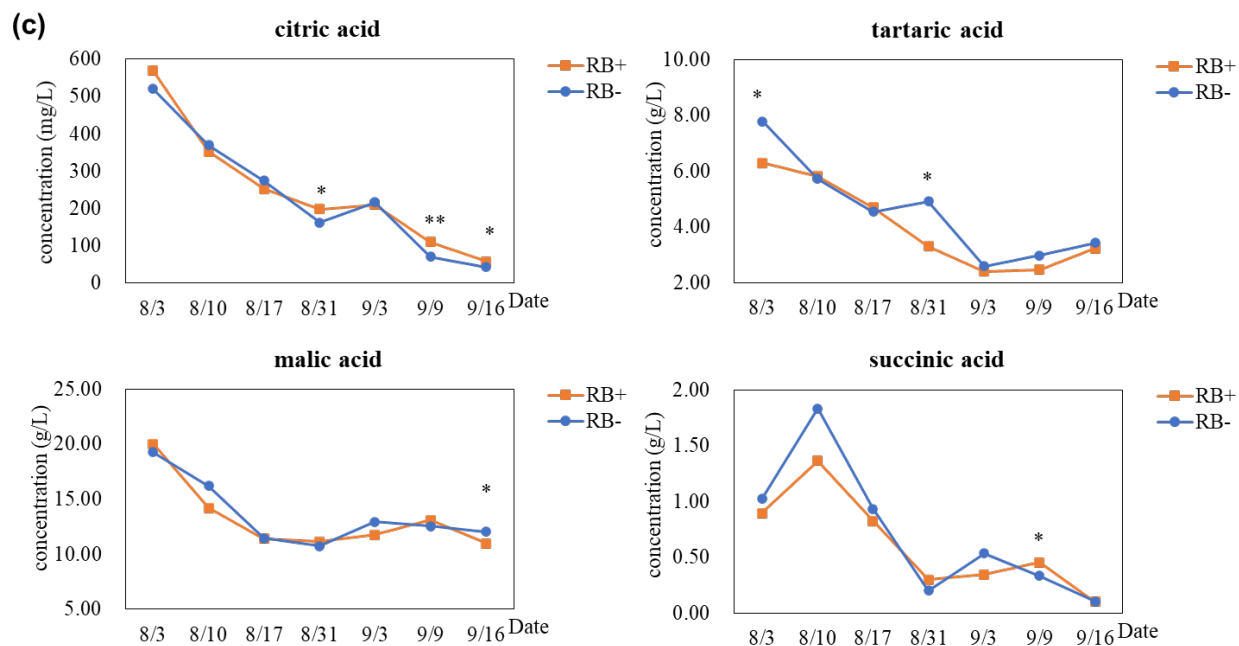
within each irrigation treatment than red blotch negative wines, indicating that disease status impacts the concentration of phenolic compounds regardless of irrigation practice.



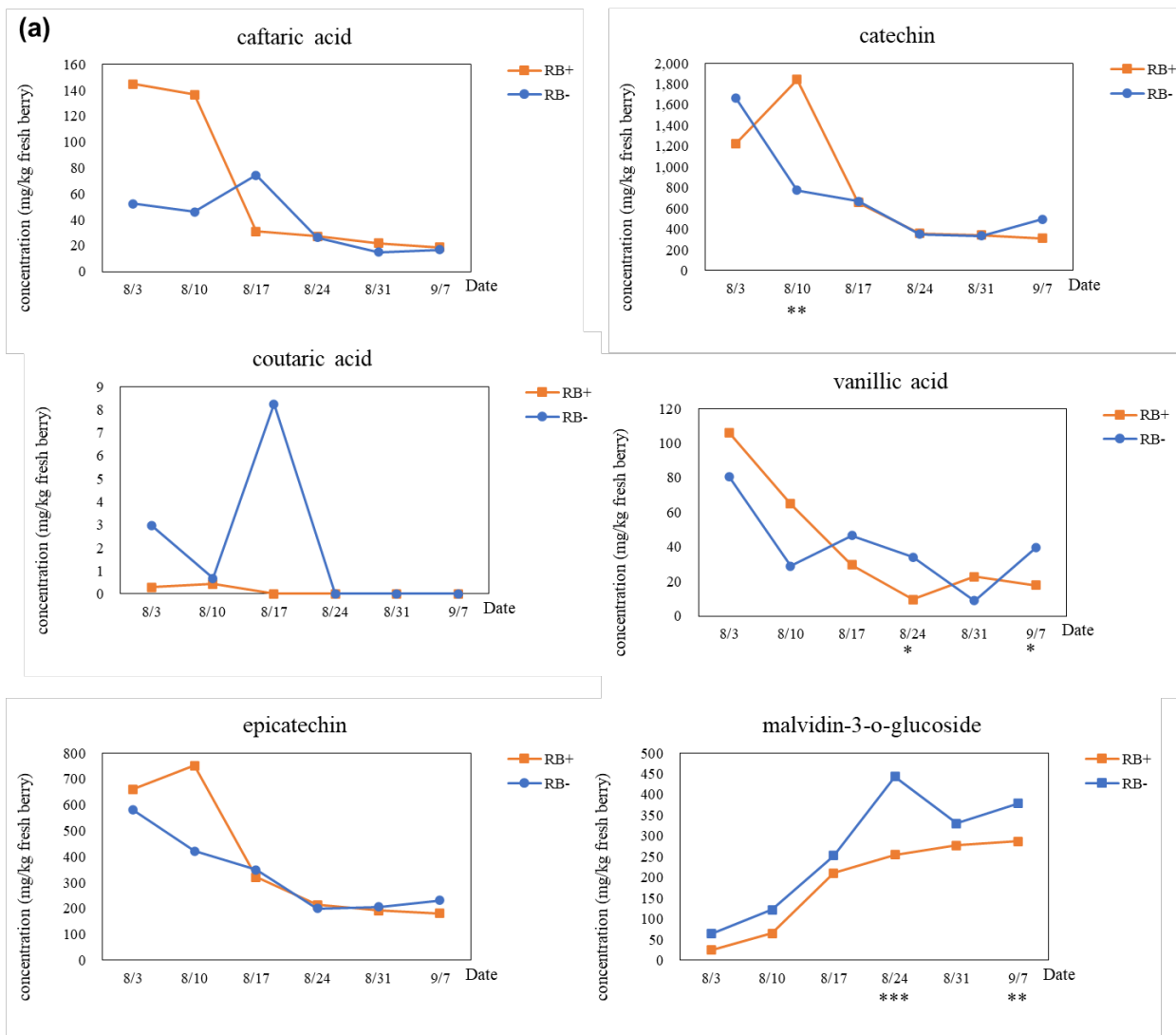
**Figure 2.** Total soluble solids of RB+/RB- Pinot noir grape juice across three years. (a): grapes harvested in 2018 (n = 4); (b): grapes harvested in 2019 (n = 4); (c): grapes harvested in 2020 (n = 4). \*:  $P < 0.05$ ; \*\*:  $P < 0.01$ .



**Figure 3a and 3b.** Organic acids of RB+/RB- Pinot noir grape juice across three years. (a): grapes harvested in 2018 (n = 4); (b): grapes harvested in 2019 (n = 4);

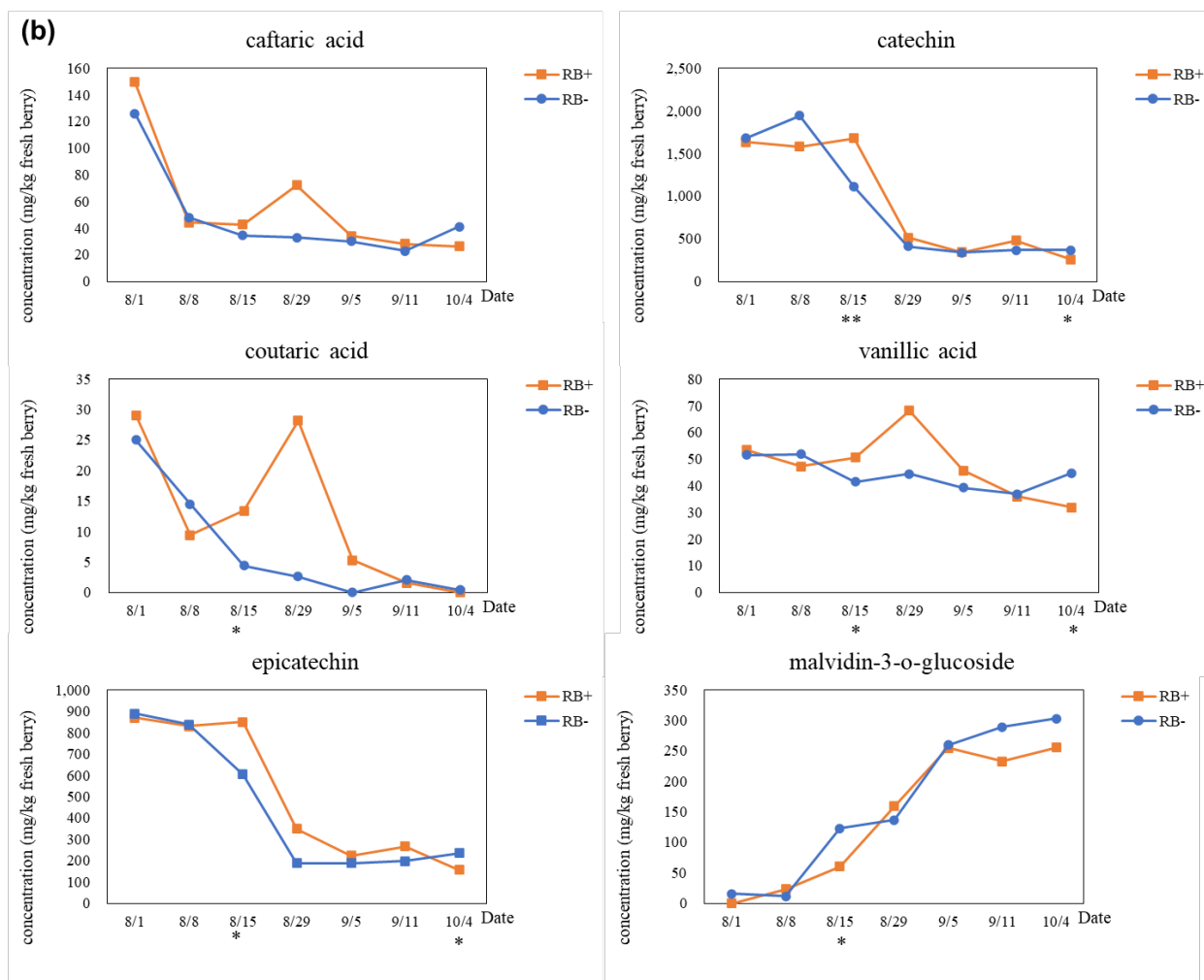


**Figure 3c.** Organic acids of RB+/RB- Pinot noir grape juice across three years.  
(c): grapes harvested in 2020 (n = 4). \*:  $P < 0.05$ ; \*\*:  $P < 0.01$ ; \*\*\*:  $P < 0.001$ .

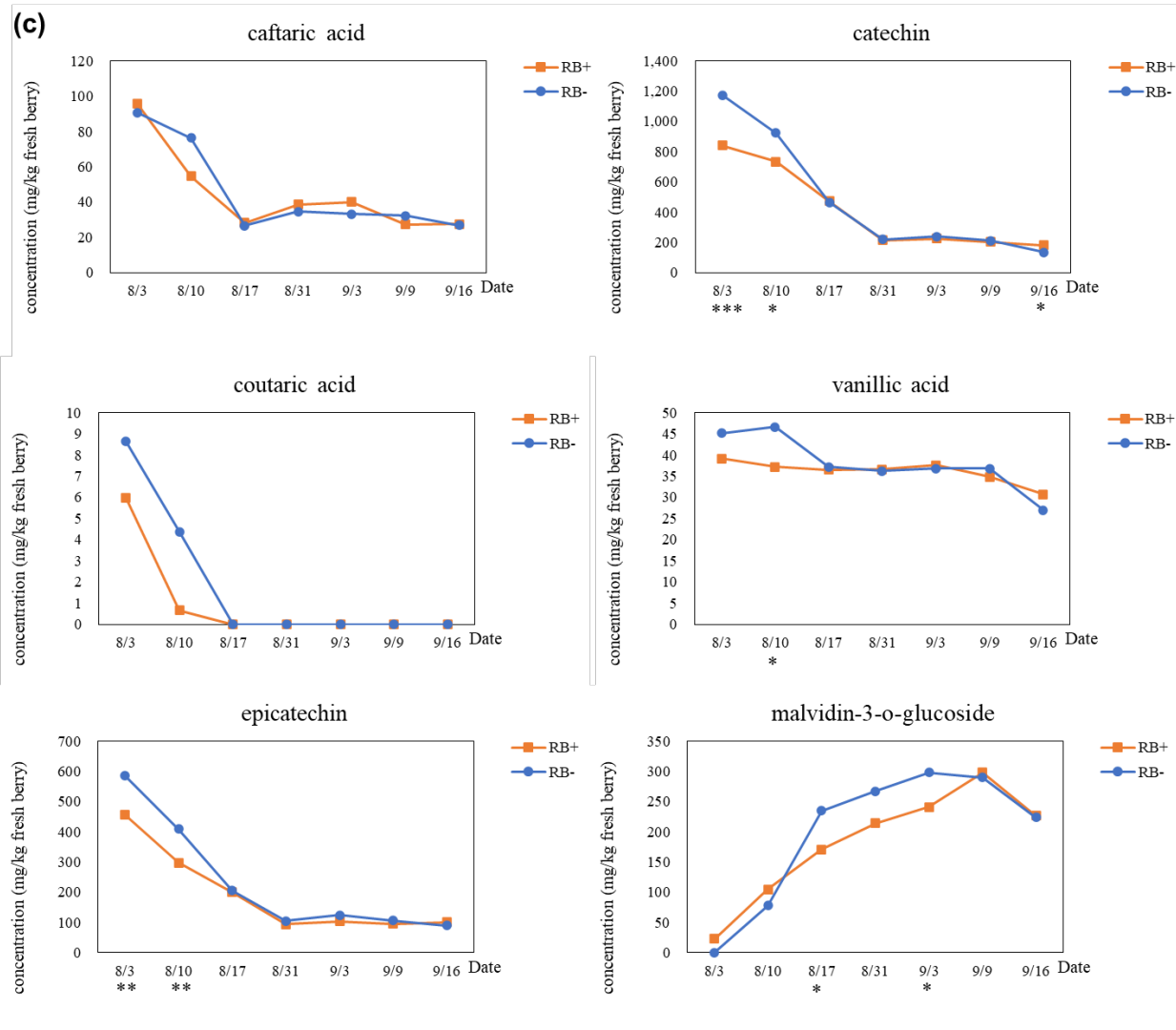


**Figure 4a.** Concentration of phenolic compounds in 2018 Pinot Noir grapes from RB+/RB- grapevines (\*:  $P < 0.05$ ; \*\*:  $P < 0.01$ ; \*\*\*:  $P < 0.001$ ).

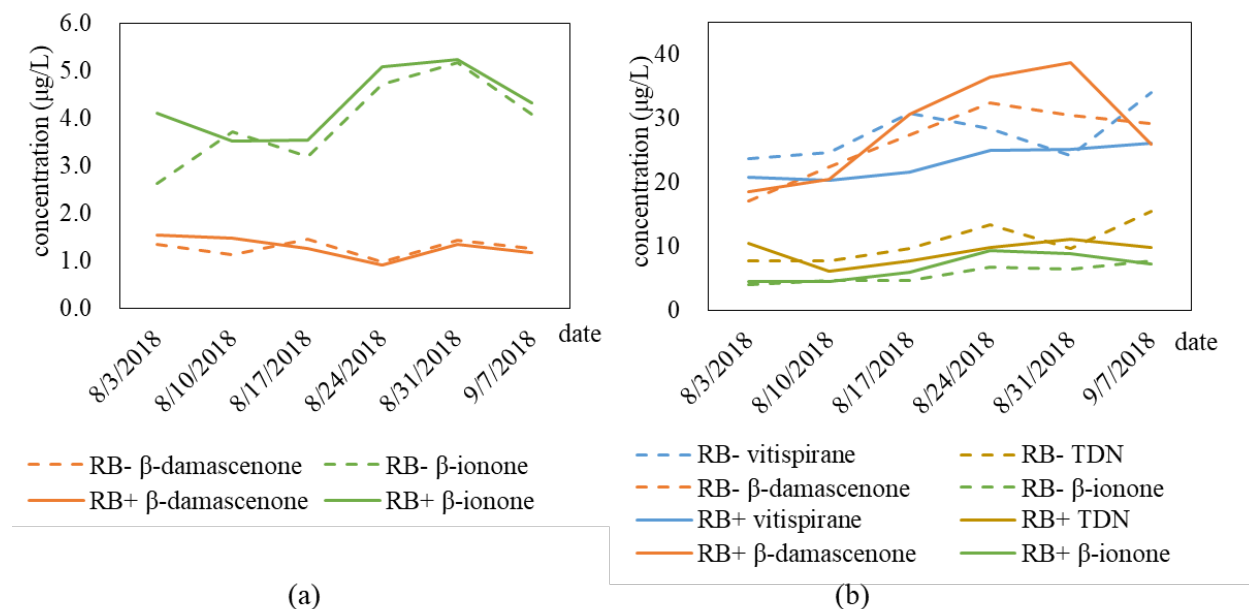




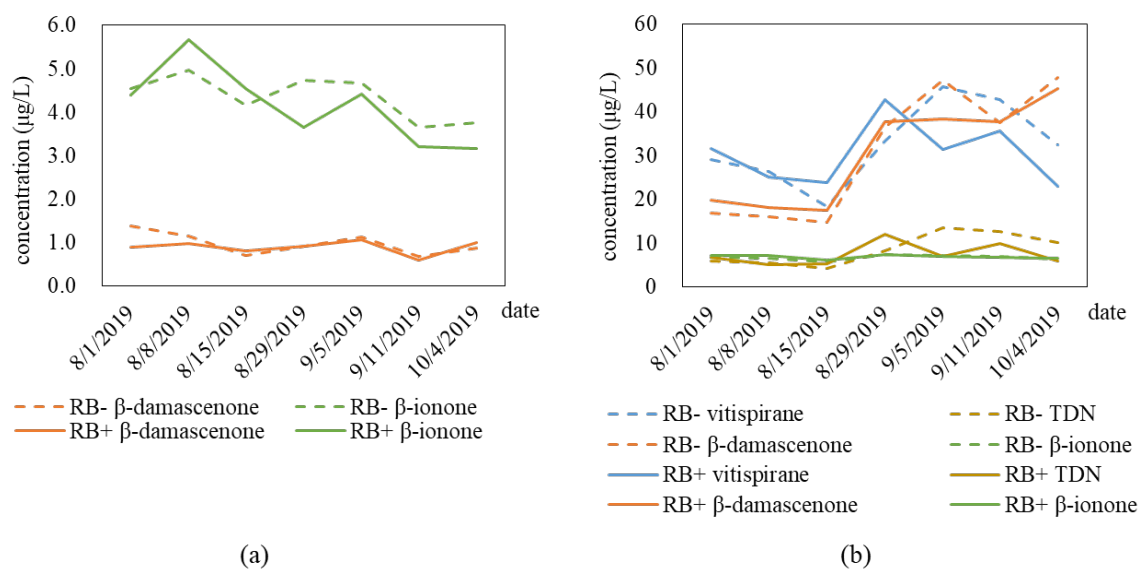
**Figure 4b.** Concentration of phenolic compounds in 2019 Pinot Noir grapes from RB+/RB- grapevines (\*:  $P < 0.05$ ; \*\*:  $P < 0.01$ ; \*\*\*:  $P < 0.001$ ).



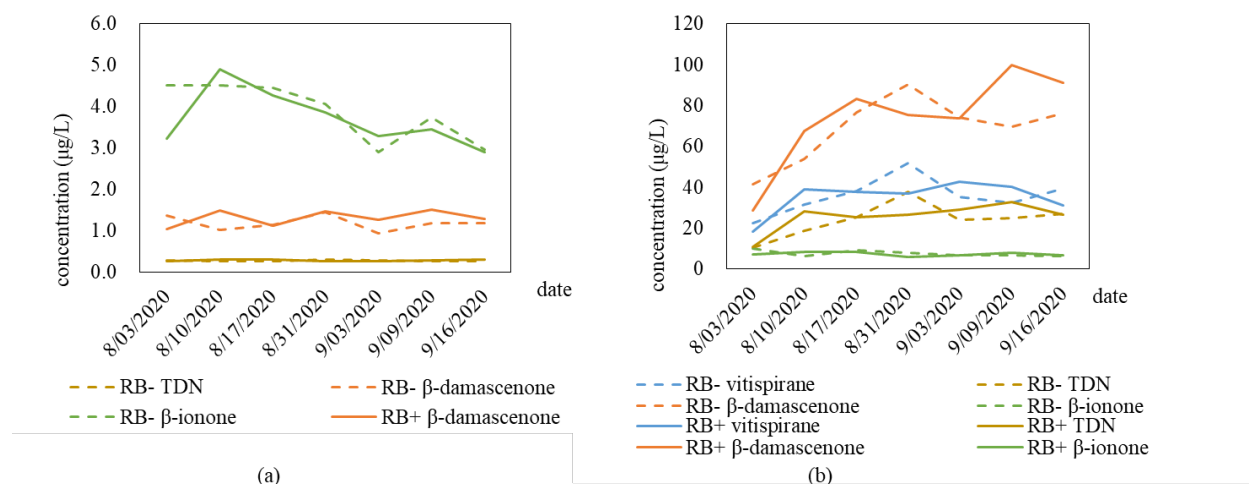
**Figure 4c.** Concentration of phenolic compounds in 2020 Pinot Noir grapes from RB+/RB- grapevines (\*:  $P < 0.05$ ; \*\*:  $P < 0.01$ ; \*\*\*:  $P < 0.001$ ).



**Figure 5.** Free form (a) and bound form (b) C<sub>13</sub>-norisoprenoids in 2018 grapes (n=4)



**Figure 6.** Free form (a) and bound form (b) C<sub>13</sub>-norisoprenoids in 2019 grapes (n=4)



**Figure 7.** Free form (a) and bound form (b) C<sub>13</sub>-norisoprenoids in 2020 grapes (n=4)

**Table 2.** Monomeric anthocyanin and total phenolic content in Pinot noir wines (mg/L).

	Year	D+	D-	W+	W-
monomeric anthocyanin	2018	31±4a	42±12a	27±5A	70±4B
	2019	129±10a	121±29a	102±14A	139±5B
	2020	178±9a	168±6a	164±11A	166±5A
total phenolic content	2018	1084±33a	1181±33a	1041±52A	1410±141B
	2019	1583±95a	1412±91a	1272±52A	1497±9B
	2020	915±53a	992±51a	944±39A	949±74A

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 in 2018 (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
caffeic 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.

**Table 4.** Concentration of major phenolic compounds with irrigation treatment in 2019 (mg/L).

Compounds	D+	D-	W+	W-
caffeoyltartaric acid	13.46±0.52a	17.08±1.39b	14.63±1.41A	18.65±0.14B
catechin	68.53±2.62a	39.64±3.86b	34.26±3.64A	33.71±0.38A
caffeic acid	4.2±0.39a	5.8±0.5b	6.75±0.57A	4.94±1.11B
epicatechin	124.07±2.92a	104.06±4.41b	100.54±11.59A	90.96±16.59A
malvidin-3-monoglucoside	203.34±3.35a	263.17±12.96b	239.42±17.14A	266.82±10.17A

Different letters indicate statistical significance ( $P < 0.05$ ) between means ( $n = 3$ ) of RB+ and RB- for each irrigation treatment.

**Table 5.** Concentration of major phenolic compounds with irrigation treatment in 2020 (mg/L).

Compounds	D+	D-	W+	W-
caffeoyltartaric acid	15.18±1.14a	18.97±0.84b	22.44±0.57A	18.87±1.1B
catechin	40.34±2.69a	42.58±3.28a	40.68±2.57A	49.81±4.09B
caffeic acid	1.29±0.12a	1.62±0.14b	1.4±0.15A	1.55±0.17A
epicatechin	13.11±1.18a	12.52±1.68a	10.72±0.84A	14.98±1.66B
malvidin-3-monoglucoside	126.66±4.41a	118.21±5.73a	116.53±5.37A	109.9±4.66A

Different letters indicate statistical significance ( $P < 0.05$ ) between means ( $n = 3$ ) of RB+ and RB- for each irrigation treatment.

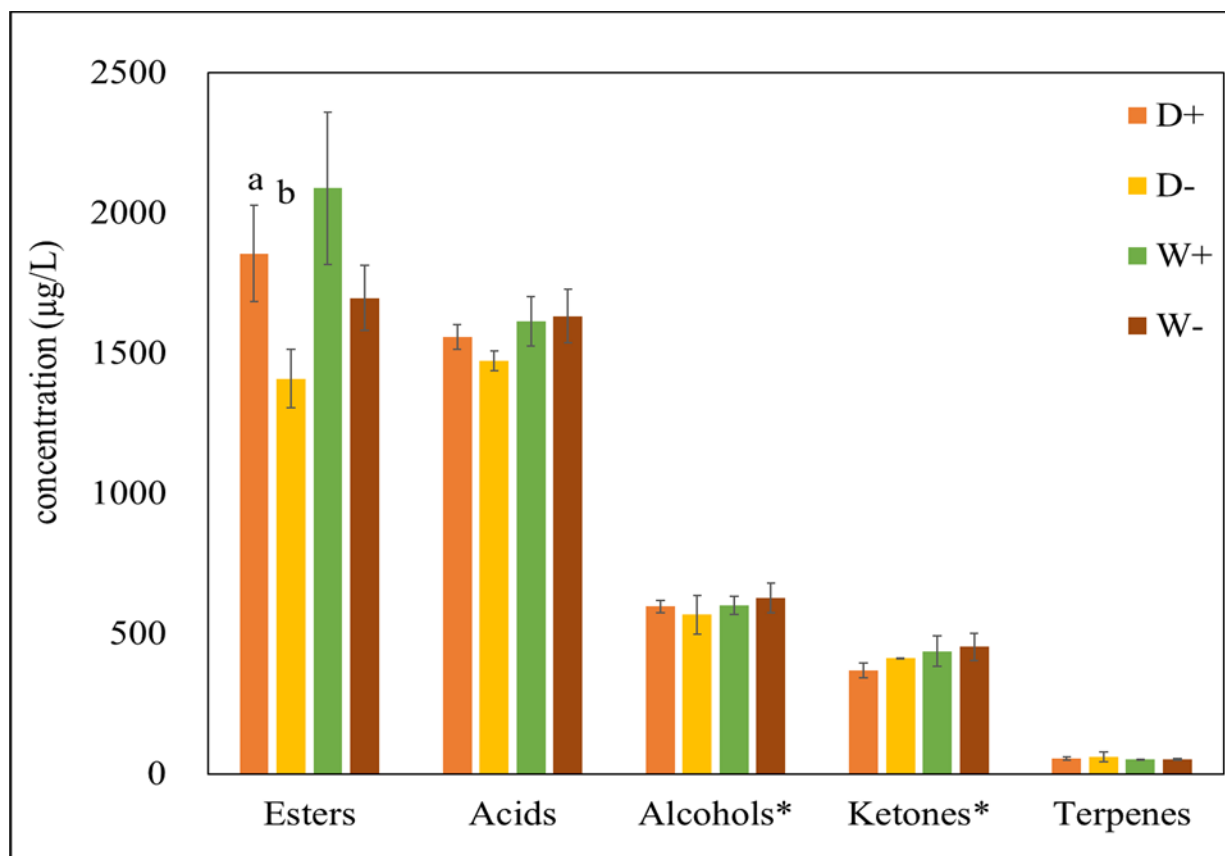
### *Wine Volatile Profiles*

The total concentration of different classes of volatile compounds in 2018 wine samples (D+, D-, W+, W-), including esters, acids, alcohols, ketones, and terpenes, is shown in Figure 8. The total esters concentration of D+ is significantly higher than D- at the 95% level, while 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 2018 wines with two disease states (Tables 6a-d). 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,  $\gamma$ -decalactone, and  $\delta$ -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,  $\gamma$ -decalactone, and  $\delta$ -undecalactone but lower levels of 1,1,6-trimethyl-1,2-dihydronaphthalene (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 TND (after hydrolysis) were lower compared to the RB- wines (W-).

In 2019, the levels of one acid, two esters, three C<sub>13</sub>-norisoprenoids, and two terpenes (octanoic acid, ethyl 2-methylbutyrate, ethyl 3-methylbutyrate, vitispirane,  $\beta$ -damascenone,  $\beta$ -ionone, linalool, and nerol) showed significant difference ( $P < 0.05$ ) between RB+ and RB- wines (D+ vs D-; W+ vs W-). Interestingly, those compounds were lower in D+ than D-, but showed opposite trends under wet treatment (Table 7), which means the content of the volatile compound can be significantly affected by irrigation treatments. In RB- wines, the concentrations of ethyl 2-methylbutyrate, vitispirane,  $\beta$ -damascenone,  $\beta$ -ionone, linalool, and nerol were significantly ( $P < 0.05$ ) higher in D- than W-. However, the concentrations of those six compounds were significantly ( $P < 0.05$ ) higher in W+ than D+ wines, which indicated wet treatment might have a positive effect on certain volatile compounds levels in RB+ wines.

In 2020, the level of decanoic acid was significantly lower ( $P < 0.05$ ) in D+ than D-, but was significantly higher ( $P < 0.05$ ) in W+ than W- wines (Table 8). In RB- wines, the concentration of decanoic acid was significantly ( $P < 0.05$ ) higher in D- than W-. However, it was significantly ( $P < 0.05$ ) higher in W+ than D+ wines. The positive effect on volatile compound level in RB+ wines of wet treatment was consistent with the data shown in 2019. The effects of red blotch on octanoic acid, (E)-2-hexenol, propanol, hexyl acetate, 6-methyl-5-hepten-2-one, vitispirane, and  $\alpha$ -terpineol concentrations were neglectable compared to irrigation treatments. Significantly higher ( $P < 0.05$ ) levels of octanoic acid, (E)-2-hexenol, and  $\alpha$ -terpineol were observed both in W- than D- and in W+ than D+ wines. Similarly, significantly lower ( $P < 0.05$ ) levels of propanol, hexyl acetate, 6-methyl-5-hepten-2-one, and vitispirane were observed both in W- than D- and in W+ than D+ wines.

Overall, both different irrigation treatments and red blotch disease status influence certain volatile aroma compounds. Wet treatment may enhance the levels of some volatile compounds in RB+ wines based on the result shown in 2019 and 2020, while the results were heavily affected by years.



**Figure 8.** Total concentration of esters, acids, alcohols\*, ketones\*, and terpenes in 2018 wines with different treatments. Units =  $\mu\text{g/L}$ , except \* =  $\text{mg/L}$ . D = dry conditions; W = wet conditions; + = red blotch affected wine; - = red blotch non-affected wine. Different letters indicate statistical significance ( $P < 0.05$ ) between means ( $n = 3$ ) of RB+ and RB- for each irrigation treatment.

**Table 6a.** Concentration of esters of 2018 wines with irrigation treatment ( $\mu\text{g/L}$ ).

Compounds	D+	D-	W+	W-
ethyl acetate*	37.3 $\pm$ 2.4	37.6 $\pm$ 1.1	40.9 $\pm$ 6.8	39.5 $\pm$ 2.7
ethyl propionate	75.1 $\pm$ 5.5	77.7 $\pm$ 2.2	77.3 $\pm$ 3.8	76.5 $\pm$ 4.6
ethyl 2-methylpropanoate	89.8 $\pm$ 11.8	74.7 $\pm$ 3.0	85.8 $\pm$ 3.2	79.1 $\pm$ 6.9
ethyl butanoate	108 $\pm$ 7	105 $\pm$ 2c	131 $\pm$ 12	113 $\pm$ 4d
isobutyl acetate	175 $\pm$ 31a	121 $\pm$ 13bc	215 $\pm$ 26a	156 $\pm$ 13bd
ethyl 3-methylbutanoate	2.30 $\pm$ 0.35a	1.65 $\pm$ 0.15bc	1.66 $\pm$ 0.43	2.06 $\pm$ 0.16d
isoamyl acetate	914 $\pm$ 134a	616 $\pm$ 104bc	1151 $\pm$ 193	869 $\pm$ 119d
ethyl 2-methylbutanoate	2.44 $\pm$ 0.44	1.91 $\pm$ 0.1	2.34 $\pm$ 0.6	2.11 $\pm$ 0.26
ethyl hexanoate	286 $\pm$ 15	274 $\pm$ 34	292 $\pm$ 39	284 $\pm$ 22
hexyl acetate	7.5 $\pm$ 6.6	7.3 $\pm$ 0.4	17.3 $\pm$ 3.9	15.3 $\pm$ 2.7
ethyl octanoate	149 $\pm$ 9ac	106 $\pm$ 19b	98 $\pm$ 6d	85 $\pm$ 6
ethyl decanoate	42.8 $\pm$ 12.0ac	20.8 $\pm$ 2.3bc	19.4 $\pm$ 0.4ad	15.3 $\pm$ 1.0bd
ethyl phenylacetate	1.21 $\pm$ 0.33ac	0.54 $\pm$ 0.15b	0.29 $\pm$ 0.15d	0.56 $\pm$ 0.20
phenethyl acetate	8.5 $\pm$ 1.1c	11.1 $\pm$ 2.2	14.8 $\pm$ 1.1d	14.0 $\pm$ 2.9
ethyl dodecanoate	0.24 $\pm$ 0.05a	0.12 $\pm$ 0.02b	0.17 $\pm$ 0.03	0.14 $\pm$ 0.03

**Table 6b.** Concentration of ketones, aldehydes, and alcohols of 2018 wines with irrigation treatment ( $\mu\text{g/L}$ ).

Compounds	D+	D-	W+	W-
<i>ketone &amp; aldehyde</i>				
acetaldehyde*	389 $\pm$ 26	441 $\pm$ 51	378 $\pm$ 49	423 $\pm$ 30
1-octen-3-one	ND	ND	ND	ND
6-methyl-5-hepten-2-one	1.08 $\pm$ 0.39	1.41 $\pm$ 0.38c	0.50 $\pm$ 0.03	0.41 $\pm$ 0.08d
<i>alcohol</i>				
propanol*	25.3 $\pm$ 1.4	23.4 $\pm$ 0.8	32.1 $\pm$ 4.4	25.0 $\pm$ 1.0
isobutyl alcohol*	199 $\pm$ 17	165 $\pm$ 26	197 $\pm$ 4	200 $\pm$ 17
isoamyl alcohol*	334 $\pm$ 7	328 $\pm$ 44	318 $\pm$ 8	368 $\pm$ 26
2-heptanol	10.2 $\pm$ 0.7	11.2 $\pm$ 1.6	10.2 $\pm$ 2.3	8.7 $\pm$ 1.0
1-hexanol	1496 $\pm$ 312	1629 $\pm$ 458	1661 $\pm$ 293	1434 $\pm$ 94
(E)-3-hexen-1-ol	86.4 $\pm$ 22.9	109.1 $\pm$ 46.3	91.2 $\pm$ 21.9	74.1 $\pm$ 24.5
(Z)-3-hexen-1-ol	16.7 $\pm$ 1.5	21.6 $\pm$ 3.4	20.3 $\pm$ 2.3	20.3 $\pm$ 1.0
(E)-2-hexen-1-ol	11.7 $\pm$ 0.2c	11.8 $\pm$ 0.2	13.1 $\pm$ 0.5d	12.0 $\pm$ 0.6
1-octen-3-ol	4.29 $\pm$ 0.89	4.02 $\pm$ 1.29c	2.53 $\pm$ 0.66	1.69 $\pm$ 0.16d
benzyl alcohol	328 $\pm$ 81	394 $\pm$ 60	381 $\pm$ 25	373 $\pm$ 8
phenyl alcohol*	36.8 $\pm$ 1.6c	41.1 $\pm$ 2.3	33.0 $\pm$ 1.7ad	39.1 $\pm$ 1.1b

**Table 6c.** Concentration of terpenes, lactones, acids, and methoxypyrazines of 2018 wines with irrigation treatment ( $\mu\text{g/L}$ ).

Compounds	D+	D-	W+	W-
<i>terpene</i>				
linalool	4.49 $\pm$ 0.41	4.99 $\pm$ 1.35	3.61 $\pm$ 0.84	4.28 $\pm$ 1.65
$\alpha$ -terpinol	1.40 $\pm$ 0.28	2.04 $\pm$ 0.42	1.30 $\pm$ 0.25	1.22 $\pm$ 0.49
citronellol	10.1 $\pm$ 1.2	12.3 $\pm$ 3.9	9.5 $\pm$ 0.4	10.4 $\pm$ 0.9
nerol	4.31 $\pm$ 0.97a	2.51 $\pm$ 0.57b	4.13 $\pm$ 0.81	3.25 $\pm$ 0.66
$\beta$ -damascenone	4.57 $\pm$ 0.44	5.96 $\pm$ 2.34	3.81 $\pm$ 1.06	4.11 $\pm$ 0.33
geraniol	28.5 $\pm$ 2.5	32.5 $\pm$ 11.2	27.5 $\pm$ 2.3	27.7 $\pm$ 3.9
$\beta$ -ionone	0.33 $\pm$ 0.04	0.32 $\pm$ 0.08	0.40 $\pm$ 0.12	0.30 $\pm$ 0.03
<i>lactone</i>				
$\gamma$ -decalactone	6.44 $\pm$ 1.24ac	2.35 $\pm$ 0.56b	2.56 $\pm$ 0.77d	2.13 $\pm$ 0.23
$\delta$ -undecalactone	1.91 $\pm$ 0.06ac	1.17 $\pm$ 0.34b	1.02 $\pm$ 0.36d	0.88 $\pm$ 0.20
<i>acid</i>				
hexanoic acid	609 $\pm$ 9	580 $\pm$ 20c	614 $\pm$ 30	633 $\pm$ 26d
octanoic acid	857 $\pm$ 45	797 $\pm$ 32	896 $\pm$ 54	894 $\pm$ 69
decanoic acid	91.8 $\pm$ 2.3	94.3 $\pm$ 11.7	103.0 $\pm$ 17.8	104.7 $\pm$ 9.1
<i>methoxypyrazine</i>				
IPMP**	1.05 $\pm$ 0.09	0.95 $\pm$ 0.13	1.02 $\pm$ 0.15A	1.15 $\pm$ 0.10B
SBMP**	18.3 $\pm$ 3.7	17.3 $\pm$ 0.9	14.8 $\pm$ 1.7	18.0 $\pm$ 4.9
IBMP**	1.77 $\pm$ 0.08	1.97 $\pm$ 0.23	1.48 $\pm$ 0.03	1.73 $\pm$ 0.15



**Table 6d.** Concentration of bound form C13-norisoprenoids of 2018 wines with irrigation treatment ( $\mu\text{g/L}$ ).

Compounds	D+	D-	W+	W-
vitispirane A #	9.72 $\pm$ 0.4c	10 $\pm$ 0.78C	7.8 $\pm$ 0.48Ad	11.77 $\pm$ 0.6BD
vitispirane B #	7.7 $\pm$ 0.25	7.93 $\pm$ 0.99	6.57 $\pm$ 0.68A	9.52 $\pm$ 0.25B
TDN	8.4 $\pm$ 0.35a	10.97 $\pm$ 0.88b	7.72 $\pm$ 0.28A	10.22 $\pm$ 0.21B
$\beta$ -damascenone	14.97 $\pm$ 1.17	15.28 $\pm$ 1.17	13.97 $\pm$ 1.35	13.63 $\pm$ 0.91
$\beta$ -ionone	0.52 $\pm$ 0.08	0.52 $\pm$ 0.08	0.57 $\pm$ 0.06	0.52 $\pm$ 0.03

Notes for Tables 6a to 6d: \* = mg/L; \*\* = ng/L; # =  $\beta$ -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-dihydronaphthalene. Different letters represent significantly ( $P < 0.05$ ) different in means ( $n = 3$ ).

**Table 7.** Concentration of volatile compounds in 2019 wines with irrigation treatment ( $\mu\text{g/L}$ ).

Compounds	D+	D-	W+	W-
<b>acids</b>				
3-methylbutanoic acid	891 $\pm$ 246a	1015 $\pm$ 49a	827 $\pm$ 189ab	860 $\pm$ 72c
decanoic acid	100 $\pm$ 12a	244 $\pm$ 16b	241 $\pm$ 54b	299 $\pm$ 54bc
hexanoic acid	933 $\pm$ 114a	970 $\pm$ 115a	948 $\pm$ 59a	776 $\pm$ 12b
octanoic acid	416 $\pm$ 90a	648 $\pm$ 68bd	663 $\pm$ 23c	560 $\pm$ 49bd
<b>alcohols</b>				
(Z)-3-hexenol	166 $\pm$ 33ab	184 $\pm$ 17ab	216 $\pm$ 19c	132 $\pm$ 32d
1-hexanol*	2.52 $\pm$ 0.42abc	2.77 $\pm$ 0.28ab	2.46 $\pm$ 0.17ac	2.07 $\pm$ 0.14d
1-octen-3-ol	10 $\pm$ 2.7a	13.7 $\pm$ 1.1b	8.6 $\pm$ 0.8a	9 $\pm$ 1.9c
benzyl alcohol	715 $\pm$ 52a	580 $\pm$ 33b	642 $\pm$ 40ab	656 $\pm$ 36c
isomyl alcohol*	167 $\pm$ 9a	178 $\pm$ 19a	152 $\pm$ 23ab	145 $\pm$ 11c
phenethyl alcohol*	15.4 $\pm$ 1.3a	20.8 $\pm$ 2.3b	14.2 $\pm$ 0.4a	14.6 $\pm$ 1.6c
propanol*	58.5 $\pm$ 3.5a	72.7 $\pm$ 9.2b	64.7 $\pm$ 11ab	61.5 $\pm$ 8.6bc
<b>aldehydes &amp; ketones</b>				
acetaldehyde*	16.1 $\pm$ 1.4a	15.4 $\pm$ 1.4a	12.1 $\pm$ 1.4c	12.6 $\pm$ 1.2c
6-methyl-5-hepten-2-one	2.84 $\pm$ 0.24a	4.75 $\pm$ 1.21b	4.39 $\pm$ 1.1b	5.47 $\pm$ 1.33bc
<b>C13-norisoprenoids (free form)</b>				
vitispirane	2.22 $\pm$ 0.02a	2.41 $\pm$ 0.14b	2.27 $\pm$ 0.01b	2.16 $\pm$ 0.08a
$\beta$ -damascenone	2.53 $\pm$ 0.05a	2.75 $\pm$ 0.09b	2.69 $\pm$ 0.08b	2.5 $\pm$ 0.05a
$\beta$ -ionone	2.94 $\pm$ 0a	3.07 $\pm$ 0.04b	2.98 $\pm$ 0c	2.96 $\pm$ 0.02a
<b>C13-norisoprenoids (bound form)</b>				
TDN (1,1,6-trimethyl-1,2-dihydronaphthalene)	20.9 $\pm$ 2.1a	21.2 $\pm$ 2.6a	17.1 $\pm$ 1c	18.6 $\pm$ 0.6b
vitispirane	64.1 $\pm$ 2.4a	70 $\pm$ 9.7a	57 $\pm$ 2.6c	70.8 $\pm$ 3.1b

$\beta$ -damascenone	51 $\pm$ 2.1a	51.3 $\pm$ 3.7a	46.8 $\pm$ 3.2ab	45.2 $\pm$ 2.5c
$\beta$ -ionone	5.29 $\pm$ 0.33a	6.13 $\pm$ 0.29b	5.51 $\pm$ 0.62ab	5.75 $\pm$ 0.34bc
<b>esters</b>				
ethyl 2-methylbutyrate	0.56 $\pm$ 0.07a	1.08 $\pm$ 0.11b	1.19 $\pm$ 0.32b	0.66 $\pm$ 0.12a
ethyl 3-methylbutyrate	1.81 $\pm$ 0.07ac	1.92 $\pm$ 0.04b	1.9 $\pm$ 0.14ac	1.69 $\pm$ 0.08d
ethyl butanoate	70.8 $\pm$ 11.8abc	80.9 $\pm$ 5.4ab	61 $\pm$ 10acd	61.4 $\pm$ 13.4cd
ethyl decanoate	134 $\pm$ 20ab	145 $\pm$ 7ab	79 $\pm$ 9c	102 $\pm$ 5d
ethyl dodecanoate	156 $\pm$ 39a	116 $\pm$ 37a	106 $\pm$ 7b	107 $\pm$ 19bc
ethyl hexanoate	247 $\pm$ 39a	167 $\pm$ 4b	171 $\pm$ 18b	155 $\pm$ 12bc
ethyl octanoate	96.44 $\pm$ 26.39a	61.7 $\pm$ 5.05b	57.84 $\pm$ 3.76b	59.22 $\pm$ 1.29bc
ethyl phenylacetate	0.91 $\pm$ 0.12a	1.22 $\pm$ 0.11a	0.68 $\pm$ 0.05c	0.73 $\pm$ 0.1c
hexyl acetate	3.28 $\pm$ 0.25a	2.4 $\pm$ 0.11b	2.95 $\pm$ 0.77ab	2.26 $\pm$ 0.11bc
isoamyl acetate	163 $\pm$ 15a	137 $\pm$ 9b	141 $\pm$ 9b	129 $\pm$ 11bc
phenethyl acetate	15.8 $\pm$ 0.1a	16.4 $\pm$ 0.6a	15.2 $\pm$ 0.4c	15.3 $\pm$ 0.2c
<b>methoxypyrazines</b>				
SBMP (2-sec-butyl-3-methoxypyrazine) **	41.1 $\pm$ 0.8a	36.5 $\pm$ 7.5a	21.3 $\pm$ 2.7c	23.1 $\pm$ 3.3c
<b>terpenes</b>				
citronellol	24.4 $\pm$ 0.2a	23.2 $\pm$ 0.1b	23.3 $\pm$ 0.1b	23.4 $\pm$ 0.2c
geraniol	4.8 $\pm$ 1.43abc	7.44 $\pm$ 0.71ab	4.5 $\pm$ 0.98acd	2.95 $\pm$ 0.36cd
linalool	5.22 $\pm$ 0.24a	13.37 $\pm$ 3.33b	8.33 $\pm$ 0.28c	6.29 $\pm$ 0.66d
nerol	2.8 $\pm$ 0.62a	6.28 $\pm$ 1.01b	4.75 $\pm$ 0.48c	3.85 $\pm$ 0.05d
$\alpha$ -terpineol	2.83 $\pm$ 0.14a	3.47 $\pm$ 0.3b	3.39 $\pm$ 0.22b	3.05 $\pm$ 0.18bc

\* = mg/L; \*\* = ng/L; Different letters represent significantly ( $P < 0.05$ ) different in means ( $n=3$ ).

**Table 8.** Concentration of volatile compounds in 2020 wines with irrigation treatment ( $\mu\text{g/L}$ ).

Compounds	D+	D-	W+	W-
<b>acids</b>				
decanoic acid	48.3 $\pm$ 6.7a	92 $\pm$ 17b	117 $\pm$ 19.3c	56.6 $\pm$ 15.7d
octanoic acid	177 $\pm$ 25ab	196 $\pm$ 34ab	281 $\pm$ 11cd	262 $\pm$ 12cd
<b>alcohols</b>				
(e)-2-hexenol	20 $\pm$ 2.6ab	18 $\pm$ 1.6ab	28.8 $\pm$ 3.8cd	30.9 $\pm$ 3.4cd
(e)-3-hexenol	193 $\pm$ 7ac	242 $\pm$ 3bd	186 $\pm$ 43acd	239 $\pm$ 28bcd
(z)-3-hexenol	76.8 $\pm$ 5.7ab	89.6 $\pm$ 10.3abd	100 $\pm$ 9.3cd	104.9 $\pm$ 14.8bcd
1-hexanol	1733 $\pm$ 53a	1892 $\pm$ 80bd	2127 $\pm$ 32cd	1909 $\pm$ 248bcd
1-octen-3-ol	8.17 $\pm$ 2.21abc	12.86 $\pm$ 3.73ab	6 $\pm$ 1.52acd	4.07 $\pm$ 1.22cd
isobutyl alcohol*	71.8 $\pm$ 0.8a	88 $\pm$ 4.8bd	88.4 $\pm$ 10.1cd	77.1 $\pm$ 11.3bcd
isomyl alcohol*	136 $\pm$ 3ac	165 $\pm$ 5b	159 $\pm$ 22acd	136 $\pm$ 18cd
phenethyl alcohol*	13.5 $\pm$ 0.2ac	15.5 $\pm$ 0.3bd	13.9 $\pm$ 0.3acd	14.3 $\pm$ 1.1bcd
propanol*	72.1 $\pm$ 0.9ab	72 $\pm$ 3.4ab	67.6 $\pm$ 2.8cd	63.9 $\pm$ 4.9cd
<b>Aldehydes &amp; ketones</b>				
acetaldehyde*	39.6 $\pm$ 2.5ac	51.9 $\pm$ 3b	36.8 $\pm$ 6.7acd	36.6 $\pm$ 4cd

6-methyl-5-hepten-2-one	2.3±0.84ab	3.53±0.64ab	0.69±0.19cd	0.89±0.55cd
<b><i>C13-norisoprenoids (free form)</i></b>				
vitispirane	0.53±0.03ab	0.54±0.01ab	0.43±0.05cd	0.48±0.03cd
β-damascenone	4.2±0.2abc	4.69±0.35ab	3.9±0.25acd	3.86±0.14cd
β-ionone	0.64±0.03ac	0.71±0.01b	0.68±0.04acd	0.63±0.03cd
<b><i>C13-norisoprenoids (bound form)</i></b>				
TDN (1,1,6-trimethyl-1,2-dihydronaphthalene)	6.43±0.41a	9.21±1.06bd	10.21±0.54cd	9.75±0.51bcd
vitispirane	18.6±0.8abc	18.9±0.2ab	17.2±0.8acd	17.3±0.3cd
β-ionone	1.26±0.09abc	1.26±0.09ab	1.15±0.15acd	1.12±0.06cd
<b><i>esters</i></b>				
ethyl 3-methylbutyrate	4.94±0.23a	5.63±0.31bd	5.48±0.34cd	5.59±0.34bcd
ethyl acetate*	40.4±4.4abc	35.4±4.2abd	36.1±0.7ac	28.5±4.3bd
ethyl decanoate	13.5±2.2ab	16.5±2.2abd	17.8±1.1cd	17.1±2bcd
ethyl dodecanoate	2.52±0.66ab	2.96±0.23abd	3.35±0.07cd	3.07±0.23bcd
ethyl hexanoate	127±8ab	183±61abd	150±12c	120±12bd
ethyl octanoate	9.8±2.2ab	14.5±3.6abd	15.5±2.6cd	14±2bcd
ethyl phenylacetate	1.86±0.3abc	2.24±0.15ab	1.58±0.05ac	1.43±0.05d
ethyl propanoate	85.3±8.2abc	86.3±9.2ab	84.3±8.9ac	63.7±9.4d
ethyl undecanoate	0.5±0.15abc	0.54±0.05ab	0.42±0.01acd	0.39±0.01cd
hexyl acetate	40.4±2ab	38.8±13.7ab	10.4±0.5c	6.8±0.8d
isoamyl acetate	367±15ac	517±76bd	523±129acd	400±100bcd
isoamyl acetate	382±34a	566±89bd	712±102cd	561±143bcd
phenethyl acetate	23.5±4.4abc	29.1±1.9ab	25.8±4.5acd	19.7±3.5cd
<b><i>methoxypyrazines</i></b>				
IPMP (2-isopropyl-3-methoxypyrazine) **	88.6±6.7ab	32.2±52.1abd	98±3c	90±4.4bd
SBMP (2-sec-butyl-3-methoxypyrazine) **	8.93±0.44ac	10.92±0.92bd	8.37±0.88acd	9.43±1.28bcd
<b><i>terpenes</i></b>				
citronellol	13.7±1ab	11.8±1.7abd	11.5±1.2cd	11.7±1.4bcd
geraniol	19.4±1.4abc	18.6±0.6ab	22±1.6acd	22.1±1.4cd
α-terpineol	8.74±1.27ab	7.02±0.61ab	13.7±1.49cd	14.54±1.77cd

\* = mg/L; \*\* = ng/L; Different letters represent significantly ( $P < 0.05$ ) different in means ( $n=3$ ).

Recent presentations include:

Ling Huang, Alexander Levin, James Osborne, Yanping L. Qian, Michael C. Qian. Impact of Grapevine Red Blotch Disease and Irrigation Treatments on Grape/Wine quality. Poster, ASEV National Conference, 2021

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## STRUCTURE-FUNCTION STUDIES ON *GRAPEVINE RED BLOTCH VIRUS* TO ELUCIDATE DISEASE ETIOLOGY

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**Reporting Period:** The results reported here are from work conducted July 2020 to November 2021.

### 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

Nearly 90 different documented viruses from 17 families infect grapes worldwide, which is far greater than the number of viruses documented in any other single perennial crop<sup>1, 2</sup>.

Geminiviruses are single-stranded (ss) DNA viruses that cause major losses to many crops throughout the world<sup>3</sup>. *Geminiviridae* constitutes the largest family of plant viruses characterized by small, circular, ssDNA genomes encapsidated in twinned (hence, the name *Gemini*) icosahedral particles. They are vector-transmissible and infect both monocotyledonous and dicotyledonous plants<sup>4</sup>. The genomes are either monopartite or bipartite with circular DNA molecules of 2.5- 5.5 kilobases. Geminiviruses possess a highly conserved core region (CR) of ~200 nucleotides containing bidirectional promoters and an inverted repeat that forms a hairpin

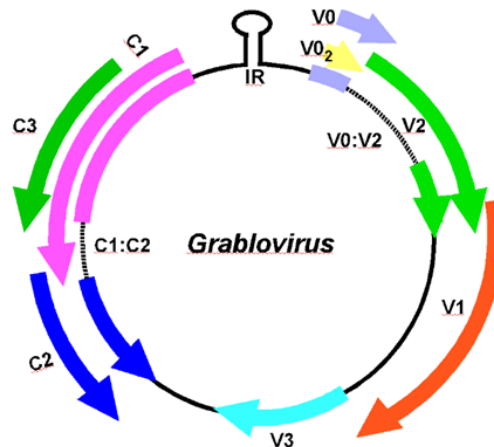
(hp) loop with an invariant 9-nt 5'-TAATATT<sup>↓</sup>AC-3' that acts as the origin of Virion (V) strand DNA replication in plant host (and some arthropod vectors), and is the target of host-mediated DNA methylation, an epigenetic transcriptional silencing immune response<sup>5</sup>. The viral gene products are required for replication and transmission<sup>6</sup>.

*Grapevine Red Blotch Virus* (GRBV) is a monopartite geminivirus in the Grablovirus genus first observed in California in 2008 as associated with Red Blotch Disease<sup>7</sup> and later proven by fulfilled Koch's postulates, including by grafting and vegetative propagation as primary inoculum, to be the causal agent of Red Blotch<sup>8</sup>. The V1 protein load was six times higher in petioles compared to leaves, which supports the notion that GRBV is phloem-restricted or phloem-limited<sup>9</sup>. 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 similar to leafroll and potassium or phosphorous deficiencies<sup>10, 11</sup>. Infected white-berried *V. vinifera* cultivars may show chlorosis and cupping, similar to *Grapevine Leaf Roll associated Virus* (GLRaV) symptoms or magnesium deficiency. Similar to other grapevine viruses, infection of GRBV in rootstocks is latent<sup>8</sup>. GRBV infection results in lower pruning mass and less winter hardiness of buds, reduced photosynthesis and stomatal conductance of leaves, delayed and uneven berry ripening, higher titratable acids, and reduced sugar, tannin, and anthocyanin contents in the berry<sup>12, 13</sup>. The impact of GRBV on foliar physiology is higher glucose and fructose, higher phenolics and terpenoids, and an altered amino acid profile<sup>14</sup>. Consequences of infections are reduced carbon translocation and impairment of fruit qualities for both table grape<sup>15</sup> and wine industries such as less alcohol, color, flavor, and aroma and increased astringency, flavonol, proanthocyanidin, and aftertaste of vegetal character<sup>16-18</sup>. Estimated price/quality penalties by GRBV for vineyard producers is estimated as high as \$68,000/ha<sup>19</sup>. Drought stress of grapevines during ripening improves fruit properties including anthocyanins and skin tannins, but not in GRBV-infected vines<sup>20</sup>.

GRBV was initially detected in ~95% of symptomatic grapevines and in ~2.7% of asymptomatic grapevines<sup>21</sup>. Highest virus titers are found in the petioles of fully expanded leaves but significantly reduced levels of virus in the shoot extremities<sup>22</sup>. Limited genetic diversity of GRBV populations in newly infected vines supports localized secondary spread within and between vineyards of 1-2% per year by a flying insect<sup>23-25</sup>. At Jacksonville in southern Oregon, 3% of vines were infected with GRBV in 2014, and GRBV incidence reached 58% of spatially associated study vines by 2016<sup>26</sup>. Bahder *et al.*<sup>27</sup> identified the three-cornered alfalfa treehopper *Spissistilus festinus* as the candidate vector that transmits GRBV under laboratory conditions, whereas Poojari *et al.*<sup>28</sup> claimed Virginia creeper leafhopper (*Erythroneura ziczac* [Walsh]), a dominant invasive species of northern California vineyards since the 1980s as the candidate vector (<http://www.ucanr.org/blogs/blogcore/postdetail.cfm?postnum=38818>).

Consistent with geminiviruses, GRBV possesses the conserved nonanucleotide sequence and seven putative overlapping open reading frames (ORF) are transcribed bidirectionally<sup>24</sup>. GRBV encodes up to five ORFs in the virion strand (*V0*, *V02*, *V1*, *V2*, *V0:V2* spliced fusion and *V3*)<sup>29</sup> and three in the complementary strand (*C1*, *C2*, *C1:C2* spliced fusion, and *C3*; **Figure 1**). Similar to mastrevirus (a monopartite geminivirus), GRBV complementary-sense ORF *C1* is predicted to encode RepA, Replication-associated protein. Another spliced transcript encompassing the *C1* and *C2* ORFs encodes Rep, the Replication protein<sup>10, 24, 30, 31</sup>. GRBV

virion-sense strand ORFs *V2* and *V3* are predicted to encode movement proteins, whereas *V1* ORF encodes coat protein. *V2* protein localizes in the nucleoplasm, Cajal bodies, and cytoplasm; the *V3* protein localizes in various unidentified subnuclear bodies. Additionally, the *V2* protein is redirected to the nucleolus upon co-expression with the nucleolus- and Cajal body-associated protein Fib2<sup>32</sup>.



**Figure 1.** Genome organization of *Grapevine red blotch virus* (GRBV)

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 develop 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<sup>33</sup>. 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<sup>34, 35</sup>.

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<sup>5</sup> (**Table 1**). The layers of complexity

employed by geminiviruses to target multiple host antiviral processes pose significant challenges to devise engineered strategies for crop viral resistance.

Early reports of engineered geminivirus resistance, which serendipitously involved host PTGS mechanisms 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<sup>36-39</sup>, whereas expression of various *Cotton leaf curl virus* genes in antisense orientations in tobacco conferred resistance<sup>40</sup>. Transient expression of a hpRNA gene of the MYMV bidirectional promoter<sup>41</sup>, ACMV-[CM] *Rep* siRNAs<sup>42</sup> and MSV *Rep* hpRNA gene<sup>43</sup> conferred resistance against the respective viruses. The hp*AC1/CI* genes conferred resistance against TYLCV in tobacco<sup>44</sup>, BGMV in common bean<sup>45, 46</sup>, and ACMV in cassava<sup>47</sup>. Transgenic expression of hpRNAs from the bidirectional promoter of ACMV in cassava<sup>48</sup> and TYLCV *CP* promoter in tomato<sup>49</sup> conferred resistance against the respective viruses. Silencing the suppressor protein by transgenic expression of hp*AC1* and hp*AC4* genes of ToLCV in tomato<sup>50</sup>, hp*AC4*<sup>51</sup> and hp*AC2* of MYMV<sup>52</sup> have proven to be a very effective strategies in conferring resistance.

Previous work on the model plant *Arabidopsis* in the PL's lab showed altered source-sink distributions of sucrose and the stress hormone abscisic acid (ABA)<sup>78</sup> interact to regulate anthocyanin accumulation via miR828, *Trans-Acting Small-interfering locus4 (TAS4)*, and their target MYeloBlastosis viral oncogene-like (v-MYB) transcription factors, viz. Vvi-MYBA6/7 and close homologues targeted by miR828 in grapevine<sup>79, 80</sup>. GRBV infections result in higher quantities of carbohydrates in symptomatic leaves<sup>28</sup>, suggesting deranged sugar signalling may play a role in the expression of red leaf symptoms. We recently characterized the conserved autoregulatory loop involving miR828 and *TAS4* down-regulates anthocyanin biosynthesis by targeting *MYB* transcription factors induced by UV light in grape<sup>81</sup>. The transcriptome profiling study of GRBV-infected host berries identified significant repression of rate-limiting ABA biosynthesis loci *NCED2/3* (first described by the PI<sup>82</sup>) in infected berries<sup>33</sup>.

Our working model is that GRBV infection interferes with the normal PTGS pathways of the host by the activity of viral-encoded suppressor proteins. The possibility exists that mixed infections of GLRaV, GRBV or other grapevine viruses like Pinot gris virus<sup>83</sup> and latent grapevine fleck virus (GFkV)<sup>84</sup> result in interactions in vectors or host causing synergistic effects and more severe damage/symptoms<sup>85-87</sup>. It is also speculated that apparent rapid spread could be driven by vector visual or olfactory cues taken from infected vines that translate to vector feeding preferences. miRNAs/tasi-RNAs/phasi-RNAs regulate a large array of host gene expression at the post-transcriptional level and transcriptional levels<sup>88</sup>. Viruses target plant miRNAs to facilitate pathogenesis, and plants have co-opted miRNAs for innate immunity<sup>89-92</sup>. Their collective changes in virus-infected and engineered transgenic tissues that results in susceptibility<sup>93, 94</sup> supports their functions as master regulators targeted by pathogens. Broader roles for plant siRNAs in evolutionary adaptations<sup>95, 96</sup> 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<sup>78, 81</sup> which fine tunes anthocyanin levels by a "rheostat" feedback<sup>81</sup>.



**Table 1.** Suppressor proteins characterized in geminiviruses and their plant targets.

<b>Virus*</b>	<b>Suppressor</b>	<b>Suppressing PTGS</b>	<b>Suppressing TGS</b>	<b>Cellular pathways</b>
MYMV	AC2	Upregulate host suppressor protein WEL1 <sup>53</sup>		
TGMV BCTV	AL2 L2	Inactivate Adenosine kinase <sup>54,55</sup> ; Upregulate rgs-CaM <sup>56</sup>		Inactivate a serine-threonine kinase SnRK1 <sup>57</sup>
BSCTV	C2	Stabilize S-adenosyl methionine decarboxylase1 (SAMDC1) <sup>58</sup>		
TGMV CaLCuV BCTV	AL2 AL2 L2		Inactivate Adenosine kinase; stabilize SAMDC1 <sup>59</sup> ; Inhibit histone Me-transferase SUVH4/KYP <sup>60, 61</sup>	
TGMV SCTV	AL2 C2			Elevation of cellular cytokinin levels <sup>62</sup>
TYLCSV	C2			Interact with CSN5 and inhibit jasmonate signaling <sup>63, 64</sup>
ICMV - SG	AC2		Upregulation of RAV2, transcription repressor <sup>65</sup>	
MYMIV	AC2	Interact with RDR6 and AGO1 <sup>66</sup>		
TYLCV	C2			Downregulate terpene synthesis <sup>67</sup> binds to ubiquitin <sup>68</sup>
ToLCTW V	C2			Suppress CMT3 expression <sup>69</sup>
ACMV	AC4	Binds ss miRNA <sup>70</sup>		
WDV	Rep	Binds ss-and duplexed 21 and 24 nt siRNAs <sup>71</sup>		
TYLCV	V2		Compete <i>NbMET1</i> for binding to histone deacetylase <sup>72</sup> ; SGS3 <sup>73</sup> ; AGO4 <sup>74</sup>	
CLCuMu V	V2		AGO4 <sup>75</sup>	
GGVA MMDaV	V2		Mechanisms not known <sup>76, 77</sup>	

\*Acronyms: *Mungbean yellow mosaic virus*: MYMV; *Tomato golden mosaic virus*: TGMV; *Beet curly top virus*: BCTV; *Beet severe curly top virus*: BSCTV; *Cabbage leaf curl virus*: CaLCuV; *Spinach curly top virus*: SCTV; *Tomato yellow leaf curl Sardina virus*: TYLCSV; *Tomato yellow leaf curl virus*: TYLCV; *African cassava mosaic virus*: ACMV; *Wheat dwarf virus*: WDV; *Mungbean yellow mosaic India virus*: MYMIV; *Indian cassava mosaic virus-Singapore*: ICMV -SG; *Cotton Leaf Curl Multan Virus*: CLCuMuV; *Grapevine geminivirus A*: GGVA; *Mulberry mosaic dwarf-associated virus*: MMDaV

**Table 2.** Analysis of publicly available transcriptome data<sup>^</sup> 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 <sup>PAL1</sup> ; 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

GRBV effects on host transcriptional profiles during berry development was reported<sup>33</sup>. **Table 2** provides preliminary evidence drawn from this publicly available berry transcriptome data which supports our model. As per our hypothesis a large (~Log2 fold-change ~ -1.46; beta = -1.01) downregulation of *Vvi-TAS4c* at veraison and post-veraison in GRBV-infected berries is seen, albeit not statistically significant (**Table 2**, tan highlights), suggesting the miR828-*TAS4*-*MYB* pathway could be a specific target of GRBV. This is supported by the strong up-regulation of *MYBA6* at harvest, the target of a deeply conserved *TAS4c* tasi-RNA 3'D4(-) along with several other *MYBs*<sup>80, 81</sup> shown to function in the phenylpropanoid/flavonol pathway and targeted by miR828. Interestingly, we observe significant up-regulation of *AGO2*, *DICER2*, and *SUPPRESSOR\_OF\_GENE\_SILENCING3* (*SGS3*) transcripts, all major effectors of the PTGS machinery required for viral resistance<sup>97, 98</sup>, and themselves subject to PTGS and spawning of amplified phasi-RNAs<sup>99-101</sup>. 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<sub>1</sub> mechanism" of transitivity<sup>102</sup> in play with *TAS4*-3'D4(-) and target *MYBA5/6/7* is novel, and its significance is not understood, unlike the known '21<sub>2</sub>' and '22<sub>1</sub> hit' mechanisms<sup>102</sup> of silencing amplification. We hypothesize a repression of silencing machinery upon virus infection, but the evidence to date 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.

## 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 microRNAs (miRNAs), *trans*-acting small interfering (tasi-) RNAs, phased-tasi-RNAs (phasi-RNAs), and effects on host target mRNAs by RNA-Seq and degradome analyses of (a) field samples, and (b) of 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 by *in vitro* and *in vivo* methods
4. a) Establish a transgenic grapevine test system and b) evaluate disease resistance to GRBV of transgenic grapevine expressing V2 and C2 hairpin silencers directed to suppressor protein transcripts

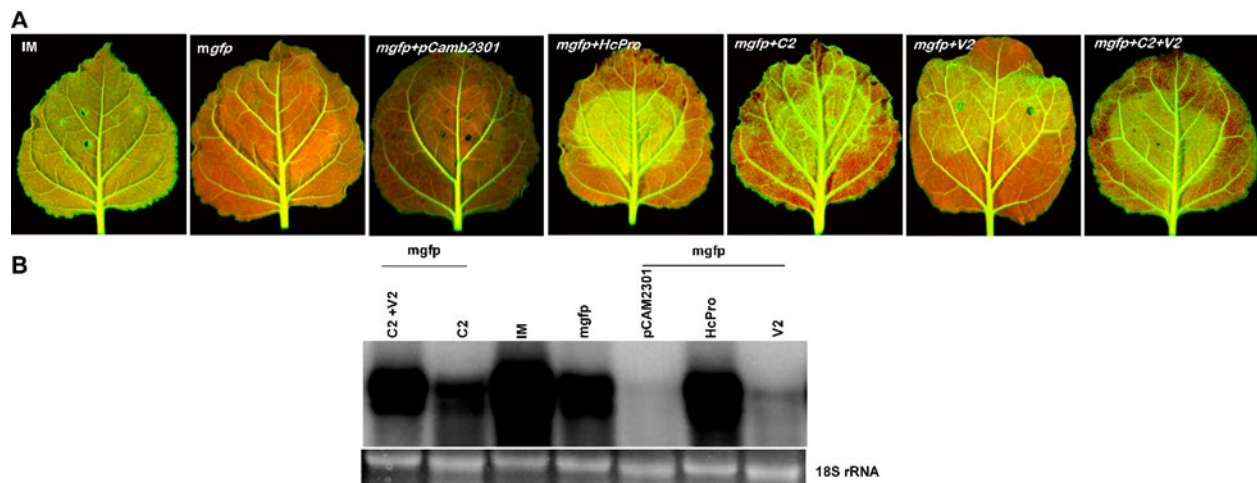
## RESULTS AND DISCUSSION

### Objective 1. Validate the Identified Candidate GRBV Suppressor Proteins C2 and V2

We proposed to characterize GRBV suppressor proteins in the initial phase of project funding. We cloned GRBV genes *V1*, *V3*, *C1* and *C3* with *HindIII/SacI* flanking sites, and *V2/C2* with *HindIII/EcoRI* sites from genomic DNAs of GRBV-infected grape leaf tissue collected in 2016 from 'Calle Contento' vineyard (cv. Merlot) in Temecula CA into the corresponding sites of pJIC-35S vector<sup>103</sup>. The pJIC-35S-ORF cassettes were subsequently cloned into the binary vector pCAMBIA2301 and electroporated into *A. tumefaciens* strain EHA105. To evaluate if GRBV possesses viral silencing suppressor proteins, *N. benthamiana* line 16c, developed in the laboratory of Sir David Baulcombe<sup>104</sup> constitutively expressing *A. victoria* jellyfish Green Fluorescence Protein (GFP) was used as the test system. In this system, RNA silencing of the *gfp* transgene can be triggered by transient expression of a *gfp(trigger)*-expressing vector. Consequently, the agroinfiltrated leaf will exhibit loss of GFP and manifest red auto-fluorescence from chlorophyll. When a silencing suppressor protein gene is co-infiltrated along with *gfp(trigger)*, the infiltrated zone will exhibit rescue of green fluorescence as marker of suppression of *GFP* RNAi silencing.

Six-week-old *N. benthamiana* 16c plants were agroinfiltrated with the *A. tumefaciens* strain harboring the p35S-*gfp* (pBI-mgfp5-ER; the 'trigger') and p35S-*gfp*+pCAMBIA-2301 with or without co-infiltration of test GRBV constructs. Potyvirus Helper component HcPro<sup>105</sup> construct co-infiltration served as positive control for silencing suppression. Five days post infiltration, local GFP silencing of infiltrated leaves was observed under long wave UV- or blue light sources as red auto-fluorescence from chlorophyll (**Figure 2A: mgfp; mgfp+pCAM2301**) which was confirmed by reduction of *gfp* transcript (**Figure 2B: mgfp; mgfp+pCAM2301**). To evaluate the silencing suppression effect of GRBV gene products, the 16c plants were agroinfiltrated with 1:1 test mixture of the *A. tumefaciens* strains harboring p35S-*gfp* (trigger) with p35S-V1, p35S-V2, p35S-V3, p35S-C1, p35S-C2, or p35S-C3, respectively. As expected, bright green fluorescence was observed in the infiltrated zones with *mgfp*+*HcPro* co-inoculation and suppression of PTGS

was confirmed by northern blot (**Figure 2A and 2B: *mgfp*+HcPro). GRBV C1, C3, V1 and V3 construct co-infiltration did not suppress the silencing triggered by *mgfp* (data not shown). In the presence of GRBV C2 and V2 expressions from co-infiltrated constructs, the infiltrated area was **not silenced** by the *gfp*(trigger)- the infiltration zones displayed green fluorescence (**Figure 2A: *mgfp*+C2; *mgfp*+V2) which was correlated by the *gfp* transcript accumulation (**Figure 2B: *mgfp*+C2; *mgfp*+V2). Thus, GRBV C2 and V2 proteins are identified as candidate suppressor proteins. This result has been repeated several times, providing compelling evidence for C2 and V2 functions as GRBV silencing suppressors (**Figure 2**). We also found evidence for additive effect of C2+V2 in suppressing silencing by co-expressing the two genes (**Figure 2A and 2B**). Although we observe conspicuous green fluorescence upon infiltration with *mgfp*+GRBV C2 and *mgfp*+GRBV V2 (**Figure 2A and 2B**), the northern results indicate the co-expression of C2+V2 together enhance the stability of *mgfp* transcript (**Figure 2B**). Mock-infiltrated (IM) sections manifested abundant *gfp* transcript compared to incomplete suppression of full length GFP transcript abundances by V2 (weaker effect) and C2 treatments alone (relative stronger suppression effect) (**Figure 2B**).******



**Figure 2.** Validation of silencing suppression by GRBV genes C2 and V2. **(A)** Agrobacterium-mediated transient expression in *N. benthamiana* leaves. **IM:** Mock-agroinfiltrated with infiltration medium; ***mgfp*:** Agroinfiltrated with the *A. tumefaciens* strain harboring p35S-gfp ‘trigger’ as control for GFP transgene silencing; ***mgfp*+pCAM2301:** Co-agroinfiltrated p35S-gfp+pCAMBIA2301 (negative control); ***mgfp*+HcPro:** Co-agroinfiltrated p35S-gfp + Potyvirus HcPro; ***mgfp*+C2:** Co-agroinfiltrated p35S-gfp + GRBV C2; ***mgfp*+V2:** Co-agroinfiltrated p35S-gfp + GRBV V2; ***mgfp*+C2+V2:** Co-agroinfiltrated p35S-gfp + GRBV C2+ GRBV V2. **(B)** Northern blot analysis with *gfp* probe. Total RNA from the infiltrated areas of mock infiltrated leaves (IM), p35S-gfp infiltrated leaves (*mgfp*), p35Sgfp+C2 (*mgfp*+C2), p35Sgfp+V2 (*mgfp*+V2), p35Sgfp+HcPro (*mgfp*+HcPro), and p35Sgfp+C2+V2 (*mgfp*+C2+V2) probed with *gfp*. The 18S rRNA portion of the ethidium bromide-stained gel is shown at bottom as control for RNA loadings. Red chlorophyll autofluorescence and green GFP fluorescence of leaves were visualized with blue light-emitting-diode source (Biorad ChemiDoc MP).

By performing the *Agrobacterium*-mediated transient expression of GRBV ORFs in *N. benthamiana* line 16c, we obtained strong evidences GRBV C2 and V2 genes encode GRBV silencing suppressors (**Figure 2**). Most of the subgrouped geminiviruses employ C2/L2/AL2 as a silencing suppressor, whereas a few (*Tomato yellow leaf curl virus*, *Grapevine geminivirus A*, and *Cotton Leaf Curl Multan Virus*) have limited evidences for V2 as a silencing suppressor (**Table 1**). However there are no significant DNA or protein sequence similarities found between GRBV C2 or V2 and the geminivirus suppressor gene sequences from **Table 1** (data not shown), consistent with the notions: that genes encoding suppressors of GRBV have evolved independently from other known geminiviral suppressor proteins. While the imaging and northern blot results were consistent with a hypothesized synergistic effect on silencing suppression (**Figure 2**), quantification of GFP fluorescence (**Figure 3**) from C2 and V2 co-infiltrated leaf zones support rather that C2 and V2 act independently to suppress GFP RNAi silencing. Quantitative analysis, despite the lack of replicates and some missing data, of unique siRNAs and mRNA reads mapping to the GFP gene in libraries made from these silencing suppression agroinfiltration experiments (**Table 3**) are conservatively consistent with results shown **Figure 3**; namely that the average silencing suppression by C2 and V2 alone and together, as measured by GFP siRNA reductions and mRNA accumulations is on par with positive control HCPro (or better) when V2 and C2 are expressed alone and co-expressed. Small RNA and RNA blot results can independently validate these preliminary results and be sufficient to prove the silencing suppressor functions of V2 and C2 going forward. It may be shown yet that V2 and C2 could interact in planta for either an additive or even synergistic effect on silencing suppression.

## **Objective 2. Elucidate by a Systems Approach the Molecular Mechanisms by Which GRBV Causes Symptoms**

We completed deep sequencing of field-collected GRBV-symptomatic and control healthy grapevine leaf sRNA libraries and mRNA transcriptome libraries. We were able to identify 13 test libraries and 17 control libraries from different locations, years, and cultivars that were collected based on clinical field symptoms of presence or absence of red leaf blotches from a total of 73 assayed samples (reported in 2020 CDFA-PD Symposium Proceedings). The vsiRNA accumulation was confirmed in the test libraries and the distribution of 20-24 nt siRNAs across the GRBV genome was plotted in the virion and complementary strand (**Figure 4**). Interestingly, the RNAseq data revealed a new splicing event involving V1 and V3 ORF with the removal of 337 nt intron seq (data not shown). We discovered mixed infections of GRBV with GLRaV strains 2 (NC\_007448.1, a ssRNA Closterovirus) and -3 (NC\_004667.1, a ssRNA Ampelovirus) in Santa Rosa and Temecula vineyard samples, respectively, collected based on visual screening for presence or absence of red blotch symptoms. Therefore we also explored our sRNA datasets for presence of reads to other emerging threat viruses Grapevine pinot gris (NC\_015782.2), fanleaf (NC\_003615.1, NC\_003623.1, NC\_003203.1), Grapevine viruses A and B (NC\_003604.2, NC\_003602.1), and fleck virus (NC\_003347.1) as well as latent viruses (MF185002.1, KF137564.1, KF137565.1, KC427107.1, ) and *Xylella fastidiosa* (AE009442.1), causal agent of Pierce's disease. The GLRaV-2 and -3 libraries are being characterized and will provide critical perspective on GRBV molecular mechanisms. GLRaV2 suppressor protein p24 has been characterized as able to bind RNA and affect miRNA activity by up-regulation of host

*AGO1* expression<sup>128</sup>, and GLRaV-3 p19.7 has been characterized as a silencing suppressor that decreases production of siRNAs, presumably prior to DCL-mediated cleavage of GFP reporter in *N. benthamiana* 16c transient assays<sup>129</sup>. Fleck virus was identified in most of the Jacksonville OR samples, but we did not observe a correlation between our GRBV sRNA DE results and presence of fleck virus (data not shown).

**Table 3.** Summary of average *GFP* silencing suppression by C2 and V2, quantified by sRNA-seq and RNA-seq deep sequencing of libraries made from *N. benthamiana* line 16c co-expression experiments shown in **Figures 2 and 3**.

Sample	assay % suppression		apparent average % suppression
	sRNA*	mRNA <sup>#</sup>	
HCPRO + mGFP positive control	42.1	15.1	29
C2 + mGFP	71 <sup>^</sup>	n.d.	
V2 + mGFP	63.1	0 <sup>&amp;</sup>	32
V2 + C2 + mGFP	81.8	27.1	54

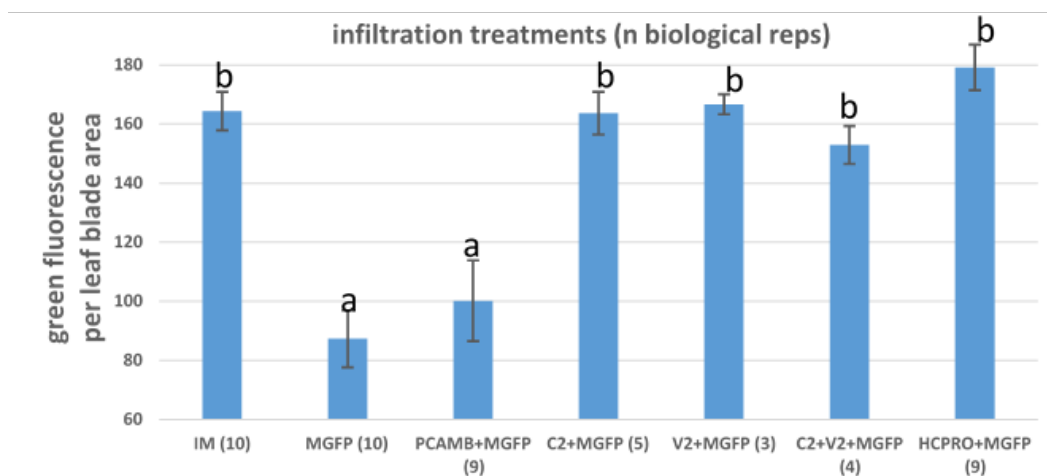
n.d.: not determined.

\* relative to control mgfp+pCAM normalized sRNAs as percentage of mRNAseq reads

<sup>#</sup> normalized GFP mRNA reads difference between test and silencing trigger controls average; dynamic range of silencing *per se* calculated as difference of silencing controls versus maximum GFP mRNA signal (control infiltration media alone= no silencing)

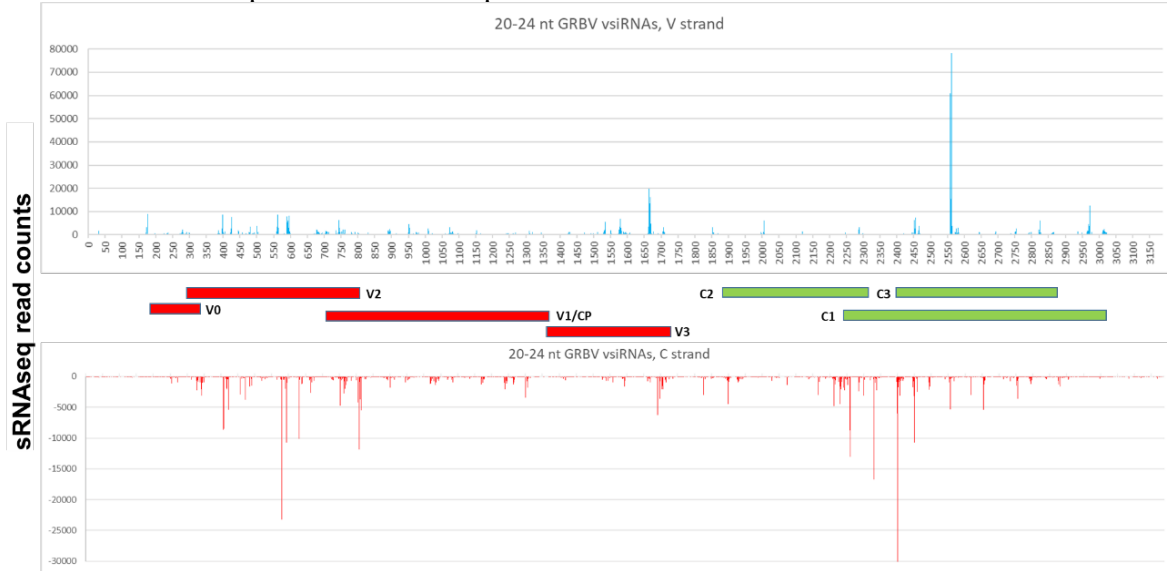
<sup>^</sup> estimated from trendline of other sRNA sample suppression, given no mRNA normalization factor

<sup>&</sup> test normalized reads lower than trigger controls; thus no evidence for suppression at mRNA level



**Figure 3.** Quantification of green fluorescence of agro-infiltrated zones on *Nicotiana benthamiana* 16c leaves. Different letters indicates significantly different between samples,  $p < 0.03$  (Student's *t* test, equal variance). Calculated Integrated density as described <https://theolb.readthedocs.io/en/latest/imaging/measuring->

[cell-fluorescence-using-imagej.html](#)) is the average of different photographic images taken for each particular test sample. Error bars are s.e.m.



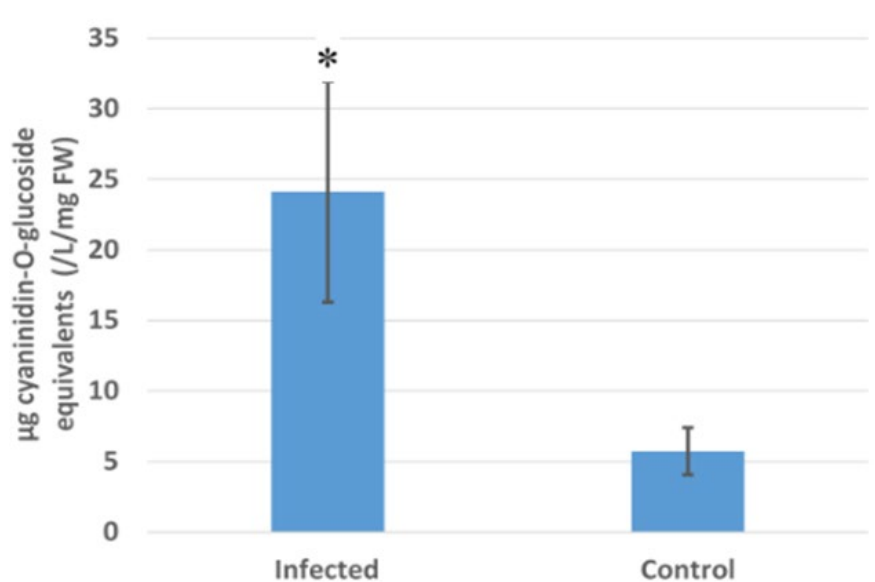
**Figure 4.** Genome-wide map of vsiRNAs from GRBV-infected grape libraries. The graph plots the number and strand specificity of 20–24 nt vsiRNA reads at each position of the GRBV genome.

Anthocyanin quantitation of 13 GRBV-infected samples showed a significant increase when compared to 16 control samples (**Figure 5**). We completed RNA-seq differential expression<sup>75</sup> (DE) analysis of eight GRBV-infected and seven uninfected leaf samples from Jacksonville, Oregon (2019) and of three GRBV-infected and five uninfected leaf samples (2019) from Santa Rosa, California. While 1,917 genes were significantly differentially regulated (983 up, 934 down) in tested Santa Rosa samples (eight biological test and seven control replicates), only 438 genes (300 up, 138 down; three test and five controls) were detected in Jacksonville Oregon samples ( $p_{\text{adj}} < 0.05$  Wilcoxon Rank Sum test, Benjamini-Hochberg adjusted)<sup>106, 107</sup>. Importantly, 160 genes were commonly and significantly up-regulated in both sets of field samples while 33 genes were reproducibly and significantly down-regulated (Pearson correlation  $R = 0.90$ ; 77% of Santa Rosa significant DE genes showed L2FC concordance with Jacksonville smaller sampling results; data not shown), thus making these common 193 loci a ‘short list’ of high-confidence GRBV differentially expressed host genes. Gene ontology analysis<sup>107</sup> of these datasets shows a Pearson correlation  $R = 0.88$  for significant up and down over-represented genes across 26 gene ontology bins. Photosynthesis and photorespiration genes were strongly down-regulated ( $p_{\text{adj}} < 10^{-20}$ ), consistent with GRBV symptoms of chlorosis<sup>106</sup>, as were protein biosynthesis and homeostasis ( $p_{\text{adj}} < 10^{-3}$ ), tetrapyrrole biosynthesis ( $p_{\text{adj}} < 0.03$ ) and redox homeostasis genes ( $p_{\text{adj}} < 0.002$ ) such as Cu/Zn superoxide dismutase, PEX3/piroxin peroxisome biogenesis effector (both targets of DE miRNAs; see below and **Table 4**), and chromatin remodeling effector<sup>123</sup> OXIDATIVE STRESS3/OXS3/*VIT\_09s0002g03340*. Of note is observed significant up-regulation of ‘stay green’ tetrapyrrole cofactor magnesium dechelatase *VIT\_02s0025g04660* and Pheophytinase/*VIT\_13s0158g00180* involved in chlorophyll degradation<sup>132</sup>, and biosynthetically concordant significant (for Santa Rosa dataset) down regulation of Mg-chelatase complex



components including GENOMES UNCOUPLED4/GUN4 involved in retrograde signalling to the nucleus, which we speculate may be affecting photosynthetic gene down-regulation directly, or indirectly by the GRBV-mediated miR2950 up-regulation (**Table 4**).

Secondary metabolism genes for chalcones/polyphenolics/flavonoids ( $p_{\text{adj}} < 10^{-11}$ ), cell wall synthesis/modification/degradation genes ( $p_{\text{adj}} < 10^{-11}$ ), a known response to virus infections affecting plasmodesmatal pore size<sup>159</sup>, and cell cycle/DNA replication genes ( $p_{\text{adj}} < 10^{-20}$ ) were strongly up-regulated in GRBV-infected leaves, like observed in cabbage leaf curl geminivirus-infected *Arabidopsis*<sup>154</sup>. Comparison of Gene Ontology over-representation analysis by PageMan<sup>107</sup> of our strong GRBV-infected Santa Rosa dataset with a publicly available geminivirus-infected *Arabidopsis* dataset<sup>154</sup> showed excellent concordance across 23 significantly over-expressed and under-expressed gene bins (Pearson of Z statistic  $R = 0.76$ ). This finding gives us confidence we are characterizing evolutionarily conserved mechanisms of geminivirus disease etiology.



**Figure 5.** Quantitation of anthocyanins in field samples used for sRNAseq differential expression analysis. Asterisk (\*) indicates significantly different than asymptomatic control samples,  $p < 0.04$  (Student's  $t$  test, equal variance). Error bars are s.e.m.,  $n = 8$ .

Genes involved in biotic stress response/disease resistance including numerous Tobacco Mosaic Virus Resistance N Receptors<sup>153</sup>/Toll/Interleukin-Receptor-like/TIR-NB-LRR domain family members (**Table 4**), cytoskeleton/microtubular network effectors ( $p_{\text{adj}} < 10^{-6}$ ), and Leucine-Rich-Repeat Kinase-IIIs ( $p_{\text{adj}} < 10^{-4}$ ) were found to be commonly up-regulated. Likewise up-regulated were abiotic stress ABA influx carrier<sup>126</sup> *AWPM19/VIT\_10s0003g02620* (2.13 L2FC,  $p_{\text{adj}} < 0.03$ ), ABA-inducible *HVA22C/VIT\_01s0026g02190* (2.98 L2FC,  $p_{\text{adj}} < 10^{-5}$ ) associated with plasmodesmata/plasma membrane<sup>127</sup>, and *PHOSPHATE-INDUCED1/VIT\_16s0022g01430* (2.34 L2FC,  $p_{\text{adj}} < 0.03$ ). On the other hand, *MLP43/VIT\_01s0011g05090* (-2.64 L2FC,  $p_{\text{adj}} <$



0.03) and *PYL8/VIT\_020025g01340* (-1.00 L2FC,  $p_{\text{adj}} < 0.03$ ) involved in ABA signaling<sup>125</sup> and several duplicated EF-hand containing *Calmodulin9-Like* genes were strongly down-regulated (data not shown). Two tetraspanins (TRN2 and TET3) and leucine-rich repeat genetic effector TRN1 associated with plasmodesmata<sup>124</sup> (thus related to virus intercellular movement) were misregulated, as were two pairs of duplicated *Sieve-Element Occlusion1-Related/SEOR* genes associated with phytoplasma movement<sup>122</sup> (**Table 4**). These links to viral host defense LRRs led to other supporting evidences drawn from co-expression analyses of unannotated genes: we observed significant down-regulation of rhodanese sulfurtransferase/N Receptor-Interacting Protein homologues (**Table 4**) which are very significantly down-regulated in geminivirus-infected Arabidopsis<sup>153, 154</sup>.

In addition another gene family strongly up-regulated but not annotated for gene ontology are known to be involved in plant viral replication; specifically Reticulon RTNLB9 family genes whose products interact with positive strand RNA viruses involved in forming the replication compartments of brome mosaic virus<sup>108</sup>. Annotated mis-regulated genes related to DNA replication (**Table 4**) were X-ray-induced DNA break repair/XRCC4, RPA70 DNA-binding subunit, and its binding partner Replication protein A-32/REPRESSOR OF ROS1/ROR1, a ssDNA-binding subunit of heterotrimeric complex involved in DNA replication, repair, and recombination. RPA32/ROR1 physically interacts with 5-methylcytosine DNA glycosylase/lyase REPRESSOR OF SILENCING1/ROS1, a repressor of RNA-dependent DNA methylation, antagonist of potyvirus HCPro silencing suppression<sup>118</sup>, and effector of flavanoid biosynthesis<sup>119</sup> and abiotic stress/pathogen resistance<sup>120</sup>. RPA32/ROR1 directly interacts with the C terminus of Mungbean yellow mosaic India geminivirus Rep protein (**Table 1**) to facilitate viral replication<sup>109</sup>. These observations are compelling support for a sRNA-mediated epigenetic model of GRBV host silencing. Concordant with this working hypothesis that GRBV disease etiology is mediated by host PTGS processes including miR828/TAS4/MYBA6/7 regulon, we find *TAS4ab* and targets *MYBA5/6/7* expressions were down-regulated albeit not statistically significantly, yet other known miR828 targets *MYB156/157* were significantly deranged in SantaRosa samples. Supporting our working model was the finding that effectors of miRNA biogenesis, activity, and virus DNA methylation (DICERs2/4, RDR6<sup>66</sup>, RDR1s, AGOs5/10, CMT1/3-2<sup>69</sup>, and DDM1<sup>155</sup>) were all significantly up-regulated in GRBV-infected samples from both Jacksonville and Santa Rosa fields (**Table 4**). Similarly, histone 2A variants involved in DNA transcriptional silencing<sup>161</sup> and damage repair<sup>160</sup> were strongly up-regulated whereas CpG reader Methyl-Binding-Domain10-11 was down regulated by GRBV infection.

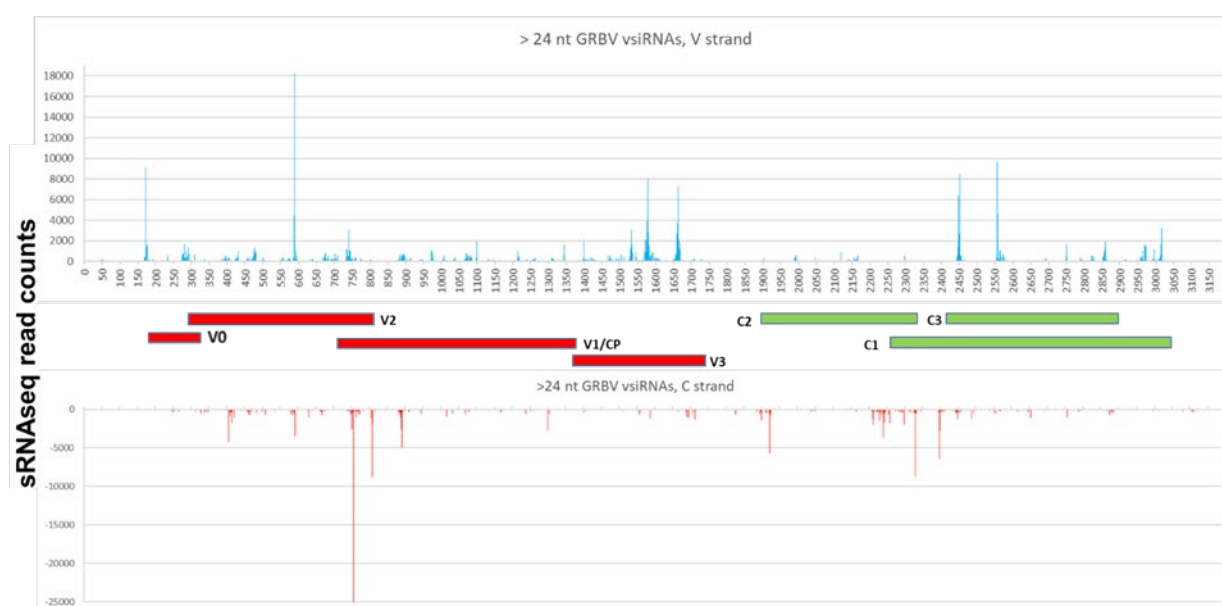
rgs-CaM is a well-characterized target of viral suppressor proteins namely TEV HcPro<sup>130</sup>, CMV 2b<sup>131</sup>, and TGMV AL2<sup>56</sup>. (**Table 1**). We observed four rgs-CaM/CaM-L37 homologs significantly up-regulated in GRBV infected field samples. rgs-CaM has recently been shown to function as an immune receptor by promoting hypersensitive responses such as  $\text{Ca}^{2+}$  fluxes, production of reactive oxygen species, and salicylic acid-mediated degradation of Cucumber mosaic virus suppressor 2b by autophagy<sup>131</sup>. rgsCaM prevents TEV HcPro and Cucumber mosaic virus suppressor 2b from binding to dsRNAs/siRNAs and reduces the suppressor protein stability by autophagy, resulting in a more potent RNAi defense against viral infection<sup>5</sup>. rgsCaM over-expressing lines were less susceptible to the virus<sup>136</sup>. Interestingly, TGMV AC2 induces a calmodulin-like protein Nb-rgsCaM<sup>56</sup> and over-expression of rgsCaM leads to an increase in viral DNA load. rgsCaM self-interaction was observed in cytoplasm while interaction with

TGMV AC2 sequestered rgsCaM to the nucleus. It was speculated that AC2-mediated localization of rgsCaM to the nucleus is the likely mechanism evolved by TGMV to evade degradation of AC2 by autophagy and thereby effectively suppress the plant defense mechanism<sup>5</sup>. A similar mechanism adapted by GRBV to evade rgsCaM-mediated autophagy cannot be discounted; four rgs-CaM homologs were significantly deranged (three up, one down) in GRBV-infected field samples (**Table 4**).

BSCTV C2 protein physically interacts with and stimulates host activity of S-adenosyl methionine (SAM) decarboxylase (SAMDC) to suppress SAM-mediated de novo methylation of viral DNA in Arabidopsis<sup>58</sup> (**Table 1**). A viral-derived siRNA (vsiRNA) was elucidated as the pathogenicity determinant in TYLCV-infected tomato where it targets by near-perfect complementarity a host long non-coding RNA involved in development<sup>137</sup>. Recently a long-sought functional connection between RNA-dependent DNA Methylation (RdDM) and antiviral defense was established by the finding that the subnuclear Cajal body is the site of methylation of TYLCV DNA by physical interaction of host AGO4 with virus V2 protein, which blocks binding of AGO4 to viral RNA and DNA<sup>74</sup>. We detected accumulation of >24 nt vsiRNAs throughout the genome in our test libraries (**Figure 6**). Dicer-independent siRNAs interacting with AGO4 and directing RDDM is known<sup>163</sup>. (Ye et al., 2016). Whether a similar mechanism operates to methylate GRBV genome is unclear but we will study with purpose the abundances of this class of PolIV/V-directed species as a lead for discovery. Another evidence for vsiRNAs as pathogenicity determinants is that RNA virus Cucumber Mosaic Virus satellite Y produces a 22nt vsiRNA targeting protoporphyrin Mg-chelatase in tobacco to impair chlorophyll biosynthesis<sup>138, 139</sup>. We characterized the GRBV field samples by deep sequencing small RNA libraries to identify miRNAs/siRNAs that are differentially expressed and hypothesized to act as pathogenicity determinants of GRBV disease.

Differential expression analysis of sRNAs quantified by ShortStack<sup>110</sup> for 29 libraries not confounded by known effectors of anthocyanin disease symptoms, specifically fanleaf virus, GLRaV, or *Xylella fastidiosa* (causal agent of Pierce's Disease), was performed by DESeq2<sup>111</sup>. We obtained 241 differentially expressed sRNAs and miRNAs (141 up, 100 down) after multiple-testing Bonferroni-Hochberg adjusted  $p_{adj} < 0.05$  for statistical significance (summarized in **Table 4**). Our data-driven approach to discovery of GRBV pathogenicity and/or symptom determinants by quantifying host sRNAs and mRNAs by deep sequencing is compelling, based on tantalizing concordant and complementary literature for host-pathogen interactions and chlorosis symptoms linked to tetrapyrrole biosynthesis effector down-regulations. Inverse/anti-concordant relationships were observed between DE miR2950 (LFC 2.94;  $p_{adj} < 10^{-5}$ ) and strong down-regulation at mRNA level of its independently validated Chlorophyllase targets *VIT\_07s0151g00110*<sup>112, 113</sup> and paralogs (**Table 4**). An inverse relationship between miR2950 and this predicted target gene was found exclusively in grapevine virus B (GVB)-infected plants<sup>141</sup>. Similar inverse relationships were observed between miR398, miR3632/482-L, miR399, miR395, miR408, and miR3624a,b and their known validated canonical and few novel<sup>81</sup> targets (**Table 4**) involved in calcium, sulfur, and phosphorus homeostasis, and reactive oxygen stress and pathogen responses. The evidence supports that miR398 strong up-regulation and miR395 down-regulations associated with GRBV infection caused targets Cu/Zn chaperone/*VIT\_02s0025g04830*, Plastocyanin/*VIT\_11s0016g05520* to be significantly down-regulated, and low affinity miR395 target Sulfate

transporter/SULTR2;1/*VIT\_18s0001g04890* to be significantly up-regulated in infected tissues (in higher quality Santa Rosa dataset), consequently in the opposite direction of the observed miRNA effector changes (**Table 4**). miR2950 and miR398 of cotton have been claimed to target the genome of monopartite geminivirus Cotton leaf curl Multan virus<sup>142</sup>, whereas overexpression of MIR2950 and MIR398 conferred resistance to the virus<sup>143</sup>. Our independent degradome analysis did not find any evidence for these or other grape miRNAs to target GRBV genome (data not shown), thus the role of the above miRNAs is likely limited to host targets in GRBV-infected plants. We did identify by PhaseTank analysis<sup>152</sup> a candidate trigger miR7122 for target *TAS-14s0081g00100 D16(+)*, which in turn targets a BURP domain-containing RD22 PHAS locus and potentially several homologs (**Figure 7**) and their respective established or predicted target effectors (**Table 4**).



**Figure 6.** Genome-wide map of vsiRNAs larger than 24 nt from GRBV-infected grape libraries. The graph plots the number and strand specificity of >24 nt vsiRNA reads at each position of the GRBV genome.

A previous study on Mexican fruit fly, *Anastrepha ludens* (Diptera: Tephritidae) by Robacker et al.<sup>134</sup> indicated Me-anthranilate is an essential insect pest attractant. Me-anthranilate is volatile and has a fruity/musky smell used in the food and perfume industries, and more importantly is known to attract insect vectors<sup>140</sup>. Consistent with this possibility is the observed significant up-regulation in GRBV-infected Santa Rosa samples of MeOH anthraniloyltransferase/*VIT\_02s0033g01030* (L2FC 5.02,  $p_{\text{adj}} = 0.02$ ) and concordant up-regulation of four pectin methylesterase/PME genes that metabolize walls to produce MeOH, the substrate for anthraniloyltransferase<sup>135</sup> (**Table 4**). PMEs are involved in the systemic spread of numerous viruses by modifying plasmodesmata<sup>156, 157, 159</sup> and can stimulate virus-induced gene silencing<sup>158</sup>. We also observed down regulation of an anthraniloyl transferase homologue

predicted to be targeted by a GRBV-derived *C3mm(-)* siRNA (**Table 4**), which is intriguing. We speculate the 'foxy' aroma and flavor coming from methyl anthranilate can serve as olfactory cues, along with the anthocyanin accumulation as potential visual cues to attract arthropod vectors as contributing factors to observed rapid spread of the GRBV in vineyards. Sequencing of degradome libraries made from GRBV-infected leaves is in process, and it is anticipated we can demonstrate slicing of host novel targets by GRBV vsRNAs (and compare with GLRaV-2 and -3 claims from our datasets) as the next advance of the project. Our data-driven approach to discovery of GRBV pathogenicity and/or symptom determinants by quantifying host mRNAs, miRNAs, and vsRNAs by deep sequencing is producing tantalizing evidences concordant with GRBV etiology/symptoms and complementary to the literature for host-pathogen interactions. For example we have identified other predicted targets of GRBV vsRNAs, several of which transcripts are down-regulated significantly in field samples and have functional relevance to chlorosis symptoms, e.g. photosynthetic gene *PSBQ* (**Table 4**).

**Table 4.** Correlations of significant miRNA differential expressions from sRNA-seq of symptomatic field samples, with observed differential expression of validated targets<sup>81</sup> in asymptomatic 2018 Jacksonville, OR field samples, and separate mRNAseq of symptomatic 2019 Santa Rosa (SR) samples. Anti-concordant mRNA target target expressions in **bold**.

miRNA/TAS	RPM	L2FC	padj	Target Mean Exprs	Target L2FC OR/SR	pval	Validated Target locus
GRBV siRNAs	5,062	10.21	10 <sup>-53</sup>	4,024	11.6/ 9.90	10 <sup>-86</sup> / 10 <sup>-32</sup>	See below for hypothesized effectors
miR2950	6.6	<b>2.94</b>	10 <sup>-5</sup>	3.5 1.0 3.4	<b>-0.77/ -3.80</b> <b>-0.68/ -3.64</b> <b>-1.30/ -2.18</b>	0.32/ 0.03 0.78/ 0.15 0.06/ 0.09	Chlorophyllases <sup>†</sup> Fboxes <sup>†</sup> 07s0151g00110 <sup>112, 113</sup> 07s0151g00250 07s0151g00190
miR3632/482-L	10.3	1.36	0.03	122	<b>-0.27/ 0.42</b>	0.16/ 0.28	PRF Disease Resistance/ 13s0067g00790
miR482 tasi-trigger D12(-) target				56 40	<b>-0.28/ 0.69</b> 1.53/ 1.99 <sup>¶</sup>	0.40/ 0.04 .00002/ .00003	NBS-LRR/18s0072g01090 RPS4/TAS5-like 18s0001g06340 <sup>81</sup>
miR398c	10.1	<b>2.48</b>	0.03	317 339 92 202	<b>-0.74/ -1.15</b> <b>-0.23/ -0.94</b> <b>-0.83/ -0.54</b> <b>-0.53/ -0.21</b>	0.01/ 10 <sup>-7</sup> 0.23/ 0.0002 0.02/ 0.21 0.02/ 0.44	Cu/Zn chaperone <sup>81</sup> 02s0025g04830 Plastocyanin/11s0016g05520 CSD1/14s0030g00830 COXVb/09s0002g06000 <sup>81</sup>
miR399i	4.0	2.48	0.04	0.1	0.59/ <b>-0.49</b>	0.64/ 0.89	PHT1 3/13s0067g03280
miR395g^	3.1	-6.22	0.05	112 370	-0.74 <sup>¶</sup> / <b>0.61</b> -0.01/ 0.01	0.03/ 0.04 0.97/ 0.98	SULTR2;1/AST68 <sup>81</sup> / 18s0001g04890 APS1/05s0020g04210 <sup>81</sup>
miR3627	27.4	1.87	0.06	439	0.41/ 0.17 <sup>¶</sup>	0.03/ 0.42	ACA10/11s0052g00320
miR3624a,b	2.6	2.80	0.07	6.7	<b>-0.21/ 0.64</b>	0.82/ 0.35	Metal bind Pro-rich/ 00s0194g00330
miR408	26.6	1.48	0.13 <i>P= .01</i>	5.2 9.0	0.23/ <b>-2.12</b> <b>-0.22/ -1.57</b>	0.70/ 0.01 0.69/ 0.08	DUF724/07s0005g02720 <sup>81</sup> Piroxin3/17s0000g02820 <sup>81</sup>
miR403a	4.5	-2.74	0.07§	244 22.5	-0.17/ <b>0.58</b> <b>0.18/ 0.87</b>	0.39/ 0.07 0.62/ 0.14	AGO2/10s0042g01150 10s0042g01180
miR7122, TAS	2.4	1.49	0.11§	13.3	<b>-1.02/ -1.06</b>	0.11/ 0.06	RD22d, PHAS 04s0008g04000

14s0081g00100D16(+) RD22 trigger, <i>TAS</i> 3'D9(-) marker	46.5	0.50	0.28§	3160	<b>-0.94/ -0.26</b>	0.004/ 0.36	04s0008g03930
GRBV C3mm(-)	1.6			3.3	<b>-0.07/ -0.53</b>	0.92/ 0.55	Me-anthraniloyl T-ase 02s0033g01070(Allen3.5)
GRBV V1-mm	1.1			225	0.19/ 0.02	0.52/ 0.96	Anthocyan permase 16s0050g00930(Allen4.0)
GRBV C2mm(-)	0.9			450	0.10/ -0.03	0.76/ 0.93	SGS3, PHAS locus 07s0130g00190(Allen4.5)
GRBV V3-mm	0.2			12.8	<b>-1.08/ 0.04</b>	0.02/ 0.95	Ankyrin 05s0029g01430 (Allen = 2.5)
GRBV V1	0.2			1620	-0.004/ 0.42	0.98/ 0.08	NADP-malic enzyme 11s0016g03210(Allen3.5)
GRBV C3(-)	0.15			2698	<b>-0.96/ -1.80</b>	0.005/ 10 <sup>-6</sup>	Oxygen-evol PSBQ 00s0904g00010(Allen4.0)
GRBV V1	0.16			17.9	<b>-0.42/ 0.30</b>	0.22/ 0.54	CHS isomerase3 19s0014g00100(Allen4.0)
GRBV V3-mm	0.15			239	<b>-0.74/ -0.04</b>	0.02/ 0.91	Ca <sup>2+</sup> sensor 17s0000g04490 (Allen = 4.0)
<b>Known/hypothesized targets/effectors of viral silencing suppressors</b>							<b>Function/VIT gene</b>
miR828 targets PHAS 17s0000g08480/MYB157 14s0066g01220/MYB156	0.4* t.b.d.	0.1	0.95	10 3.8	-0.14/2.93 -0.45/-1.76	0.92/ 0.0001 0.58/ 0.05	Anthocyanin biosynth? Anthocyanin biosynth?
<i>TAS4a</i>	3,708	0.76	0.21	3.0	-0.05/ -0.15	0.95/ 0.90	Anthocyanin repress
<i>TAS4b</i>	300	-0.55	0.24	10	0.3/ -1.1	0.64/ 0.15	Anthocyanin repress
<i>TAS4c</i>	35.6	-2.31	0.11	n.d.			Anthocyanin repress
MYBA6, <i>TAS4</i> target	1.3	-0.52	0.61	0.5	-0.1/ -0.56	0.99/ 0.81	Anthocyanin biosynthesis
MYBA7, <i>TAS4</i> target	0.9	n.a.		0.7	-0.34/ -2.4	0.91/ 0.41	Anthocyanin biosynthesis
MYBA5, <i>TAS4</i> target				0.3	-0.51/ -0.02	0.86/ 0.99	Anthocyanin biosynthesis
1-deoxy-D-xylulose-5- phosphatesynthaseDXS				52	-1.24/-1.72	0.002/0.00001	isopren biosynth; binds HCPPro <sup>133</sup> 00s0218g00110
SAMDCs 01s0010g00990 11s0037g00950				494 88	-0.40/0.94 0.44/ 2.2	0.008/ 0.002 0.39/ .00001	viral methylation <sup>58</sup>
Pectin methylesterases 12s0035g01900 16s0098g01900 09s0002g00320 14s0060g01960				69.4 68.4 16.2 13.2	2.67/ 2.34 1.56/ 1.51 3.29/ 4.61 3.70/ 4.83	10 <sup>-7</sup> / 10 <sup>-6</sup> 0.005/ 0.02 10 <sup>-6</sup> / 10 <sup>-8</sup> 10 <sup>-10</sup> / 10 <sup>-7</sup>	Me-anthranilate biosynth <sup>135</sup> ; vector attractant <sup>134</sup> ; viral silencing <sup>158</sup> ; promote viral movement <sup>156-159</sup>
Mg chelatasers				587 4884	-0.58/-0.90 -0.63/-0.57	0.08/ 0.04 0.14/ 0.06	05s0102g00310/GUN4 08s0007g08540
Mg dechelatase Pheophytinase				658 827	-0.04/1.37 0.47/ 1.51	0.87/ 0.003 0.13/ 0.0001	02s0025g04660 13s0158g00180
rgs-CaM-L41-like <sup>56</sup>				114	0.32/2.95	0.24/ .00001	18s0001g11830
rgs-CaM-L5-like				7.4	2.06/2.52	0.001/ 0.001	05s0102g00450
rgs-CaM-L25-like				403	-1/13/ -1.44	0.001/ 10 <sup>-7</sup>	18s0001g03880
rgs-CaM-L18-like				6.7	0.82/ 1.44	0.07/ 0.02	16s0039g01880
rgs-CaM-L23-like				55.8	-0.14/ 0.48	0.67/ 0.37	04s0006g01400
XRCC4/18s0041g00370				1601	-1.22/ -2.16	0.001/ 10 <sup>-24</sup>	DNA repair, recomb.
TRN2/02s0012g01410 TET3/06s0004g03390 TRN119s0090g01650				30 502 49	3.14/ 4.41 -0.15/ -0.70 1.82/ 2.23	10 <sup>-9</sup> / 10 <sup>-12</sup> 0.53/ 0.01 0.0002/ 10 <sup>-9</sup>	Plasmodesmata- associated <sup>121,124</sup>
SEOR1/01s0137g00390 01s0137g00400 14s0066g00130				17.5 6.0 8.4	2.93/ 4.76 2.70/ 3.56 2.58/ 4.17	10 <sup>-5</sup> / 10 <sup>-9</sup> 0.0003/0.0003	Sieve element occlusion <sup>122</sup>

14s0066g00120				142	2.42/ 5.30	0.0002/ 10 <sup>-5</sup> 0.0001/ 10 <sup>-23</sup>	
OXS3/09s0002g03340				27.3	-0.98/ -3.81	0.04/ 10 <sup>-9</sup>	Chromatin remodelling <sup>123</sup>
DCL2/04s0023g00920	t.b.d.			251	0.29/1.26	0.31/ 0.00001	Viral siRNA biosynth
DCL4/11s0149g00120				138	0.58/ 0.88	0.03/ 0.002	Viral siRNA biosynth
RDR6/ 04s0008g05430				104	-0.20/ 0.84	0.46/ 0.002	Viral siRNA biosynth <sup>66</sup>
RDR1/ 01s0011g05880				518	0.27/ 0.94	0.30/ 0.001	Viral siRNA biosynth
RDR1/ 01s0011g05870				10.6	0.91/ 1.51	0.05/ 0.009	
AGO5-L/ 06s0061g01040				157	0.75/ 0.73	0.01/ 0.01	Viral siRNA activity
AGO10/ 05s0020g04190				9.2	0.92/ 1.16	0.09/ 0.11	Viral siRNA activity
CMT3-2/ 06s0004g01080				3.7	2.27/ 3.07	0.001/ 0.007	Viral replication <sup>69</sup>
CMT1/08s0007g06800				9.7	1.12/ 0.81	0.02/ 0.22	
DDM1/ 04s0023g01610				6.0	1.89/ 1.53	0.004/ 0.03	Viral DNA silencing <sup>155</sup>
H2A.Z/00s0179g00340				2.4	3.48/ 4.57	10 <sup>-4</sup> / 0.001	DNA silencing <sup>155, 161</sup>
H2A.X/07s0104g00960				4.1	2.14/ 4.64	0.001/ 10 <sup>-4</sup>	DNA damage repair <sup>160</sup>
MBD10/09s0002g0680				1015	-0.16/ -1.54	0.72/ 0.001	Binds m-CpG; silencing
RTNLB9/01s0011g0403				66	4.47/ 6.37	10 <sup>-14</sup> / 10 <sup>-20</sup>	Viral replication <sup>108</sup>
17s0000g03260				3.1	1.59/ 2.82	0.11/ 0.005	
12s0028g03190				2.1	2.12/ 3.12	0.05/ 0.009	
RPA32/ROR1 04s0008g02570				2.4	3.02/ 3.40	0.0006/ 0.02	Viral replication <sup>109</sup>
RPA70/17s0000g07440				11.1	3.45/ 3.31	10 <sup>-6</sup> / 10 <sup>-5</sup>	Viral replication <sup>109</sup>
TMV-N Recptr TIR-LRRs 18s0089g00050 18s0089g00040 18s0089g00080 18s0089g00090				3.2 5.1 24.4 30.4	1.79/ 4.98 1.82/ 2.86 1.23/ 2.56 1.35/ 2.42	0.03/ 0.0003 0.03/ 0.002 0.02/ 10 <sup>-5</sup> 0.02/ 0.001	Host defense <sup>153</sup>
Rhodanese sulfur-Tases 08s0007g06200 00s0684g00030 17s0000g09620 08s0105g00310				137 436 56.3 49.3	-0.77/ -1.05 -1.14/ -1.02 -0.67/ -0.93 -0.36/ -1.90	0.009/ 10 <sup>-4</sup> 0.009/0.005 0.02/ 0.01 0.35/ 10 <sup>-4</sup>	Viral replication <sup>153, 154</sup>
n.d.: not detected; n.a.: not analyzed; *: miR828star species only detected. §not Bonferroni adjusted. † F-box targets 18s0072g00820, 19s0014g01860 not yet validated; Allen scores 4.5, 3.0 resp. CHLs Allen= 4.0 ‡ concordant expression with miRNA effector LFC ^ other family members have star species dominant							

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 binary constructs (Objective 1 findings) a transgenic tobacco line that overexpresses the Arabidopsis target of *TAS4* siRNA: *AtMYB90/PRODUCTION\_OF-ANTHOCYANIN\_PIGMENT2*<sup>114</sup>. Towards this we established axenic tissue-cultured control, hemizygous and homozygous transgenic plants that have been super-transformed with empty binary vector -pCAMBIA2301 (control) or with binary vector harboring the GRBV ORFs C2/V2 (pCAM-C2/ pCAM-V2). The leaf discs transformed were selected on shooting media containing cefotaxime 250 mg/L and kanamycin 100 mg/L. The regenerated shoots have been established on rooting media (**Figure 8**; representative picture of pCAM-C2 super-transformant).



**Figure 8.** Potted heterozygous AtMYB90 tobacco plant super-transformed with pCAM-C2.

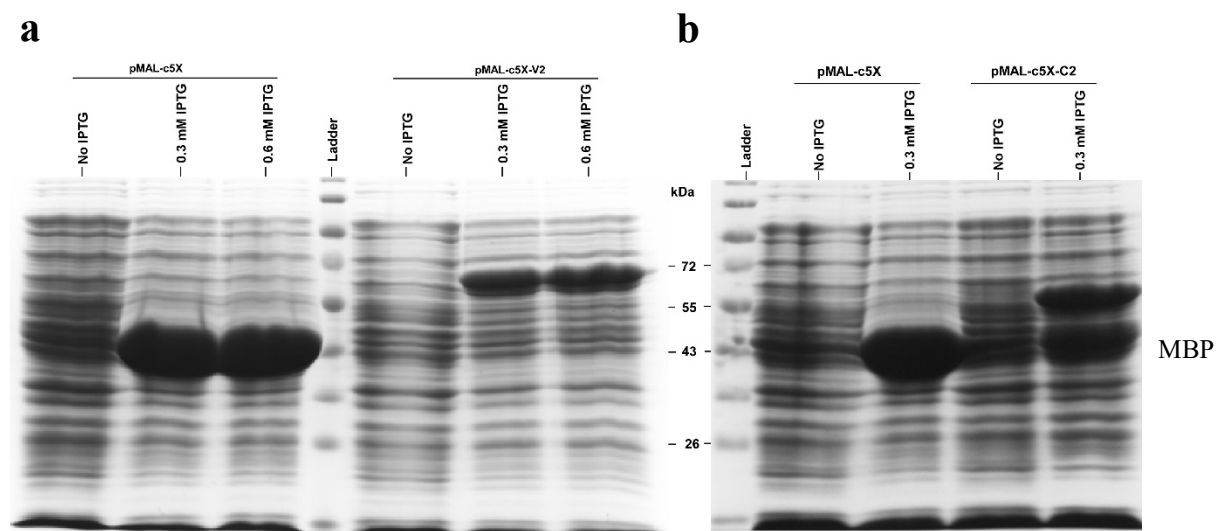
**Objective 3. Identify the host grapevine targets of GRBV suppressor proteins C2 and V2 by *in vitro* and *in vivo* methods.**

To elucidate if the mechanism of silencing suppression is by binding miRNA/siRNA as is known for other viral silencing suppressor proteins (e.g. AC4 of ACMV<sup>70</sup> and HcPro of Potyvirus), we have proposed to purify the suppressor proteins C2 and V2 using pMAL<sup>™</sup> Protein Fusion & Purification System (New England Biolabs). Towards this we have PCR-amplified the GRBV C2 and V2 genes as blunt end fragments in the 5' end and with *Sbf*I restriction site in the 3' end which were cloned into the pMAL-c5X vector digested with *Xmn*I and *Sbf*I. The clones were confirmed by restriction digestion (data not shown) and Sanger sequencing. We re-transformed the clones into *E. coli* strain ER2523 (NEB Express) for protein expression. As a pilot experiment, cells were grown to 0.5 OD at 37°C, induced with 0.3 mM IPTG for four hours. The cells were re-suspended and protein extracts run on an SDS-PAGE gel. The maltose binding protein (MBP) was observed at 42.5 kDa upon inducing cells transformed with pMAL empty vector (**Figures 9a and 9b**). The pMAL-V2 fusion protein was observed at 61.5 kDa (**Figure 9a**) and pMAL-C2 protein at 59.4 kDa (**Figure 9b**) as expected since the calculated size of C2 is 16.8 kDa. However, in addition to the pMAL-C2 fusion protein, MBP (a highly stable protein) was also observed at 42.5 kDa in pMAL-C2 induced cells (**Figure 9b**). This is likely due to proteolysis of the fusion protein.

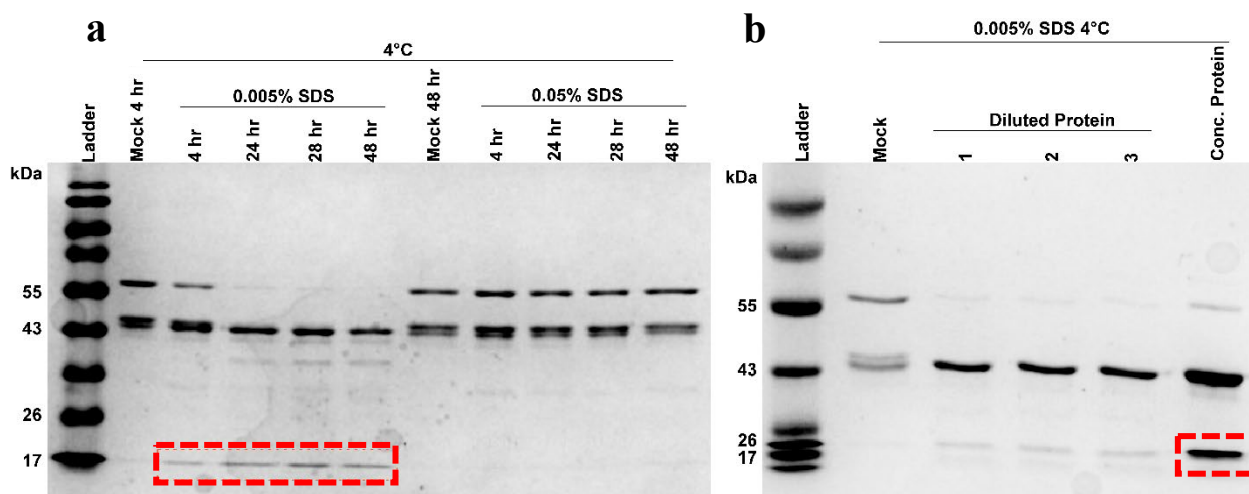
To overcome this limitation, we retransformed pMAL-C2 vector in a protease-deficient strain (T7 Shuffle). The cells were grown at 37°C, induced with 0.3 mM IPTG for four hours and checked for induction using SDS-PAGE. We again observed proteolysis of induced C2 fusion protein (data not shown).

To reduce the observed proteolysis we induced pMAL-C2 in T7-shuttle and in NEB Express cells at 18°C for 18 hours with 0.3 mM IPTG. Proteolysis of induced protein was observed in

T7-shuttle cells as well as in NEB-Express cells (data not shown). However, the proteolysis was less in NEB-Express in comparison to T7-shuttle cells. Hence, pMAL-C2 in NEB-Express was induced with 0.1 mM IPTG at 18°C for 18 hours. The proteolysis of C2 fusion protein was reduced and hence large scale induction of pMAL-C2 was performed using the above condition.



**Figure 9.** pMAL protein expression **a)** pMAL-c5x-V2 **b)** pMAL-c5x-C2. MBP mW is 42.5 kDa; V2 calculated mW19.2 kDa; C2 calculated mW is 16.8 kDa.



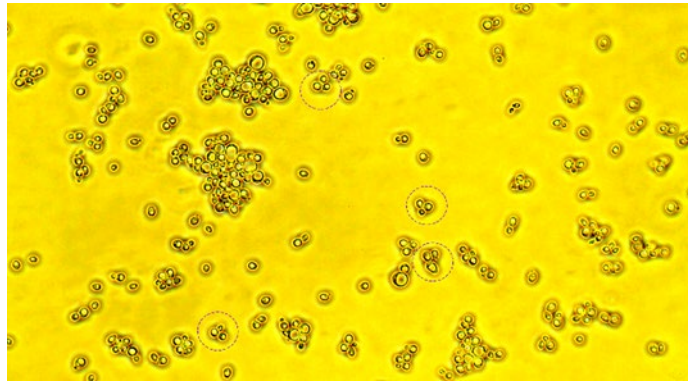
**Figure 10.** Factor Xa Cleavage of MBP:C2 fusion protein. **a)** pMAL-c5x-C2 MBP cleavage optimization **b)** Purified pMAL-c5x-C2 protein MBP tag cleavage confirmation. Red dotted box: C2 protein, calculated mW=16.8 kDa.



The large scale induced MBP-C2 protein was purified using amylose resin. The purified protein was dialysed to remove reductant  $\beta$ -mercaptoethanol. A pilot experiment with 20  $\mu$ L of purified protein and 200  $\mu$ g/mL of Factor Xa was done at room temp to standardize cleavage of fusion protein. We could not observe any cleavage when incubated in room temp and sampled at four time points namely 4 hr, 24 hr, 28 hr and 48 hr (data not shown). We also tested cleavage of fusion protein at 28°C, 37°C and 4°C at different time points without any success. To increase accessibility of the cleavage site of fusion protein, three different concentrations of sodium dodecyl sulfate/SDS (0.01%, 0.05% and 0.005%) or 6M Guanidine hydrochloride (GuHCl) was added and incubated with Factor Xa at room temp, 28°C, 37°C and 4°C. Varying concentrations of SDS or GuHCl did not facilitate cleavage of fusion protein tested at room temp, 28°C or at 37°C. While fusion protein cleavage was not observed at 4°C with 6M GuHCl or with 0.01% SDS (data not shown) or 0.05% SDS (**Figure 10a**), complete cleavage of fusion protein was observed in 0.005% SDS treated protein in 48 hrs at 4°C (**Figure 10a**). We used the conditions from pilot experiment to cleave the MBP tag from large scale purified fusion protein extracts (**Figure 10b**). The cleaved protein was dialysed against 20 mM Tris-HCl, 25 mM NaCl, pH 8.0 buffer and then passed through strong anion exchange column HiTrap Q FF. A gradient of 25 mM NaCl to 500 mM NaCl in 20 mM Tris-HCl, pH 8.0 (25 mL each) was used to elute the protein based on electrostatic competition with NaCl and 1 mL fractions were collected. We confirmed MBP tag eluted in 125 mM NaCl fraction (Factor Xa elutes at about 400 mM NaCl). We will perform large scale purification of V2 protein. We could not detect C2 protein in any of the fractions eluted, or in the flow through void volume. The likely cause is that our protein of interest is trapped in the column, a known phenomenon for proteins with an extended isoelectric region, according to vendor-documented guidelines and the literature<sup>162</sup>. As part of troubleshooting, we calculated the isoelectric point (pI, neutral net surface charge) of GRBV C2 using ExPASy pI software to be pH 8.4, which is near pH of the elution buffer (V2 pI is pH 5.0; MBP pI is pH 5.1; factor Xa pI is pH 5.7/mW 54.7 kDa). Thus the highly basic C2 protein may have an extended isoelectric region and not interact typically with the strong Q anion column under standard elution conditions. After consultation with vendor technical experts, we plan to modify the pMAL protein purification of C2 using a cation exchange column instead, since the proscribed pH 8.0 elution buffer is at the upper limit for preventing protein denaturation and aggregation. The C2 protein should bind and elute from a cation exchange column at neutral pH 7.2, conditions which safeguard against C2 protein denaturation/aggregation. The purified C2 and V2 proteins will be used for ss- and dsRNA *in vitro* binding assays as the next step. The large-scale production of purified C2 and V2 will open the prospects in the future to contract fee-for-service production of polyclonal antibodies against V2 and C2. For example the anti-C2/V2 antibodies could prove useful for RNA-Immuno-Precipitation-seq (RIP-seq) from GRBV-infected plant materials as an independent *in planta* test of hypothesized C2/VP RNA-binding properties, as well as discovering sequence-specific host- and/or viral nucleic acids and proteins (by mass-spectrometry) bound by C2 or V2 in host cells.

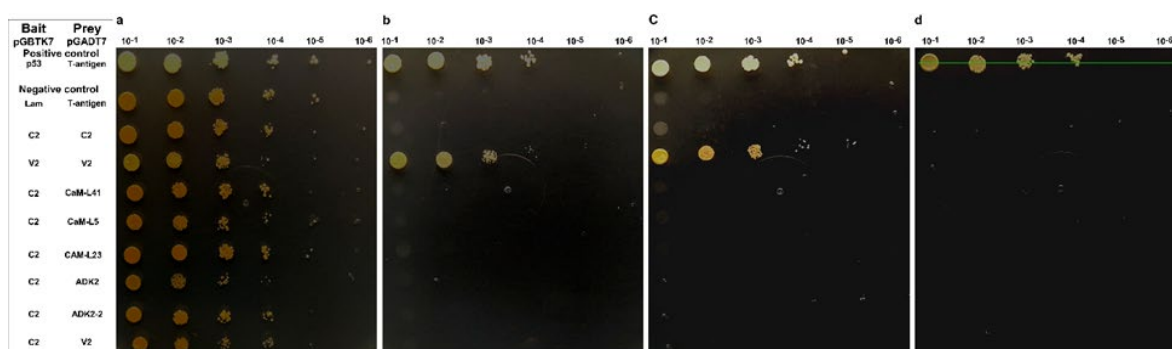
We proposed yeast two-hybrid (Y2H) screen as an unbiased alternative approach to discover host proteins that bind physically to GRBV C2 and V2. Towards this objective suppressor genes C2 and V2 were cloned in a bait vector pGBTK7-BD (data not shown). We have made grape cDNA library of  $2.9 \times 10^8$  clone complexity ( $\sim 10,000$ -fold greater than the grape proteome complexity) using Mate & Plate library system (Takara) to identify plant targets of GRBV C2 and V2. High quality RNA extracted (RIN:8) from GRBV infected- and uninfected leaf samples,

*in vitro* UV treated and untreated grape plantlets and six-week post veraison berry skin samples exposed and unexposed to natural solar radiation<sup>81</sup> were used for cDNA preparation. The mating of pGBTK7-C2 with Mate and Plate grape cDNA library was confirmed by visualizing the zygote formation under phase contrast microscope (40X) (**Figure 11**). We are in the process of identifying the targets of GRBV C2 and V2 in grape cDNA library by Y2H mating.



**Figure 11.** Phase contrast microscopic image (40x magnification) of yeast zygote. Zygotes (circled) has a three-lobed structure where the lobes represent the two haploid parental cells and the budding diploid cell.

Based on our transient co-infiltration results (Objective 1) that co-expression of C2+V2 together had higher suppression activity (**Figure 2A, B**), we are testing C2-V2 viral proteins for physical interaction by cloning C2 and V2 in prey vector pGADT7-AD. We also proposed to test grape homologs of ADK, SAMDC, rgsCaM, KYP, HAD6, AGOs, SNF1-related kinase, and RPS27 (**Table 1**) for hypothesized interaction with GRBV suppressor proteins C2 and V2. Towards this, we have cloned GRBV C2, and V2 and grape homologs rgs-CaM-L41, rgs-CaM-L5-like, rgs-CaM-L23, ADK2, and ADK2-2 in the prey vector pGADT7-AD. The clones were confirmed by restriction digestion (data not shown) and sequencing. pGBTK7-C2 or V2 bait vector and prey vectors (pGADT7-C2/V2/rgs-CaM-L41/ rgs-CaM-L5-like/rgs-CaM-L23-like/ADK2/ADK2-2) were co-transformed into Y2H gold strain and selected on SD/-Leu/-Trp-double drop out (DDO) media for transformation and on SD/-Leu/-Trp-His-triple drop out (TDO) media for functional interactions. To rule out false positive interactions, the colonies from DDO were screened on SD/-Leu/-Trp-His-Ade quadruple dropout media and on SD/-Leu/-Trp+Aureobasidin A (DDO+A).



**Figure 12.** Y2H assay **a)** SD/-Leu/-Trp-double drop out (DDO) media for screening transformants **b)** SD/-Leu/-Trp-His-triple drop out (TDO) media for screening interaction **c)** SD/-Leu/-Trp-His-Ade quadruple dropout media for ruling out false positive interaction **d)** SD/-Leu/-Trp+Aureobasidin A (DDO+A) for ruling out false positive interaction.

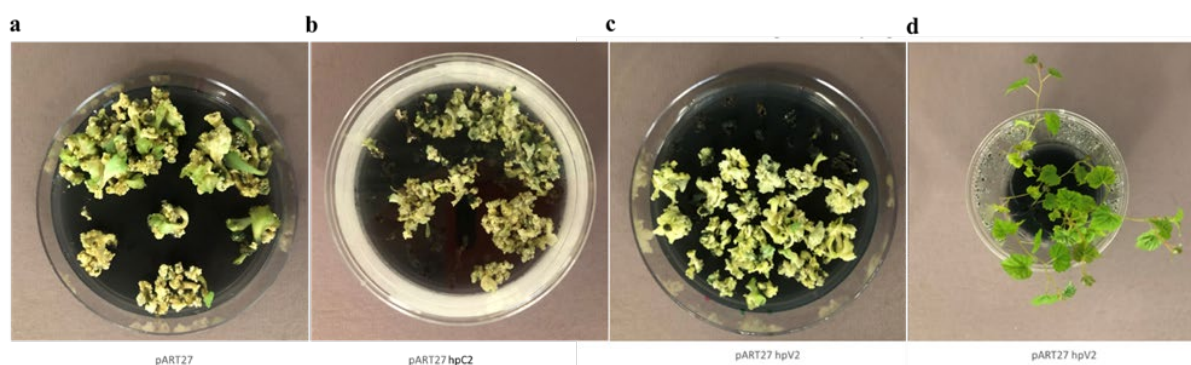
Positive (pGBTK7-p53 and pGADT7-AD-T) and negative (pGBTK7-Lam and pGADT7-AD-T) control vectors were used for assay validation. The co-transformation of bait and prey vectors were confirmed by screening serially diluted colonies on DDO media (**Figure 12a**). We observed positive control colonies on TDO, QDO and DDO+A (**Figures 12b-d**). While the test bait and prey constructs co-transformation interactions were confirmed on DDO media, only pGBTK7-V2 and pGADT7-AD-V2 grew on TDO and QDO (**Figures 12b and c**). pGBTK7-V2 co-transformation with empty prey vector pGADT7-AD also yielded colonies on TDO media (data not shown), thus a plausible interpretation is that pGBTK7-V2 exhibits auto-activation. pGBTK7-C2 did not exhibit self-interaction or interaction with V2 (**Figures 12b-d**). GRBV C2 did not exhibit interaction with tested grape homologs rgs-CaM-L41, rgs-CaM-L5-like, rgs-CaM-L23-like, ADK2 and ADK2-2 (**Figures 12b-d**).

The auto-activation of pGBTK7-V2 prevents testing this construct for interaction against other viral proteins and grape homologs. A low probability 9 amino acid activation domain is observed in the C-terminal region of GRBV V2 protein. We will construct a new bait vector by removing the putative activation domain of GRBV V2 to overcome this plausible complication/limitation.

#### **Objective 4.a) Establish a transgenic grapevine test system and b) evaluate disease resistance to GRBV of transgenic grapevine expressing V2 and C2 hairpin silencers directed to suppressor protein transcripts.**

We proposed to construct hpRNA vectors targeting GRBV C2 and V2 genes for stable transformation and regeneration of grapevine rootstock 101-14. We confirmed the C2 and V2 genes are highly conserved across 93 known GRBV isolates by multiple sequence alignment (data not shown)<sup>83</sup>. In making the hpRNA constructs, C2 and V2 genes were cloned in sense and antisense orientation of pHANNIBAL vector<sup>115</sup> to obtain the hpRNA vector pHANNIBAL-hpC2 or pHANNIBAL-hpV2 (data not shown). The hpRNA gene cassette comprising the hpC2 or hpV2 was cloned in the T-DNA binary vector pART27<sup>116</sup>, which harbors the *neomycin phosphotransferaseII* gene as the plant transformation selectable marker under control of the nopaline synthase promoter and terminator. The binary vector was mobilized into *A. tumefaciens*

strain EHA105 by electroporation. The vector strains were shipped to Cooperator Tricoli for 101-14 transformation in March 2020. Three independent transformations were initiated with pART27-empty vector, pART27-hpC2 and pART27-hpV2, respectively. The current status of transformation events is listed in **Table 5**. Regeneration of plantlets is in process under duly executed and invoiced fee-for-service contract # C15297 with UC Davis Plant Transformation Facility. **Figure 13** shows representative images of the status of regenerated grapevine shoots emerging from transgenic somatic embryos. The USDA-APHIS BRS permit for interstate movement of the transgenic rooted grapevine plantlets is in process and will issue within 45 days.

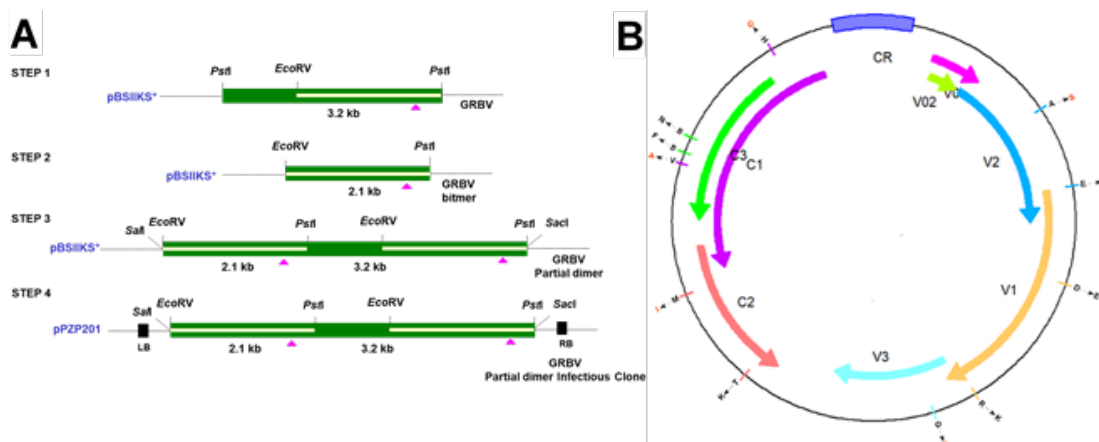


**Figure 13.** Status (Dec. 1, 2021) of regeneration of grape 101-14 plantlets from somatic embryos derived from anther explants and transformed with binary vector a) pART27; b) pART27-hpC2; c, d) pART27-hpV2.

**Table 5.** Status of grapevine rootstock 101-14 transformation experiments in process at UC Davis Transformation facility

Date	Cultivar	Agro Strain	PL Construct Code	PTF Code	Plant Selection	Explant
16-Apr-20	101-14	EHA105 Gelvin	pART27	AT20046	kanamycin	immature embryos
16-Apr-20	101-14	EHA105 Gelvin	pART27 hpC2	AT20047	kanamycin	immature embryos
16-Apr-20	101-14	EHA105 Gelvin	pART27 hpV2	AT20048	kanamycin	immature embryos
21-Apr-20	101-14	EHA105 Gelvin	pART27	AT20046	kanamycin	immature embryos
21-Apr-20	101-14	EHA105 Gelvin	pART27 hpC2	AT20047	kanamycin	immature embryos
21-Apr-20	101-14	EHA105 Gelvin	pART27 hpV2	AT20048	kanamycin	immature embryos
21-Jun-19	101-14	EHA105 Gelvin	pART27	AT20046	kanamycin	immature embryos
21-Jun-19	101-14	EHA105 Gelvin	pART27 hpC2	AT20047	kanamycin	immature embryos
21-Jun-19	101-14	EHA105 Gelvin	pART27 hpV2	AT20048	kanamycin	immature embryos

The ability to infect and invade is a fundamental requirement for a successful pathogen. To test the transgenic plants from Objective IVa for disease resistance we have initiated cloning of GRBV viral clones for agroinfection. GRBV full length genomic sequence of 3.2 kb was cloned into pBSII-KS+ to yield pBS-GRBV vector following rolling circle amplification (RCA) (GE Healthcare) and restriction digestion with *Pst*I enzyme of RCA product from field-infected grape leaf samples from Santa Rosa (**Figure 14A** below, STEP 1). The clones were confirmed by restriction digestion and Sanger sequencing. We have confirmed the full-length sequence of the virus and all the viral ORFs from Santa Rosa isolate (**Figure 14B**). Our viral isolate displayed 99.8% identity towards Washington isolate (MF795176.1) and 99.5% identity towards New York type member isolate (JQ901105.2). We have completed cloning of bitmer fragment as *Eco*RV/*Pst*I in pBSII-KS+ (**Figure 14A**, STEP 2), and completed construction of the partial dimer (**Figure 14A**, STEPS 3-4) and are transferring the DNA to *Agrobacterium* wild type strain Ach5 for infectivity assay. This particular strain is used for tumor formation that facilitates replication of the virus in mitotically active host plant cells. We will test the infectivity of our partial dimer clone with greenhouse-grown grapevine rootstock 101-14 plants (and tobacco from Objective 2b, above) as the next steps.



**Figure 14.** Cloning of GRBV viral partial dimer constructs for agroinfection functional assays. **A)** Schematic representation of partial dimer cloning. **B)** Genome map of Santa Rosa GRBV isolate. Point mutations when compared to JQ901105.2 in all ORFs denoted in black. Mutations identical to MF795176.1 denoted in red.

## CONCLUSIONS

We identified GRBV C2 and V2 as suppressors of post-transcriptional gene silencing. The comprehensive analysis of sRNA and mRNA libraries from GRBV-infected grape field samples identified potential plant targets in grape. A two pronged approach involving protein binding assay and Yeast Two Hybrid interaction is underway to identify the plant targets of GRBV suppressor protein C2 and V2. Somatic embryos transformed with hpC2 and hpV2 are currently regenerating. The partial dimer viral clone is ready for infectivity assay and will be used for testing hpV2 and hpC2 plants for GRBV disease resistance/tolerance.

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## IMPROVED UNDERSTANDING OF VIRUS TRANSMISSION OF GRAPEVINE RED BLOTCH VIRUS

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**Reporting Period:** The results reported here are from work conducted July 2020 to June 2021.

### LAYPERSON SUMMARY

Controlled greenhouse and laboratory infestation trials were conducted to determine the ability of treehopper populations to transmit grapevine red blotch virus (GRBV). Only one positive test of petiole tissue from a leaf basal to the feeding leaf occurred following a 24-hour IAP. Results showed no evidence following successive years of testing that GRBV can be transmitted by source populations of *St. basalis* or *T. albidosparsus*. A single case of transmission of GRBV by *Sp. festinus* was observed. Our data therefore illustrate that it is possible for *S. festinus* to vector Red blotch virus, but that this transmission happens in rare cases. None of the other tested treehopper species were able to vector Red Blotch virus to grapevines within the current timeframe. Additional virus testing is planned for spring 2021 in order to confirm these results. Currently, the available data points to the fact that either there has to be other arthropod species possibly vectoring the disease, or that the lag period before virus presence can be detected. This is however beyond the current experimental period. For this reason, we have asked for a no-cost extension of the current grant, which will enable us to determine possible lag effects. Additional testing of the vine plant materials will be conducted during 2021.

### 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 tenfold increase of virus incidence across three seasons (Dalton et al. 2019). One species of treehopper (Hemiptera: Membracidae), *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 2016-2018, *Tortistilus* species were consistently found feeding on grape shoots and leaves in Oregon vineyards where virus spread are documented. Evidence from previous controlled transmission experiments strongly suggests that these insects are vectors of GRBV.





**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 virus status of plants subjected to transmission biology assays from 2016-2021.

To elucidate the roles that Oregon treehopper populations may have in GRBV epidemiology, greenhouse and laboratory-based bioassays were conducted from 2016-2018. In 2016, a GRBV transmission bioassay occurred in the greenhouse using adults of *St. basalis* and *T. albidosparsus* (hereafter the 2016 greenhouse GRBV transmission bioassay). A GRBV transmission bioassay was conducted in the greenhouse in 2017 using adults of *St. basalis* and *T. albidosparsus*, but with modified methodology (2017 greenhouse GRBV transmission bioassay). Immature 3<sup>rd</sup>- or 4<sup>th</sup>-instar *St. basalis* nymphs were used in 2018 for a greenhouse GRBV transmission bioassay (2018 greenhouse GRBV transmission bioassay). Laboratory tests investigating the immediate migration of GRBV particles within *V. vinifera* occurred in 2018 using adults of *Sp. festinus*, *St. basalis* and *T. albidosparsus* (2018 laboratory GRBV transmission bioassay).

## RESULTS AND DISCUSSION

### Objective 1. Determine the Virus Status of Plants Subjected to Transmission Biology Assays from 2016 to 2021

**2016 Greenhouse GRBV Transmission Bioassay.** While most grapevine inoculum source materials tested positive for GRBV infection, no treatment or negative control vines developed a diagnostic GRBV infection from tissue samples collected during 2016-2019, as determined by qPCR analysis. Of the inoculum source materials that were exposed to *St. basalis*, 11 out of 15 tests of leaf petiole tissue (73.3%) resulted in a positive reading. Of the inoculum source materials provided to *T. albidosparsus*, tests of petiole tissues resulted in a positive reading in 11 out of 19 assays (57.9% of vines).

**2017 Greenhouse GRBV Transmission Bioassay.** Inoculum source material vines hosting only *St. basalis* (n=6), only *T. albidosparsus* (n=5), and both species concurrently (n=3) during the AAP were tested using qPCR for presence of GRBV particles. GRBV infection was identified in

12 out of 14 inoculum source material vines in 2017, but no treatment or negative control vines tested positive for GRBV from 2017-2019 (tested through 2020).

**2018 Greenhouse GRBV Transmission Bioassay.** Virus assays conducted from plant materials kept from 2018 through 2021 on grapevines that had been infested by *St. basalis* nymphs failed to identify GRBV infection in negative control vines or vines infested with *St. basalis* nymphs. Of the inoculum source material, 3 of 4 vines subjected to qPCR assays returned at least two samples with measurable Ct and a Tm value in line with the expected values (**Figure 2**). By the conclusion of the IAP, 5 insects had died on Schwarzmänn vines (6.7%), and 7 insects had died on Wädenswil vines (9.3%). Two adult male insects emerged on Wädenswil vines during the IAP.

**2018 Laboratory GRBV Transmission Bioassay.** Vegetative tissues were harvested in 2018-2021 from uninfested negative control vines of Schwarzmänn and Wädenswil vines that had been infested with individual treehopper adults immediately before sampling (**Figure 3**). Of all tissues, only two results from qPCR assays suggested potential transmission of virus particles from the insect to its host grapevine (Table 1). Nucleic acid samples from potentially affected tissues were subjected to confirmatory analysis using ddPCR. Results showed that the petiole below the feeding leaf that was infested for 24 hours with a male *Sp. festinus* (sample e of vine 18663) contained droplets containing GRBV particles on the same order of magnitude as positive controls. Root tissue of a vine that was infested for 72 hours with a male *St. basalis* (sample g of vine 18670) tested negative for GRBV particles using ddPCR. Positive and negative controls performed as expected (Table 2).

All samples containing plant materials from the above bioassays were kept under greenhouse conditions. Additional testing of plant materials were conducted during spring 2021, in order to possibly eliminate lag effects of GRBV virus particles becoming systemic, resulting in more consistent testing results. No samples showed additional positive transmission during Spring 2021 (data not shown).

GRBD is an emerging viral disease of economic importance in Oregon wine grape production systems, and observed spread in Oregon vineyards suggested field transmission by a mobile vector (Dalton et al. 2019). Multiple species of insects have shown the ability to uptake GRBV, but in-depth studies have yielded inconsistent and even contradictory results (Poojari et al. 2013, Bahder et al. 2016b, Cieniewicz et al. 2018b). The *Geminiviridae* represent a diverse group of plant viruses containing at least nine genera, many of which are transmitted by specific insect vectors (Zerbini et al. 2017). The treehopper *M. malleifera* is the only known vector of *Tomato pseudo-curlytop virus*. Given the similarities between the genome organization of genera *Topocuvirus* and *Grablovirus*, of which the type species GRBV is purportedly transmitted by *Sp. festinus*, the likelihood is that at least one treehopper species is responsible for field spread of GRBV (Bahder et al. 2016). Archived voucher specimens of *Sp. festinus* collected from sites in western Oregon suggest the historical presence of the species; however, specimens were decades old, and the prevalence of GRBV in viticultural regions cannot be explained by the known distribution of *Sp. festinus* as determined through recent collections (Dalton et al. 2020). On the other hand, the widespread potential distribution of *Sp. festinus* in western Oregon cannot be discounted. Dozens of adults were collected from 2016-2018 in Jackson County in proximity to a

vineyard block that was recently removed due to high incidence of GRBV infection (Stowasser et al. 2020, Dalton et al. 2019). Moreover, a single adult *Sp. festinus* was collected in August 2019 in a vineyard in close proximity to YV and CV study sites (J. Lee, *personal communication*).

While our GRBV transmission bioassays were mostly unsuccessful, it is possible that the biotype of the potential vector might impact transmission efficiency. In a seminal study on transmission of whitefly-transmitted geminiviruses, it was found that populations of *Bemisia tabaci* (Gennadius) differentially transmitted viruses from diverse geographic regions, and with variable efficiency on different plant hosts (Bedford et al. 1994). Biotypes of *Sp. festinus* from distinct geographic regions of the southern United States were recently differentiated using DNA metabarcoding of the insect *mt-COI* gene and the nuclear internal transcribed spacer 2 region (Cieniewicz et al. 2020). While in some cases there may be no considerable differences of virus transmission in vector populations, as was found in an assessment of vector-pathogen specificity of the GLRaV complex (Tsai et al. 2010), it remains to be seen whether any differences of epidemiological importance exist between Oregon and California populations of *Sp. festinus*. Furthermore, plant host tissue could have an effect on the ability of an insect to transmit a virus. Free-roaming *Sp. festinus* successfully transmitted GRBV to test plants (Bahder et al. 2016b), whereas in our studies treehoppers were restricted to leaf petioles of treatment vines. This is an important distinction because feeding-induced girdling of a leaf petiole might inhibit the further translocation of GRBV particles (Andersen et al. 2002). Partial girdles of petioles, or alternatively stem girdles, may allow more efficient translocation of GRBV, but this hypothesis needs verification. The girdling status of the plant that tested positive in the 2018 laboratory GRBV transmission bioassay is unknown because the leaf petiole was harvested before girdling could develop.

In the 2016 greenhouse GRBV transmission bioassay, the 2-day AAP could partially explain the apparent lack of transmission of GRBV. A latent period inside vector insects must be satisfied prior to transmission of geminiviruses (Gray et al. 2014). Immature *St. basalis* were repelled from *Brassica rapa* L. var. *silvestris* and survived up to eight days without feeding on host plants (D.T. Dalton *unpubl. data*). If insects in the greenhouse or laboratory GRBV transmission bioassays did not feed on inoculum source materials, then the possibility exists that no uptake of virions could have occurred. To maximize the likelihood of feeding, subsequent infestation trials used AAP of 6 days. In the current work, inoculum source materials and treehoppers of all source populations tested positive for GRBV; thus, it is likely that test plants were injected with GRBV particles during the IAP. Notably, the highest incidence of GRBV in insects following bioassays was found in *Sp. festinus*, lending further support to its potential role as a vector of GRBV. While transmission of GRBV by *Sp. festinus* occurred at low frequency in the laboratory, field transmission remains to be shown. The comprehensive suite of greenhouse and laboratory GRBV transmission bioassays conducted in the current study was unable to show transmission by other populations of smiliine treehoppers.

**Table 1** Results of quantitative polymerase chain reaction assays of 'Pinot noir' clone 828 on Schwarzmann rootstock and self-rooted 'Pinot noir' clone Wädenswil. Oregon populations of treehopper insects (Hemiptera: Membracidae) were provided inoculation access periods (IAP) of either 24 or 72 hours in the laboratory. Bolded numbers indicate vines showing a potentially positive finding.

Insect species	24-hour IAP						72-hour IAP					
	Schwarzmann			Wädenswil			Schwarzmann			Wädenswil		
	Vine	(+)	N	Vine	(+)	N	Vine	(+)	N	Vine	(+)	N
<i>Tortistilus albidosparsus</i>	18678	0	6									
	18681	0	6									
	18682	0	6									
	18688	0	6									
<i>Spissistilus festinus</i>	18702	0	6	18650	0	6	18704	0	6	18645	0	6
	18703	0	6	18655	0	6	18705	0	6	18648	0	6
	18707	0	6	<b>18663</b>	<b>1</b>	<b>6</b>	18706	0	6	18656	0	6
	18710	0	6	18672	0	6	18708	0	6	18668	0	6
	18711	0	6	18696	0	6	18709	0	6	18669	0	6
<i>Stictocephala basalis</i>	18677	0	6	18640	0	6	18679	0	6	18644	0	6
	18683	0	6	18643	0	6	18680	0	6	18660	0	6
	18686	0	6	18658	0	6	18684	0	6	18667	0	6
	18687	0	6	18659	0	6	18685	0	6	<b>18670</b>	<b>1</b>	<b>6</b>
	18690	0	6	18665	0	6	18689	0	6	18671	0	6
Uninfested Control	18697	0	1	18642	0	1	18691	0	1	18661	0	1
	18698	0	1	18646	0	1	18692	0	1	18655	0	1
	18699	0	1	18649	0	1	18693	0	1	18656	0	1
	18700	0	1	18664	0	1	18694	0	1	18663	0	1
	18701	0	1	18666	0	1	18695	0	1	18696	0	1

**Table 2** Results of droplet digital polymerase chain reaction assays on ‘Pinot noir’ grapevine. Two dilutions (1% and 100%) of DNA were tested. Previously tested Grapevine red blotch virus (GRBV)-positive and GRBV-negative (+ and -) control vines are indicated. Percent values indicate the fraction of positive droplets from duplicate runs of each sample (no additional positive samples found in spring 2021).

Vine	1% dilution		100% dilution		Determination
	Pct (+) droplets	total droplets	Pct (+) droplets	total droplets	
17357	0.1%	36,909	>0.1%	37,019	negative
17360	0.1%	35,571	0.0%	34,114	negative
17395	0.1%	35,305	>0.1%	35,921	negative
17400	0.1%	34,947	0.1%	35,986	negative
18663e	4.3%	36,594	91.6%	33,895	positive
18670g	>0.1%	38,620	0.0%	36,072	negative
990 (+)	2.7%	33,358	95.4%	30,753	positive
1027 (+)	16.0%	34,967	53.7%	25,081	positive
1049 (+)	4.0%	34,485	99.3%	30,951	positive
651 (-)	0.0%	32,748	0.0%	37,562	negative
687 (-)	0.2%	31,077	0.1%	35,954	negative
698 (-)	0.1%	31,563	0.1%	32,548	negative

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## BIOLOGY AND ROLE OF TREEHOPPERS IN GRAPEVINE RED BLOTCH DISEASE

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**Reporting Period:** The results reported here are from work conducted July 2018 to June 2021.

### LAYPERSON SUMMARY

The results of this project added significant new knowledge towards better understanding the role of the three-cornered alfalfa hopper and other vineyard treehoppers in the epidemiology of GRBV, including management of virus spread, by determining 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, and phloem-feeding leafhoppers found in vineyards where GRBV is spreading has also been studied. This essential information will contribute to the management of 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 ssDNA 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 this disease (Yepes et al. 2018). Because of its adverse effect on wine quality and resulting revenue loss, GRBV has become 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 treehopper (3CAH), *Spissistilus festinus* Say, 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 *S. festinus* adults caught on yellow sticky traps that tested positive for GRBV by PCR indicated that this membracid is the most likely vector of significance to virus epidemiology (Cieniewicz et al. 2018). Transmission of GRBV by *S. festinus* in a greenhouse study has been independently

validated by Flasco et al. (2021) subsequent to the initiation of this study. Our studies on GRBV transmission using 3CAH in the laboratory and greenhouse has not produced consistent results, and we had never documented field transmission in a controlled study at the beginning of this project. For example, our attempts to document field transmission of GRBV using 3CAH in an experimental vineyard at the Armstrong Tract UC Davis Plant Pathology Research Farm in Davis, initiated in 2015, has yet to show successful transmission. In the case of geminiviruses, members of the genus *Begomovirus* are transmitted by a whitefly, *Bemisia tabaci*, (Hemiptera: *Aleyrodidae*). Recently, *B. tabaci* has been recognized as a cryptic species complex within which three biotypes have been recognized (Jiu et al. 2017). An added problem is differential transmission specificity by cryptic species (Polston et al. 2014)

In California's North Coast and in southern Oregon, the Zalom and Sudarshana labs at UC Davis and Dr. Vaughn Walton's lab at Oregon State University have found colonization of grapevines by other treehoppers of the genera *Tortistilus* and *Stictocephala* in vineyards where virus spread is occurring (Dalton et al. 2019). However, the status of these and other treehopper species as vectors of GRBV has not been determined and preliminary attempts to transmit GRBV by field-collected *Tortistilus* have not been successful to date. There remains a need to study related treehopper species that have been found in vineyards where virus spread is occurring in Oregon and California, as well as 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." Subsequently, we began a collaboration with Dr. Dennis Kopp, an expert taxonomist on Membracidae at the Smithsonian Natural History Museum in Washington D.C., to unravel the identification of *Tortistilus* treehoppers, study their seasonal biology, and determine their possible role in GRBV transmission.

Understanding the biology of treehoppers as potential GRBV vectors is critical to their successful management. Treehopper feeding symptoms are easy to recognize on grapevines and appear as girdled young shoots and petioles. Even though 3CAH had been considered a minor pest of grapevines because of girdling damage, it had not previously been determined if grape is a reproductive host for 3CAH. Cindy Kron (nee Preto) completed her dissertation research in our lab conducting studies that identified feeding and reproductive hosts of 3CAH. The study identified cover crops and common weeds which serve as their feeding and reproductive hosts (Preto et al., 2018a), suitability of grapevines as a reproductive host for 3CAH (Preto et al., 2018b), population dynamics of 3CAH in vineyards (Preto et al., 2019a), and sustainable management guidelines (Preto et al., 2019b). Her studies served as the basis for developing a model (Bick et al. 2020) that would enable use of management of orchard floor vegetation to reduce resident 3CAH populations in vineyards late spring to reduce their populations later season. The approach could potentially be adapted for management of other treehopper or leafhopper species that might serve as GRBV vectors as well.

## 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.

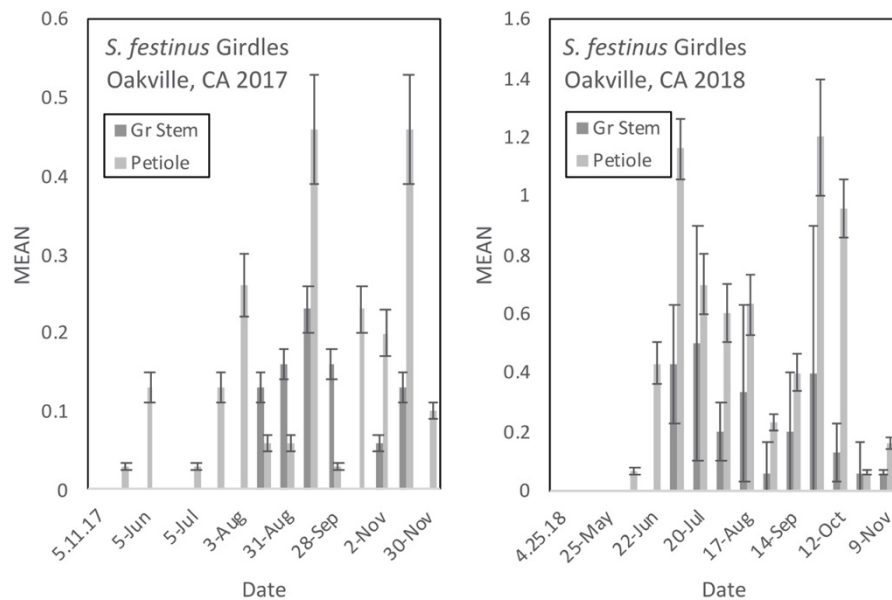
## RESULTS AND DISCUSSION

### Objective 1. Determine the Timing of Treehopper Girdling in Relation to Red Blotch Incidence in Vineyards

We documented 3CAH phenology from 2016-2018 in a GRBV-infected Cabernet Sauvignon block 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 CA-29 near Oakville (Oak-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 3 m 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 Oak-1 vineyards to obtain a second year of data. We also began to count girdles in a replanted Cabernet Sauvignon vineyard located along Oakville Cross Rd. just east of Oakville (Oak-2) that is close to a riparian area. Girdle counts from these vineyards were taken every 2 weeks beginning three weeks after bud break until Fall leaf drop from six rows containing five vines each located within the same vineyard. Girdles (Figure 1) 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.** Girdles on petioles from feeding by *Spissistilus festinus* (left) and *Tortistilus* spp. (right).

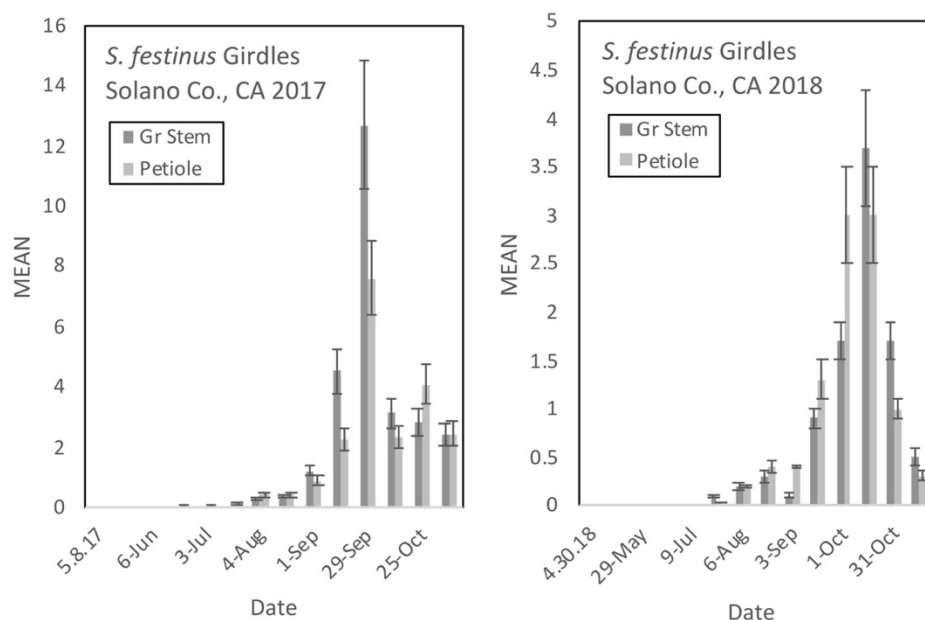


**Figure 2.** Mean  $\pm$  SEM *Spissistilus festinus* girdles per Cabernet Sauvignon grapevine (n=30) from biweekly sampling at the Oakville 1 CA site in 2017 (left) and 2018 (right). Sampling was terminated following the November 9, 2018 sampling date as this entire block was removed because of high red blotch disease incidence.

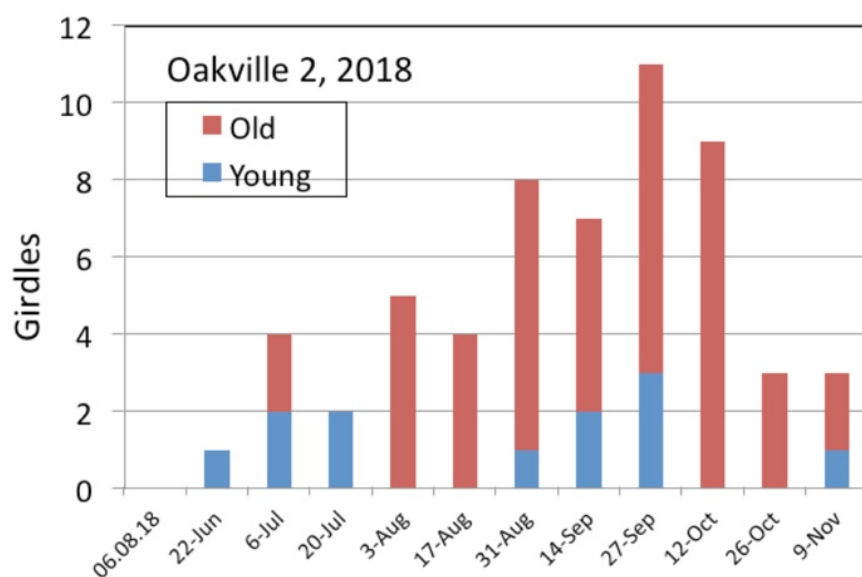
Figure 2 presents the results of our girdle sampling in the Oakville-1 vineyard. Similar to our results from 2017 (which were collected on a previous PD/GWSS-sponsored project), girdles were first observed in June with peaks of new girdles occurring in July and late September, coinciding with 3CAH adult emergence. More girdles were found on petioles than apical shoots.

Figure 3 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 15 four-year-old recipient Cabernet Sauvignon grapevines [quantitative PCR (qPCR) tested GRBV negative] between the established field vines. The 15 interplanted and adjacent vines were sampled for treehopper girdles every two weeks. The first girdle was found on June 22 (on a young vine), with peak new girdles occurring in late September (Figure 4). 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 3.** Mean  $\pm$  SEM *Spissistilus festinus* new girdles per Cabernet Sauvignon grapevine (n=30) sampled biweekly at the Solano Co., CA site in 2017 (left) and 2018 (right).



**Figure 4.** Total number of new petiole and apical shoot girdles found on established (old) and interplanted (young) vines at the Oakville 2 vineyard.

UC Davis MS student Michael Bollinger has summarized these data as part of his MS Thesis (Bollinger et al. 2021), and we are preparing a manuscript on results of the biweekly monitoring of treehopper girdles in these vineyards.

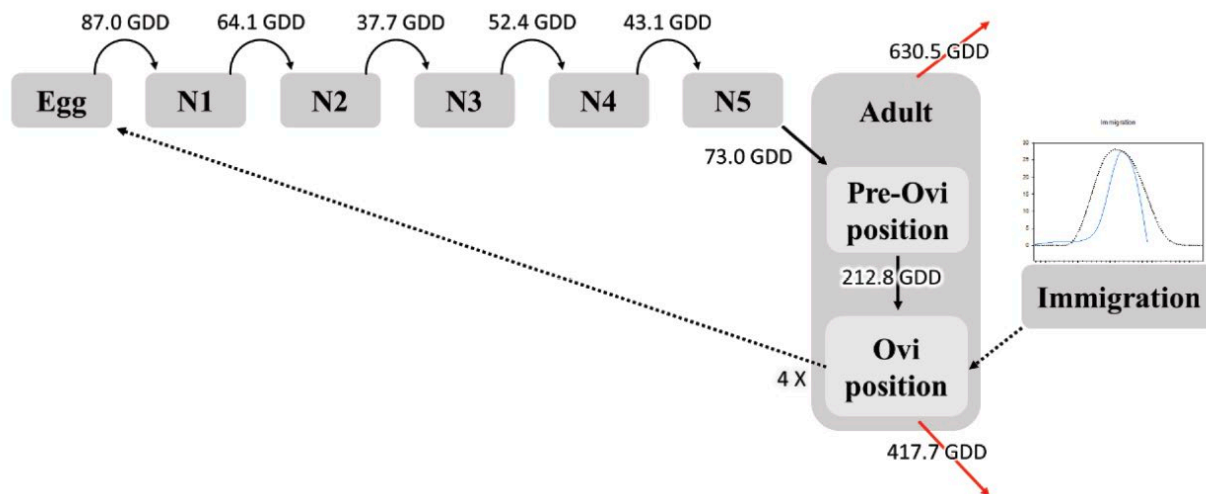
We continued to sample treehoppers in vineyard groundcover prior to budbreak and through late June in 2019, 2020 and 2021 to determine if GRBV can be detected in the overwintering 3CAH or in first generation adults. This will be useful information to determine if adults present in vineyards through the winter could be a source of GRBV for the following year, or if first generation 3CAH, which are usually found on groundcover, acquire GRBV at this period.

### ***Spissistilus festinus* management model**

In late winter, during grapevine dormancy, *S. festinus* migrate into vineyards to feed and reproduce on cover crop and weed hosts. Tilling of planted and native vegetation into the soil would provide growers an opportunity to strand, starve and mechanically destroy early instars that are relatively immobile, effectively reducing the first-generation population of *S. festinus* in vineyards. Presence of nymphs is generally difficult to recognize. First through third instars were not detected in sweep net samples in our two-year biweekly sampling study, while fourth and fifth instar nymphs were first found on the same sample date as emerging adults. Using our sampling and girdling data, a degree-day model was developed to predict when early *S. festinus* instars are present in the vineyard to aid in exploiting the time period when *S. festinus* would be most susceptible to cultural control measures.

Cover crops and ground cover in vineyard rows are traditionally tilled under in springtime, but recommended timing of this practice is not precisely defined. However, the earlier that cover crops are tilled under the fewer units of nitrogen are added to the soil for vine uptake (Hirschfeld 1998, Miller et al. 1989). Legumes left in the vineyard on which *S. festinus* mature to adults provide an ideal location for the treehoppers to complete their life cycle and increase in prevalence (Preto et al. 2018a). Preto et al. (2019a) showed that *S. festinus* have one to two generations per year in a California vineyard, therefore enhancing mortality of immatures would significantly reduce the seasonal population of resident treehoppers. To facilitate this strategy by assisting growers in timing when to till under the cover crop/resident vegetation before first generation nymphs are able to complete their development, a stage-structured degree day model that simulates *S. festinus* immigration, reproduction, and mortality was developed to predict the population dynamics of *S. festinus*, specifically 1<sup>st</sup> – 3<sup>rd</sup> instar nymphs (Figure 5). The model (Bick et al. 2020) tracks *S. festinus* stage-structure (time in each instar) and population by simulating daily time steps (24 h increments) for a duration of 135 days that allows the simulation of initial immigration, all nymphal stages, and the first adult generation that emerges in the vineyard.

To validate this model, *S. festinus* occurrence at additional vineyard sites in Napa and Sonoma Counties groundcover sampling has been conducted from February to June in both 2020 and 2021 in collaboration with recently appointed UC Cooperative Extension Integrated Pest Management Advisor Cindy Kron. To confirm the insects collected were all *S. festinus*, DNA was extracted from whole bodies of individual insects for CO1 analysis by PCR, and qPCR tests were conducted to test for GRBV using the primers described by Bahder et al. (2016). All insects tested negative for GRBV by qPCR.



**Figure 5.** Diagram representing the *Spissistilus festinus* stage-structured degree day model that includes the immigration, reproduction, and mortality used to predict the population dynamics of *S. festinus*. N1-N5 refers to 1<sup>st</sup> through 5<sup>th</sup> instar, respectively. Growing degree days (GDD) required to move from one life stage to the next are listed between each stage.

## 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

### Methodology developed 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 (VCLH, *Erythroneura ziczac*) 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 Acquisition Access Period (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 speed 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, and DNA isolated using the Qiagen DNeasy blood and tissue kit. In this particular assay, none of the salivary glands 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 used to evaluate other potential GRBV vector candidates in subsequent studies associated with the project as described in this report.

### **Field Spread and Transmission Studies**

**a) UC Davis Armstrong 3CAH field transmission study.** Five years ago, we 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. Adjacent to this block, we also planted an alfalfa strip to serve as an untreated reservoir for potential vectors. On September 8, 2018, we planted 10 rooted GRBV-infected Zinfandel vines from the Amador County vineyard between our established Cabernet Sauvignon GRBV-free vines to provide a virus source within the established vineyard. Half of the vines were infected with clade I (ACU-I) and the other half with clade II (ACU-II) GRBV isolates. Petiole samples collected from the neighboring established vines were qPCR tested for GRBV in July 2019, but all tested negative for the virus. In fall 2020, a visual inspection showed no symptomatology associated with GRBV, but feeding damage by 3CAH can be easily found across the block. Eight neighbor vines to the positive control vines tested negative late fall 2020 by qPCR. We plan to continue testing vines in this block as long as the vineyard is available to us after the conclusion of this project to determine if GRBV spread is occurring.

**b) UC Davis Oakville Experimental Station spread study.** The original block A at the Oakville Station was removed in 2015 due to high incidence of GRBV incidence, and replanted with Cabernet Sauvignon grapevines in 2016. 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 6). 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. In fall 2019, we again visually assessed all of the vines for red blotch symptoms. Petioles from grapevines exhibiting suspicious foliar reddening were collected from 12 grapevines as well as petioles from 3 symptomless grapevines. All these samples were tested



by qPCR for GRBV and all of them tested negative. As control for this test, we also collected two red blotch symptomatic samples from a neighboring block, and these tested positive. We have continued to monitoring this block for symptom expression to determine the earliest time when infection can be recognized. In our fall 2020 evaluation, we observed one vine with recognizable of Grapevine Red Blotch Disease symptoms. We collected petioles from this vine, and it tested positive for GRBV. This is the first vine that became infected in this block. This vine had tested negative in the previous year, and we had recorded girdles on the vine in our initial survey. We will keep monitoring this block and test selected vines to identify symptomless infections and spread pattern. It is important to note that the block has been treated annually since 2017 with insecticides that target potential GRBV vectors.

**c) *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. albidosparsus* treehoppers (Figure 7). This species tends to be more abundant in cooler hillside areas of vineyards in contrast to 3CAH which tends to be in warmer vineyard areas. 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 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 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 previously reconfirmed by qPCR testing in April 2019. Individual mesh cages containing 10 insects each were placed onto each of our healthy (Figure 8a) and GRBV-positive block vines (Figure 8b). 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.

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.

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			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	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	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						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			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 6.** Map of treehopper girdles on the Cabernet Sauvignon block (A block) planted in 2016 at the UC Davis Oakville Experimental Station. (G = vine with girdles, \* = rootstock only, and dark rectangle = missing vine.)



**Figure 7.** Morphs of *Tortistilus albidosparsus* (left) and infestation of *Tortistilus albidosparsus* feeding on Cabernet Sauvignon shoots in the Gordon Valley vineyard (right).



**Figure 8a (left) and 8b (right).** qPCR-tested grapevines that were uninfected (left) or GRBV-infected (right), showing AAP and IAP cages with *Tortistilus albidosparsus*.

The salivary gland dissection and virus-testing method previously described was evaluated on 20 adult *T. albidosparsus* (17 horned and 3 unhorned individuals) 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.

The GRBV status of the potted vines from these field studies by qPCR tests was negative at 6 months after IAP. The next qPCR testing for round of GRBV status is planned for Fall 2021.

### **Greenhouse Transmission Studies**

#### **a) 3CAH greenhouse transmission study using GRBV positive clade-I and clade-II isolates from the Sierra foothills area in Amador County, CA, as source vines.**

We have monitored a Zinfandel vineyard in Amador County each year from 2015 through 2020 where the number of GRBV-infected grapevines have increased dramatically (Wunderlich et al. 2017) indicating a very rapid spread of the virus similar to what has been observed in some Oregon vineyards (Dalton et al. 2019). From 2016-2018, GRBV spread in vineyard blocks I, II and III, which are located nearest the vineyard's edge, increased by 18.2%, 19% and 7.1%, respectively. Another block (VI) only had an overall incidence of 3.8% symptomatic vines when visually assessed for symptoms in 2016, and the number of vines increased at a much lower rate (2.2%) though 2019. We qPCR-tested all 206 vines from block VI for GRBV and found that only 10 of the 14 symptomatic vines tested positive for GRBV as did one asymptomatic vine. Of course, finding GRBV-like symptomatic plants to be negative by qPCR can be due to the accuracy of the visual inspections, even by experienced observers, but it is also possible that GRBV genetic variants that could not be detected by the qPCR test might be present. Therefore, we intend to sequence additional GRBV isolates from this block and conducted rolling circle amplification tests to detect GRBV in fall 2020.

Cuttings from this vineyard infected with either GRBV clade-I (ACU-I) or GRBV clade-II (ACU-II) were self-rooted to serve as source vines for our future transmission studies, supplementing the Cabernet Sauvignon source vines that we had been using exclusively for our transmission studies prior to the start of this project. In Fall 2018, 500 adult 3CAH's were collected in an organic alfalfa field near Davis, CA, divided equally and transferred into three insect cages containing either a GRBV clade-I vine (ACU-I), a GRBV clade-II (ACU-II), or a tested (GRBV-free) vine. After a 48 hr AAP, these insects were transferred individually into clip cages fastened onto the oldest leaf of ten qPCR-tested GRBV-negative recipient vines for each treatment. Ten replicate grapevines for each of the three treatments were established. Petioles collected from these vines tested negative for GRBV in June 2019 and December 2019. Additional qPCR tests are planned for fall 2021 after 36 months of IAP.

#### **b) *Tortistilus* sp. transmission study using GRBV isolates of clade I (Ghv-377) and clade II (Ghv-392) and as GRBV source vines.**

In June 2018, 500 adult *T. albidosparsus* were collected from vetch growing 100 ft away from GRBV-infected grapevines in Pope Valley, CA, and equally separated into four 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. The insects from each of these groups were allowed to feed for a 48 h AAP, then transferred to 15 tested GRBV-negative recipient vines for each group for a 48 h IAP. Petioles samples from all 60 of these recipient vines were collected and qPCR-tested for GRBV in August 2019, but all samples were negative for the virus to that time. These plants were transplanted to the field in Armstrong Tract D block soon thereafter in order to be subjected to field dormancy. We were unable to test the plants in late summer 2020 due to Covid-restrictions in our lab, but GRBV status will be evaluated in late summer 2021, following two field dormancy cycles, instead.

**c) Transmission studies using two distinct *Spissistilus* genotypes.**

In fall 2019, we discovered a treehopper morphologically indistinguishable from *S. festinus* that exhibited distinctive behavioral differences at a commercial vineyard site where GRBV spread had been documented. CO1 barcoding of this treehopper revealed that the population differed from *S. festinus* by about 10%. We initiated a transmission assay with this population using qPCR-tested GRBV-free Cabernet Sauvignon grapevines in our Armstrong Tract vineyard at UC Davis as recipient plants. We collected about 100 adults of the new genotype by sweep netting the commercial vineyard where the new population was found, and caged 50 of them on a qPCR-tested GRBV-negative vine in the vineyard and the remaining 50 on a qPCR-tested GRBV-positive vine in the vineyard. After the AAP, the surviving insects were transferred to vines in our Armstrong Tract vineyard. At least four insects that had fed on healthy grapevines were caged on each of eight vines, while seven vines were exposed to at least two insects that had fed on the GRBV positive grapevines. The treehoppers were retrieved from the cages after the IAP and analyzed for GRBV presence in the salivary glands and abdomen, or in the whole body. Total nucleic acids (TNA) extracted from the insects were used for barcoding of the CO1 gene to confirm their genotype. In spring and fall 2020, and again in spring 2021 (6, 12 and 17 months following the IAP period, respectively), petiole samples from all of the recipient vines were collected, TNA extracted using MagMax, and tested for GRBV by qPCR. All recipient plants tested negative for the virus. Vines will be tested again in late fall 2021

Due to COVID restrictions and wildfires in California, our ability to initiate new GRBV transmission assays has been significantly constrained. However, we were able to initiate a few transmission assays with this new 3CAH genotype in the greenhouse and also in the field in Amador County between July and October 2020. The vines tested negative for GRBV by qPCR in mid-November 2020 and again in April 2021. We plan to test them again in Fall 2021.

As part of a field validation of the *S. festinus* model described previously, Dr. Cindy Kron has been collecting adult *Spissistilus* in a biweekly survey of North Coast vineyards in Napa and Sonoma counties during spring (May-June) 2020 and 2021, and we have been determining species of these treehoppers using CO1 as well as their GRBV status. The 2020 samples proved to be *S. festinus* and none tested positive for the virus. We have not evaluated the status of the insects collected in 2021 as of the time this report was being prepared.

**d) Transmission studies comparing shorter vs longer AAP.**

Over 1,000 adults of the common central valley population of 3CAH were collected in a Solano County alfalfa field in December 2019. Six hundred were caged on a qPCR-tested GRBV-positive potted-grapevine (Cabernet Sauvignon Ghv-392), and 300 were caged on two tested GRBV-negative vines. Two AAPs were tested (AAP-1: 4 days; and AAP-2: 14 days) using a 15-day IAP for both treatments. For AAP-1, 10 vines were caged with at least 15 3CAH adults from the GRBV-positive donor vine (Ghv-392), and 5 plants were caged with at least 15 adults of 3CAH each from a GRBV-negative donor vine. Fewer adults survived within the longer AAP-2 cohort. Therefore, 8 vines were caged with at least 10 3CAH each from the GRBV-positive donor vine (Ghv-392) while 5 vines were caged with at least 10 3CAH each from a GRBV-negative donor. At 5, 10, and 15 months post IAP, the recipient vines and controls were tested by qPCR for GRBV status and all tested negative. The vines will be tested again late Fall 2021 and in Summer 2022. Salivary glands and abdomens of all retrieved alive 3CAHs at the end of the

IAP were dissected and subjected to qPCR test for GRBV. From the AAP-1 cohort, just one salivary gland tested positive out of 69, and all abdomens tested negative. However, from the AAP-2 cohort, the virus was detected in 10 abdomens out of 17, but all salivary glands tested negative.

### **GRBV detection studies with Auchenorrhyncha and alternate hosts**

During 2014 and 2015, we collected insects by sweep-netting and light-trapping at the UC Davis Oakville Experimental Station in Napa County, and the captured insects were tested by qPCR for GRBV presence. Transmission studies were conducted, when possible, for those species that were found to have GRBV present in their bodies. These studies suggested that 3CAH as well as several other insects were candidates as vectors of GRBV (Bahder et al. 2016). Because of our inability to date to confirm 3CAH transmission of GRBV in the field, we reinitiated day and night insect collections in vineyards where GRBV spread is occurring with a specific focus on insects in the Hemiptera suborder Auchenorrhyncha (which includes treehoppers, leafhoppers, planthoppers, and psyllids) in January 2019. Specimens of Auchenorrhyncha were collected by sweep-netting in vineyards in Amador, Santa Barbara, San Luis Obispo and Solano counties and analyzed for the presence of GRBV.

In 2020, we focused our vector detection work on collecting and analyzing *S. festinus* from vineyards in Amador and Napa Counties, alfalfa fields in Solano County near Davis, and the riparian area along Putah Creek in Solano and Yolo counties where GRBV spread could be documented. The insects collected were analyzed for presence of GRBV from nucleic acid extracted from the salivary glands and abdomen of individual insects. Additionally, a subset of *S. festinus* from some of the sites were also allowed to feed on GRBV positive source vines, then tested for retention of the virus in the salivary glands. Our preliminary results are shown in Table 1.

#### **a) Insect collections in vineyards for GRBV detection.**

Of the many Auchenorrhyncha specimens collected two leafhopper species from Amador County, including the one we had collected in our earlier studies, were particularly notable because of the relatively high percentage of individuals that tested positive for GRBV by qPCR. We subsequently initiated transmission assays with both species and sequenced their CO1 gene.

*Leafhopper 1.* Over 400 Leafhopper-1 adults were collected in October 2019 by sweep-net were caged on either a GRBV-positive vine or a GRBV-negative vine. After allowing the leafhoppers to feed on these vines for a 48 h AAP, at least 15 of the leafhoppers were transferred to each of 29 caged GRBV-negative Cabernet Sauvignon potted vines. Fifteen replications represented leafhoppers from the GRBV-positive donor vine and 14 replications were from the GRBV-negative vine. All of the leafhoppers transferred to the recipient vines were dead after the 48 h IAP. The dead insects were retrieved and analyzed for GRBV presence in a whole-body assay. After 5 months, the vines were tested by qPCR for the presence of GRBV and were negative. The recipient vines will be tested again in fall 2020, 12 months after the IAP. CO1 barcoding enabled us to identify the leafhopper as *Ceratagallia* sp. As the *Ceratagallia* sp. population started decreasing, we collected them by sweep-net and caged them on GRBV positive and negative vines as previously described for acquisition, and then released them into cages on two GRBV-negative vines in our Armstrong Tract Cabernet Sauvignon vineyard that served as



recipient plants. There was no defined IAP, rather insects were allowed to feed for approximately a month until the majority had died. The surviving insects from both recipient vines were transferred to potted GRBV-negative CS plants to determine if the virus had been retained during that time and the insects were still capable of virus transmission. The recipient vines tested negative for GRBV after 5 months post IAP. An additional batch of 200 *Ceratagallia* sp. adults were collected in the same vineyard as the first study. Half were transferred to a cage containing a GRBV-negative Cabernet Sauvignon potted grapevine and the other half to a cage containing a GRBV-positive Cabernet Sauvignon potted grapevine (Ghv-392) for acquisition. At least 15 insects from the GRBV positive and negative donor vines were transferred to each of 10 caged GRBV-negative Cabernet Sauvignon potted recipient grapevines (ten vines in all). The insects were qPCR-tested for GRBV either in abdomen, head or the whole body (WB) (Table 2). From the insects captured and exposed to a negative donor plant, 23 out of 166 tested positive, indicating they were carrying the virus acquired in the field. Testing of insects exposed to a positive donor plant showed 23 out of 45 heads were positive, while 134 out of 161 tested positive in either their abdomen or whole body. That heads tested positive could be the result of virus particles present on mouthparts or salivary glands. The recipient grapevines tested negative 5, 12 and 17 months post IAP (Table 3), and will be tested again in fall 2021 and Summer 2022.

*Leafhopper 2.* The second leafhopper species (Leafhopper-2) occurred at densities far lower than that of Leafhopper-1 which has limited our transmission studies with this insect to a few observations. CO1 barcoding enabled us to identify the leafhopper as *Acinopterus* sp. So far, at least five wild captured Leafhopper-2 adults from one collection were released onto a GRBV-negative plant in the greenhouse; 11 Leafhopper-2 adults from another collection were caged on a GRBV-positive grapevine in the vineyard and then transferred to a qPCR-tested GRBV-negative potted grapevine. Finally, five Leafhopper-2 adults caged initially on a GRBV-negative Cabernet Sauvignon potted grapevine and one Leafhopper-2 adult initially caged on a GRBV-positive Cabernet Sauvignon (GHV 24-392) potted donor vine were then released into individual cages containing a single potted GRBV-negative Cabernet Sauvignon potted recipient grapevine. qPCR analysis of the insects for GRBV, indicated that the virus was present in heads and abdomens or whole bodies (Table 2). The recipient grapevines tested negative for GRBV by qPCR after 5, 12 and 17 months (Table 3), and will be tested again in fall 2021 and Summer 2022.

Analysis of salivary glands and abdomens of many other Auchenorrhyncha specimens are currently in progress. It is our intention to summarize these data into a manuscript at the conclusion of this season.

**Table 1.** Summary of GRBV presence in salivary glands or whole bodies of *Spissistilus festinus* genotypes.

ID	County	Collection site	Fed on GRBV source vine	Salivary gland Positive/Total	Abdomen Positive/Total	Whole body Positive/Total
1	Amador	Vineyard	no	1/5	1/5	N/A**
2	Amador	Vineyard	no	9/20	NT*	4/14
3	Amador	Vineyard	yes	11/12	NT	13/13
4	Solano	Alfalfa	no	0/10	NT	N/A
5	Solano	Alfalfa	no	7/10	10/10	N/A
6	Solano	Alfalfa	no	0/10	0/10	N/A
7	Solano	Alfalfa	no	0/24	0/24	0/24
8	Solano	Alfalfa	yes	1/69	0/69	N/A
9	Solano	Alfalfa	no	0/17	0/17	N/A
10	Solano	Alfalfa	yes	0/17	10/17	N/A
11	Napa	Vineyard	no	2/16	1/16	N/A
12	Yolo	Riparian Area	no	0/2	0/2	N/A
13	Calistoga	Vineyard	no	N/A	N/A	0/9
14	Healdsburg	Vineyard	no	N/A	N/A	0/2
15	Geyersville	Vineyard	no	N/A	N/A	0/2
16	Amador	Vineyard	yes	N/A	N/A	1 /2
17	Amador	Vineyard	no	N/A	N/A	4/23
18	Amador	Vineyard	yes	13/18	18/18	N/A
19	Solano	Riparian Area	yes	0/2	2/2	N/A
20	Amador	Vineyard	yes	1/10	10/10	N/A
21	Amador	Vineyard	no	1/4	4/4	N/A
22	Amador	Vineyard	no	N/A	N/A	7/10
23	Solano	Riparian Area	no	N/A	N/A	0/12
24	Amador	Vineyard	no	N/A	N/A	1/1
25	Solano	Riparian Area	yes	1/2	2/2	N/A
		Total		47/248	58/204	30/110
		Percentage positive		19%	28%	27%

NT = not tested; \*\* N/A = not available

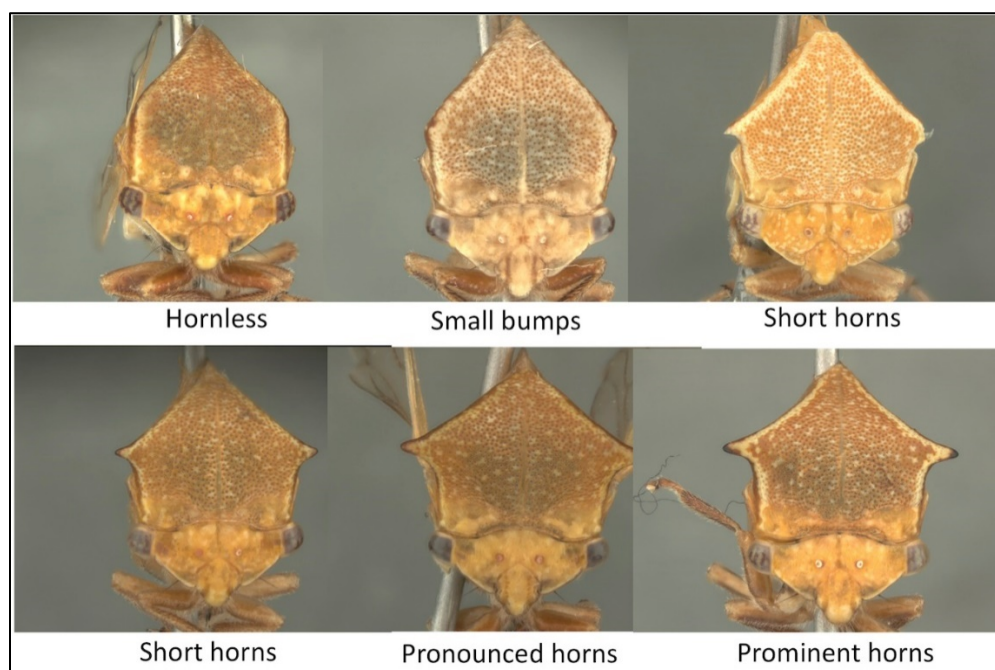
**Table 2.** Status of transmission studies with leafhopper species 1 and 2 caught in vineyards.

Species	No. of plants	Fed on GRBV source vine	Head Positive/Total	Abdomen or WB Positive/Total	Vines tested at five months Positive/Total
<i>Ceratagallia</i>	21	no	0/5	23/166	0/21
	23	yes	23/45	134/161	0/23
<i>Acinopterus</i>	3	no	6/10	7/13	0.3
	3	yes	7/11	9/11	0/3



**Table 3.** Transmission studies with leafhopper species 1 and 2 collected from vineyards.

Species	No. of assays	No. of plants used per assay	No. of insects per plant
1	4	1 to 15	4 to 30
2	3	1 each	1 to 11

**Figure 9.** Clinal variation of suprahumeral horns in females from a single collection of *T. albidosparsus* from a Napa County vineyard.**Table 4.** 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

### **b) Testing of wild grapes near Russell Ranch**

Putah Creek and its riparian area are an important natural habitat between Yolo and Solano Counties. In this area, it is common to find wild cane berries and grapevines. In order to assess potential reservoirs for GRBV along this riparian corridor, wild grapevines growing in this area were tested by qPCR. As part of our vegetation survey, eleven wild grapevines from the Putah Creek area near Winters were sampled in June 2019 and tested negative for GRBV. From October and November 2019, 7 of 49 wild grapevines from the Putah Creek area between Stevenson Bridge and UC Davis's Russell Ranch tested positive for GRBV. The virus from these samples was then amplified and partially sequenced, and indicated a genome similarity of 98% with Clade II GRBV isolates. Our testing results were shared with Dr. Maher Al Rwhanih of UC Davis Foundation Plant Services. We did not conduct additional sampling of wild grapes in this area after the establishment of COVID-19 restrictions in March 2020, and the vines have subsequently been removed.

### ***Objective 3. Taxonomic identification and seasonal monitoring of *Tortistilus* spp. populations in California vineyards and surrounding landscapes***

In spring 2016, we found high densities of treehoppers on grapevines grown on hillsides in Napa County where virus spread was occurring that we identified to belong to the genus *Tortistilus*. The presence of *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, Dr. Vaughn 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 Co. vineyard and found morphs of brown and green color both with and without horns from the same host plants on the same day. These insects were tentatively identified as *Tortistilus albidosparsus*, *Tortistilus pacificus* and *Tortistilus wickhami* based on the presence or absence of a suprahumeral horn characteristic (Figure 9) 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, Dr. Dennis Kopp at the Smithsonian Natural History Museum in Washington DC. He identified the four morphs; brown horned, green horned, brown unhorned and green unhorned, as being the same species based on microscopic observations of genitalia and the characteristic spots on the front of their head.

### **Taxonomic identification of male *T. albidosparsus* by 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) (Table 4). 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*. Morphological similarity together with these results indicate that the morphs indeed all belong to a single species which according to zoological nomenclature priority would be *Tortistilus albidosparsus* (Stål) (originally '*albidosparsus*') that was previously thought to only have horned individuals.

Interestingly, the Swedish authority who named the species, Stål (1860), designated a specimen from San Francisco, CA as its holotype.

Traditional identification of *Tortistilus* species (and most other treehoppers) is primarily done based on morphological characters of male genitalia. However, taxonomic determination of the *Tortistilus* species of interest as potential GRBV vectors was met with challenges. Among these was that 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 10A-H), confirming our biological observations as well as the results of the CO1 sequencing, and adding further confirmation that they represent a single species.

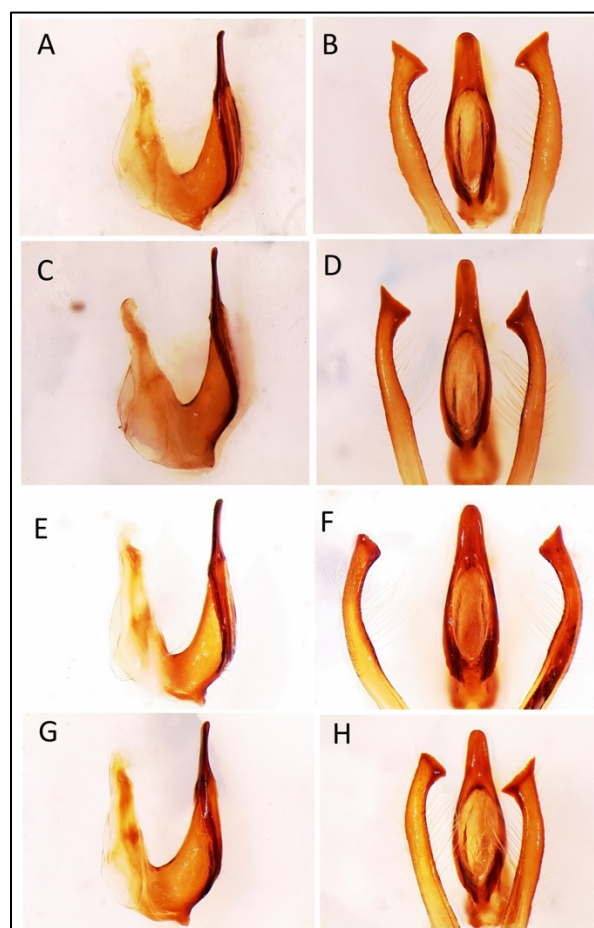
***Tortistilus albidosparsus* mating study with the 4 *Tortistilus* morphotypes.**

On April 25, 2018, we collected 152 third and fourth instar *T. albidosparsus* nymphs from purple vetch (*Vicia americana*) growing adjacent to a confirmed GRBV infested vineyard in Pope Valley, Napa Co. The nymphs were then returned to UC Davis where they were transferred into individual clip cages placed on potted vetch plants in pop-up cages. The surviving nymphs matured to become adults in mid-June. As adults emerged, the *T. albidosparsus* in individual clip cages were classified as horned or unhorned, and sex was determined. Finally, the adults were combined into six groups consisting of three males and five females based on the presence or absence of subhumeral horn. One group consisted of horned males and horned females, one group of unhorned males and unhorned females, and four groups consisted of either horned males and unhorned females or vice versa. Each of the six groups were then placed into six separate nylon insect mesh cages fastened onto a potted blue oak tree (*Quercus douglasii*) with purple vetch growing at its base. Each of the six potted blue oak trees were then placed into a larger mesh insect cage and transferred into a UC Davis greenhouse and were not disturbed other than being watered twice a week. Oviposition scars (Figure 11) were noted on the blue oak tree seedlings.

Matings of all combinations of horned and unhorned morphotypes collected as sexually immature nymphs in late spring 2018 produced offspring that matured to adults from April to May 2019. Of the six mating groups, three produced both horned and unhorned progeny (Table 5). This is the first report of successful mating of the horned and hornless morphs and the production of offspring.

**Table 5.** Number of *Tortistilus albidosparsus* progeny resulting from the mating of 3 males and 5 females of the designated combinations of morphotypes in cages containing a blue oak, *Quercus douglasii*, seedling and purple vetch, *Vicia americana*.

Group	Male parent with horns?	Female parent with horns?	Male progeny		Female progeny	
			Horns	No horns	Horns	No horns
1	Y	Y	11	none	3	none
2	Y	N	none	none	none	none
3	Y	N	1	8	none	8
4	N	Y	none	none	1	1
5	N	Y	none	none	none	none
6	N	N	none	none	1	2



**Figure 10.** A, C, E, and G are profiles of male *Tortistilus 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 80°C for 40 minutes. Images were taken with a digital JVC camera mounted onto a Leica MZ 16A dissecting microscope at 110X magnification.



**Figure 11.** *Tortistilus albidosparsus* oviposition scar on oak twig in the mating study using both horned and hornless insects.

### **Novel genotypes of *Spissistilus*.**

Because transmission of GRBV by 3CAH has proven to be inconsistent, we have been curious if it is possible that some GRBV isolates are more likely to be insect transmitted or if there are biotypes of 3CAH that are more competent vectors. Therefore, we began sequencing 3CAH collected from different sites where GRBV spread is observed and additional locations where spread has not been observed. In summer, 2019, we identified a population of *S. festinus* from a vineyard where we have documented spread in which the CO1 gene substantially differs from that found in the 3CAH most commonly found in alfalfa fields near Davis and at many other vineyard locations. To determine if this genetic difference corresponds to another species, or a particular genotype of 3CAH, we sent vouchers of both populations to our cooperator Dr. Dennis Kopp, a specialist on the treehopper family Membracidae at the Smithsonian Natural History Museum. He confirmed that the two populations appear morphologically indistinguishable. We are conducting studies to characterize these populations including genomic comparisons with archival (museum) specimens, its presence in other grapevine production areas, and their potential involvement in GRBV transmission. We are also preparing a manuscript on our findings to date.

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## MONITORING VINE MEALYBUG RESISTANCE TO IMIDACLOPRID

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**Reporting Period:** The results reported here are from work conducted July 2019 to October 2021.

### ABSTRACT

The vine mealybug (*Planoccocus 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 infesting a 10 cm tall grape seedling planted in a 20 ml pot with 5 vine mealybug nymphs that were in the 2<sup>nd</sup> instar. The plant was then treated with an 8 ml drench of a solution containing a known quantity of imidacloprid. After 6 days, number of nymphs surviving was determined. For each set of assays, 6 doses of imidacloprid were evaluated, with 10-20 replicates completed for each dose. Three colonies initiated from collections made in the San Joaquin Valley (1 from Stanislaus County, 1 from Tulare County, and 1 from Kern County) during 2019 and 2020 and a reference laboratory colony have been evaluated. An additional 6 collections were made during fall of 2021 (2 from Stanislaus County, 1 from Fresno County, and 3 from Tulare/Kern County) and will be subjected to testing once colonies have reached a sufficient population size. For all colonies tested, application of 8 ml of imidacloprid at a dose > 1 ug [AI]/ml was required to observe mortality, with occasional survivors at doses of 100 and 1,000 ug [AI]/ml. Survivors at high doses (>1 ug[AI]/ml) displayed delayed development and were less vigorous than survivors at lower doses. To determine if mortality was dependent on assay length, a subset of tests compared mortality of mealybugs in tests conducted over 12 versus 6 days, with similar results. While survivors at high doses were observed 12 days post-treatment, given their lack of vigor additional testing is required to determine if such survivors can complete development and reproduce. To determine how doses applied to seedlings relate to field doses, all plants from

assays were frozen and the quantity of imidacloprid per gram of leaf tissue will be determined using high performance liquid chromatography (HPLC) based methods. For reference, leaves from vineyards treated with imidacloprid were collected throughout the summer of 2020 and 2021 and will be assayed to determine peak quantities of imidacloprid in field treated vines. Processing of leaf samples for HPLC testing will be conducted during fall and winter of 2021.

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## ABBREVIATIONS

3CAH	threecornered 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	threecornered 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 <i>Vitis</i> virus 1
<i>Xf</i>	<i>Xylella fastidiosa</i>