

# Pierce's Disease Control Program

## RESEARCH PROGRESS REPORTS

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

~ DECEMBER 2017 ~



**CALIFORNIA  
PD/GWSS BOARD**

*Partnership for Winegrape Pest Solutions*



# California Department of Food & Agriculture



**Research Progress Reports**

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

- December 2017 -

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**Note to Readers:**

The reports in this document have not been peer reviewed.

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# **Section 1:**

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



## EVALUATING POTENTIAL SHIFTS IN PIERCE'S DISEASE EPIDEMIOLOGY

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**Reporting Period:** The results reported here are from work conducted July 1, 2017 to October 27, 2017.

**ABSTRACT**

Extensive blue-green sharpshooter (*Graphocephala atropunctata*) trapping in Napa and Sonoma during 2016 and 2017 has indicated that populations are currently low, and follow spatial and temporal patterns somewhat expected based on previous surveys. Insects occur more frequently near source habitats such as riparian zones. However, the data showed significant differences in trends between Napa and Sonoma valleys. In addition, due to the observation of Pierce's disease hotspots away from blue-green sharpshooter habitat, we have initiate surveillance efforts targeting spittlebugs. This work was initiated in 2017, so there is limited data for any conclusion. One question raised by reviewers of this project was associated with the possibility of a novel Pierce's disease genotype/strain being responsible for the recent epidemic on the North Coast and elsewhere. We collected and sequenced over 100 *Xylella fastidiosa* isolates from five grape-growing regions in California. The results show clustering of isolates based on region, indicating absence of a sweep through the state; these are based on preliminary data.

**LAYPERSON SUMMARY**

A Pierce's disease epidemic emerged in Napa and Sonoma counties. Very high Pierce's disease prevalence was reported throughout the region, with a large number of stakeholders reaching out to University of California Cooperative Extension Farm Advisors. In summer 2015, the project team held a series of joint meetings/field visits with the Farm Advisors. Two observations have been made that raised our concern about the problem. First, high prevalence of Pierce's disease in the North Coast is usually below 1-2% per vineyard; several vineyards visited had over 25% of vines symptomatic. Second, historically Pierce's disease is closely associated with riparian zones in the North Coast; we have visited several vineyards where Pierce's disease does not appear to be associated with riparian zones. We have observed these greater rates of disease incidence and dissociation with riparian areas throughout Napa and Sonoma counties; they are not district specific. The goal of this proposal is to determine what factors are driving this epidemic, so that ecology-based disease management strategies can be devised and immediately implemented, as was successfully done in the past when disease drivers appear to have been different.

**INTRODUCTION**

Pierce's disease of grapevine has reemerged in Napa and Sonoma counties, where disease incidence has been much higher than usual and the distribution of sick vines within vineyards often does not fall within expectations. These field observations taken together with the very high number of vineyards affected in the region indicate that a Pierce's disease epidemic is emerging. The goal of this proposal is to determine what factors are driving this

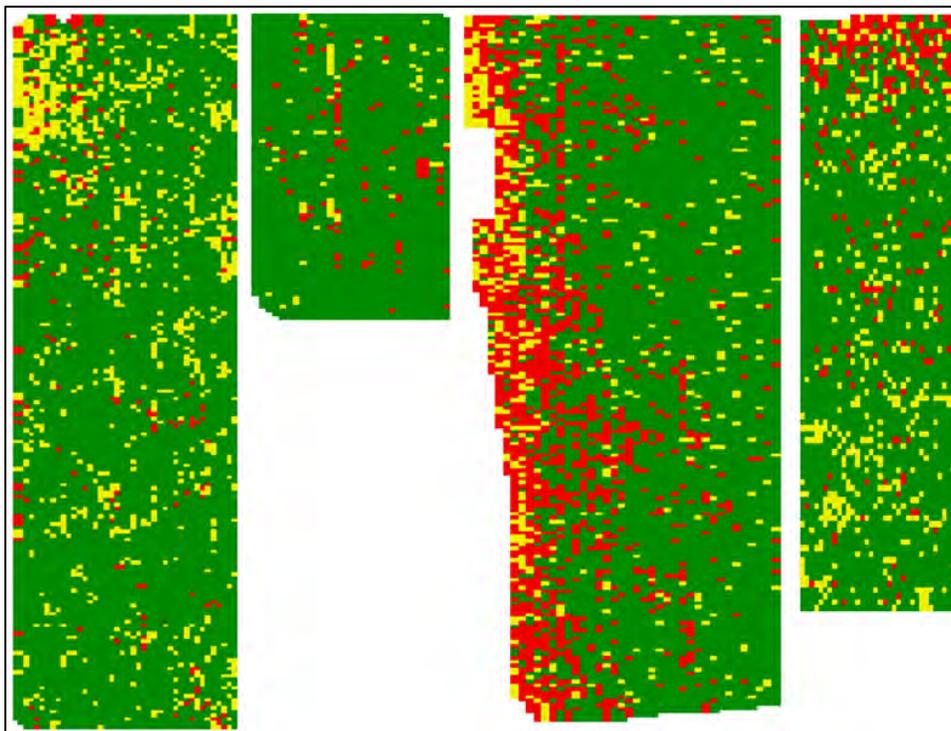
epidemic, so that ecology-based disease management strategies can be devised and immediately implemented, as was successfully done in the past when disease drivers appear to have been different. This report summarizes activities associated with Pierce's disease ecology and *Xylella fastidiosa* (*Xf*) population genomics. We present Results and Discussion sections together addressing each original objective. Furthermore, we note that limited amount of data analyses has been done, primarily because efforts have focused on collecting data, and not spending time generating/interpreting preliminary results.

## OBJECTIVES

1. Vector, pathogen, and host community surveys to inform the development of a quantitative model to assess future Pierce's disease risk and develop integrated management strategies.
2. *Xf* colonization of grapevines and the role of overwinter recovery in Pierce's disease epidemiology.
3. Determine the role of spittlebug insects as vectors of *Xf*.
4. Data mine and disseminate existing information on vector ecology, vegetation management, and efficacy of pruning.
5. Develop a larger extension and outreach footprint with additional seminars, extended interviews made available on the web, and an update to the *Xf* website, the main online resource for Pierce's disease information.

## RESULTS AND DISCUSSION

This report will focus on recent results obtained for Objective 1. Prior results are available in previous reports. As part of Objective 1, we have now conducted two years of Pierce's disease surveys in 32 vineyards throughout Napa and Sonoma counties, in the fall of 2016 and again the fall of 2017. As a first step toward understanding the condition changes that may have triggered the recent Pierce's disease epidemic in the North Coast, we have initiated a set of spatial analyses to describe the patterns of disease at each site at the outset of the study. Here, we summarize the results of those analyses for four representative vineyards in the fall of 2016 (**Figure 1**). Two sites are located in Napa County ("CDV" and "TREF"), have no nearby riparian habitat, and were estimated to have less than 5% Pierce's disease (**Table 1**). Two other sites located in Sonoma County ("NEWS" and "V7") are adjacent to riparian corridors, with Pierce's disease prevalence ranging between approximately 8 and 20% (**Table 1**).



**Figure 1.** Mapping results for Pierces disease at four representative sites in the fall of 2016. Red pixels denote vines with Pierce's disease, yellow are dead, missing, or replant vines, and green denotes apparently healthy vines. Sites (L to R) are: CDV, TREF, NEWS, V7. Maps are on the same approximate scale, but each is oriented arbitrarily. For NEWS and V7, riparian habitat is located to the left and above, respectively.

In the fall of 2016 we surveyed all of the vineyards, inspected each vine in the block, noted the status of each vine as: apparently healthy, Pierce’s disease, dead, replant, or missing, and collected tissue samples from up to 20 Pierce’s disease vines to confirm infection by *Xf*. The mapped distributions of initial disease prevalence (**Figure 1**) were then subjected to a suite of analyses to look for (1) non-random distribution (i.e. clustering) of Pierce’s disease cases, (2) spatial association between Pierce’s disease cases and other non-healthy disease categories (i.e. dead, missing, or replant vines), and (3) non-uniform distribution of Pierce’s disease cases over the block (i.e. anisotropic gradients in disease).

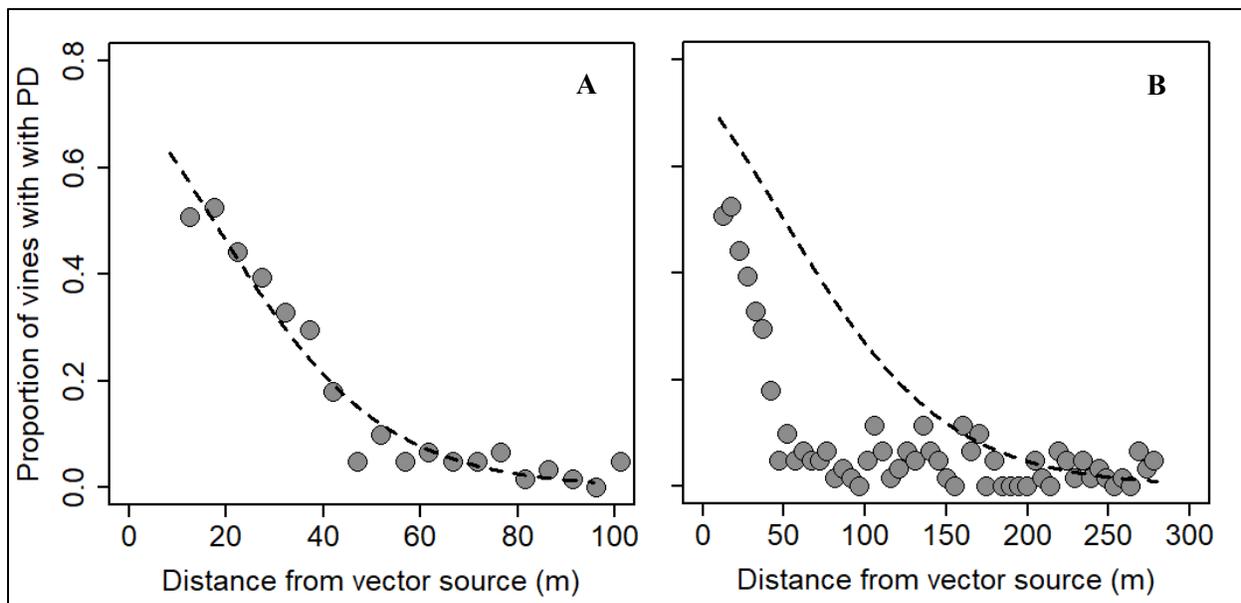
For the first two analyses, we used a pair of point pattern analyses to look at the strength and scale of clustering in non-healthy vines (Dale and Fortin 2014). In the first, we used an L means test on just vines showing evidence of Pierce’s disease (Brunson and Comber 2015). The tests were significant for all four of the sites (**Table 1**). This suggests significant clustering of Pierce’s disease cases at all sites, though the scale of clustering varied from below 5 vine spaces for site TREF to over 15 vine spaces at site NEWS. Next, a similar L means test was used for Pierce’s disease vines versus other non-healthy vines to look for co-clustering (Brunson and Comber 2015). This second set of tests indicated variability among the sites, with three sites showing significant co-clustering while the fourth (TREF) was non-significant (**Table 1**). In other words, at the three significant sites (CDV, NEWS, V7), Pierce’s disease vines are more likely to be found near dead, missing, or replant vines than expected by chance.

**Table 1.** Summary statistics for Pierce’s disease at four representative sites in the fall of 2016, including whether they are adjacent to riparian habitat, total number of vines surveyed, percent of vines showing Pierce’s disease symptoms, L means test for clustering of Pierce’s disease cases, L means test for co-clustering between Pierce’s disease cases and missing, dead, or replant vines, and test for uniformity in the distribution of Pierce’s disease cases across the vineyard block (i.e. no disease gradient).

Site	Riparian	# vines	% PD	PD clustering		Co-clustering		Uniformity		
				<i>u</i>	<i>P</i>	<i>u</i>	<i>P</i>	$\chi^2$	df	<i>P</i>
CDV	N	7406	2.85	144.17	0.01	6.670	0.01	1.0172	2	0.6013
TREF	N	2220	4.68	37.158	0.01	5.050	0.12	1.7144	2	0.4243
NEWS	Y	6608	20.11	256.4	0.01	17.832	0.01	9.6049	2	0.0082
V7	Y	3355	8.29	107.45	0.01	0.5741	0.01	21.663	2	<0.0001

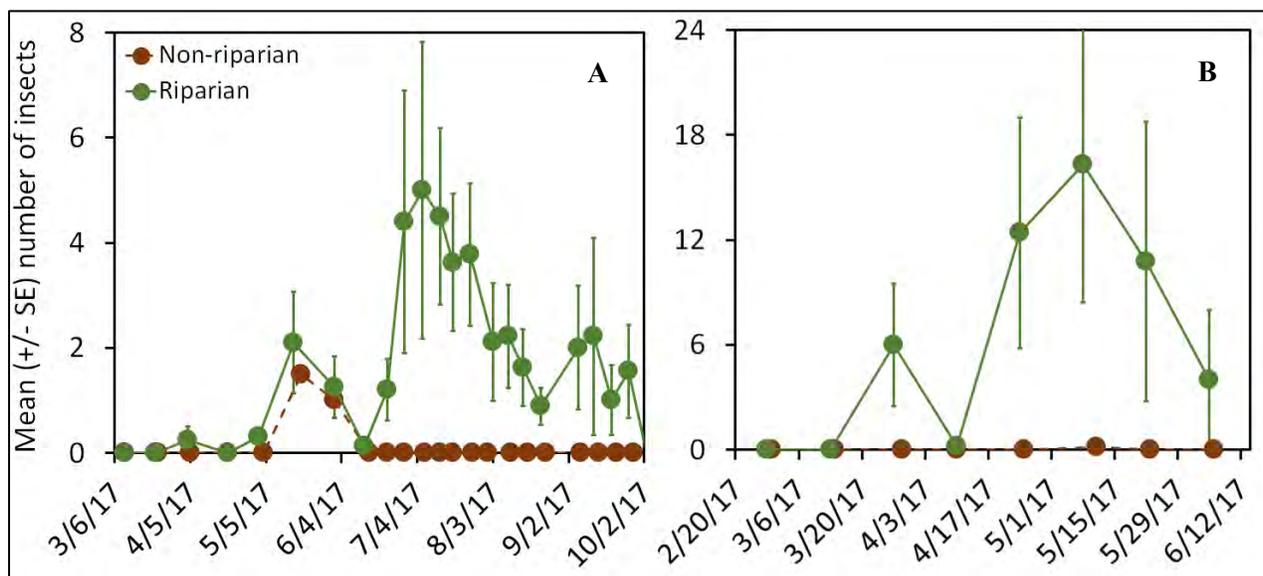
In the third analysis of Pierce’s disease patterns at each site, we used Guan’s test for uniformity (package spTest() in the R programming language; Weller 2016) to determine whether there were gradients in Pierce’s disease across the vineyard block. For this test, a significant value (i.e.  $P < 0.05$ ) indicates anisotropy, which was followed up with a generalized linear mixed-effects model (GLMM) to quantify the nature of that gradient. Specifically, we used a GLMM binomial error, a fixed effect of distance from potential vector source habitat (i.e. nearby riparian habitat), and a random effect of vine number nested within row number to account for spatial autocorrelation. The results of the test for uniformity showed evidence of significant gradients at the two riparian sites, but not the non-riparian sites (**Table 1**). For the two riparian sites the likelihood of a vine having Pierce’s disease declined significantly at greater distances, with most cases within approximately 60 m of the riparian corridor but with still a handful of cases at much greater distances (**Figure 2**).

In addition to mapping Pierce’s disease, we have been monitoring vector populations at each of the vineyards on a regular basis. This monitoring is intended to address aspects of Objectives 1 and 3, but clarifying the diversity, abundance, and distribution of vector populations. Our monitoring includes using yellow sticky traps primarily for blue-green sharpshooter (*Graphocephala atropunctata*). In addition, this season we used sweep-net sampling to track populations of other potential vectors (i.e. leafhoppers and spittlebugs) on the vineyard floor. Starting toward the end of winter, every two weeks we conducted 15 sets of sweeps at each site on ground vegetation located along the border of the vineyard or between vineyard rows. All collected insects were identified, preserved in ethanol, and will be tested for the presence of *Xf*. Sweep net sampling continued through the end of the spring or longer if sufficient live vegetation was present.



**Figure 2.** Gradients in Pierce's disease prevalence as a function of distance from riparian habitat. Sites: (A) NEWS, (B) V7. Points reflect proportions of vines with Pierce's disease of 50-100 vines at different binned distances. Dashed lines denote model fit.

Over the season a total of 331 potential vectors were collected in sweep-net sampling at Sonoma sites, and 256 in sampling at Napa sites. Notably, the composition differed substantially among sites and between the two counties. At Sonoma sites, blue-green sharpshooter was the most common (44.4% of insects), the red-headed sharpshooter (*Xyphon fulgida*) was nearly as common (31.4%), followed by meadow spittlebug nymphs or adults (*Philaenus spumarius*; 18.1% total), and an unidentified leafhopper species (*Pagaronia* sp.) was rarer (2.7%). Meanwhile at Napa sites no blue-green sharpshooters or red-headed sharpshooters were collected, while meadow spittlebug and especially the *Pagaronia* sp. were relatively more common (36.3% and 62.9%, respectively). There is also a clear effect of surrounding habitat type on vector abundance in the sweep-net sampling, with substantially greater abundance at riparian sites compared to non-riparian sites, especially after late spring (**Figure 2**).



**Figure 3.** Total number of leafhoppers and spittlebugs collected in sweep net sampling at riparian and non-riparian sites in (A) Sonoma and (B) Napa counties. Scale of axes differ between panels.

Another component of this report is associated with our effort to address one question: was the current epidemic due to the emergence of a new *Xf* genotype? To answer this question we collected isolates from grapevines with Pierce's disease symptoms from five regions in 2016; Napa, Sonoma, Bakersfield, Temecula, and Santa Barbara. Approximately 120 *Xf* genomes were cultured from plants, triple-cloned in the laboratory, and had their genomes sequenced. Preliminary analyses indicate that isolates from each region sampled clustered together, with a few exceptions, providing no evidence to support the hypothesis of an emerging genotype of *Xf* causing Pierce's disease. Analyses are ongoing to better understand factors affecting the structuring of populations in California.

## **CONCLUSIONS**

Our ongoing analysis of patterns of Pierce's disease at the vineyard sites has shown three notable results thus far. First, there is significant spatial clustering of Pierce's disease cases within vineyard blocks. Such clustering is not an uncommon feature of infectious diseases, including some vector-borne pathogens. The scale of that clustering is helpful for guiding future investigations of the nature of pathogen spread at these sites. Second, there appears to be significant co-clustering of Pierce's disease cases nearby other dead, missing, or replant vines. This result suggests that other non-healthy vines may reliably reflect older cases of Pierce's disease, meaning that the total losses due to Pierce's disease are more substantial than just those vines showing evidence of disease at any given time. Third, thus far analyses of disease patterns at riparian sites, as expected, show gradients in disease over fairly substantial distances from vector sources. Meanwhile non-riparian sites exhibit clustering of Pierce's disease cases without any evidence of gradients from an obvious vector source, suggesting the potential for other mechanisms (e.g. alternative vectors, reservoir hosts) to contribute to Pierce's disease hotspots in certain contexts. Finally, in addition to documenting the distribution and abundance of known important vectors at each site, such as blue-green sharpshooter, we've begun to document the diversity and abundance of alternative vectors at each site. Such information on the broader vector assemblage may yield insights into why certain sites show high Pierce's disease prevalence despite not having obvious blue-green sharpshooter source habitat nearby. The next step for analysis, after describing the initial spatial patterns of disease at all 32 vineyard sites, is to quantify interannual change in Pierce's disease prevalence (i.e. 2016 to 2017) to better understand the factors driving Pierce's disease incidence (e.g. surrounding plant community composition, blue-green sharpshooter abundance and dispersal, other vector abundance).

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# THE EPIDEMIOLOGY OF NOVEL *PdRI* RESISTANT GRAPEVINES: EPIDEMIC AND VECTOR MOVEMENT MODELS TO SUPPORT INTEGRATED DISEASE MANAGEMENT

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**Reporting Period:** The results reported here are from work conducted July 1, 2017 to October 27, 2017.

## ABSTRACT

Resistant cultivars of agricultural crops are integral to sustainable integrated disease management strategies. Our previous work indicated that grapevines that express the *PdRI* gene exhibit resistance against *Xylella fastidiosa* (*Xf*) and are likely to slow the spread of *Xf* among vineyards. In the current project we are testing the generality of our previous results by testing multiple *PdRI* resistant and susceptible genotypes in our vector transmission experiments and integrating greater biological detail into our epidemic modeling work. While *PdRI* resistant grapevines provide promising resistance, it remains unclear how growers may incorporate these hybrid plants into their production. Growers may be able to benefit from *PdRI* resistant cultivars without planting all of their acreage to them. We will explore the implications for *Xf* spread and Pierce's disease severity from planting adjacent blocks of *PdRI* resistant and susceptible grapevines through bio-economic modeling. Finally, our modeling efforts rely on assumptions on insect vector dispersal within and among vineyards, yet our knowledge of sharpshooter dispersal has been limited by the difficulty of experimentally measuring dispersal. We will use large spatio-temporal data sets of vector abundance for both blue-green sharpshooter (*Graphocephala atropunctata*) and glassy-winged sharpshooter (*Homalodisca vitripennis*) and hierarchical statistical models to estimate dispersal directly from field data. Taken together, our project will provide clearer recommendations for disease management strategies using *PdRI* and related resistant grapevines.

## LAYPERSON SUMMARY

Sustainable management of Pierce's disease will rely on developing grape cultivars that are resistant to *Xylella fastidiosa* (*Xf*). We found previously that grape cultivars that express the *PdRI* gene exhibit intriguing levels of delayed resistance against *Xf* that reduced transmission rates by vector insects. We propose to expand on this work by testing multiple lines of *PdRI* resistant grapes for transmission rates by vector insects. We will then use statistical models to estimate vector movement into vineyards. Finally, we will integrate vector transmission and movement information to predict *Xf* spread through *PdRI* and susceptible cultivars using mathematical models.

## INTRODUCTION

Resistance against pathogens in agricultural crops is one of the more successful strategies to effectively manage agricultural diseases (Mundt 2002). This includes vector-borne pathogens. Though insecticide suppression of vectors is a common practice, previous research has called into question the efficacy of insecticides and highlighted the risks of evolved resistance against them (Perring et al. 2001, Erlanger et al. 2008).

However, while plant resistance traits are often effective at suppressing pathogen spread, this is certainly not the case with tolerance traits. Where resistance traits alleviate disease symptoms by reducing pathogen burden, tolerance traits alleviate symptoms with negligible effects on pathogen burden (Roy and Kirchner 2000). For vector-borne pathogens, the influence of resistance traits on pathogen spread and disease prevalence can differ dramatically from tolerance traits (Zeilinger and Daugherty 2014, Cronin et al. 2014). Introducing resistance traits into a host population will generally reduce pathogen spread, whereas tolerance traits can have the opposite effect.

Specifically, when vectors of a pathogen avoid feeding on diseased (i.e. symptomatic) hosts, introducing tolerant hosts will enhance pathogen spread (Zeilinger and Daugherty 2014). Because the primary sharpshooter vectors of *Xylella fastidiosa* (*Xf*) in California (blue-green sharpshooter and glassy-winged sharpshooter) preferentially avoid feeding on Pierce's disease symptomatic plants (Daugherty et al. 2011), tolerance traits in grapevines could increase the risk of *Xf* spread within and among vineyards.

Ongoing efforts to identify resistance to *Xf* in native *Vitis* spp. has resulted in hybrid plants that express the *PdRI* locus (Walker and Tenschler 2016). These hybrid vines do not suffer from Pierce's disease symptoms to the same extent as susceptible lines (Krivanek and Walker 2005, Krivanek et al. 2006). Furthermore, from our previous results, *PdRI* resistant grapevines appear to reduce insect vector transmission rates. As such, they are likely to reduce spread of *Xf* within and among vineyards.

## OBJECTIVES

The overall goal of this project is to assess the epidemiological consequences of managing Pierce's disease with resistant grapevines expressing the *PdRI* locus (Walker and Tenschler 2016). Specifically, we ask, under what conditions and spatial arrangements will the use of *PdRI* vines reduce *Xf* spread and maximize economic benefits to growers? The research consists of three objectives:

1. Test the effects of *PdRI* resistant plants on vector feeding preference and transmission of *Xf*.
2. Model the optimal mixture of *PdRI* and susceptible grapevines to reduce *Xf* spread and maximize economic return.
3. Estimate dispersal of insect vectors from field population data.

## RESULTS AND DISCUSSION

As the project recently began, we have no results to report at this time.

## CONCLUSIONS

Conclusions are pending, concomitant with results.

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## DEVELOPMENT OF A BIOLOGICAL CONTROL FOR PIERCE'S DISEASE

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### ABSTRACT

*Xylella fastidiosa* (*Xf*) is an important phytopathogen that infects a number of important crops including citrus, almonds, and coffee. The *Xf* Temecula 1 strain infects grapevines and induces Pierce's disease. We deleted the *Xf* PD1311 gene and found that the mutant strain was avirulent. Based on sequence analysis, PD1311 is predicted to encode an acyl-CoA synthetase, which is a class of enzymes involved in numerous processes including secondary metabolite production. We characterized  $\Delta$ PD1311 and found that it expresses *in vitro* phenotypes that are consistent with reduced virulence, is avirulent in grapevines, and reduces the virulence of wild-type *Xf*. Therefore, we propose that the  $\Delta$ PD1311 has potential as a biological control for Pierce's disease.

### LAYPERSON SUMMARY

We discovered that deleting the *Xylella fastidiosa* (*Xf*) gene, PD1311, resulted in a strain that does not induce Pierce's disease. Additionally, we have evidence that the PD1311 mutant has potential as a biological control. When grapevines were inoculated with the mutant prior to wild-type *Xf*, disease development became significantly reduced. Given the agricultural importance of Pierce's disease, it is critical to understand how PD1311 exerts its effects. Options for managing Pierce's disease are limited, so developing new control strategies are critically important. Our results expand the understanding of Pierce's disease and provide information in relation to controlling the disease.

### INTRODUCTION

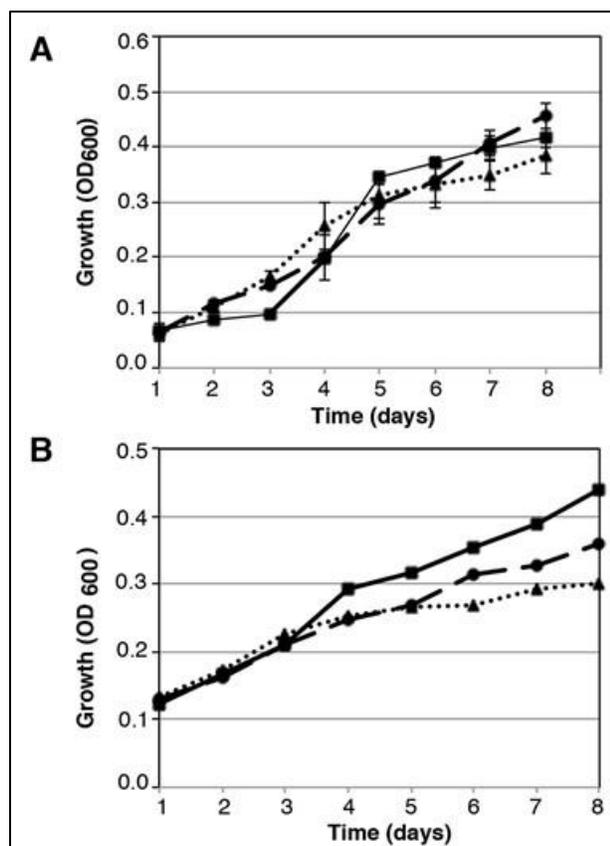
*Xylella fastidiosa* (*Xf*) is a Gram-negative, xylem-limited bacterium that causes Pierce's disease in grapevines (Chatterjee et al. 2008). *Xf* is transmitted to plants by insect vectors and once in the xylem, *Xf* is postulated to migrate, aggregate, and form biofilm that clogs the vessels leading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

We deleted the *Xf* PD1311 gene ( $\Delta$ PD1311), a putative acyl-CoA synthetase (ACS), as we were interested in genes potentially involved in secondary metabolite production. ACSs catalyze long-chain fatty acyl-CoAs (Black et al. 1992), and they are involved in numerous processes including pathogenicity (Barber et al. 1997). We recently published our work on this gene, which includes showing it has potential to function as a Pierce's disease biocontrol (Hao et al. 2017).

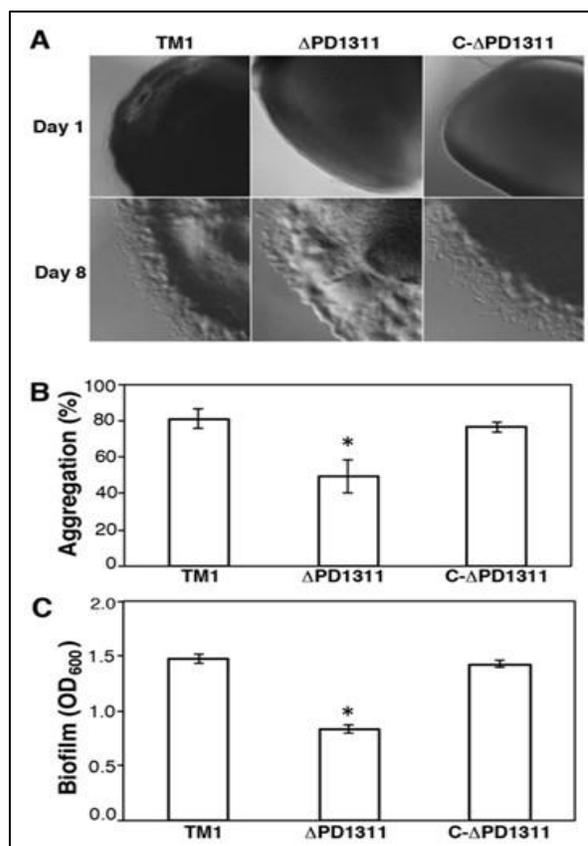
We found that PD1311 is a functional enzyme (data not shown), and that  $\Delta$ PD1311 grows in PD2 and *Vitis vinifera* sap (**Figure 1**) (Hao et al. 2017). In addition, motility, aggregation, and biofilm production are key behaviors of *Xf* that are associated with Pierce's disease (Chatterjee et al. 2008).  $\Delta$ PD1311 is reduced in type IV pili-mediated motility on periwinkle wilt (PW) plates and is non-motile on sap agar (**Figure 2**) (Hao et al. 2017). In comparison to wild-type cells (Temecula 1),  $\Delta$ PD1311 is reduced in aggregation and biofilm production. We

therefore hypothesized that  $\Delta$ PD1311 is less virulent in plants, as mutants with similar phenotypes have been shown to have reduced virulence or be avirulent (Cursino et al. 2009, Cursino et al. 2011, Guilhabert and Kirkpatrick 2005, Killiny et al. 2013). We found that  $\Delta$ PD1311 was avirulent and was not able to cause Pierce's disease, even at 24 weeks post-inoculation (**Figure 3**).

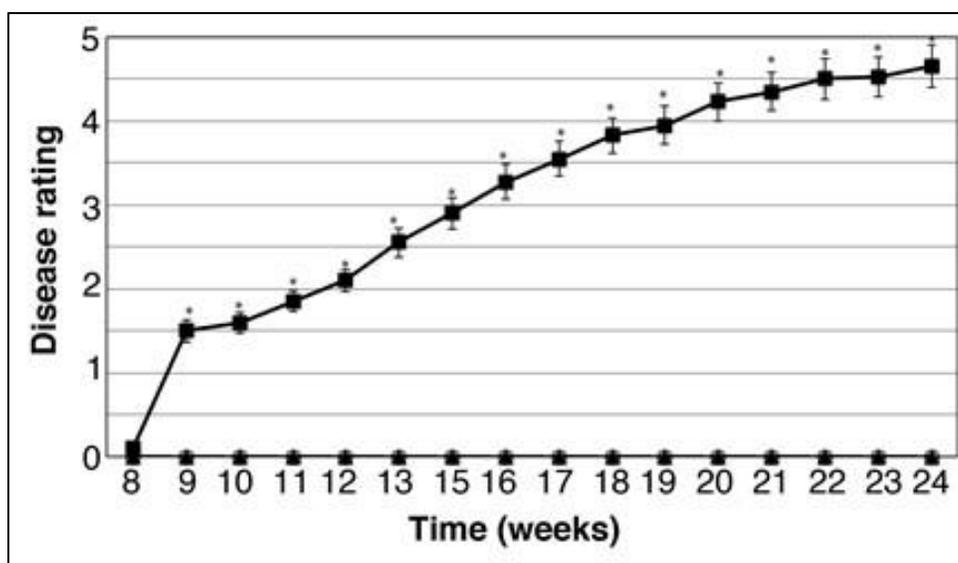
The weakly virulent *Xf* elderberry strain EB92-1 has been studied as a potential Pierce's disease biological control (Hopkins 2005, Hopkins 2012). Other approaches towards controlling Pierce's disease include resistant rootstocks (Cousins and Goolsby 2011) and transgenic vines (Dandekar 2014, Gilchrist et al. 2014, Gilchrist and Lincoln 2014, Kirkpatrick 2014, Lindow 2014, Powell and Labavitch 2014). Continued research of Pierce's disease controls is warranted. We had initial results that  $\Delta$ PD1311 lowered the incidence of wild-type-induced Pierce's disease. Given the avirulent phenotype of  $\Delta$ PD1311 and its ability to limit wild-type-induced Pierce's disease, this strain provides potential for development of a new biological control.



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



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



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

## OBJECTIVES

The overall goal is to optimize  $\Delta$ PD1311 as a biological control for Pierce's disease and to understand the mechanisms of disease inhibition that will facilitate commercialization.

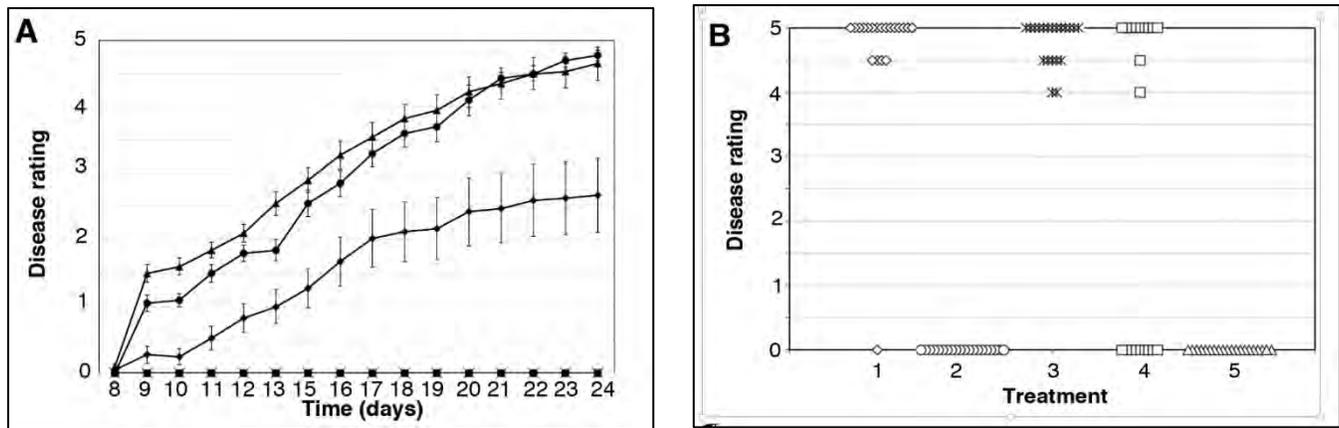
1. Examine aspects of  $\Delta$ PD1311 Temecula 1 strain as a biological control of Pierce's disease.
  - a. Optimize application timing and conditions for the  $\Delta$ PD1311 strain.
  - b. Determine if over-wintered  $\Delta$ PD1311 inoculated plants maintain Pierce's disease resistance.
  - c. Explore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

## RESULTS AND DISCUSSION

### Objective 1. Examine Aspects of $\Delta$ PD1311 Temecula 1 Strain as a Biological Control of Pierce's Disease

#### Objective 1a. Optimize Application Timing and Conditions for the $\Delta$ PD1311 Strain

To examine if the *Xf*  $\Delta$ PD1311 Temecula 1 strain could act as a potential biocontrol, we inoculated *V. vinifera* cv. Cabernet Sauvignon vines per standard procedures (Cursino et al. 2011) and recorded development of Pierce's disease using the five-scale assessment (Guilhabert and Kirkpatrick 2005). We created three different inoculation conditions: i) wild-type *Xf* after a two-week pre-treatment with  $\Delta$ PD1311 [following procedures used in *Xf* elderberry EB92.1 strain biocontrol studies (Hopkins 2005)], ii) wild-type and  $\Delta$ PD1311 co-inoculated, and iii) controls (wild-type-only,  $\Delta$ PD1311-only, buffer). We previously found that inoculating  $\Delta$ PD1311 after a two-week pre-treatment with the wild-type strain did not limit Pierce's disease (data not shown). Our controls included vines inoculated with wild-type Temecula 1,  $\Delta$ PD1311, or buffer (Hopkins 1984). We found that pre-treatment with  $\Delta$ PD1311 inhibited Pierce's disease, while co-inoculation did not alter disease development (**Figure 4**) (Hao et al. 2017).



**Figure 4.** ΔPD1311 inoculation to grape prior to TM1 suppressed Pierce's disease development. **A.** Weekly mean disease ratings of vines inoculated with TM1-only (triangles), TM1 and ΔPD1311 simultaneously (circles), ΔPD1311 two weeks prior to TM1 (diamonds), ΔPD1311-only (squares) and buffer (x marks) respectively. Error bars represent standard errors. Ten plants were included for each experiment and the assay was repeated twice. **B.** Disease rating for each vine at 24 w.p.i. 1 = TM1-only, 2 = ΔPD1311-only, 3 = co-inoculation with TM1 and ΔPD1311 simultaneously, 4 = ΔPD1311 two weeks before TM1, and 5 = buffer. TM1 = wild-type *Xf* Temecula 1, ΔPD1311 = *Xf* Temecula 1 deleted of the PD1311 gene.

In 2016 (May to October), we investigated the effectiveness of ΔPD1311 as a Pierce's disease biological control. To test the impact of inoculation timing, we inoculated vines with ΔPD1311 at two days, one week, and two weeks (previous conditions successful in disease inhibition as described in **Figure 4**) prior to inoculation with wild-type at the same inoculation point. To determine if inoculation location impacts Pierce's disease control, we inoculated the base of selected green shoots (~ 50 cm tall plant) with ΔPD1311 as described above and then two weeks later with wild-type *Xf* into vines at 5 cm or 30 cm above the initial inoculation site. Our control treatments included the above treatments except with buffer instead of ΔPD1311, in order to exclude any possible effects on plants caused by wounding prior to wild-type. In addition, vines inoculated with wild-type-only, ΔPD1311-only, and buffer (Hopkins 1984) were also included as disease positive and negative controls. The results from these trials are shown and discussed in the Conclusion section.

**Objective 1b. Determine If Overwintered ΔPD1311 Inoculated Plants Maintain Pierce's Disease Resistance**

In 2014 we had *V. vinifera* plants infected with wild-type *Xf* or ΔPD1311 two weeks prior to wild-type *Xf*. These vines were cut back and placed in nursery storage for the 2015 winter. The plants were then grown in the greenhouse in spring 2015 to follow potential Pierce's disease development. Preliminary results showed that wild-type *Xf* could overwinter and cause Pierce's disease in the following year. Plants treated with ΔPD1311 followed by wild-type *Xf* did not show symptoms either year and enzyme-linked immunosorbent assay (ELISA) did not detect *Xf* (Temecula 1 or ΔPD1311) in year 2 (**Table 1**). This data suggests that ΔPD1311 protection may last overwintering. However, we did not explore whether biocontrol treatment in year 1 would protect against a fresh wild-type inoculation in year 2. If found, this result would indicate that the ΔPD1311 biocontrol may have long-lasting protection in the field. If symptoms do develop in year 2 in the ΔPD1311-treated plants, this result would indicate that reapplication of the biocontrol will be necessary to maintain Pierce's disease suppression.

The 2015-treated plants were stored in a cold-room overwinter. These included wild-type-only, ΔPD1311-only, ΔPD1311 two weeks before wild-type, and buffer-only plants. Half of the overwintered plants were regrown without further treatment to determine if symptoms appear. The other half were allowed to grow for 1.5 months and then received new wild-type *Xf* inoculations at the base of the re-growing shoots. These results are shown and discussed in the Conclusions section.

**Objective 1c. Explore Leafhopper Transmission of the ΔPD1311 Strain**

Xylem-sap feeding leafhopper vectors transmit *Xf* from plant to plant (Chatterjee et al. 2008). The bacterium utilizes adhesins, such as FimA, HxfA, and HxfB, to attach and form biofilms on insect foreguts, which then becomes a source of inoculum for further disease spread (Killiny and Almeida 2009, Killiny et al. 2010). This

interaction with insects is a known key step for *Xf* to accomplish its life cycle. For development of  $\Delta$ PD1311 as a commercially viable biological control agent and for future field studies, it will be necessary to understand its insect transmissibility. Because  $\Delta$ PD1311 has reduced aggregation and biofilm (**Figure 2**), we hypothesized that  $\Delta$ PD1311 is altered in its ability to be insect vectored. As an initial assay, we wanted to examine the adhesion of the mutant strain to the hindwing of the leafhopper vector, as this assay has been found to mimic adhesion to the foregut region owing to similar chitinous nature of the cuticles (Killiny et al. 2010). Our preliminary data showed that  $\Delta$ PD1311 attached to insect wings at a level similarly to the wild-type strain (**Figure 5**).

**Table 1.** *Xf* ELISA results in overwintered plants.<sup>a</sup>

Treatment Year 1 <sup>b</sup>	Symptoms Year 1 <sup>c</sup>	Symptoms Year 2 <sup>c</sup>	0 cm <sup>cd</sup>	30 cm <sup>cd</sup>	150 cm <sup>cd</sup>
wild-type	+	+	+/1 <sup>e</sup>	+/1	+/1
		-	-/3	-/3	-/3
$\Delta$ PD1311 then wild-type	-	-	-/2	-/2	-/2

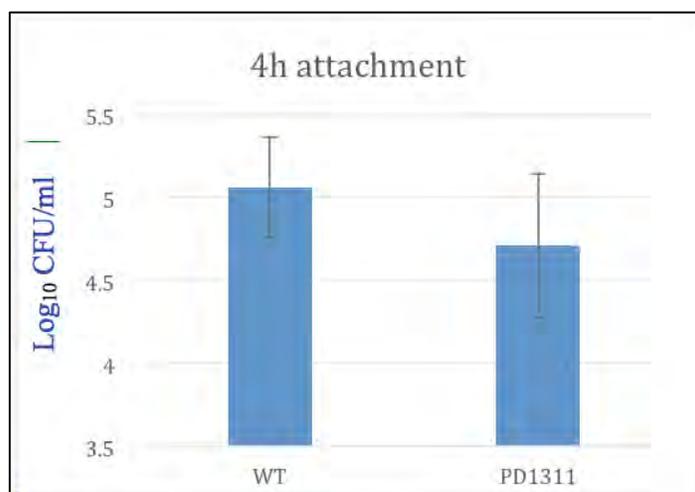
<sup>a</sup> Plants overwintered in cold storage between year 1 and 2.

<sup>b</sup> Plants were given no further inoculations in year 2.

<sup>c</sup> “+” = Pierce’s disease symptoms; “-” = no Pierce’s disease symptoms.

<sup>d</sup> Sample distance up from inoculation point in year 2.

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



**Figure 5.** The  $\Delta$ PD1311 strain attached to leafhopper hind wings similarly to the wild-type strain. The attachment assay was performed as described previously (Baccari et al. 2014). The experiment was performed once with eight replicates included for each strain.

#### **Objective 1d. Develop a Clean Deletion Strain of $\Delta$ PD1311 that Would Be Suitable for Commercialization**

Construction and deletion of a mutant of gene PD1311 was halted once it was noted that results in 2016 were not consistent with previous years. This would be an important step once the status of current  $\Delta$ PD1311 and wild-type strains are determined.

#### **Objective 2. Determine the Function of the PD1311 Protein and the Mechanism By Which $\Delta$ PD1311 Acts as a Biological Control**

##### **Objective 2a. Elucidate the Role of PD1311 Protein**

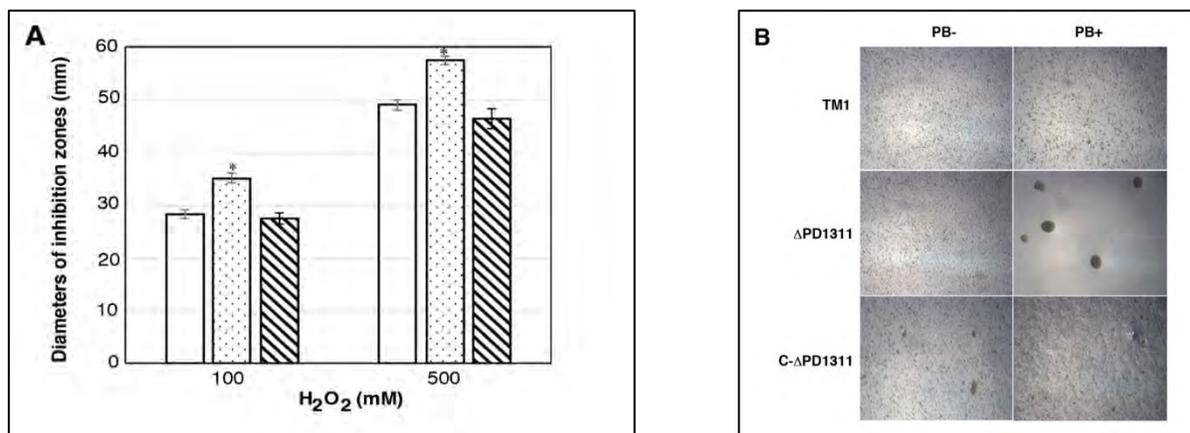
The *Xf*PD1311 gene has motifs suggesting it encodes an ACS protein (acyl- and aryl-CoA synthetase) (Chang et al. 1997, Gulick 2009). ACS metabolite intermediates are involved in beta-oxidation and phospholipid biosynthesis. ACS proteins have also been implicated in cell signaling (Korchak et al. 1994), protein transportation (Glick and Rothman 1987), protein acylation (Gordon et al. 1991), and enzyme activation (Lai et

al. 1993). Importantly, ACSs are involved in pathogenicity (Banchio and Gramajo 2002, Barber et al. 1997, Soto et al. 2002).

ACS proteins metabolize fatty acids through a two-step process to form a fatty acyl-CoA precursor utilized in any downstream metabolic pathways (Roche et al. 2013, Watkins 1997, Weimar et al. 2002). To confirm enzymatic activity, we expressed and purified a PD1311-His tag protein, and we tested it for ligase activity using acetate as the substrate. Acetate is the simplest substrate for fatty acid synthetase reaction, as a two-carbon (C2) chain length molecule. We used a standard colorimetric assay that measures acyl-CoA production (Kuang et al. 2007). The PD1311 protein exhibited a functional ATP/AMP binding domain that performed the following reaction: ATP + acetate + CoA is converted to AMP + pyrophosphate + acetyl-CoA (data not shown). Therefore we confirmed that the protein is functional.

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

Considering the avirulent phenotype of  $\Delta$ PD1311 on grapevines, PD1311 may be involved in lipid A biosynthesis or membrane production. Therefore, the  $\Delta$ PD1311 cells may be more sensitive to environmental stresses such as oxidative stress and cationic antimicrobial peptide polymyxin B (PB). When wild-type and  $\Delta$ PD1311 cells were exposed to hydrogen peroxide on agar plates in a Kirby-Bauer type assay, the zone of inhibition was greater for the mutant strain than wild-type cells (**Figure 6A**) (Hao et al. 2017). In addition,  $\Delta$ PD1311 cells were more sensitive to PB than wild-type or  $\Delta$ PD1311 complement cells. While both wild-type and  $\Delta$ PD1311 complement cells grew on plates supplemented with 16  $\mu$ g/mL PB, almost all  $\Delta$ PD1311 cells were killed when plated on PW agar supplemented with 1  $\mu$ g/mL PB (**Figure 6B**).



**Figure 6.** Relative sensitivity of  $\Delta$ PD1311 to H<sub>2</sub>O<sub>2</sub> and polymyxin B (PB). **A.** Mean diameters of inhibition zones of TM1 (empty bars),  $\Delta$ PD1311 (dotted bars), and C- $\Delta$ PD1311 (dashed bars) exposed to 100 or 500 mM of H<sub>2</sub>O<sub>2</sub> on PD2 agar plates. Error bars represent standard deviations. Three replicates were included for each experiment and the assay was repeated twice. \* represents a significant difference of p<0.01. **B.** Growth of TM1 and C- $\Delta$ PD1311 on PD2 plates amended with 16  $\mu$ g/mL PB and growth of  $\Delta$ PD1311 on PD2 plates with 1  $\mu$ g/mL PB. Images were taken under a Stemi-2000C dissecting microscope with a magnification of 3.2X. The assay was repeated four times with similar observations. TM1 = wild-type *Xf* Temecula 1,  $\Delta$ PD1311 = *Xf* Temecula 1 deleted of the PD1311 gene, C- $\Delta$ PD1311 =  $\Delta$ PD1311 complement strain.

## Objective 2b. Examine Impact of the $\Delta$ PD1311 Strain on Wild-Type *Xf* In Vitro and In Planta

To have better grounding on why  $\Delta$ PD1311 acts as a biological control, we needed to explore the mechanism by which the mutant strain impacts wild-type cells. We have results showing that the wild-type induced disease can be limited only when  $\Delta$ PD1311 was inoculated two weeks before the pathogen (Fig. 4). Therefore, we wanted know how the two strains spread through the plant when both are inoculated.  $\Delta$ PD1311 does not secrete a toxin that affects wild-type populations (Table 2); we grew wild-type cells in supernatant from  $\Delta$ PD1311 cells and found no growth changes (data not shown). Understanding how the mutant cells impact wild-type *Xf* is important for understanding not only how the biological control is achieved but also how the treatment would be most effectively applied in the field.

Table 2. Wild-type *Xf* detection by ELISA in petioles 24 w.p.i.<sup>a</sup>

Treatment	PD Symptom	Trial	Distance above inoculation point (cm)		
			0	30	150
$\Delta$ PD1311 then TM1 <sup>b</sup>	-	1	- <sup>c</sup> /3 <sup>d</sup>	-/3	-/3
		2	-/5	-/5	-/5
	+	1	n.d. <sup>e</sup>	n.d.	+/6
		2	n.d.	n.d.	+/4
TM1 + $\Delta$ PD1311	+	1	n.d.	n.d.	+/5
		2	n.d.	n.d.	+/4
TM1 only	+	1	n.d.	n.d.	+/5
		2	n.d.	n.d.	+/4

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

<sup>a</sup> w.p.i. = weeks post-inoculation.

<sup>b</sup> TM1 = wild-type; TM1 was inoculated two weeks after  $\Delta$ PD1311.

<sup>c</sup> “+” or “-” indicates positive or negative for *Xf*, respectively

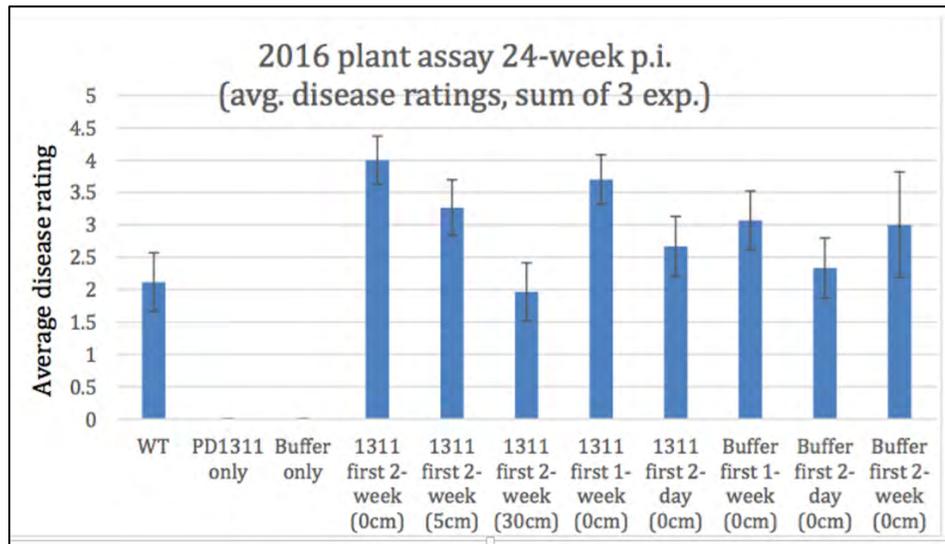
<sup>d</sup> Number is the number of plants tested by ELISA.

<sup>e</sup> n.d. = not assessed as no petioles left due to disease.

## CONCLUSIONS

Concerning objective 1, results from the 2016 inoculation experiments are shown in Figure 7. Although  $\Delta$ PD1311 was again confirmed as avirulent, we were unable to further verify the Pierce’s disease suppression by  $\Delta$ PD1311. Plants inoculated with wild-type *Xf*-only developed about 40% infection which was much lower than in past years where infection level was close to 100%. The reason for this difference is unknown. We do not expect conditions in the greenhouse were involved as the grapevines were growing well and the internal climate was similar to past years. For treatments where  $\Delta$ PD1311 was applied prior to wild-type the level of disease was frequently higher than the inoculation with wild-type alone. No disease developed when  $\Delta$ PD1311 was applied alone. Differences were mostly observed when  $\Delta$ PD1311 was applied two weeks prior to wild-type and was inoculated 30 cm above the wild-type inoculation point. For that treatment Pierce’s disease was less than when  $\Delta$ PD1311 was inoculated at the point of wild-type inoculation of 5 cm above. We had not attempted the treatments of 5 cm or 30 cm previously. Disease suppression was observed over the past three years when wild-type was applied at the same inoculation site as  $\Delta$ PD1311.

The significant differences that were observed in 2016 with regard to Pierce’s disease suppression by  $\Delta$ PD1311 could also be due to a modification in the  $\Delta$ PD1311 strain. Before going ahead with research on  $\Delta$ PD1311 it would be essential to explore the possibility that the strain became altered in storage. Initially it would be important to test previously reported  $\Delta$ PD1311 phenotypes including biofilm formation, aggregation, and motility on synthetic media and sap agar. If it appears that the strain has changed from its original behavior, we would check additional stocks or remake the mutant. This research was not funded in 2015, however, we were able to conclude the experiments because of being granted a no-cost extension of funds that remained from 2015.



**Figure 7.** Effect of  $\Delta$ PD1311 on Pierce's disease development, 2016. Specific methods and conditions used for the different treatments are explained in the text above.

The experiments to test the effect of  $\Delta$ PD1311 on Pierce's disease development in overwintered plants was inconclusive because there was great variability in disease across all categories of plants. Some that showed disease during the summer of 2015 did not develop disease in 2016 regardless of being treated with  $\Delta$ PD1311 or not. It would be necessary to repeat the experiments on overwintered plants once factors that were involved in overall reduced disease and reduced inhibition of  $\Delta$ PD1311 in the 2016 experiments were determined.

Preliminary data suggests that  $\Delta$ PD1311 attaches to insect hindwings at an equal level as observed for wild-type cells. Therefore, in nature  $\Delta$ PD1311 could possibly be distributed by the vector.

For objective 2, our preliminary results showed that the mutant had greater sensitivity to chemical environments (hydrogen peroxide, antimicrobial peptides), which may contribute to its avirulent phenotype and help explain the role of the protein in the bacterium. Much of our work in relation to this grant has been recently published (Hao et al. 2017). Overall, this work will help further our understanding of disease development and prevention. It has also identified a key Pierce's disease virulence factor, PD1311, that will be important in future research to understand the mechanism by which *Xf* causes Pierce's disease. Additional work on this essential putative enzyme is highly warranted.

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

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**Reporting Period:** The results reported here are from work conducted July 2016 to September 2017.

## ABSTRACT

This research is a continuation of the field evaluation of chimeric anti-microbial protein (CAP; Dandekar et al. 2012a) and polygalacturonase-inhibiting protein (PGIP; Agüero et al. 2005, 2006) expressing rootstocks that enable trans-graft protection of scion varieties of grapevine from developing Pierce's disease after infection with *Xylella fastidiosa* (*Xf*). Rootstocks (Thompson Seedless, TS) expressing these proteins individually were evaluated in the field; this part of the study was concluded in winter 2017. TS rootstock lines expressing either CAP or PGIP show promise in their ability to transgraft protect a scion variety (also TS) against Pierce's disease, validated with in-field inoculations. The lines expressing CAP showed the highest efficacy in protecting grafted transgenic grapevines from developing Pierce's disease. The ongoing testing involves evaluating novel CAP lines in commercially relevant rootstocks 101-14 and 1103 (Christensen 2003). The original neutrophil elastase – cecropin B (NE-CB) CAP construct (Dandekar 2012a) was improved by identifying grapevine-derived components (Chakraborty et al. 2013, 2014b). The surface binding NE component was replaced with P14a protein from *Vitis shuttleworthii* that also displays serine protease activity (Chakraborty et al. 2013, Dandekar et al. 2012c, 2013). The antimicrobial component CB was replaced with HAT52 and/or PPC20 that were identified using novel bioinformatics tools developed by us (Chakraborty et al. 2013, 2014a) and the efficacy of the selected peptides were verified for their ability to kill *Xf* cells (Chakraborty et al. 2014b). In addition to the original NE-CB CAP (CAP-1), five additional CAP constructs included in the current round of testing are VsP14a (CAP-2), VsP14a-CB (CAP-3), VsP14a-HAT52 (CAP-4), VsP14a-PPC20 (CAP-5), and 35s OM/RAMY/Flag CAP (CAP-6; Dandekar et al. 2012c, 2013, 2014). Transformation of these six CAP constructs into the 101-14 and 1103 rootstock backgrounds was initiated in 2015 and greenhouse testing was started in fall 2016, with field introductions planned for spring of 2018. The field introduction of these rootstocks is aimed at evaluating different lines to identify those with good efficacy in protecting the grafted, sensitive scion cultivar Chardonnay from developing Pierce's disease.

## LAYPERSON SUMMARY

This project is a continuation to evaluate the field efficacy of transgenic grapevine rootstocks expressing a chimeric anti-microbial protein (CAP) or a polygalacturonase-inhibiting protein (PGIP) to provide protection to the grafted scion variety from developing Pierce's disease. We concluded a field evaluation where four CAP and four PGIP expressing Thompson Seedless (TS) were tested as rootstocks to protect grafted wild-type TS scions. These plants were infected with *Xylella fastidiosa* in 2012, 2013, 2014, and 2015 and evaluated each year for their ability to provide resistance to Pierce's disease. Our conclusion is that the transgenic rootstocks were able to provide transgraft protection to the scion. They showed less symptoms, higher survival, and harbored a lower titer of the pathogen than grafted untransformed controls. Since TS is not a commercially relevant rootstock we have now begun testing the field efficacy of this strategy by expressing different CAP proteins in the commercially relevant rootstocks 110-14 and 1103. Greenhouse evaluations were initiated in 2018 and field evaluations will begin in spring of 2018. Elite rootstock lines identified in this project will be good candidates for commercialization.

## INTRODUCTION

The focus of this study is to evaluate the rootstock-based expression of chimeric antimicrobial proteins (CAP; Dandekar et al. 2012a) and polygalacturonase-inhibiting protein (PGIP; Agüero et al. 2005, 2006) to provide transgraft protection of the scion grapevine variety against Pierce's disease. Rootstocks (Thompson Seedless, TS) expressing these proteins individually are currently being evaluated in the field; this part of the study was concluded this year. Since TS is not a rootstock these genes must be tested in a commercially relevant rootstock.

Methods to successfully transform two commercially relevant rootstocks (101-14 and 1103; Christensen 2003) were successfully developed (Dandekar et al. 2011, 2012b) and the method was further improved by David Tricoli in the UC Davis Plant Transformation Facility. The original neutrophil elastase - cecropin B (NE-CB) CAP construct (Dandekar 2012a) was improved by identifying grapevine-derived components (Chakraborty et al. 2013, 2014b). The surface binding NE component was replaced with P14a protein from *Vitis shuttleworthii* that also displays serine protease activity (Chakraborty et al. 2013, Dandekar et al. 2012c, 2013). The antimicrobial component CB was replaced with HAT52 and/or PPC20 that were identified using novel bioinformatics tools developed by us (Chakraborty et al. 2013, 2014a) and the efficacy of the selected peptides was verified for their ability to kill *Xylella fastidiosa* (*Xf*) cells (Chakraborty et al. 2014b). In addition to the original NE-CB CAP (CAP-1), five additional CAP constructs were developed that contained VsP14a (CAP-2), VsP14a-CB (CAP-3), VsP14a-HAT52 (CAP-4), VsP14a-PPC20 (CAP-5), and 35s OM/RAMY/Flag CAP (CAP-6; Dandekar et al. 2012c, 2013, 2014). These transgenic CAP-expressing rootstocks testing in the greenhouse and field started in fall 2016. The additional CAP constructs that will be tested are aimed at addressing the concern that the protein components of the present CAP-1 have a non-plant origin. Transformation of these five CAP constructs into the 101-14 and 1103 rootstock backgrounds was initiated in 2014. Greenhouse testing was initiated in 2016, with field testing 2018 onward. The field introduction of these rootstocks is aimed at evaluating different lines to identify those with good efficacy in protecting the grafted, sensitive scion cultivar Chardonnay from developing Pierce's disease.

## OBJECTIVES

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

## RESULTS AND DISCUSSION

### Activity 1. Complete and Conclude Testing of the Current Round of Plants in the Field

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



**Figure 1.** Left: Solano County field trial grafted transgenic grapevines inoculated in spring 2014 and spring 2015 (photo taken in fall 2016). Right: Terminated Solano County field trial (photo taken in spring 2017).

**Table 1.** Solano County field trial grape field map, color-coded by *Xf* inoculation date, from 2012 to 2015.

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

↑ North

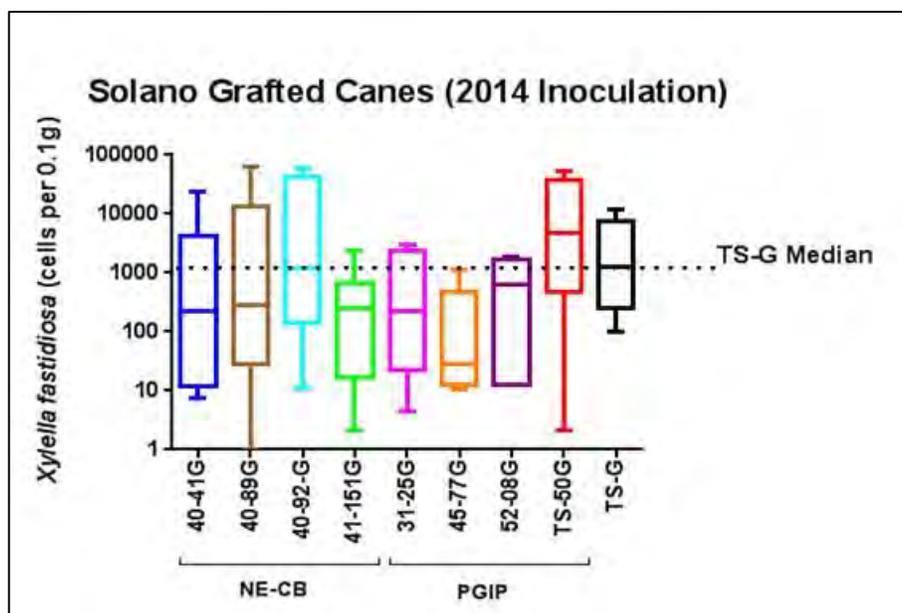
■ Grapevine inoculation with *Xf* (Temecula:Stag's leap mix, 60:40) at 250,000 per 20ul on 5/29/2012.

■ Grapevine inoculation with *Xf* (Temecula) 250,000 per 20ul on 6/17/2013.

■ Grapevine inoculation with *Xf* (Temecula:Stag's leap mix, 60:40) at 500,000 per 20ul on 5/27/2014.

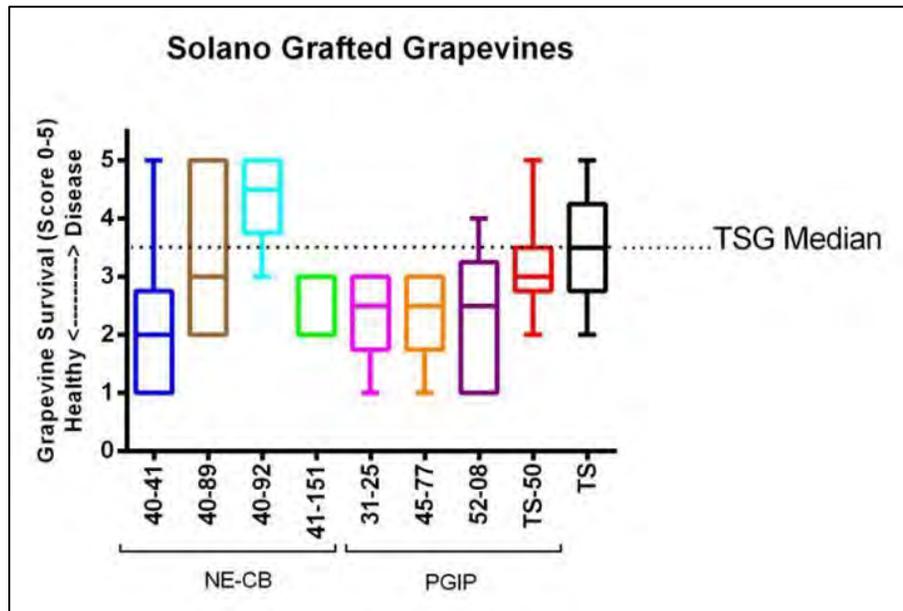
■ Grapevine inoculation with *Xf* (Temecula) at 500,000 per 20ul on 5/27/2015.

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



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

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

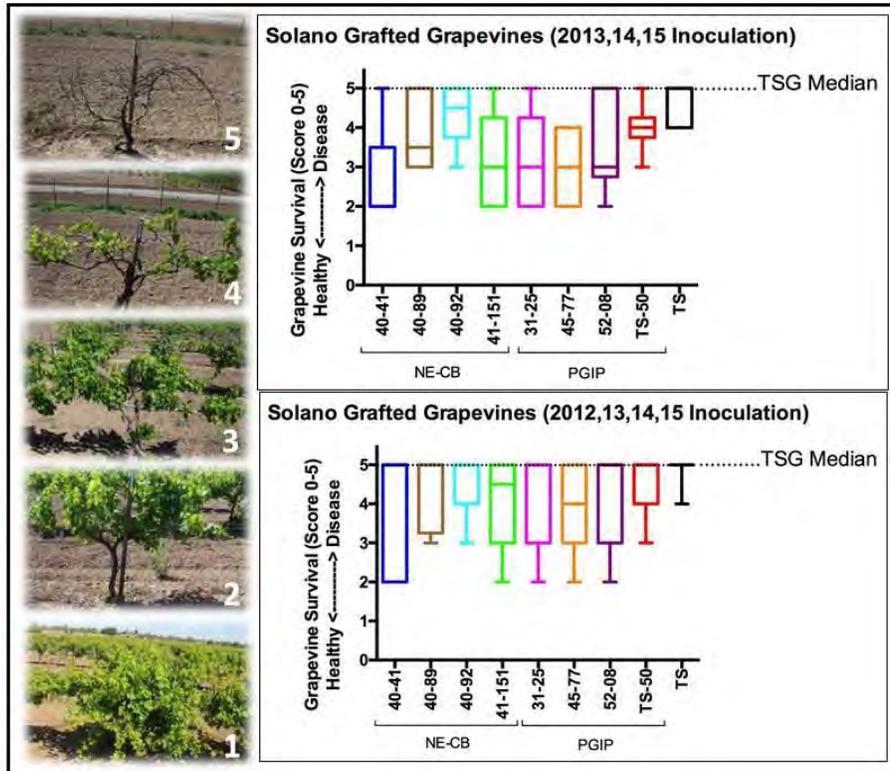


**Figure 3.** Severity or absence of Pierce’s disease symptoms for all Solano County field trial grafted inoculated grapevines in fall 2015.

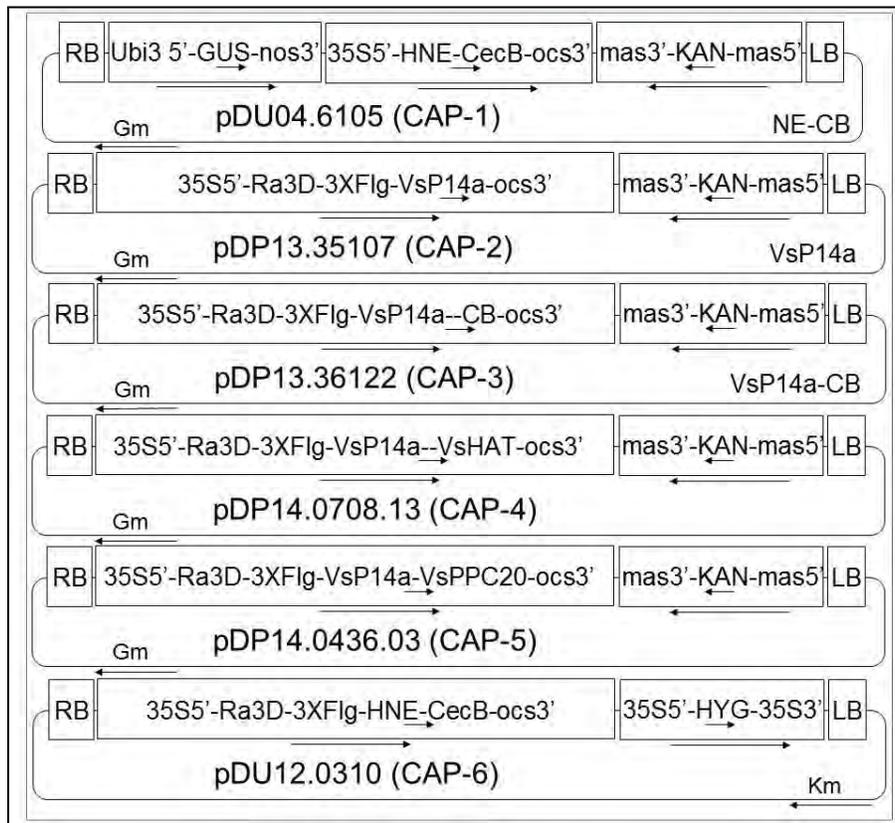
Grapevine survival of grafted transgenic grapevines that were inoculated in 2014-2015 was assessed on October 6, 2016 using a 1 to 5 score, where 1 = very healthy and vigorous grapevine, 2 = healthy grapevine and slightly reduced vigor, 3 = slightly reduced spring growth, 4 = much reduced spring growth, and 5 = dead grapevine (**Figure 4**). The grapevine survival rate was greater in most grafted inoculated transgenic lines using either strategy than in grafted untransformed controls, with the greater efficacy seen in CAP lines. The Solano County field trial was terminated in the summer of 2017.

## Activity 2. Conduct Greenhouse and Field Evaluation of CAP-Expressing 101-14 and 1103 Rootstocks

This activity focused on greenhouse and field testing of six vector constructs that are in the plant transformation pipeline on two commercially relevant rootstocks, 101-14 and 1103 (Christensen 2003). The components present in these constructs are shown in **Figure 5** below. The construction of CAP-1 was described earlier (Dandekar et al. 2012a), and the components, mostly from grapevine, and construction of CAP-2, CAP-3, CAP-4, CAP-5, and CAP-6 shown in **Figure 5** have been previously described (Chakraborty et al. 2014b, Dandekar et al. 2012c, Dandekar et al. 2013, Dandekar et al. 2014a). The grapevine transformation methods for the 101-14 and 1103 rootstocks have been described previously (Dandekar et al. 2011, Dandekar et al. 2012b) but were further improved by David Tricoli at the UC Davis Plant Transformation Facility, who did the transformation of all of the binary vector constructs shown in **Figure 5**. The transgenic plants obtained from the facility and propagated for testing are described in detail below. The transformation of the two rootstock species with all six CAP constructs was initiated in 2014 and the selection and regeneration of plants is ongoing. The field testing of these rootstocks is aimed at evaluating their efficacy in protecting the grafted sensitive Chardonnay grapevine variety from developing Pierce’s disease.



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



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

Transformation of the first construct (CAP-1) yielded thirty 101-14 and four 1103 derived transgenic lines. Since the yield for 1103 lines transformed with CAP-1 was low a new transformation was initiated back in August 2015. In addition, in summer 2016 we began receiving 110-14 and 1103 lines transformed with the other constructs (CAP-2 to CAP-6) and the numbers and distribution of these lines is indicated in **Table 2**.

**Table 2.** Pierce's disease resistance greenhouse testing of CAP-expressing transgenic rootstocks.

CAP	Binary Vector	Transgenic Plants Received		Greenhouse Testing		Advancing For Field Testing	
		101-14	1103	101-14	1103	101-14	1103
Designation							
CAP-1	pDU04.6105	30	4	30	4	6	0
CAP-2	pDP13.35107	8	2	8		In progress	
CAP-3	pDP13.36122	6	1	6		In progress	
CAP-4	pDP14.0708	11	6	10		2	
CAP-5	pDP14.0436.03	8	6	8		In progress	
CAP-6	pDU12.0310		10		10		In progress

A propagation/testing pipeline has been successfully developed to test the efficacy of both 101-14 and 1103 grapevines and the transgenic lines for Pierce's disease resistance in the greenhouse. The 101-14 and 1103 transgenic rootstocks lines are first screened for the presence of CAP transgene using PCR. Those 101-14 and 1103 plants that are PCR-positive are clonally propagated for greenhouse testing. The clones are trained into a two-cane system and inoculated on one of the canes with *Xf*. Plants are inoculated with 20 uL of *Xf* at roughly three nodes above the fork in the canes and eight leaves below the top of the cane. Then the plant is turned over and inoculated with another 20 uL of *Xf* directly behind the first inoculation. The *Xf* inoculum is prepared as described earlier (Dandekar et al. 2012a).

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

All 34 CAP-1 transgenic lines have been analyzed and six have been identified for field testing. All six were 110-14 transgenic. Of the six 110-14 transgenic lines selected one was an elite line, presented no Pierce's disease symptoms, and got a score of 1. The remaining five 101-14 plant lines got a score of 2, which look very promising and were considerably less sick than the untransformed 101-14 control which was scored a 5 (**Figure 6**). All 1103 lines scored bad and received a score of 5. The six 101-14 transgenic rootstocks expressing CAP-1 that scored a 1 or 2 have been clonally propagated from the uninfected mother plants.

Nine out of ten CAP-4 transgenic events expressing VsP14a-VsHat22 in the 101-14 background that screened PCR positive were clonally propagated and infected with *Xf*, and two have been identified for field testing. All other plants in the 101-14 and 1103 backgrounds that have been confirmed PCR positive are in the cloning-growing-inoculating pipeline for inoculation with *Xf* (**Figure 7**). Plants of each background continue to be produced at the UC Davis Plant Transformation Facility; as plants emerge they are propagated for greenhouse and field testing.

A more detailed scoring system was recently developed for the analysis of Pierce's disease symptoms during greenhouse screening. A scoring system of 0 to 5 was used to score each leaf with values of 0 = no visible disease symptoms, 1 = disease symptoms just appearing with < 10% of the leaf scorched, 2 = 10-25% of the leaf scorched; 3 = 25-50% of the leaf scorched, 4 = 50-75% of the leaf scorched, and 5 = 75-100% of the leaf scorched or only the petiole remaining (**Figure 8**). Pierce's disease symptoms for the CAP-4 plants in the 101-14

background were scored using the detailed score system. Results of the screening process of CAP-4 plants in the 101-14 background are shown in **Figure 9**.



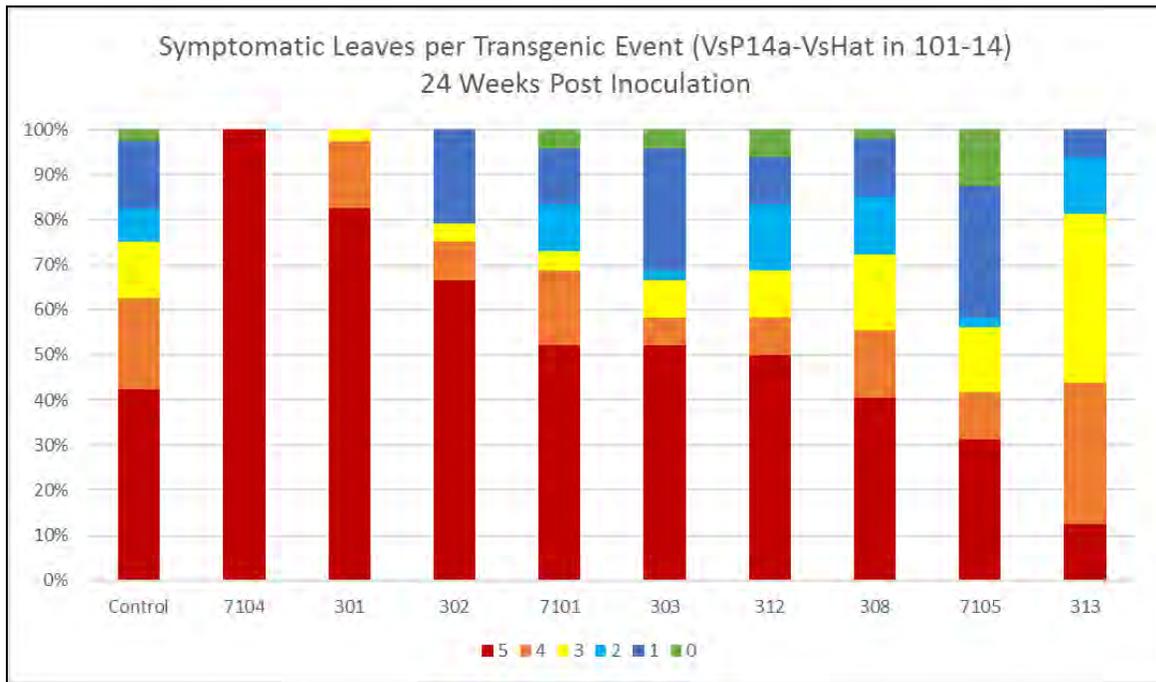
**Figure 6.** Infected two-cane vines with the left uninfected and the right infected. (A) WT 101-14 grapevines with disease symptoms running the entire length of the infected cane. (B) The elite CAP-1 transgenic line of 110-14 that showed no symptoms 18 weeks post inoculation.



**Figure 7.** Transgenic 110-14 and 1103 lines expressing CAP-2 to CAP-6 are in the cloning-growing-inoculating pipeline for greenhouse inoculation with *Xf*.



**Figure 8.** Pierce's disease symptoms scoring system of 0 to 5. Top left to right: 0, 1, and 2; Bottom left to right: 3, 4, and 5.



**Figure 9.** Last data point collected while screening the 101-14 transgenic rootstocks expressing CAP-4. Plants are scored weekly after the Pierce's disease symptoms begin to show.

## CONCLUSIONS

We have successfully concluded field-testing of Thompson Seedless (TS) as a rootstock expressing CAP or PGIP. Grapevine survival of grafted transgenic grapevines inoculated between 2012 and 2015 was assessed and the survival rate of most grafted inoculated transgenic TS lines using both strategies was greater than in untransformed controls, with the CAP lines most efficient in protecting against Pierce's disease. The phenotypic disease data corresponded to the bacterial titer estimations using qPCR, which revealed lower bacterial titers in transgenic plants as compared to the wild-type susceptible TS plants. Severity or absence of Pierce's disease symptoms on all Solano County field trial grafted transgenic grapevines inoculated between 2012 and 2015 was also assessed and Pierce's disease symptom severity scores were lower in most grafted inoculated transgenic lines using either strategy than in grafted untransformed controls. The field-testing data confirm that TS rootstock lines expressing either CAP or PGIP are able to provide protection against Pierce's disease. We have developed a successful propagation and two-cane testing pipeline to evaluate sixty-two 101-14 and fourteen 1103 transgenic rootstocks expressing various CAP constructs. Field testing will be initiated in spring of 2018.

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

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**Reporting Period:** The results reported here are from work conducted April 1, 2017 to October 30, 2017.

**ABSTRACT**

This field project began in 2010 to evaluate grapevines expressing potential Pierce's disease suppressive transgenes under field conditions. The second phase of this project will evaluate transgenic rootstocks for protection of untransformed scions against Pierce's disease. The new rootstocks with two transgenes each will be evaluated first in the laboratory and then in the greenhouse before moving to the field. The highest expressing rootstocks will be grafted to susceptible non-transgenic Chardonnay scions to assess potential cross graft protection against Pierce's disease by the respective transgenes. All plants will be located in a secured, USDA APHIS approved area in Solano County. The disease will be introduced into the cordon trained plants by mechanical injection of *Xylella fastidiosa* into stems after the first year of growth beginning in 2018. The plants are to be monitored regularly for quantity and movement of the bacteria, along with symptoms of Pierce's disease. Test plants include transgenic plants expressing genes from Dandekar, Powell, Lindow, and Gilchrist projects, compared with non-transgenic Pierce's disease susceptible Thompson Seedless and Freedom rootstock plants as controls. In addition, transgenic rootstocks expressing some of the test genes grafted to untransformed Pierce's disease susceptible scions were introduced in 2011 and 2012. The results in 2016 indicated that the mechanical inoculations introduced the bacteria into the plants, with subsequent appearance of classic foliar symptoms and cane death within 24 months in susceptible controls. There is no evidence of spread of the bacteria to uninoculated and uninfected susceptible grape plants adjacent to infected plants, confirming tight experimental control on the pathogen and symptoms. Each of the transgenes tested suppress the symptoms of Pierce's disease inoculated vines to varying degrees, including protection of untransformed scions on the grafted plants. This first phase of field research has been terminated and is now moving forward with the a second generation of two new transgenic rootstocks (1103 and 101-14) expressing pairs of the disease suppressive genes in a gene stacking approach, with the genes paired together by differential molecular function.

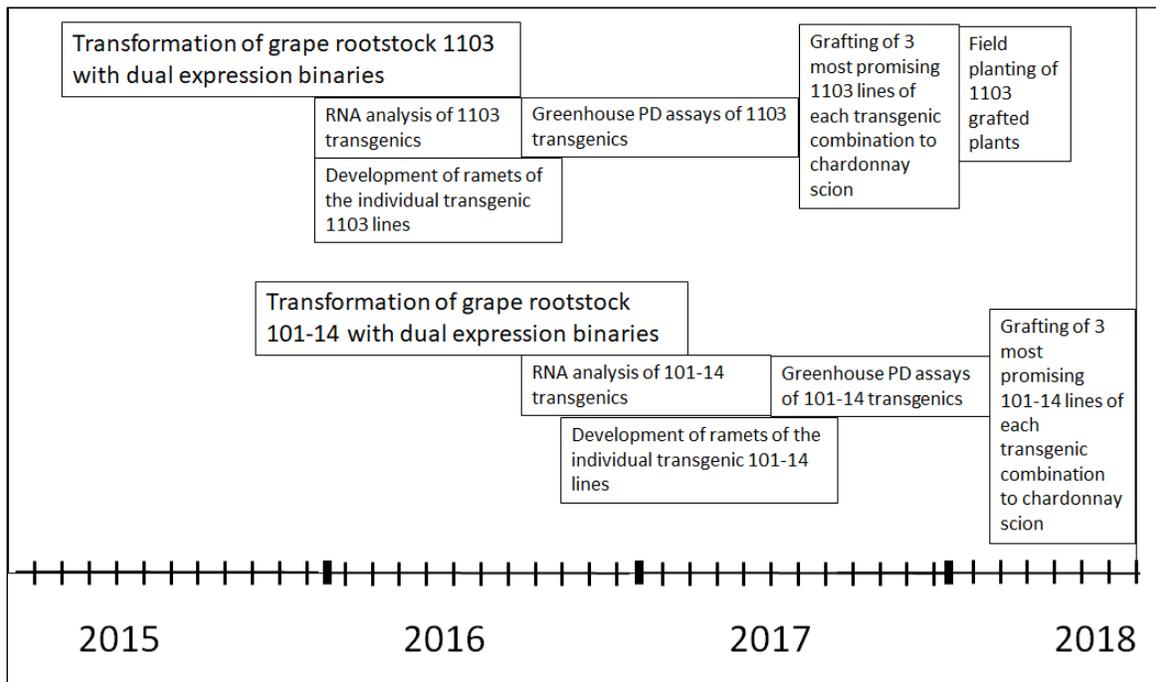
**LAYPERSON SUMMARY**

This first phase field project begun in 2010 to evaluate grapevines expressing potential Pierce's disease suppressive transgenes under field conditions was terminated in 2017. A second phase field experiment will continue evaluation of resistance to Pierce's disease in transgenic grape and grape rootstocks by expressing dual combinations of five unique transgenes under field conditions. The evaluation continues in a USDA APHIS regulated Solano County site where the plants are mechanically injected with *Xylella fastidiosa*. Pierce's disease symptoms, including classical foliar symptoms and cane death, occur within 24 months. The initial field tests have shown positive protection against Pierce's disease by five different DNA constructs. A new planting is in progress that will consist of untransformed Pierce's disease susceptible scions grafted to transgenic rootstocks (1103 and 110-14) expressing the paired constructs of the five genes to assess cross-graft protection of a non-transformed scion that is otherwise highly susceptible to Pierce's disease.

## INTRODUCTION

Genetic strategies for disease suppression and information characterizing the bacterial-plant interaction are high priority areas in the Pierce's disease research program. Projects from laboratories of Dandekar, Powell, Lindow, and Gilchrist have been tested extensively under greenhouse and field conditions in USDA APHIS approved field environments in Riverside and Solano counties. Two types of genetically modified plants bearing single constructs of test genes have been evaluated under disease conditions: Whole plant transgenics and graft-transmissible transgenes in which transgenic rootstocks were grafted to non-transformed Pierce's disease susceptible scions. Positive and promising results from both types of transgenic strategies provided the necessary impetus to move this program forward to the next logical step in which combinations of the transgenes will be introduced into individual rootstocks adapted to California grape growing regions.

The anticipated research and implementation timeline is shown below (**Figure 1**). The individual laboratories of the principal investigator and co-principal investigators have established transgenic plants and field tested the following genes as transgenes in a commercial grape rootstock and a commercial grapevine variety. Each of the genes were selected based on laboratory, greenhouse, and field data to address and disrupt known functions related to virulence of the bacteria or key factors triggering the susceptible response in the grape host. There is strong evidence that each of these genes can protect, but to differing levels, as transgenes, and each appears to be able to exert suppressive action on the symptoms of Pierce's disease in cultivated grapes.



**Figure 1.** Anticipated timeline for evaluation, propagation, and planting of dual construct/susceptible scion combinations, fully transformed rootstock control, and untransformed susceptible control plants.

## OBJECTIVES

1. Destruction of existing planting was begun in the fall of 2016. All posts and wires were removed in November but early rains prevented the removal of the plants. Mechanical undercutting of the base of the plants and roots was followed by moving the plant material to piles. Final burning occurred on June 6, 2017 and the ashes scattered prior to disking and leveling (**Figure 2**). Following the complete destruction, the field will be fumigated to ensure no living grape vegetative material remains, which will complete the USDA APHIS requirements for removal and destruction of all transgenic material.
2. Establish a new planting area within the current USDA APHIS approved site (**Figure 3**) to contain a new set of lines bearing paired, Pierce's disease suppressive DNA constructs, referred to as stacked genes. The stacked genes have been transferred to two adapted rootstocks (1103 and 101-14). These rootstocks will be grafted to an untransformed Pierce's disease susceptible Chardonnay scion prior to field planting. The goal is

to assess the potential of cross graft protection against Pierce's disease of a non-transgenic scion. Planting is to begin in 2018 and be completed by 2019.

## RESULTS AND DISCUSSION

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

The field experiment that began in 2010 was terminated under objective 1 of this proposal according to the regulations specified in the USDA APHIS permit (**Figure 2**). This will be followed by establishment of the second phase approved by the Product Development Committee to develop transgenic rootstocks incorporating stacked genes (dual constructs) to be grafted to non-transformed Pierce's disease-susceptible Chardonnay scions to test for potential cross-graft protection against Pierce's disease (objective 2).

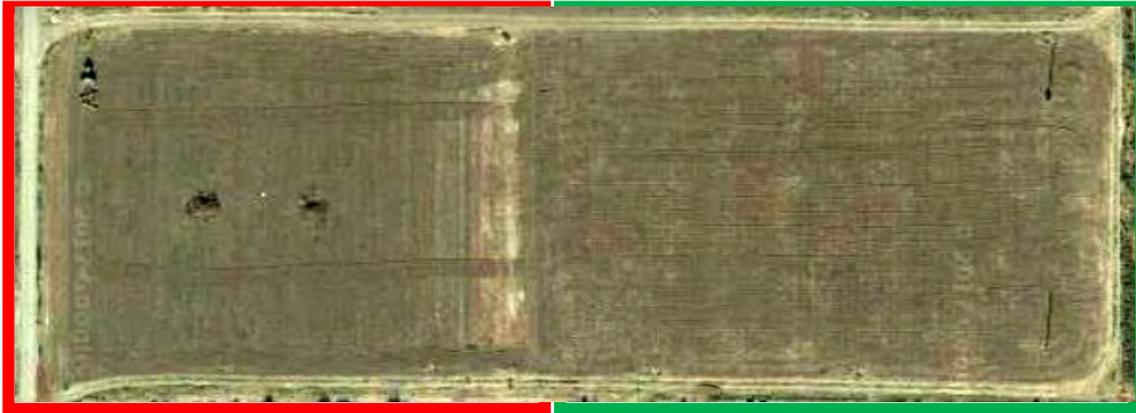
Destruction of the existing planting was begun in the fall of 2016. All posts and wires were removed in November but early rains prevented the removal of the plants. The plant removal, burning of the plants, and fumigation of the area to permit future use was accomplished as soon as the field dried in the spring.



**Figure 2.** Final destruction of the plants at the Solano County field trial site by burning on June 7, 2017 following removal of poles and wires, undercutting, and piling of plants.

Establishment and management of new planting in relation to the 2010 planting is shown in **Figure 3** and will be guided by Josh Puckett and Deborah Golino of Foundation Plant Services at UC Davis, working with principal investigator Gilchrist to produce clones for grafting non-transgenic scions, grafting the scions, field planting, trellising, and plant management to reflect commercial production standards. The design will enable experimental *Xylella fastidiosa* (*Xf*) inoculations and pathogen and disease assessments, as well as grape yield. Land preparation and planting of the experimental area will be sufficient to accommodate and manage 900 new plants. Row spacing will be nine feet between rows with six feet between plants. This spacing permits 32 rows of 28 plants each (up to 896 plants total) and includes a 50-foot open space around the planted area as required by the USDA APHIS permit. The planting pattern will permit a two-bud pruned bilateral cordon system of sufficient lengths for inoculation, real time sampling of inoculated tissue, and determination of the fruit yield by the untransformed Chardonnay scions. Total fenced area occupied by plants and buffer zones as required by the USDA APHIS permit will be ~ 3.4 acres (**Figure 2**). All plants will be maintained under a newly installed drip irrigation system. An example of row spacing and drip irrigation is shown in **Figure 4**.

The development of the stacked gene rootstock transgenics is in progress, with a preliminary greenhouse evaluation of the transgenic rootstocks for expression of the transgenes and response of the rootstocks to inoculation with *Xf* prior to grafting and establishment in the new field area.



**Figure 3.** Solano County planting area. Future area (green box) available to plant the next generation of transgenic plants expressing the dual constructs or new single genes. This area is 300 ft x 470 ft for planting, which equals 1.8 acres accommodating up to 38 new rows (excluding the 50-ft buffer areas surrounding the plots). The new area will accommodate ~ 900 new plants in 2016-18. Current area (rows) now planted to grapes: 300 ft x 370 ft, equaling 1.6 acres, including the 50-ft buffer areas surrounding the plots.



**Figure 4.** Planting configuration for the dual constructs. The design follows the description in the objectives section. The insert illustrates the new plantings, which will be watered by drip irrigation, as shown.

### **Protocols to Be Followed as the Planting Proceeds**

- a. Experimental design will be a complete randomized block with six plants per each of five entries (replications), including all controls. Each plant will be trained as a single trunk up the wood stake as with the existing planting. When the shoot tip reaches about 12 inches past the cordon wire it will be topped to just above a node that is about two to three inches below the wire. Then, the laterals that push will be used to establish the bilateral cordons. The plants will be allowed to grow vertically, or close to vertical, rather than tying them while green, which reduces their elongation and tends to force more lateral growth. Metal nine-foot highway stakes, inserted three feet into the ground every 18 feet, will support the wires, including catch wires. A single 11-gauge wire will be used for the cordons and 13-gauge wire for the catch wires. Two pairs

of moveable catch wires will be installed to tuck and position the shoots vertically for optimizing bacterial inoculation, bacterial analysis, and fruit production. The catch wires will be installed initially or after the first year of growth using 13-gauge wire to support the drip irrigation wire, about 18 inches off the ground.

- b. After the first year, the canes will be tied down during the dormant season and trimmed to the appropriate length or shorter if the cane girth is not over 3/8 inches in diameter. The shoots that push will be suckered to remove double shoots and to achieve a shoot (and hence spur position) spacing of about four to five inches between them.
- c. Grape fruit yield will be measured after the second or third year depending on the fruit set.
- d. Evaluation of the experimental plants for plant morphology, symptoms of Pierce's disease infection, and the presence of the bacteria will follow past protocols. Each parameter will be determined over time by visual monitoring of symptom development and detection of the amount and movement of the bacteria in plant tissues (mainly leaves and stems) by quantitative polymerase chain reaction (qPCR) assays. The analysis will be done in the Gilchrist lab by the same methods and laboratory personnel as has been done with the current planting. A comparative quantitative determination by qPCR of the presence of *Xf* in non-transgenic scions and grape rootstocks will be compared with conventional grape and grape rootstocks.
- e. Both symptom expression and behavior of the inoculated bacteria will provide an indication 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.
- f. The area is adjacent to experimental grape plantings that have been infected with Pierce's disease for the past two decades, with no evidence of spread of the bacteria to uninfected susceptible grape plantings within the same experiment. Hence, there is a documented historical precedent for the lack of spread of the bacteria from inoculated to uninoculated plants, an important consideration for the experiments carried out for this project and for the granting of the USDA APHIS permit. The field area chosen has never had grapes planted therein, which is to avoid any potential confounding by soil-borne diseases, including nematodes.
- g. Irrigation and pest management, primarily for powdery mildew, weeds, and insects, will be coordinated by principal investigator Gilchrist and conducted by Bryan Pellissier the field superintendent employed by the UC Davis Department of Plant Pathology. The field crew work closely with Gilchrist to determine the timing and need for each of the management practices, including pruning and thinning of vegetative overgrowth as necessary.
- h. Regular tilling and hand weeding will maintain a weed-free planting area. Plants were pruned carefully in March of each year, leaving all inoculated/tagged branches and numerous additional branches for inoculation and sampling purposes in the coming year. All pruning material was left between the rows to dry, then flail chopped and later rototilled to incorporate the residue per requirements of the USDA APHIS permit.
- i. Application of the fungicides Luna Experience and Inspire will be alternated at periodic intervals to maintain the plants free of powdery mildew. Leafhoppers and mites will be treated with insecticides when needed. Neither powdery mildew nor insect pressure has been observed with these ongoing practices throughout the past five growing seasons.

#### **Research Timetable for the New Planting of Dual Constructs and Untested Single Constructs**

Four years beginning with the initial planting in 2018 (**Figure 4**) to be followed by additional plantings as experimental plants become available in the second and third years. Inoculation and evaluation will begin when the plants have been in the ground for one year, and will continue annually until the field planting is terminated. Funding for completion of the fourth and any following years will be proposed in the 2018-2020 funding cycle and will depend on the results of the field evaluation up to that point. The field area has been designated legally available for planting the specified transgenic grapes by USDA APHIS under permit number 7CFRE340 that is held by co-principal investigator Dandekar. The protocols for managing the existing and the new plantings with the dual constructs have been used successfully over the past five years (Gilchrist 2016). These protocols include

plant management, inoculation with *Xf*, development of classical symptoms of Pierce's disease exhibiting the range from foliar symptoms to plant death, and the assessment of protection by a set of transgenes selected by molecular techniques to suppress the symptoms of Pierce's disease and/or reduce the ability of the pathogenic bacteria to colonize and move within the xylem of the grape plant.

## **CONCLUSIONS**

The current planting of transgenic grapes was fully terminated in the spring of 2017 per the USDA APHIS agreement by dismantling trellising, uprooting the plants, and burning all grape plant material on site. The complete removal of the plants was followed by cultivation, and the area will be fumigated when conditions permit to ensure no living grape vegetative material remains. The field research using Pierce's disease suppressive transgenes is moving forward with the generation of new transgenic rootstocks expressing pairs of the disease suppressive genes in a gene stacking approach, with the genes paired together by differential molecular function. The new rootstocks with two transgenes each will be evaluated first in the laboratory and then in the greenhouse before moving to the field. The highest expressing rootstocks will be grafted to susceptible non-transgenic scions to assess potential cross graft protection against Pierce's disease. The field area has been permitted by the USDA APHIS for this experiment. The protocol for constructing the rootstocks and grafted scions and planting and management of the vines is in place and will be coordinated by Josh Puckett and Deborah Golino. Initial planting will begin in 2018 and will be followed by additional plantings as experimental plants become available in the second and third years. Inoculation and evaluation will begin when the plants have been in the ground for one year and will continue annually until the field planting is terminated. Funding for completion of the fourth and any following years will be proposed in the 2018-2020 funding cycle and will depend on progress of the field evaluation up to that point.

## **REFERENCES CITED**

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

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

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**Reporting Period:** The results reported here are from work conducted April 1, 2017 to October 30, 2017.

### ABSTRACT

Collectively, a team of researchers (S. Lindow, A. Dandekar, J. Labavitch/A. Powell, and D. Gilchrist) identified, constructed, and advanced to field evaluation five novel DNA constructs that, when engineered into grapevines, suppress symptoms of Pierce's disease by either (a) reducing the titer of *Xylella fastidiosa* (*Xf*) in the plant, (b) reducing systemic spread of the bacteria, or (c) blocking *Xf*'s ability to trigger Pierce's disease symptoms. Each of the five transgenes, when expressed as single genes, reduced the disease levels under field conditions, both as full plant transgenics and in transgenic rootstocks grafted to a non-transformed Pierce's disease susceptible scion. This initial field trial consisting of single gene constructs was discontinued at the end of the 2016 growing season, to be replaced with a second field trial designed to evaluate untransformed scion protection by rootstocks bearing paired combinations of the five constructs. If successful, the obvious benefit would be that any unmodified (non-transgenic) varietal winegrape scion could be grafted to and be protected by transformed rootstock lines. This approach involves "stacking" a combination of distinct protective transgenes in a single rootstock line, which is intended to foster not only durability but also more robust protection of the non-transformed scion against Pierce's disease.

### LAYPERSON SUMMARY

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

### INTRODUCTION

Briefly, we describe information on the history and impact of the genes deployed as single transgenes that were in the initial field study in USDA Animal and Plant Health Inspection Service (APHIS) approved field trials, where test plants were mechanically inoculated with *Xf*. The experimental materials of this project are five specific DNA

constructs (**Table 1**) that were shown to be effective in Pierce's disease suppression under field conditions as single gene constructs, and also appear to have potential in cross-graft-union protection described by S. Lindow, A. Dandekar, and D. Gilchrist in previous reports and noted in the references.

**Table 1.** Genes selected to evaluate as dual genes in the second generation field evaluation for suppression of Pierce's disease in grape (gene names, abbreviation used, and presumed function).

Gene	Code	Function
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

### **Chimeric Antimicrobial Protein and Polygalacturonase-Inhibiting Protein (Abhaya Dandekar)**

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

### **rpff and Diffusible Signal Factor (Steven Lindow)**

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

### **PR1 and microRNA UT456 (David Gilchrist)**

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

## OBJECTIVES

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

Additionally, there could be favorable synergistic protection when two or more resistance-mediating DNA constructs are employed. There are data indicating synergism in other crops. For example, the paper, “Field Evaluation of Transgenic Squash Containing Single or Multiple Virus Coat Protein Gene Constructs for Resistance to Cucumber Mosaic Virus, Watermelon Mosaic Virus 2, and Zucchini Yellow Mosaic Virus” (Tricoli et al. 1995), describes the stacking of several genes for virus resistance in squash. [Note: David Tricoli, the lead author in this paper, will be doing the stacking transformations in this project.] Additionally, the Dandekar laboratory has successfully stacked two genes blocking two different pathways synergistically to suppress crown gall in walnut (Escobar et al. 2001). Experiments proposed here will evaluate potential synergism in suppression of Pierce’s disease symptoms and in reducing *Xf* titer for inoculations distant from the graft union.

1. Complete introduction pairs of protective paired constructs via the dual insert binary vector into adapted grapevine rootstocks 1103 and 101-14 for a total of 20 independent transgenic lines to be evaluated with at least 10 paired combinations from each rootstock line delivered by the transformation facility.
2. Conduct extensive analysis, both by Northern analysis and polymerase chain reaction (PCR) and reverse transcription quantitative PCR (RTqPCR) experiments, of each transgenic plant to verify the presence of the two stacked genes in the genome, the full RNA sequence, and the expression level of each of the mRNAs expected to be produced by the inserted genes, before they are subjected to grafting and greenhouse assays for transgene movement and resistance to Pierce’s disease.
3. The second major step in the process after verification of the genotypic integrity of the transgenic plants is production of the clonal ramets of each plant line to enable two cane growth development of the rootstocks and grafting of the Chardonnay scions. [Note: this step is being eliminated once it was clear that the PCR confirmation of successful dual transformation, but not foliar symptoms, was successful. There were discernable differences among the individual plants in preliminary pathogenicity tests based on bacterial counts within each of the 10 dual combinations.]
4. A total of five independent transgenic lines of each dual construct in the two rootstocks will be advanced to the lathe house for overwintering. Early spring, cuttings will be made, rooted, and bud-grafted with non-transformed Chardonnay. Up to six copies of each rootstock/scion combination will be prepared for field planting in the spring of 2018 at the USDA APHIS approved site in Solano County.

## RESULTS AND DISCUSSION

### Construction of Dual Gene Expression Binaries

The strategy is to prepare dual plasmid constructs bearing a combination of two of the protective genes on a single plasmid with single selectable marker, as described previously (Gilchrist and Lincoln 2016). The binary backbone is based on pCAMBIA1300 (Hajdukiewicz et al. 1994). Binaries were constructed to express two genes from two 35S promoters. The DNA fragments containing transcription units for expression of the transgenes are flanked by rare cutting restriction sites for ligation into the backbone. The nt-PGIP used in these constructs is a modified version of the Labavitch PGIP that was constructed in the Dandekar laboratory to include a signal peptide obtained from a grapevine xylem secreted protein (Aguero et al. 2006). Binary plasmids capable of expressing two genes from the same TDNA were constructed by James Lincoln (Gilchrist et al. 2016).

All plasmids were transformed into *Agrobacterium* strain EHA105, the preferred transformation strain for grape plants. As a check on the integrity of the dual binary plasmid the plasmid was isolated from two *Agrobacterium* colonies for each construct, and the plasmid was used to transform *Escherichia coli*. Six *E. coli* colonies from each *Agrobacterium*-isolated plasmid (for a total of 12 for each construct) were analyzed by restriction digest to confirm that the plasmid in *Agrobacterium* is not rearranged. **Table 2** shows transformations by the UC Davis Plant Transformation Facility. To ensure optimum recovery of the transgenic embryos two versions of the plasmid, with different antibiotic selectable markers, were delivered to the transformation facility. Hence, the dual

inserts can now be subjected to two different selections that enable transformation to move forward in the fastest manner, depending on which marker works best for each dual or each rootstock. Each plasmid containing the dual protective DNA sequences is introduced into embryogenic grapevine culture in a single transformation event, rather than sequentially as would normally be the conventional strategy at the transformation facility. The new transgenic dual-gene-expressing grape plant lines exhibit a phenotype indistinguishable from the untransformed wild-type rootstock (**Figure 2**). The transformation progress, following verification of insert integrity, for each line is shown in **Table 2**.

**Table 2.** Transcript profiling of the dual construct transformed transgenic rootstocks.

Genotype	Construct Code	Construct	# Plants Both Transcripts	Dual Transcript Analysis
1103	AB	pCA-5oP14HT-5oUT456	8	Complete
101-14	AB	pCK-5oP14HT-5oUT456	8	Complete
1103	AC	pCA-5fCAP-5oP14HT	10	Complete
101-14	AC	pCK-5fCAP-5oP14LD	0	
1103	AD	pCA-5PGIP-5oP14HT	10	Complete
101-14	AD	pCK-5PGIP-5oP14LD	9	Complete
1103	AF	pCA-5oP14HT-5orpFF	0	
101-14	AF	pCK-5oP14LD-5orpFF	4	
1103	BC	pCA-5fCAP-5oUT456	10	Complete
101-14	BC	pCA-5fCAP-5oUT456	1	
1103	BD	pCA-5PGIP-5oUT456	0	
101-14	BD	pCK-5PGIP-5oUT456	12	Complete
1103	BF	pCA-5oUT456-5orpFF	1	
101-14	BF	pCK-5oUT456-5orpFF	4	
1103	CD	pCA-5PGIP-5FCAP	4	
101-14	CD	pCK-5PGIP-5FCAP	4	
1103	CF	pCA-5fCAP-5orpFF	10	Complete
101-14	CF	pCK-5ofCAP-5orpFF	0	
1103	DF	pCA-5PGIP-5orpFF	12	Complete
101-14	DF	pCK-5PGIP-5orpFF	12	Complete

### Analysis of the Transgenic Rootstocks to Confirm Dual Insertions Transcripts

This analysis is performed by isolating the RNA from transgenic grape leaves and purifying by a modification of a cetyl trimethylammonium bromide (CTAB) protocol, and includes LiCl precipitation. The RNA is converted to cDNA by oligo dT priming and reverse transcriptase. PCR reactions are set up using the synthesized cDNA as template and specific pairs of primers designed against each of the five putative transgenes. The goal is to identify five independently transformed lines bearing the dual sets of the five transgenes to confirm the genotype of each rootstock to be placed in the field, with six replications of each line. The aforementioned analysis indicated that the successful insertion of two genes into a given transgenic plant was 64 percent of the total plants provided by the transformation facility (**Table 3**). This underscores the need for dual transcript verification prior to moving plants forward to grafting and subsequent analysis for product movement across a graft union and symptom suppression of the untransformed Chardonnay. These assays, while time consuming and tedious, will ensure that each plant will have a full phenotypic and genotypic analysis prior to inoculating them in the field.

**Table 3.** Frequency of dual gene transcripts as confirmed in transgenic plants delivered by the UC Davis Plant Transformation Facility by reverse transcription and PCR analysis.

Transgene Transcripts	Number of Plants	Percent of Plants
2	169	64
1	84	32
0	11	4

## Production of Two-Cane Growth Development of Each Plant Line to Enable Collection of Rootstock Cuttings for Grafting of the Chardonnay Scions

Following verification of the genotypic integrity of the transgenic rootstock plants, clonal copies of each plant line were made to enable two-cane growth development for production of rootstocks to be grafted with Chardonnay scions (**Figure 2** and **Figure 3**).

Preliminary inoculations were initiated in the greenhouse and selections made based on qPCR analysis of *Xf* titre in the tissue above the inoculation site. These tests will be repeated after the scions are inoculated in the field. In total, over the two years of transgenic rootstock delivery and greenhouse evaluations, there will be approximately 7,000 molecular analyses conducted to minimize time and maximize the likelihood correlating the field results on bacterial dynamics with Pierce's disease symptom scoring. The timeframe from receipt of plants, analysis, and selection of the individuals for field planting has been 9 to 13 months. The total number of plants to screen if all plants are verified transgenics will be at least 1,070, including 70 untransformed control plants.

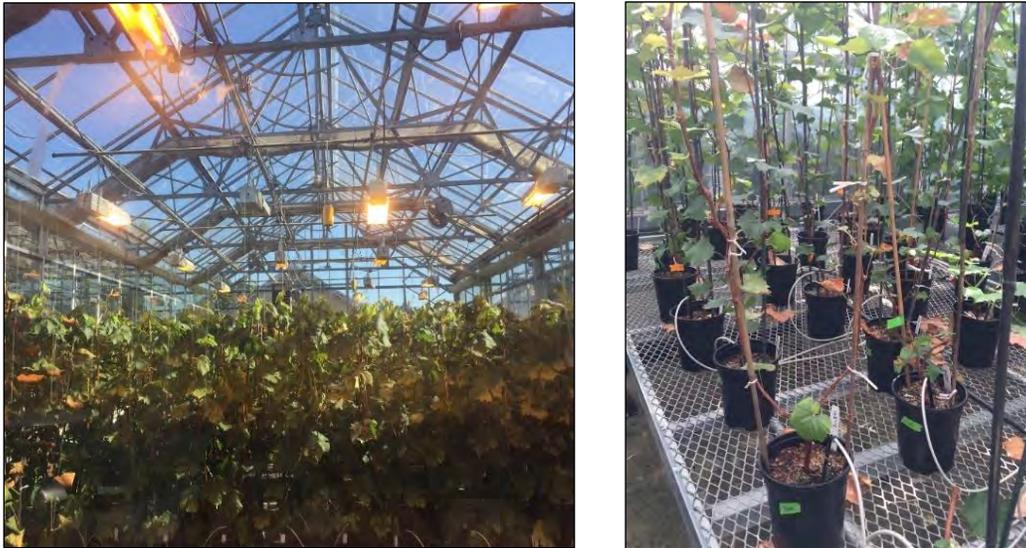
The following images illustrate the status of the dual construct transgenic plants as they are managed in the greenhouse (**Figures 1** and **2**). Each plant is staked to support vegetative growth for inoculation, symptom expression, and sampling. Each pot is individually irrigated with a nutrient solution, and plants are trimmed as necessary to avoid excessive branching under these growth conditions. *Xf* Inoculation of the first transgenic lines of 1103 in the greenhouse are illustrated in **Figure 3**. Within the inoculation experiment, samples are taken to determine the population of bacteria at the inoculation site and 10 cm and 30 cm from the inoculation site. Unfortunately the foliar symptoms are not reliably diagnostic of relative bacterial titer in the inoculated canes. Hence, we have found the more reliable indicator was the insert-dependent suppression of bacterial titre. **Table 4** and **Figure 4** show the selected lines now in the lathe house for final stem development prior to rooting of the transformed rootstock and prior to grafting.



**Figure 1.** Transgenic grape plants growing in the greenhouse. Left side of image shows plants right before inoculation with *Xf* and cuttings are taken.



**Figure 2.** Transgenic grape plants growing in the greenhouse. Image shows newly-potted cuttings.



**Figure 3.** Results of *Xf* inoculation of greenhouse grown grapes containing inserts of dual DNA constructs capable of expressing suppression of Pierce’s disease symptoms. Symptom expression was not reliable but bacterial population analysis was differential and used under these controlled conditions to select transgenic rootstocks for grafting and field evaluation.

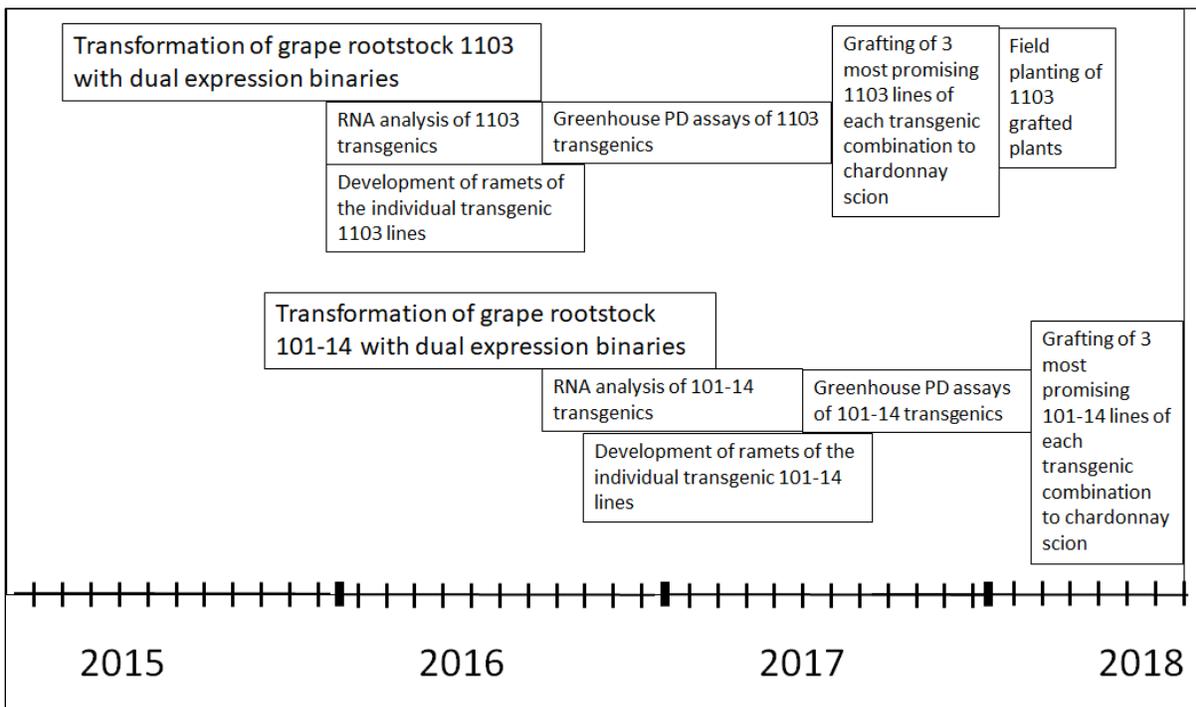


**Figure 4.** Plants selected as rootstock source material. Image shows selected dual construct containing plants in lath house as final site to produce material for rootstock development, for grafting of non-transgenic scions and field evaluation.

**Table 4.** Dual construct transformed 1103 and 101-14 rootstocks now in a lath house for making rooted cuttings prior to grafting.

1103 Rootstocks						101-14 Rootstocks	
AB15-01	AC35-01	AD13-04	BC36-03	CF07-02	DF108-03	BD23-05	DF85-01
AB15-02	AC62-01	AD13-06	BC36-05	CF07-03	DF108-07	BD58-01	DF85-02
AB15-04	AC62-02	AD13-07	BC36-06	CF07-04	DF108-08	BD58-02	DF85-04
AB15-05	AC62-04	AD33-01	BC36-09	CF07-05	DF108-09	BD58-08	DF85-06
AB15-06	AC62-06	AD33-02	BC36-11	CF07-06	DF108-10	BD80-05	DF85-08
AB 15-03	AC35-05	AD13-02	BC36-13	CF07-12	DF108-04	BD23-01	DF85-10

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



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

## CONCLUSIONS

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

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

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# FUNCTIONAL CHARACTERIZATION OF *PILG*, A KEY VIRULENCE GENE, AND EVALUATION OF THE EFFECTS OF ANTI-VIRULENCE INHIBITORS ON THE VIRULENCE OF *XYLELLA FASTIDIOSA*

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**Reporting Period:** The results reported here are from work conducted October 2016 to September 2017.

## ABSTRACT

Type IV pili of *Xylella fastidiosa* (*Xf*) are regulated by *pilG*, a response regulator protein putatively involved in chemotaxis-like operon sensing stimuli through signal transduction pathways. To elucidate the role of *pilG* in the pathogenicity of *Xf*, the *pilG*-deletion mutant *Xf*Δ*pilG* and complemented strain *Xf*Δ*pilG*-C were generated. Results demonstrated that *Xf*Δ*pilG* showed significant reduction in cell-matrix adherence and biofilm production compared with wild-type *Xf* and *Xf*Δ*pilG*-C. *In planta* experiments showed that no Pierce's disease symptoms were observed in grapevines inoculated with *Xf*Δ*pilG*, whereas grapevines inoculated with the wild-type *Xf* and complemented strain of *Xf*Δ*pilG*-C developed typical Pierce's disease symptoms. These results indicate that *pilG* has a role in *Xf* virulence. To evaluate the effect of anti-virulence molecules on the target gene, a chemical library consisting of putative small molecular inhibitors and their analogs was screened. Our preliminary results indicate that some of the small molecules exhibit effective suppression on twitching motility and virulence traits under *in vitro* and *in planta* evaluation. This study provides a new target-basis strategy to combat Pierce's disease of grapevines.

## LAYPERSON SUMMARY

*Xylella fastidiosa* (*Xf*) causes Pierce's disease of grapevines. To understand pathogenicity of *Xf* genetic analyses were conducted to compare phenotypes of wild-type and a mutant strain of *Xf* with defective *pilG*, a virulence gene that is predicted to play a key functional role for Pierce's disease. Greenhouse experiments indicated that grapevines inoculated with the mutant strain showed no Pierce's disease symptoms compared to grapevines infected with *Xf* wild-type. This study confirms that *pilG* is a key virulence gene that is required to develop Pierce's disease in grapevines. Anti-virulence molecular screening identified some anti-virulence molecules that effectively suppressed virulence traits of *Xf*, suggesting that this approach could provide a new target-basis strategy to combat Pierce's disease of grapevines.

## INTRODUCTION

*Xylella fastidiosa* (*Xf*) is a Gram-negative non-flagellated bacterium and is limited to the water-conducting xylem vessels. Pierce's disease of grapevines results in the blockage of xylem vessels, water stress, and nutritional deficiencies (Hopkins 1989). The twitching motility of *Xf*, a means of flagellum-independent bacterial movement through extension, attachment, and retraction of the polar type IV pili (Mattick 2002), has been microscopically characterized in a fabricated microfluidic chamber (Li et al. 2007, Meng et al. 2005). The colonization of xylem vessels is dependent on the ability of *Xf* to move within xylem vessels (Meng et al. 2005). The *pilB*, *pilQ*, and *pilR* mutants resulting in the defect of type IV pili and non-twitching phenotypes showed reduced disease symptoms in grapevines (Li et al. 2007, Meng et al. 2005). These suggest that twitching motility provides *Xf* not only a means for long-distance intra-plant movement and colonization, but also contributes toward virulence.

The activity of twitching motility of *Xf* is controlled by a chemotaxis-like regulatory system (Cursino et al. 2011), Pil-Chp operon, similar to that in *Pseudomonas aeruginosa* and *Escherichia coli* (Fernandez et al. 2002, Fulcher et al. 2010). Like the *P. aeruginosa* CheIV (Pil-Chp) cluster, *Xf* possesses a single predicated chemosensory system, Pil-Chp operon that regulates the twitching motility of type IV pili (Fulcher et al. 2010, Simpson et al. 2000). The Pil-Chp operon of *Xf* encodes proteins involved in signal transduction pathways including *pilG*, *pilI*, *pilJ*, *pilL*, *chpB*, and *chpC* as in *P. aeruginosa* and *E. coli* (Cursino et al. 2011, Fulcher et al. 2010). Upon binding of the chemical stimuli in the periplasmic domain, the transmembrane chemoreceptors activate a signalling cascade in the cytoplasmic portions and ultimately control bacterial twitching motility (Cursino et al. 2011). A phospho-

shuttle protein, PilG, in the Pil-Chp operon of *Xf* is homologous to CheY, a response regulator in chemotaxis systems of *P. aeruginosa* and *E. coli*, in which CheY interacts with the flagellar motor proteins (Fernandez et al. 2002, Fulcher et al. 2010). Recent studies indicated that the homologue of the chemotaxis regulator PilG is required for the twitching motility of *Xf*, since the *pilG*-deleted *Xf* strain was deficient in twitching motility (Shi and Lin 2016). The critical roles of the Pil-Chp operon in the virulence of *Xf* were examined recently (Cursino et al. 2011). However, the contributions of *pilG* in Pil-Chp chemotaxis operon toward the pathogenicity of *Xf* are not clear. In this project, the functional roles of the chemotaxis regulator PilG involving biofilm, cell adherence, and pathogenicity are discussed.

The mobility mediated by pili genes was reported to play important roles in the pathogenicity of animal and human bacterial pathogens including *Vibrio cholera*, *Neisseria meningitidis*, and alkaliphilic *Bacillus* strains (Hung et al. 2005, Mehta et al. 2015, Sugiyama et al. 1998). Recently, small molecule inhibitors targeting bacterial motility were reported (Hung et al. 2005, Mehta et al. 2015, Sugiyama et al. 1998). These molecules specifically bind target domains and suppress virulence factors. For example, a small molecule amiloride was found to be able to target the extracellular Na<sup>+</sup>-driven flagellar motor, resulting in the inhibition of the motility of alkaliphilic *Bacillus* strains (Sugiyama et al. 1998). Rasmussen et al. (2011) demonstrated that the small molecule quinazoline and its analogs effectively inhibited the expression of cholera toxin and toxin-coregulated pilus responsible for motility, but did not affect the cell growth *in vitro*. In *Xf*, the twitching motility mediated by type IV pili contributes toward virulence via long-distance intra-plant movement and colonization. Thus, the disruptions of the functions of the type IV pilus genes via small molecule inhibitors to block the twitching motility of *Xf* could be a promising strategy to disarm pathogenicity and prevent and/or block disease development. Here we functionally characterized the effect of the small molecule inhibitors on the twitching motility and pathogenicity of *Xf* on the plant. The goal of this project is to identify (characterize) potent small molecule inhibitors against *Xf*.

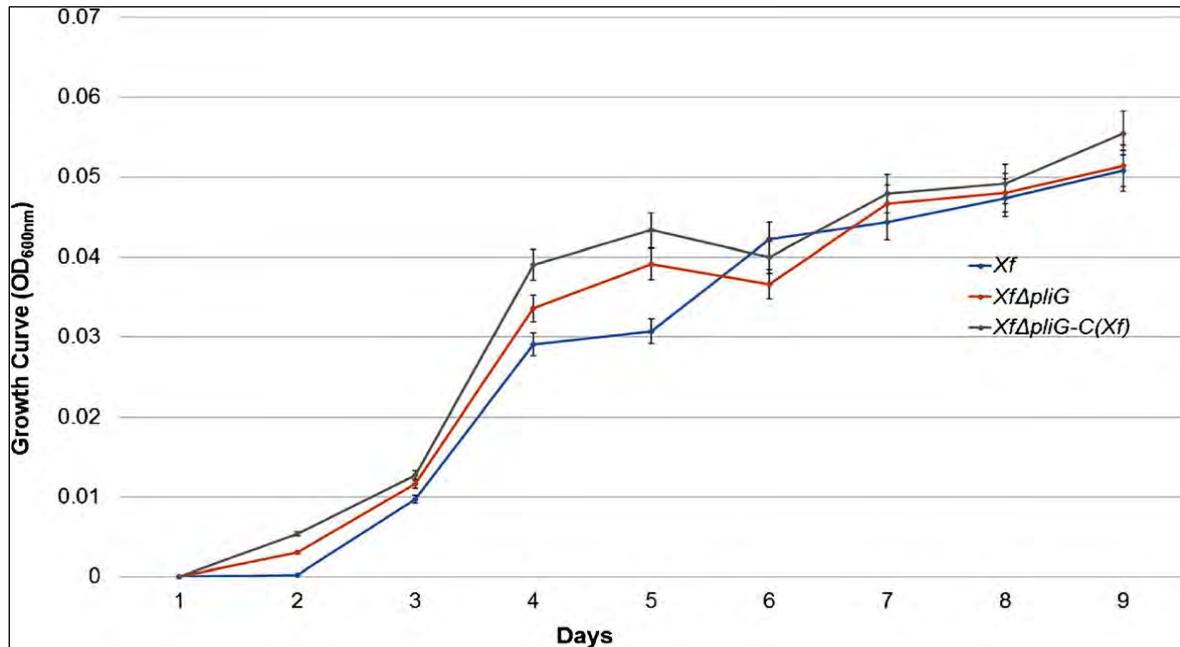
## OBJECTIVES

1. Functional characterization of the roles of *pilG* in cell growth, attachment, biofilm formation, and pathogenicity.
2. Evaluation of the effects of small molecular inhibitors on the twitching motility and pathogenicity of *Xf*.

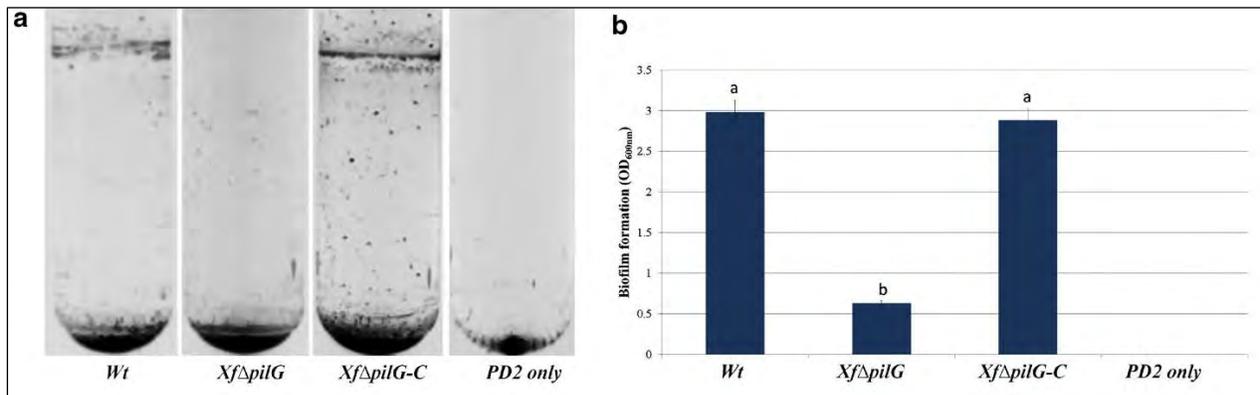
## RESULTS AND DISCUSSION

### Objective 1. The Roles of *pilG* in Cell Growth, Attachment, Biofilm Formation, and Pathogenicity

*pilG*-knock-out strain *Xf* $\Delta$ *pilG* and complemented strain *Xf* $\Delta$ *pilG*-C were obtained as described previously (Shi and Lin 2016). The expression of *pilG* was not detected in *Xf* $\Delta$ *pilG* but was detected in complemented *Xf* $\Delta$ *pilG*-C (data not shown). *Xf* $\Delta$ *pilG* showed a similar growth curve as wild-type when both were grown in PD2 medium (**Figure 1**), indicating that the *pilG* mutant does not affect cell growth. Results from *in vitro* studies showed that deletion of *pilG* caused significant reductions in cell attachment and biofilm formation, whereas the complemented strain *Xf* $\Delta$ *pilG*-C restored wild-type phenotypes (**Figures 2a** and **2b**). *In planta* pathogenicity assessment further confirmed that grapevines inoculated with *Xf* $\Delta$ *pilG*-C developed typical Pierce's disease symptoms with severity comparable to wild-type. In contrast, grapevines inoculated with *Xf* $\Delta$ *pilG* exhibited no visible symptoms in greenhouse experiments (**Figure 3**). The titers of three strains of *Xf* were well correlated with the severity of disease symptoms (**Figure 4**). Previous reports showed that *Xf* $\Delta$ *pilG* was deficient in IV pilus-dependent twitching motility (Shi and Lin 2016). Twitching motility is one of the important virulence factors. Several *Xf* twitching motility-associated mutants have been reported (Li et al. 2007, Meng et al. 2005). Most of these were associated with only partial reductions in virulence and Pierce's disease symptoms (Cursino et al. 2009, Meng et al. 2005). More recently, Cursino et al. (2009) reported that *tonB1* mutant showed about 30% of reduction in virulence when compared to its wild-type *Xf*, although the *tonB1* mutant caused motility deficiency. In this study, however, we found that the pathogenicity was completely knocked-out in *Xf* $\Delta$ *pilG*. To this regard, based on our *in vitro* and *in planta* data, we conclude that *pilG* could have critical roles involving multiple regulatory functions and pathogenicity. Therefore, it is a central virulence factor in mediating Pierce's disease development.



**Figure 1.** Growth curves of *Xf* wild-type, *XfΔpilG* mutant, and *XfΔpilG-C* complement strains in PD2 broth were measured over nine days with a spectrophotometer. Data are the average of three replications. The experiments were repeated three times.

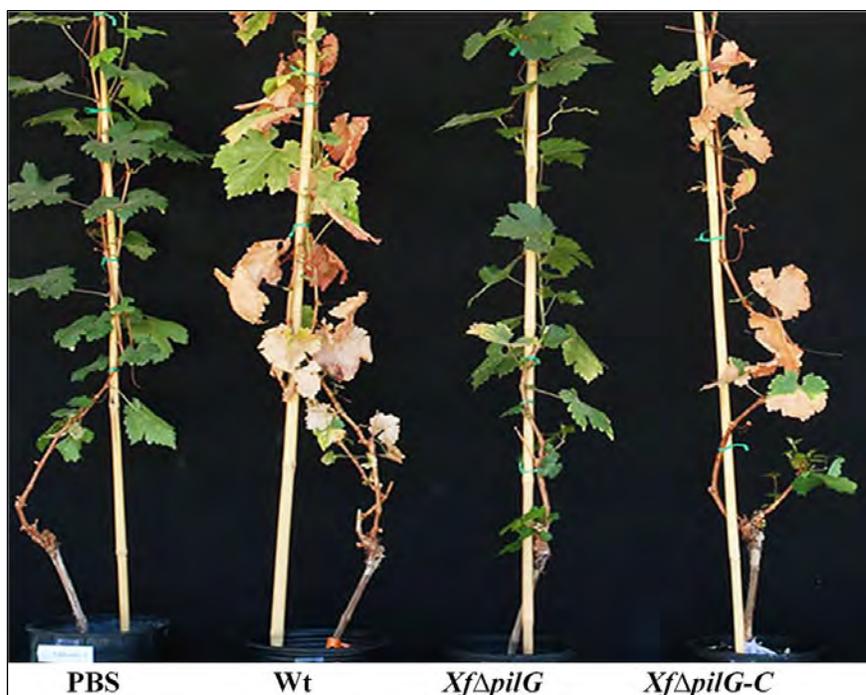


**Figure 2.** Cell attachment and biofilm formation analysis of *Xf* wild-type, *XfΔpilG*, and *XfΔpilG-C* in PD2 broth. (a) *Xf* cells attached to the inside wall of the glass tubes forming a ring. (b) Quantitative measurement of biofilm formation of *Xf* wild-type, *XfΔpilG* and *XfΔpilG-C* strains. Data are the average of three replications, with error bars indicating standard deviation. Bars with the same lowercase letter are not significantly different ( $P < 0.01$ ). The experiments were repeated three times.

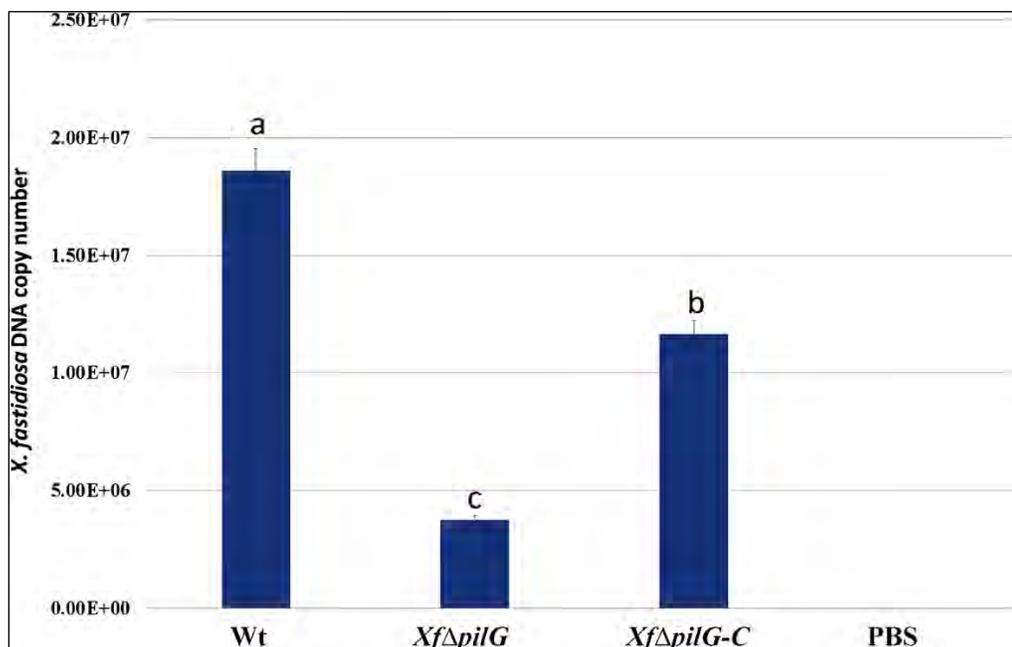
## Objective 2. Evaluation of the Effects of Small Molecular Inhibitors on the Twitching Motility and the Pathogenicity of *Xf*

Previous studies showed that several small molecule inhibitors had functional roles in inhibiting the pilus assembly and suppressing bacterial motility (Rasmussen et al. 2011, Syed, et al. 2009, Mehta et al. 2015). For example, the inhibition of motility with phenamil in *V. cholera* has been shown to have effects on virulence gene expression (Hase 2001) and mitigation of disease development (Syed et al. 2009). These findings suggest that small molecule inhibitors could exert antimicrobial action on virulence traits of pathogenic bacteria. We have previously demonstrated that *pilG* mutant exhibited deficiency in twitching motility, reduction in biofilm formation, and virulence (Shi and Lin 2016). In this study we constructed a custom chemical library consisting of putative small molecule inhibitors and evaluated the effect of inhibitors (anti-virulence compounds) on the twitching motility and virulence traits of *Xf*. Since the peripheral fringe is indicative of type IV pilus-mediated twitching motility by the bacteria (Shi and Lin 2016), in this study we assessed the inhibitory effect of small molecules on the peripheral fringe morphologies of *Xf*. Among the small molecular screenings we have identified

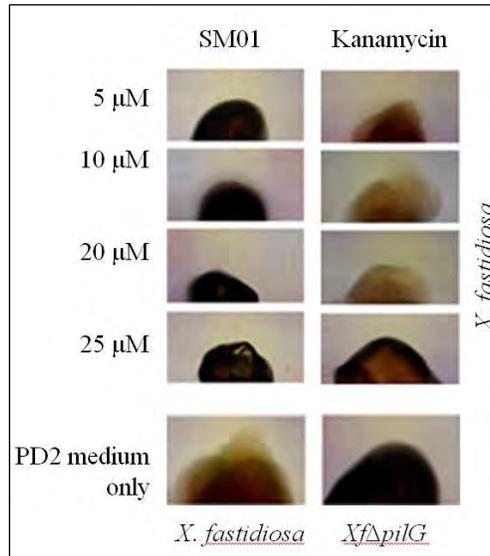
several compounds that showed promising inhibition effects on bacterial twitching motility. For example, one of the small molecular compounds, SM01, exerts effective inhibition on peripheral fringes at a concentration of as low as 5  $\mu$ M (**Figure 5**). A time-lapse microfluidic chamber recording system was used to further confirm the suppression of twitching motility with anti-virulence molecule supplemented in flow PD2 medium (data not shown).



**Figure 3.** Pathogenicity assays on Chardonnay grapevines inoculated with phosphate-buffered saline (negative control), *Xf* wild-type, *Xf* $\Delta$ *pilG*, and *Xf* $\Delta$ *pilG-C* 20 weeks post-inoculation in the greenhouse. Grapevines inoculated with wild-type and *Xf* $\Delta$ *pilG-C* developed typical Pierce's disease systems. The experiments were repeated three times.

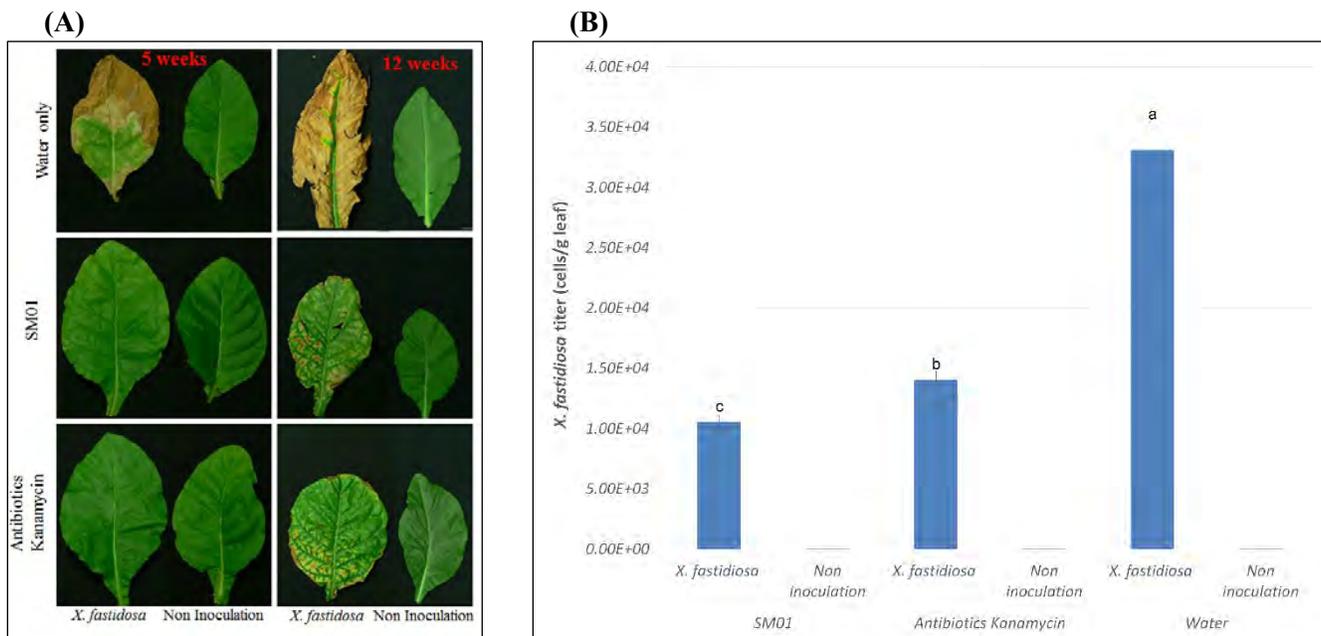


**Figure 4.** Populations of *Xf* wild-type, *Xf* $\Delta$ *pilG*, and *Xf* $\Delta$ *pilG-C* from Chardonnay grapevine petioles were estimated by quantitative polymerase chain reaction 20 weeks post-inoculation. Data represent the means from five replications. Different letters indicate statistical significance at  $P < 0.05$ .



**Figure 5.** The peripheral fringes were observed in *Xf* colonies grown on PD2 agar. *pilG* mutant *Xf* $\Delta$ *pilG* showed smooth colony morphology. When PD2 medium was supplemented with 5  $\mu$ M, 10  $\mu$ M, 20  $\mu$ M, and 25  $\mu$ M of small molecular inhibitor (SM01), no peripheral fringes were observed on *Xf* colonies. In contrast, the effective concentration on suppression of peripheral fringe structure was observed on medium supplemented with at least 25  $\mu$ M kanamycin. The experiments were repeated three times.

To further evaluate the effect of small molecular inhibitors on the pathogenicity of *Xf*, greenhouse-grown *Xf*-infected tobacco plants were foliar-sprayed with selected inhibitor compounds including SM01. Chlorosis and necrosis developed on the tobacco leaves infected with *Xf* wild-type, while plants treated with SM01 alleviated the effects of *Xf* infection (**Figure 6A**). SM01 treatment also resulted in lower bacterial titers compared to untreated tobacco plants (**Figure 6B**).



**Figure 6.** Pathogenicity assays on tobacco plants inoculated with *Xf*. (A) Progressive development of leaf symptoms on the experimental tobacco plants five weeks and 12 weeks after inoculation with *Xf* and foliar-sprayed with water, SM01, or the antibiotic kanamycin, respectively, by foliar spray, once per week at 50  $\mu$ M for four weeks in the greenhouse. Tobacco from each treatment demonstrates disease symptoms ranging from healthy to severe. Greenhouse experiments were repeated three times. (B) *Xf* concentrations from tobacco plant leaves were estimated by enzyme-linked immunosorbent assay two months post-inoculation. Data are means from five replications. Different letters indicate statistical significance ( $P < 0.05$ ).

## CONCLUSIONS

We have demonstrated that *pilG* plays a critical role involving regulatory hierarchy governing the pathogenicity of *Xf*. *In vitro* experiments have characterized anti-virulence molecules that have potent inhibition on virulent traits of *Xf*. The preliminary results presented here suggest that this strategy could provide a new approach to manage Pierce's disease.

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

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### ABSTRACT

*Paraburkholderia phytofirmans* strain PsJN<sup>1</sup> was found to be capable of extensive growth and movement within grape after both needle or spray inoculation. The population size of *Xylella fastidiosa* (*Xf*) is greatly reduced in plants in which *P. phytofirmans* is either co-inoculated at the same time and location, inoculated at the same time but at other nearby locations, and even inoculated at other locations either three weeks before or after that of the pathogen. The dramatic reductions in population size of *Xf* are observed in all grape varieties tested. Reductions in pathogen population are similarly large when *P. phytofirmans* is inoculated by spraying in a suspension containing 0.2% Break-thru, an organosilicon surfactant with very low surface tension, as when directly inoculated into plants using a needle. While *P. phytofirmans* can achieve quite large population sizes in inoculated grape within three to four weeks after inoculation, and spread up to one meter away from the site of point inoculation, its population size then often decreases with further time after inoculation. The very large decrease in population size of *Xf* in plants inoculated with *P. phytofirmans* even after that of the pathogen is suggestive of a mechanism by which this antagonistic microorganism sensitizes the plant to the presence of the pathogen, thereby initiating a plant disease resistance reaction. Support for such a model was provided by evidence of up-regulation of the expression of the PR1 and ETR1 genes in grapes inoculated both with *P. phytofirmans* and *Xf* but not that of the pathogen alone.

### LAYPERSON SUMMARY

A naturally occurring *Paraburkholderia* strain capable of production of diffusible signal factor-like molecules that is also capable of growth and movement within grape has been found that can confer increased resistance to Pierce's disease. We are exploring the biological control of disease using this strain. The movement of *Xylella fastidiosa* (*Xf*) within plants and disease symptoms are greatly reduced in plants in which this *Paraburkholderia* strain was inoculated either simultaneously with, prior to, or even after that of *Xf*. The biological control agent can be applied either by direct introduction into the xylem by droplet puncture or by spray application to foliage using a penetrating surfactant. These results are quite exciting in that they reveal that biological control of Pierce's disease using *P. phytofirmans* is both robust and may be relatively easy to employ by various ways of inoculation.

### OBJECTIVES

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

### RESULTS AND DISCUSSION

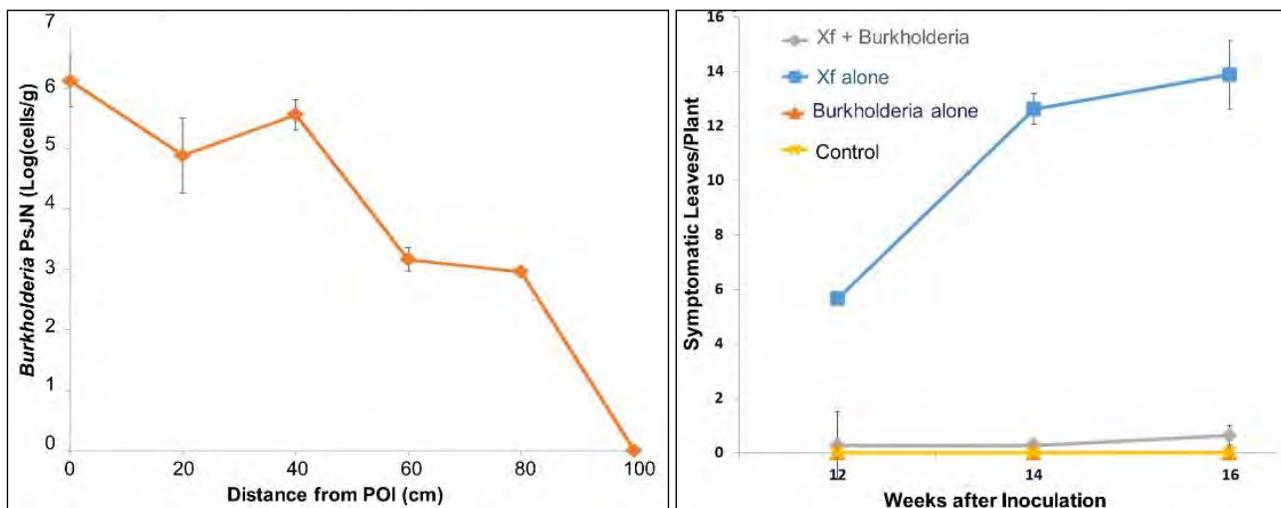
#### Objective 1. Biological Control with *Paraburkholderia phytofirmans* PsJN<sup>1</sup>

While the biological control of Pierce's disease with endophytic bacteria that would grow within grape and produce diffusible signal factor (DSF) has been an attractive strategy, until recently we have been unable to find bacteria capable of exploiting the interior of grape. All of hundreds of strains isolated from within grape by our group as well as that of Bruce Kirkpatrick exhibited no ability to grow and move beyond the point of inoculation

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<sup>1</sup> *Burkholderia. phytofirmans* strain PsJN has recently been renamed *Paraburkholderia phytofirmans* due to the recognition that it is genetically unrelated to other *Burkholderia* strains which are potentially human or plant pathogens, and is thus genetically similar to a variety of environmental strains known not to be plant pathogens.

when re-inoculated. We have recently, however, found that *Paraburkholderia phytofirmans* strain PsJN, which had been suggested to be an endophyte of grape seedlings, multiplied and moved extensively in mature grape plants (**Figure 1**). Its population size and spatial distribution in grape within six weeks of inoculation was similar to that of *Xf* itself, suggesting that it is an excellent grape colonist. Furthermore, DSF production has been demonstrated in certain other *Paraburkholderia* species and the genome sequence of *P. phytofirmans* revealed that it has a homologue of *Xf rpfF*. While we have no evidence for its production of a DSF species to which *Xf* could respond, the promiscuous nature of RpfF in *Xf* and other species suggested that it might make DSF species to which *Xf* would respond under some circumstances, such as when growing within plants. Preliminary results suggest that co-inoculation of *Xf* and *P. phytofirmans* resulted in greatly reduced disease symptoms compared to plants inoculated with *Xf* alone. Whereas the number of infected leaves of plants inoculated with *Xf* alone increased rapidly after week 12, very little disease was observed in plants inoculated with *Xf* and *P. phytofirmans* (**Figure 1**).

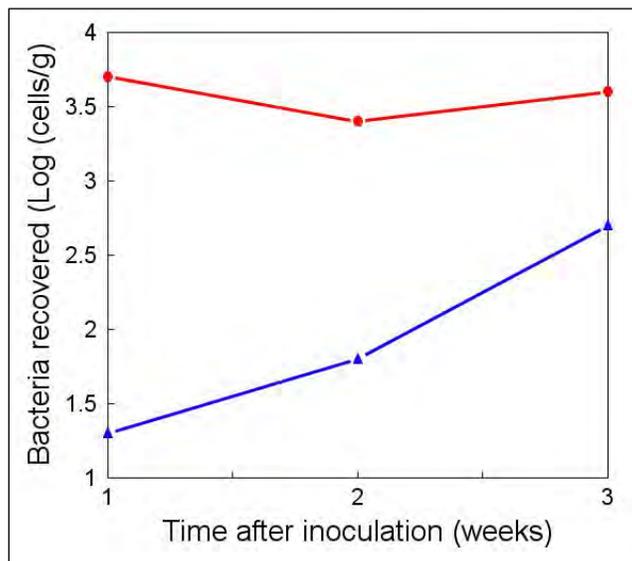


**Figure 1.** (Left). Population size of *Paraburkholderia* (formerly *Burkholderia*) *phytofirmans* in Cabernet Sauvignon grape at various distances from the point of inoculation after six weeks incubation. (Right). Severity of Pierce's disease of Cabernet Sauvignon at various times after inoculation with *Xf* alone (blue) or when co-inoculated with *P. phytofirmans* (gray) or when inoculated with *P. phytofirmans* alone (red).

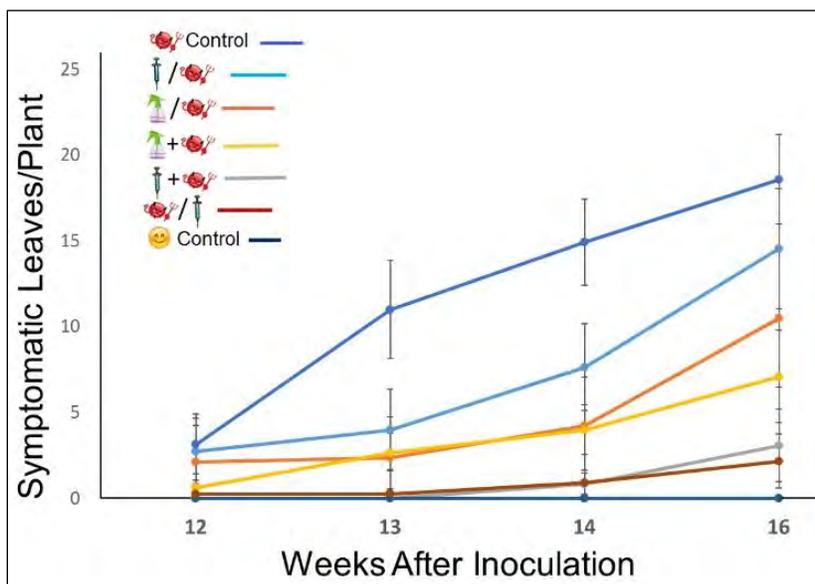
While the droplet puncture method used in **Figure 1** to introduce *P. phytofirmans* is an effective way to introduce bacteria into the xylem, we have investigated the potential to introduce *P. phytofirmans* into the vascular tissue by topical application to leaves using 0.2% Break-thru, an organo-silicon surfactant with sufficiently low surface tension that spontaneous invasion of plant tissues can be achieved. The population size of *P. phytofirmans* in the petioles of leaves distal from the leaf on which cell suspensions in Break-thru ( $10^8$  cells) have been applied were used as a measure of growth and movement potential from such an inoculation site. Substantial numbers of cells of *P. phytofirmans* could be recovered from petioles within one or two weeks after topical application to leaves in the presence of Silwet L-77 or Break-thru (**Figure 2**). Very few cells were present within petioles when the bacterium was applied without a penetrating surfactant. Topical application of such an endophyte thus appears to be a very practical means of inoculating plants in the field.

Given the promising results of the reduction of severity of Pierce's disease in grape treated with *P. phytofirmans* we performed additional experiments in which *Xf* was co-inoculated with *P. phytofirmans* as well as when *P. phytofirmans* both preceded or followed inoculation of plants with *Xf* by 30 days. As observed before, the severity of Pierce's disease of plants co-inoculated with *P. phytofirmans* and *Xf* was greatly reduced at all times after inoculation compared to that on plants inoculated with the pathogen alone (**Figure 3**). Importantly, the severity of Pierce's disease was also substantially less on plants in which inoculation with *P. phytofirmans* followed inoculation with the pathogen by 30 days than on control plants inoculated only with the pathogen (**Figure 3**). Almost no disease was observed on plants inoculated with *P. phytofirmans* 30 days after inoculation with the pathogen (**Figure 3**). These results are quite exciting and confirmed that *P. phytofirmans* can confer high

levels of disease resistance in grape, both when co-inoculated with the pathogen and also when inoculated into plants already infected with *Xf*. It might have been anticipated that pre-inoculation of plants with *P. phytofirmans* would have yielded the largest degree of disease resistance. However, this and other studies have shown that disease incidence and severity is reduced whenever *P. phytofirmans* and *Xf* are present together in the plant. Inoculation of plants with *P. phytofirmans* after that of the pathogen would, by definition, place them both in the plant together while pre-inoculation could result in a situation where the biological control agent may not be present in a plant, particularly if it did not continuously colonize the plant.

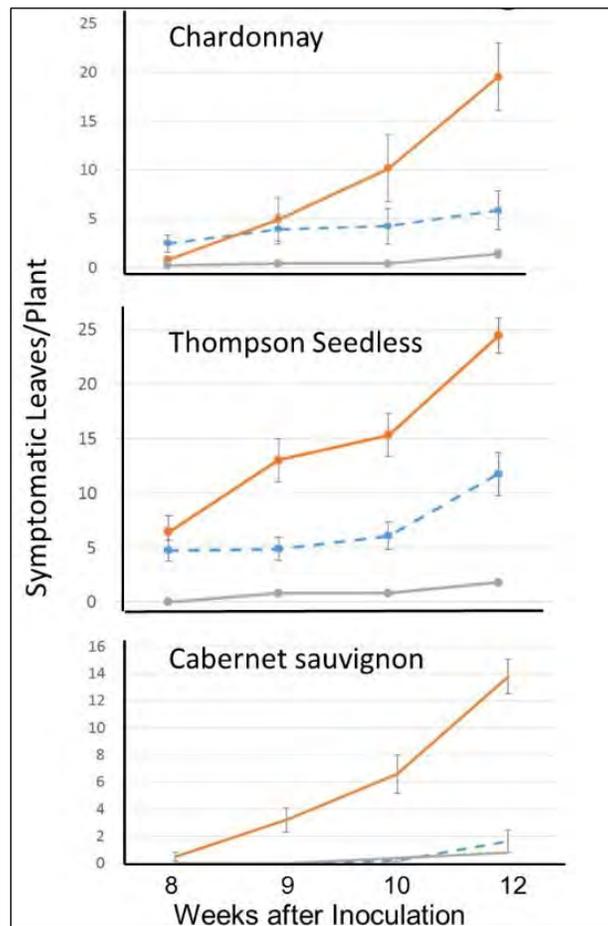


**Figure 2.** Population size of *P. phytofirmans* in petioles of Cabernet Sauvignon of plants sprayed with this strain alone (blue line) or this strain applied with 0.2% Break-thru (red line).



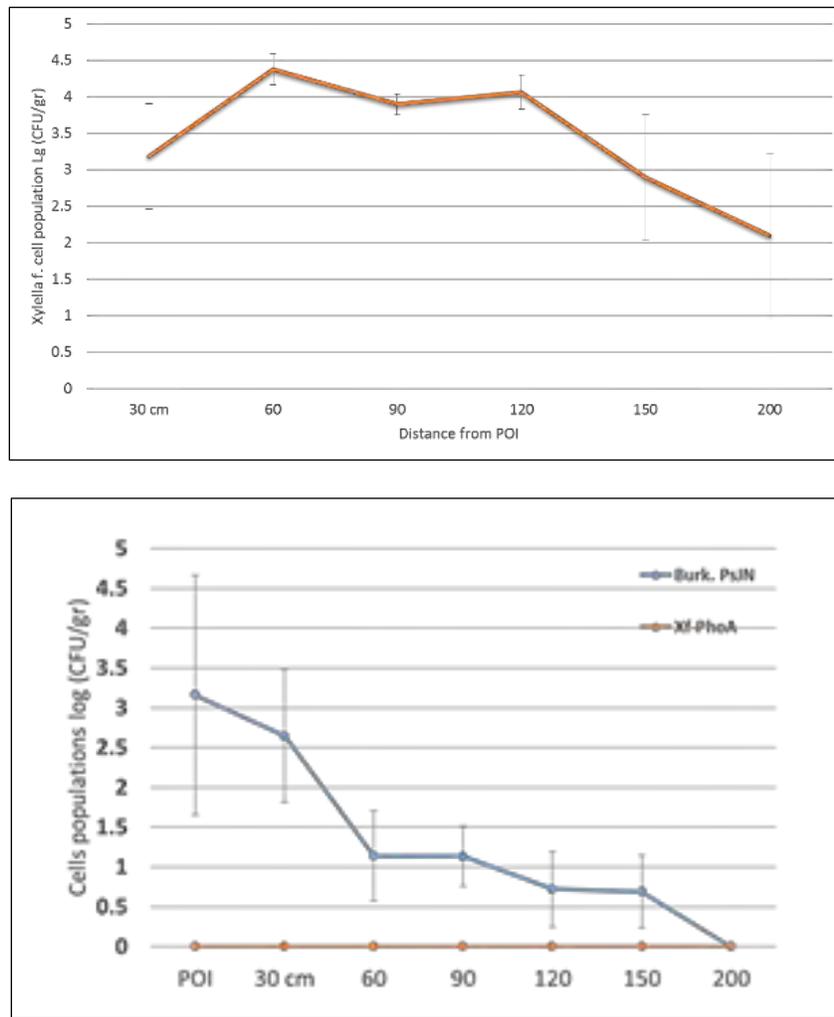
**Figure 3.** Severity of Pierce's disease symptoms (number of symptomatic leaves/vine) on Cabernet Sauvignon plants needle inoculated only with *P. phytofirmans* (dark blue line), only with *Xf* (medium blue line), or co-inoculated with *Xf* and *P. phytofirmans* (yellow line). Also shown is disease severity on plants needle inoculated with *P. phytofirmans* 30 days before inoculation with *Xf* (light blue line) or sprayed with *P. phytofirmans* in a solution of 0.2% Break-thru 30 days before inoculation with *Xf* (orange line), as well as on plants needle inoculated with *Xf* 30 days after inoculation with *P. phytofirmans* (maroon line). The vertical bars represent the standard error of the determination mean disease severity.

*P. phytofirmans* was able to inhibit Pierce’s disease development in all grape varieties in which it was evaluated. When inoculated simultaneously into different grape varieties (although not at the same location, but within about one cm of the site of inoculation with the pathogen), the progression of Pierce’s disease was greatly suppressed compared to that of plants inoculated with *Xf* alone (**Figure 4**). While the greatest reduction in disease severity was conferred in Cabernet Sauvignon, a variety somewhat more resistant to Pierce’s disease than either Thompson Seedless or Cabernet, *P. phytofirmans* conferred a very high level of disease resistance (**Figure 4**). It thus appears that the beneficial effect of *P. phytofirmans* is not variety specific, and that it should confer high levels of resistance in all grape varieties.



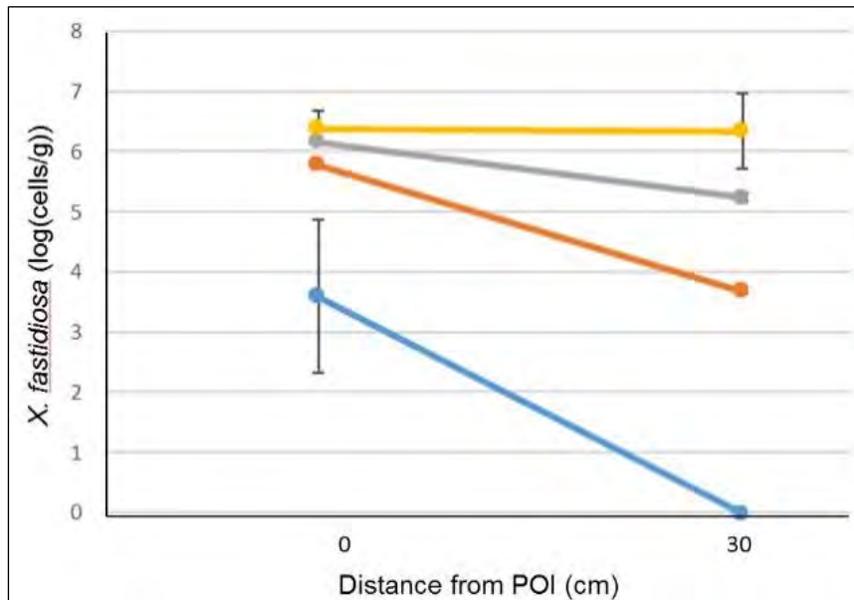
**Figure 4.** Severity of Pierce’s disease observed in different grape varieties needle inoculated at the same time but at different locations with *Xf* and *P. phytofirmans* (blue line) compared to that inoculated only with *Xf* (orange line), or with *P. phytofirmans* alone (gray line). The vertical bars represent the standard error of the determination mean disease severity.

While the mechanism by which *P. phytofirmans* reduces the severity of Pierce’s disease remains somewhat unclear, the biological control activity conferred by this bacterium is associated with its ability to reduce the population size of *Xf* in inoculated plants. Relatively high population sizes of *Xf* were recovered from stem segments collected from 30 to 300 cm away from the point of inoculation in plants inoculated only with the pathogen (**Figure 5**). As expected, the highest population sizes were seen within the first 120 cm, but population sizes greater than 100 cells per gram were observed as much as 200 cm away from the point of inoculation. In contrast, the population size of *Xf* was much lower at a given distance away from the point of inoculation in plants co-inoculated with *Xf* and *P. phytofirmans* (**Figure 5**). Whereas population sizes of the pathogen were usually in excess of  $10^4$  cells per gram in stem segments within 120 cm of the point of inoculation in plants inoculated with the pathogen alone, the pathogen population sizes were much lower, decreasing from a high of  $10^{2.5}$  to less than 10 cells per gram in plants co-inoculated with *P. phytofirmans* (**Figure 5**).



**Figure 5.** (Top). Population size of *Xf* in the stems of grapes at various distances from the point of inoculation of the pathogen alone when measured 12 weeks after inoculation. (Bottom). Population size of *Xf* in the stems of grapes at various distances from the point of inoculation of the pathogen when co-inoculated with *P. phytofirmans* (blue) or populations of *P. phytofirmans* (orange). The vertical bars represent the standard error of the mean population size/g.

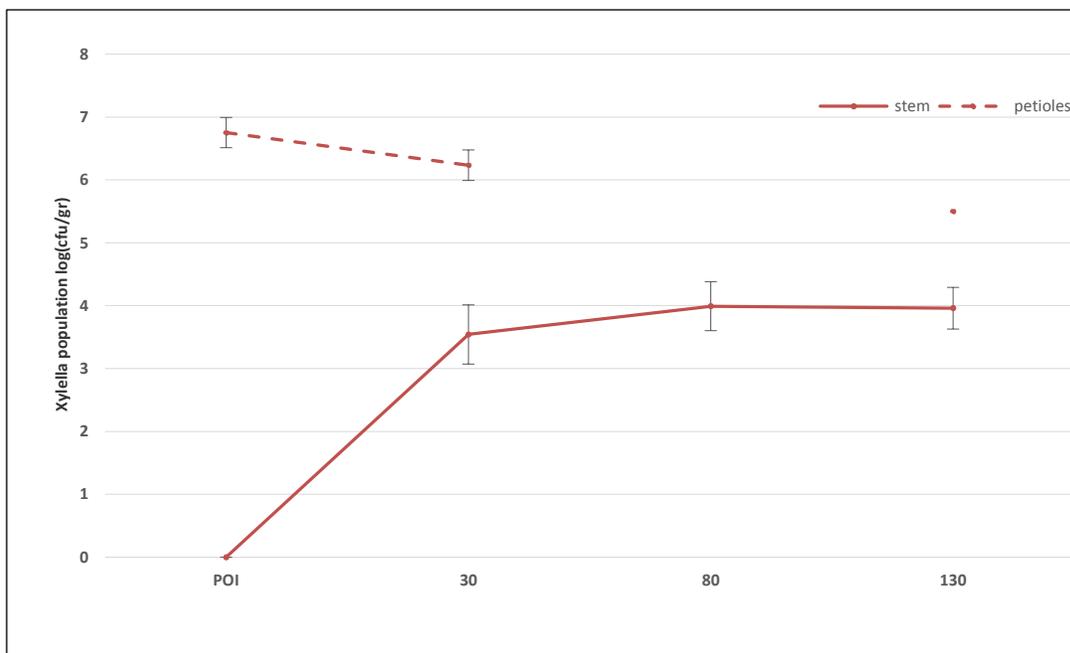
Surprisingly, we have frequently observed that while *P. phytofirmans* rapidly achieves high population sizes and spreads extensively with plants after inoculation, when assessed several weeks after inoculation its population sizes in inoculated plants, irrespective of whether *Xf* was also inoculated into the grape plants, is often quite low. These results suggest that the interactions of *P. phytofirmans* with either the plant or *Xf* occur early in the infection process. The fact that the effect of inoculation of plants with *P. phytofirmans* reduces population sizes of *Xf* most at sites distal to the point of inoculation suggest that it had reduced the motility of the pathogen. Such an effect would be expected if it stimulated DSF-mediated quorum sensing. That is, the behavior of *Xf* in plants treated with *P. phytofirmans* was similar to that seen in transgenic plants harboring *Xf rpfF* that produce DSF. It is curious, however, that the population size of *Xf* is often lower even near the point of inoculation in plants also treated with *P. phytofirmans* (**Figure 6**). This suggests that in addition to any effect that *P. phytofirmans* has on changing the signaling behavior of *Xf*, possibly by altering DSF signaling, that it might also be either directly antagonistic to the pathogen in the plant or, more likely, triggering a host defensive reaction that inhibits the growth or survival of the pathogen. Experiments are under way to distinguish these different possibilities.



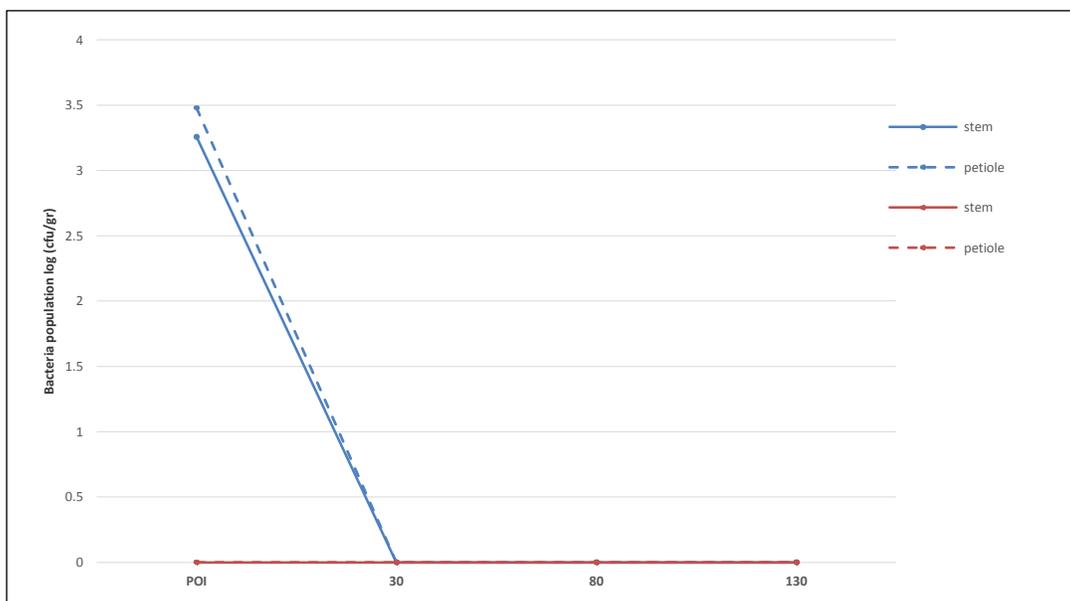
**Figure 6.** Population size of *Xf* three weeks after inoculation of plants with the pathogen alone (yellow line), plants sprayed with *P. phytofirmans* on the same day that it was needle inoculated with the pathogen (gray line), plants needle inoculated with *P. phytofirmans* on the same day that it was needle inoculated with the pathogen at a nearby site (orange line), and plants needle inoculated with *P. phytofirmans* three weeks prior to being needle inoculated with the pathogen at a nearby site (blue line). The vertical bars represent the standard error of the determination of log-transformed population sizes.

The dramatic reductions in both the population size of *Xf* as well as Pierce's disease symptoms, both in plants in which the pathogen and *P. phytofirmans* were simultaneously inoculated (either together as a mixture or in close proximity) as well as when inoculated at different times relative to one another in grape, raise the question as to whether the pathogen and *P. phytofirmans* had to be coincident for biological control to occur or whether the presence of *P. phytofirmans* was mediating a distal effect in the plant. That is, could the presence of *P. phytofirmans* in the plant be having an effect on *Xf* even at a distance, perhaps by initiating a host-mediated defense against the pathogen, perhaps on a systemic level? Experiments were conducted to provide evidence to distinguish between these possibilities. In this experimental design, the pathogen and *P. phytofirmans* were inoculated simultaneously but at spatially distant locations in the plant to ascertain whether a systemic resistance to the growth and movement of *Xf* or disease symptoms could be conferred by *P. phytofirmans* inoculated many centimeters away from the pathogen. The two bacteria, *Xf* and *P. phytofirmans*, were either co-inoculated or inoculated in the same grape plant at the same time but 30 centimeters from each other. The experiment used rooted cuttings of Cabernet Sauvignon inoculated when the plants were approximately 50-70 cm tall. Grapes were either needle droplet puncture inoculated with *P. phytofirmans* alone, with *Xf* alone, or with an equal mixture of the two bacteria as in earlier studies. However, in addition, in one treatment plants were inoculated at their base with *Xf* while *P. phytofirmans* was inoculated 30 cm towards the distal portion of the stem at the same time. In the converse treatment, *P. phytofirmans* was inoculated at the base of the plant while *Xf* was inoculated at the same time 30 cm distal along the stem. The population size of both *P. phytofirmans* and *Xf* was determined at eight weeks post inoculation in petioles collected at various points on the plant, as well as at various locations in the stem. As has been seen in all experiments, the population size of the pathogen was greatly reduced at all locations in the plant when co-inoculated with *P. phytofirmans* (compare **Figure 7** and **Figure 8**). While *Xf* reached population sizes of over  $10^4$  cells/g in the stem even at distances of 130 cm from the point of inoculation when inoculated alone in plants (**Figure 7**), its populations were undetectably low at all stem locations when co-inoculated with *PP* (**Figure 8**). It is noteworthy that *P. phytofirmans* populations were low at most locations in the plants when measured eight weeks after inoculation (**Figure 8**), although much higher populations were detected earlier in the experiment (data not shown). In contrast to the great reduction in populations of *Xf* seen when co-inoculated with *P. phytofirmans*, population sizes of the pathogen were only modestly reduced when *P. phytofirmans* was inoculated either 30 cm towards the base or 30 cm towards the apex of the grape plant relative to that of the pathogen (**Figure 9** and **Figure 10**). In both cases, however, the population sizes of *Xf* were reduced greatly at locations furthest from the point of inoculation of the pathogen (**Figure 9** and **Figure 10**),

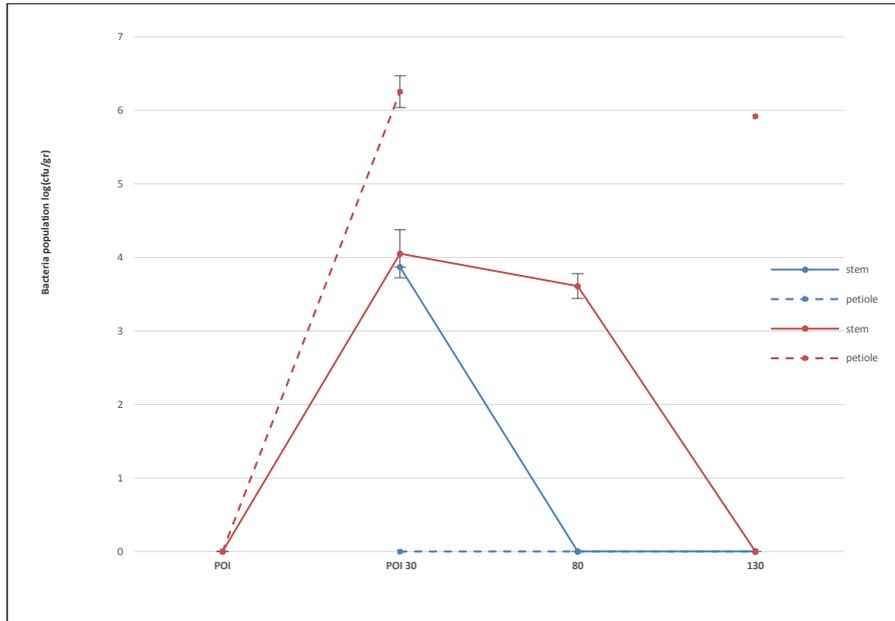
indicating that the growth and movement of the pathogen was strongly influenced by *P. phytofirmans* but that such inhibition was context-dependent in that it apparently was maximal in locations distal from the point of the separate inoculations, where these two strains would have been expected to have been coincident in the plant. These preliminary results suggest that inoculation of grape with *P. phytofirmans* does not lead to a strong, systemic resistance to the colonization of the plants by *Xf*, and thus to symptom development. Instead, it suggests a plant response may be occurring. Studies to test this hypothesis will be discussed below.



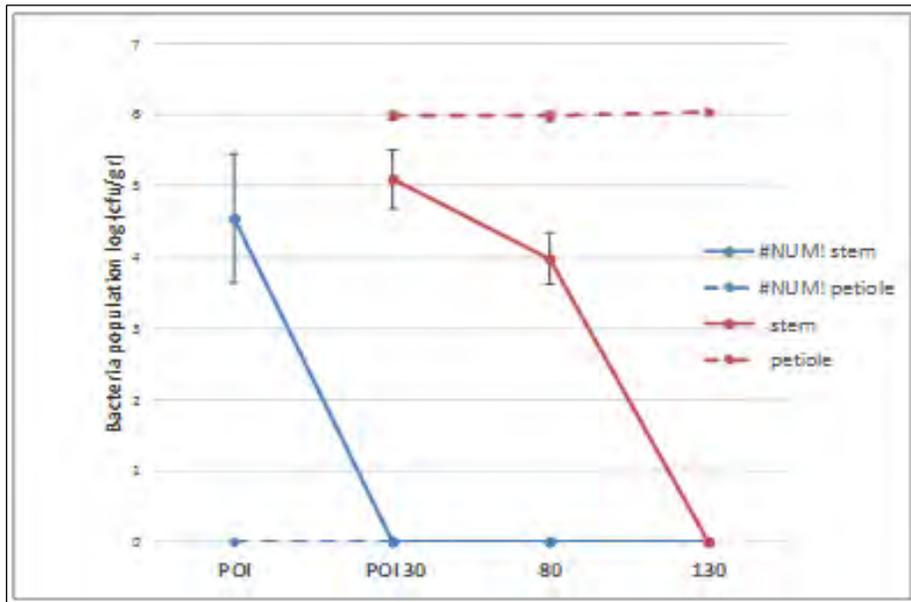
**Figure 7.** Population size of *Xf* in grape plants inoculated only with the pathogen. The solid red line represents the bacteria populations in the stem while the dashed line represents pathogen populations in the petioles in samples taken at different centimeter locations from the point of inoculation shown on the abscissa. The vertical bars represent the standard error of log transformed population size per gram.



**Figure 8.** Population size of *Xf* (red lines) and *P. phytofirmans* (blue lines) in grape plants co-inoculated with the pathogen and *P. phytofirmans* at the same location. The solid lines represent bacteria populations in the stem while the dashed lines represent populations in the petioles in samples taken at different centimeter locations from the point of inoculation shown on the abscissa.



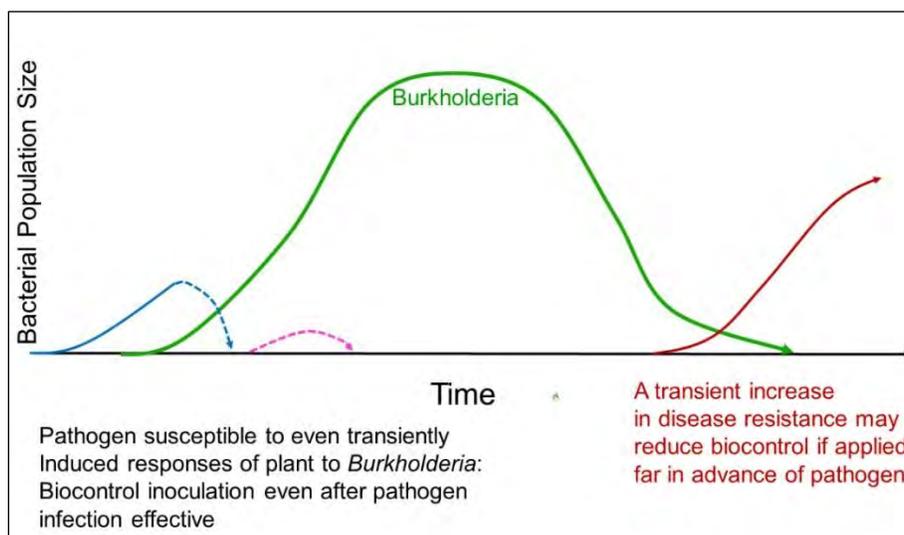
**Figure 9.** Population size of *Xf* (red lines) and *P. phytofirmans* (blue lines) in grape plants inoculated at their base with the pathogen while *P. phytofirmans* was inoculated 30 cm distal to the point of inoculation at the same time. The solid lines represent bacteria populations in the stem while the dashed lines represent populations in the petioles in samples taken at different centimeter locations from the point of inoculation shown on the abscissa. The vertical bars represent the standard error of the determination of log-transformed population sizes per gram.



**Figure 10.** Population size of *Xf* (red lines) and *P. phytofirmans* (blue lines) in grape plants inoculated at their base with *P. phytofirmans* while *Xf* was inoculated 30 cm distal to the point of inoculation at the same time. The solid lines represent bacteria populations in the stem while the dashed lines represent populations in the petioles in samples taken at different centimeter locations from the point of inoculation, shown on the abscissa. The vertical bars represent the standard error of the determination of log-transformed population sizes per gram.

We have observed in the many experiments in which grape has been inoculated with *P. phytofirmans* that population sizes of this biological control agent are maximal in plants within a few weeks after inoculation, but that populations in the plant seem to decrease thereafter. For example, when measured four to six weeks after inoculation, very large *P. phytofirmans* populations are often observed a meter or more away from the point of

inoculation (**Figure 1**). However, we have often observed that when measured many weeks after inoculation, such as in the experiments described in **Figures 7-10**, *P. phytofirmans* population sizes throughout the plant are much lower than they had been earlier. Intensive experiments are under way to systematically examine the temporal and spatial dynamics of *P. phytofirmans* populations in grape. We will be testing the hypothesis that *P. phytofirmans* is a very efficient colonizer of grape, but one that may be self-limiting. Specifically, we hypothesize that the plant may locally recognize and respond to the colonization of *P. phytofirmans* in a way that leads to a reduction in its population size. In fact, it may be this response of the plant to *P. phytofirmans* that is also responsible for the dramatic reductions in *Xf* populations in plants inoculated with *P. phytofirmans*. If, as we hypothesize, such a host response is relatively local to the plant region colonized by *P. phytofirmans*, the patterns of biological control that we have observed could be explained. Specifically, biological control of Pierce's disease would be expected if *P. phytofirmans* was applied either before or after that of the pathogen (such as was seen in experiments described in **Figure 3**) if the rapid movement of *P. phytofirmans* throughout the plant mediated a defensive reaction either before the plant had been colonized by *Xf* or before the pathogen had achieved population sizes sufficient to incite disease symptoms. In this model, the spatial movement and persistence of *P. phytofirmans* in the plant would be of great importance to the efficacy of biological control (**Figure 11**). Our ongoing studies to investigate the spatial movement and temporal persistence of *P. phytofirmans* in plants after inoculation relative to that of the pathogen when inoculated at different times and locations are central to our understanding of how to optimize biological control of Pierce's disease.



**Figure 11.** A model describing the expected temporal growth and persistence of *P. phytofirmans* in grape plants after inoculation (green line) and the expected effects on population sizes of *Xf* inoculated at various times relative to that of *P. phytofirmans* (blue, pink, and red lines) based on the hypothesis that *P. phytofirmans* mediates a local inhibitory effect on pathogen populations.

## Objective 2. Mechanisms of Biological Control

As discussed in objective 1, it seemed possible that *P. phytofirmans* may alter the behavior and survival of *Xf* by inducing changes in grape plants themselves, such as by stimulating innate plant immunity. Plant innate immunity serves as an important mechanism by providing the first line of defense to fight against pathogen attack. While grape apparently does not successfully recognize and therefore defend against infection by *Xf*, it might be possible that plants could be “primed” to mount a defense against *Xf* by another organism such as *P. phytofirmans*. Certain beneficial microorganisms such as *P. phytofirmans* PsJN have been shown to prime innate defenses against various pathogens in model plant system such as *Arabidopsis*, and a recent study suggest that it could also do so in grapevine. Further, the bacterium induces plant resistance against abiotic stresses, apparently by changing patterns of gene expression in host plants. We are thus exploring whether the reduced disease symptoms and lower pathogen population seen in plants inoculated with *P. phytofirmans* either before or after that of *Xf* is mediated by the activation of plant innate immunity. To test this hypothesis we measured the expression of several defense related genes in three groups of plants: (1) Control plants with no treatment, (2) plants injected with the *P. phytofirmans* strain alone, (3) plants injected with both *P. phytofirmans* and *Xf* strains simultaneously,

and (4) plants inoculated only with *Xf*. A comparison of gene expression patterns in grape from these three treatments should enable us to determine whether *P. phytofirmans* alone can alter gene expression patterns in grape or, instead, may “prime” the plant to respond to *Xf*. Tissue samples were collected every week for five weeks and included stem segments, petioles, and a leaf blade tissue starting from the point of inoculation and continuing every 10 cm up to 50 cm from the point of inoculation.

As we had seen in previous experiments, the population size of *P. phytofirmans* increased rapidly with time at the site of inoculation and quickly could be detected as much as 40 cm away from the point of inoculation, although at somewhat lower population sizes that also tended to increase with time (Figure 12 and Figure 13). As we have consistently seen, *Xf* could not be detected in plants that were co-inoculated with *P. phytofirmans* at any time (Figure 12 and Figure 13). In contrast, the population size of *Xf* increased rapidly with time and by three and five weeks could be detected 40 cm away from the point of inoculation (Figure 12 and Figure 13). Because of the design of this experiment it was possible to systematically examine the population dynamics of *P. phytofirmans* as a function of time after it was inoculated into plants. An examination of Figure 12 and Figure 13 reveal that its population size at a given site in the plant typically increased for two to three weeks before dropping by week five (Figure 14). This pattern is most apparent when one considers its population size at the point of inoculation as a function of time (Figure 15). It thus appears that *P. phytofirmans* increases rapidly within the plant but its population sizes then drop thereafter, suggesting that it may be somewhat self-limiting in its colonization capacity of grape. Its population and dynamics are quite different from that of *Xf*, which increased continually with time at a given site within the plant (Figure 14 and Figure 15). The study is being repeated so as to allow us to monitor population sizes of the pathogen and *PP* in plants for a longer period of time after inoculation.

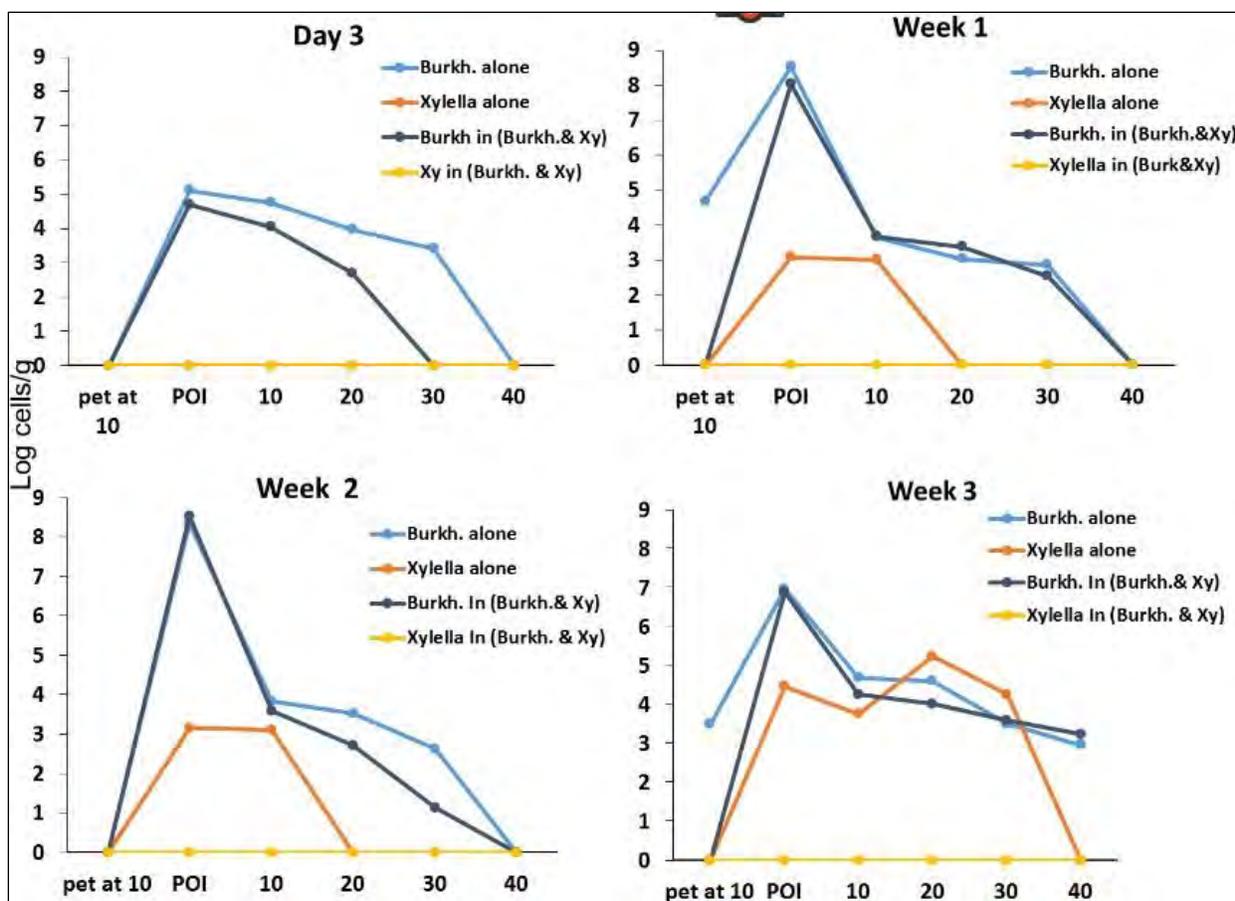
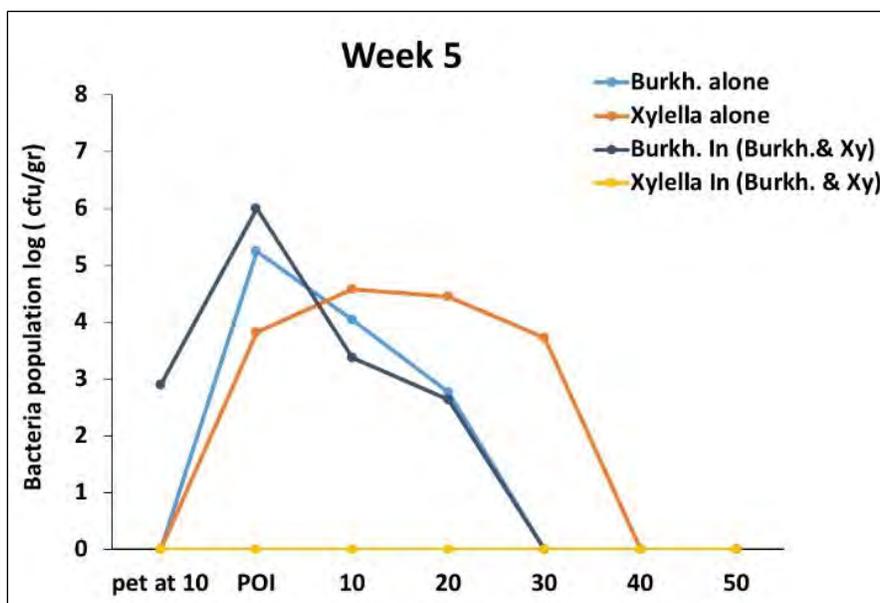
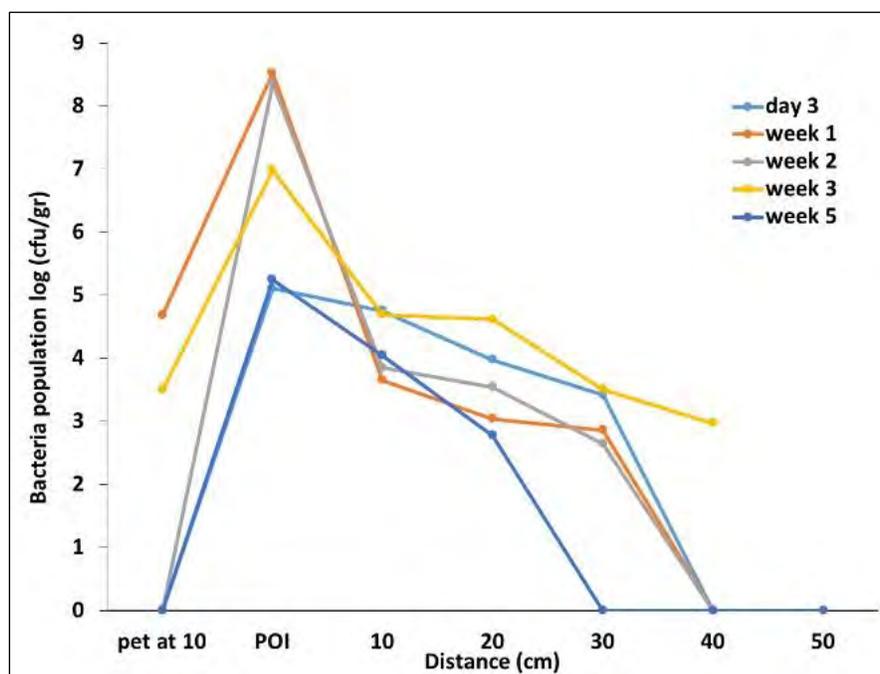


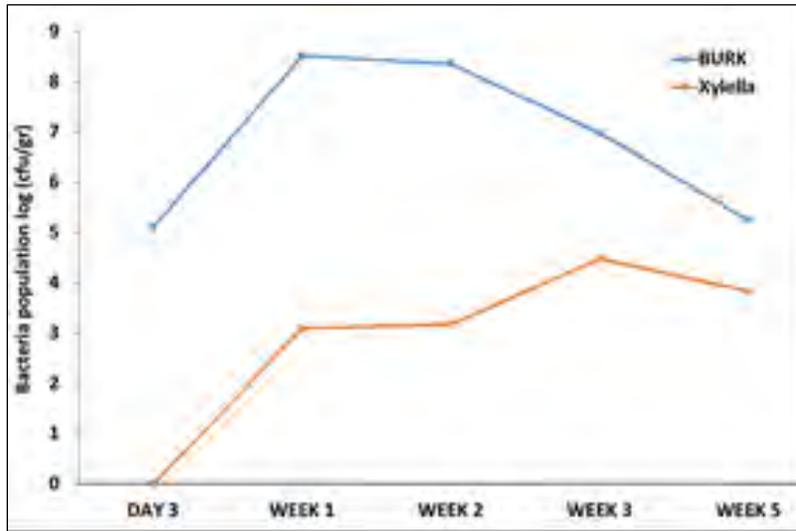
Figure 12. Population size (log cells/gram) of *P. phytofirmans* in plants inoculated only with this strain (light blue lines), *P. phytofirmans* in plants co-inoculated with *Xf* (dark blue lines), *Xf* alone inoculated (orange lines), and *Xf* in plants co-inoculated with *P. phytofirmans* (yellow lines). Samples were collected at the different times shown on each graph in stem segments at the point of inoculation (POI) as well as at different distances (in cm) distal to the point of inoculation shown on the abscissa. Samples were also collected from petioles (pet) located 10 cm distal from the point of inoculation (pet at 10).



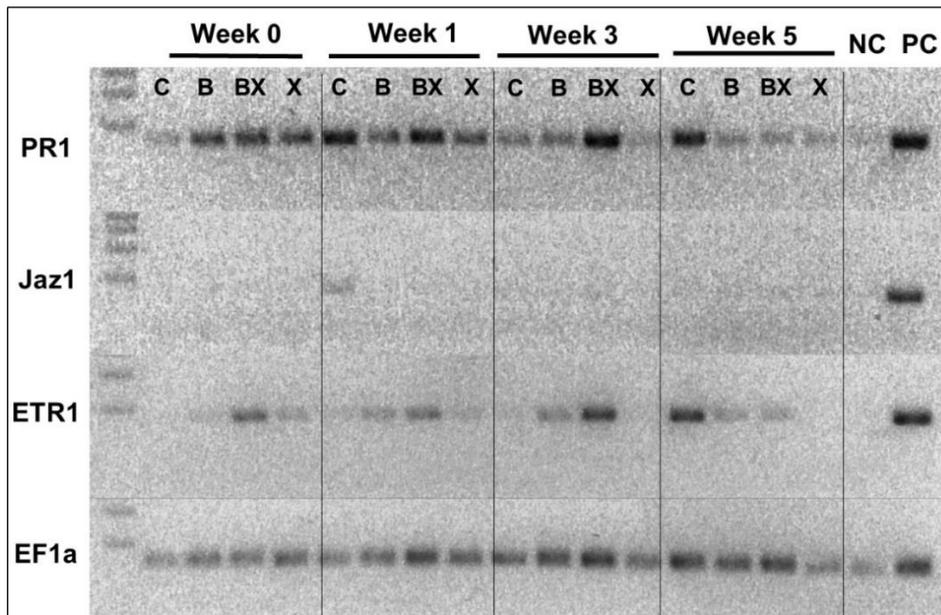
**Figure 13.** Population size (log cells/gram) of *P. phytofirmans* in plants inoculated only with this strain (light blue line), *P. phytofirmans* in plants co-inoculated with *Xf* (dark blue line), *Xf* alone inoculated (orange line), and *Xf* in plants co-inoculated with *P. phytofirmans* (yellow line). Samples were collected five weeks after inoculation in stem segments at the point of inoculation (POI) as well at different distances (in cm) distal to the point of inoculation shown on the abscissa. Samples were also collected from petioles located 10 cm distal from the point of inoculation (pet at 10).



**Figure 14.** Population size (log cells/gram) of *P. phytofirmans* in plants inoculated only with this strain when sampled three days (light blue line), one week (orange line), two weeks (gray line), three weeks (yellow line), and five weeks (dark blue line) from stem segments collected at the point of inoculation (POI) as well as at different distances (in cm) distal to the point of inoculation shown on the abscissa. Samples were also collected from petioles located 10 cm distal from the point of inoculation (pet at 10).



**Figure 15.** Population size (log cells/gram) of *P. phytofirmans* in plants inoculated only with this strain (light blue line) or with *Xf* alone (orange line) in stem segments collected at the point of inoculation at the various times shown on the abscissa.



**Figure 16.** PCR amplification products obtained after PCR amplification of cDNA obtained from RNA that had been subjected to reverse transcriptase that was isolated from grape plants that were (C) not inoculated, (B) inoculated with *P. phytofirmans* alone, (BX) inoculated with both *P. phytofirmans* and *Xf*, and (X) inoculated with *Xf* alone. Shown are bands corresponding to amplification products of PR1, Jaz1, ETR1, and EF1a from RNA sampled from plants harvested at the various times shown above each panel.

Not only were populations of *P. phytofirmans* and *Xf* measured in each of the samples, but total RNA was extracted and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) performed to measure the expression of several key genes in the defense-signaling network of grape. Among them are PR1 (salicylic acid related), Jaz1 (Jasmonic acid related), and ETR1 (ethylene related) genes. EF1 $\alpha$  was used as an internal control, as it is typically constitutively expressed in plants. While the expression of these various genes involved in plant defense were typically very low and not influenced by inoculation by *P. phytofirmans* alone, *Xf* alone, or co-inoculation with *P. phytofirmans* and *Xf* (data not shown), we did find evidence of induced expression of PR1 and ETR1 within one to three weeks after inoculation in plants co-inoculated with *P. phytofirmans* and *Xf* but not in plants inoculated with either of these strains alone, especially those petioles near the point of inoculation (Figure 16). We interpret these results to suggest that the presence of *P. phytofirmans* somehow primed a host

defense reaction toward *Xf*, but that the pathogen alone was not capable of inducing such defenses. The induction of defense in such a successful pathogen would not have been expected. Because of the different anatomical structure of stem tissue compared to petiole tissue, it may be that there was less living tissue in contact with either of these bacteria than in petioles, thus limiting our ability to measure such a defense reaction even if it had happened in the stem tissue. Given that we did not see evidence of induction of PR1 and ETR1 at distances distal from the point of inoculation, it suggests that host defenses are induced primarily locally in the presence of both *P. phytofirmans* and *Xf*. We will be repeating these results to confirm that at least one of the effects of inoculation with *P. phytofirmans* is to induce host defenses. It is possible that such an induction of host defenses is also leading to its own demise in the plant with time.

### Objective 3. Field Efficacy of Biological Control of Pierce’s Disease

While we have already obtained strong evidence of effective biological control of Pierce’s disease in the greenhouse, and further details of how this process can be exploited will be addressed in objective 1, it will be important to demonstrate that the process of biological control is robust under field conditions, since greenhouse plants and field plants could differ. Therefore, we are evaluating the extent to which the factors which control the efficacy of biological control in the greenhouse are directly applicable to a field setting. The study would also allow us to evaluate the effectiveness of spray application of *P. phytofirmans* relative to that of direct needle inoculation. An extensive field study has been initiated in which we will (1) challenge plants of three different grape varieties (Chardonnay, Cabernet Sauvignon, and Pinot Noir) with *Xf* relatively soon after needle inoculation or topical treatment with *P. phytofirmans*, (2) challenge plants with *Xf* several weeks after inoculation with *P. phytofirmans* in different ways, (3) inoculate *P. phytofirmans* into plants in different ways only after challenge inoculation with *Xf* to assess the potential for “curative effects” after infection has occurred, and (4) challenge inoculate plants treated with *P. phytofirmans* with *Xf* on multiple occasions, spanning more than one growing season, to reveal the persistence of the biological control phenomenon. Greenhouse studies in our current project have indicated that topical applications of a DSF-like molecule, palmitoleic acid, with a penetrating surfactant can also confer disease resistance. This treatment will therefore be compared with biological control treatments. Studies are being done in a replicated field site managed by the Department of Plant Pathology at the University of California, Davis. Each treatment consists of 10 plants for a given grape variety. The experimental design is as follows:

May 2018	June 2018	July 2018	May 2019
Needle <i>Paraburkholderia</i>	<i>Xf</i>		
Spray <i>Paraburkholderia</i>	<i>Xf</i> <i>Xf</i> control		
Needle <i>Paraburkholderia</i>			
Spray <i>Paraburkholderia</i>			
Needle <i>Paraburkholderia</i>		<i>Xf</i>	
Spray <i>Paraburkholderia</i>		<i>Xf</i> <i>Xf</i> control	
Needle <i>Paraburkholderia</i>	<i>Xf</i>	<i>Xf</i>	<i>Xf</i>
Spray <i>Paraburkholderia</i>	<i>Xf</i> <i>Xf</i> <i>Xf</i> <i>Xf</i>	<i>Xf</i> <i>Xf</i> Needle <i>Para</i> Spray <i>Para</i>	<i>Xf</i> <i>Xf</i>
Needle <i>Paraburkholderia</i>			<i>Xf</i>
Spray <i>Paraburkholderia</i>			<i>Xf</i> <i>Xf</i> control
<i>Paraburkholderia</i> rootstock	<i>Xf</i>		
Rootstock control	<i>Xf</i>		
10 mM palmitoleic acid + 0.2% Break-thru	<i>Xf</i>		
0.2% Break-thru control			
Uninoculated control			

So-called “Uber” plants for the study were generously provided by Duarte Nurseries and were planted in late April 2017 (due to the presence of wet soils) at the UC Davis field site. These large “Uber” plants are growing rapidly and should allow for rapid establishment of plants in the field trial, enabling experimentation to proceed as planned starting in the spring of 2018. A permit from the USDA Animal and Plant Health Inspection Service to allow the field use of *P. phytofirmans* apparently will require us to demonstrate the presence of microorganisms closely related to *P. phytofirmans* in California and nearby states. We thus are in the process of collecting plant and soil samples which will be interrogated for the presence of full-length 16S ribosomal RNA genes identical to or very closely related to that of *P. phytofirmans*.

## **CONCLUSIONS**

The studies under way directly address practical strategies of control of Pierce’s disease. Our results reveal that *Paraburkholderia phytofirmans* continues to provide levels of biological control under greenhouse conditions that are even greater than what we would have anticipated, and the encouraging results of practical means to introduce this strain into plants such as by spray applications as well as the fact that it seems to be active even when not co-inoculated with the pathogen is a very promising result that suggests that this method of disease control might also be readily implemented. 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.

## **FUNDING AGENCIES**

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

# FIELD EVALUATION OF PIERCE'S DISEASE RESISTANCE OF VARIOUS DIFFUSIBLE SIGNAL FACTOR PRODUCING GRAPE VARIETIES AS SCIONS AND ROOTSTOCKS

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**Reporting Period:** The results reported here are from work conducted July 1, 2016 to October, 2017.

**ABSTRACT**

Transgenic plants of several different winegrape and rootstock varieties in which the *rpfF* gene encoding the diffusible signal factor (DSF) synthase from *Xylella fastidiosa* is expressed under the control of a strong constitutive promoter, as well as a variant of *rpfF* encoding a protein with sequences that should direct the enzyme to the chloroplast of plants, are being made in an effort to produce significant levels of DSF in plants. The presence of high concentrations of DSF should cause abnormal behavior of the pathogen such that its virulence to plants will be greatly reduced. The majority of the transgenic plants have now been produced, and most of these plants have now been tested for disease resistance in greenhouse studies. A greenhouse malfunction has delayed the testing of the remaining plants, although all testing should be completed by early 2018. Initial planting of these transgenic grape varieties should commence in early 2018, with most planted by the end of 2018.

**LAYPERSON SUMMARY**

*Xylella fastidiosa* coordinates its behavior in plants in a cell density-dependent fashion using a diffusible signal factor (DSF) molecule which acts to suppress its virulence in plants. Artificially increasing DSF levels in grape by introducing the *rpfF* gene which encodes a DSF synthase reduces disease severity in greenhouse trials. We are generating and testing five different DSF-producing grape varieties both as own-rooted plants as well as rootstocks for susceptibility to Pierce's disease. The majority of these transgenic grape varieties have now been produced at the Plant Transformation Facility at the University of California, Davis and are under evaluation under greenhouse conditions at UC Berkeley to determine those particular transgenic lines that have the highest disease resistance. Additional gene constructs will be made to generate transgenic plants in which the DSF synthase is directed to a cellular environment in which higher levels of DSF production can be expected in those few grape varieties in which such expression has not yet been successful. While some of the transgenic varieties will be available for establishment in the field plot as own-rooted plants or as rootstocks of plants with a normal Cabernet Sauvignon scion in spring 2018, most of the remaining plants for the field trial will not be available for planting until later in 2018. Disease severity and population size of the pathogen will be assessed in the plants after their establishment in the field as a means of determining their susceptibility to Pierce's disease after artificial inoculation.

**OBJECTIVES**

1. Determine the susceptibility of diffusible signal factor (DSF)-producing grape as own-rooted plants as well as rootstocks for susceptible grape varieties to Pierce's disease.
2. Determine population size of the pathogen in DSF-producing plants under field conditions.

**RESULTS AND DISCUSSION**

This is a continuing project that exploits results we have obtained in the project 14-0143-SA titled "Comparison and Optimization of Different Methods to Alter DSF-Mediated Signaling in *Xylella fastidiosa* in Plants to Achieve Pierce's Disease Control" which was funded by the CDFR Pierce's Disease and Glassy-winged Sharpshooter Board. One of the major objectives of that project was to compare DSF production and level of disease control conferred by transformation of *Xylella fastidiosa* (*Xf*) RpfF into several different grape cultivars. This and other projects in the previous eight years had described a cell density-dependent gene expression system in *Xf* mediated by a family of small signal molecules called diffusible signal factor which we have now characterized as 2-Z-tetradecenoic acid (hereafter called C14-cis) and 2-Z-hexadecenoic acid (C16-cis). The accumulation of DSF attenuates the virulence of *Xf* by stimulating the expression of cell surface adhesins such as HxfA, HxfB, Xada, and FimA that make cells sticky and hence suppress its movement in the plant while down-

regulating the production of secreted enzymes such as polygalacturonase and endoglucanase which are required for digestion of piths and thus for movement through the plant. Artificially increasing DSF levels in transgenic plants expressing the gene for the DSF synthase from *Xf* was found to be highly effective in reducing disease severity of inoculated plants when used as scions and to confer at least partial control of disease when used as rootstocks. Nearly all of the work had been done in the Freedom rootstock variety, and the goal of project 14-0143-SA was to transform a variety of other winegrape and rootstock varieties to determine the robustness of this strategy of disease control. The majority of these transgenic plants have now been generated and extensive greenhouse testing to identify the most persistent lines is getting closer to completion. The work of this new continuing project is to establish field trials in 2018 and subsequent years where these lines can be compared with each other for Pierce's disease control when used as both scions and rootstocks.

### **Objective 1. Disease Susceptibility of Transgenic DSF-Producing Grape in Field Trials**

As part of a continuing part of project 14-0143-SA, the grape variety Thompson Seedless as well as the advanced rootstock varieties 1103, 101-14, and Richter were transformed with the *rpjF* gene from *Xf*. In addition to untargeted expression of RpfF, we produced plants in which RpfF is targeted to the chloroplast of grape by fusing the small subunit 78 amino acid leader peptide and mature N-terminal sequences for the *Arabidopsis* ribulose biphosphate carboxylase (which is sufficient to target the protein to the chloroplast) to RpfF. This RpfF fusion gene product should be directed to the chloroplast, where it presumably has more access to the fatty acid substrates that are required for DSF synthesis (chloroplast-targeted). While the genetic constructs were made at UC Berkeley, transformation of the various grape varieties is being conducted at the Ralph M. Parsons Foundation Plant Transformation Facility at UC Davis.

Our goal was to obtain between five and ten individual transformants for each variety/construct combination. As will be summarized below it has been both slow and difficult to obtain sufficient numbers of transformants for certain of these combinations. Because the expression of *rpjF* in a given transformant of a given plant line will vary due to the chromosomal location of the randomly inserted DNA it is necessary to identify those lines with the highest levels of expression. To determine the disease susceptibility of each line they were grown to a sufficiently large size that vegetative clones could be produced (three months) and then each cloned plant was propagated and assessed for disease susceptibility (five additional months). At least 12 vegetative clones each of the lines were produced from green cuttings of plants developing from each transgenic plant selected in the assays above. These plants, as well as an untransformed control plant of a given variety (ca. 30 cm high), are being inoculated with *Xf* by droplet needle puncture as in earlier studies. Disease severity is being assessed visually weekly after inoculation. In this process we are able to identify the transformant from each variety/construct combination that is most highly resistant to Pierce's disease, and thus suitable for field evaluation.

The following table (**Table 1**) indicates the number of individual independently transformed plants of each combination that have been delivered to UC Berkeley. Nearly all have been successfully propagated and vegetative clones produced to enable testing for disease susceptibility. Disease susceptibility has been completed from the majority of the transgenic lines, although a few of the lines have been inoculated but disease assessments are still being made under greenhouse conditions at UC Berkeley.

**Table 1.** Number of individual independently transformed plants delivered to UC Berkeley.

Variety	Untargeted RpfF	Gene Introduced Chloroplast-targeted RpfF
Thompson Seedless	23	2
Richter 110	6	none
Paulsen 1103	6	none
Milardet et de Grasset 101-14	13	none

Certain of the varieties such as Chardonnay could not successfully be transformed at UC Davis. Furthermore, others such as Richter 110 and Paulsen 1103 have proven to be somewhat more difficult to transform than other varieties, yielding fewer transformants than other grape varieties. Although the reason is unclear, the kanamycin resistance determining construct in which the chloroplast targeted RpfF is being delivered has yielded relatively

few transformants, with none being recovered for three of the varieties being investigated. These transformations will again be repeated with a fresh *Agrobacterium*/vector combination. A modification of this vector is also being developed to determine if it will be more successful. As noted above, screening for disease resistance of the non-targeted RpfF plants already delivered is underway. Unfortunately, there was a major greenhouse malfunction in August 2017 which blocked watering of the plants for a couple of days. This malfunction unfortunately also happened during a relatively warm period in Berkeley, and the plants suffered substantial damage. The plants had been inoculated for a period of about 10 weeks at that point and were on the verge of being assessed for visual symptoms of disease severity. Because the plants were so severely damaged they had to be cut back to the soil level and the newly emerging tissues have now been re-inoculated. This unfortunate setback will delay the final assessment of the disease resistance of these plants until early 2018. We might also expect complications with assessing disease symptoms during the winter months of 2017 and 2018, as we typically have the best results for disease assessment studies during spring and summer months. Overall the process of evaluating the various lines for disease resistance has proved to be slower than expected because the plants obtained from UC Davis often arrived during winter months and thus were both very small and very slow to grow under these winter growing conditions. This has lengthened the time needed to obtain the vegetative clones required for disease susceptibility testing. We have however now obtained sufficient number of plants from each of the four newly-transformed grape varieties to evaluate the relative efficacy of expression of RpfF, and thus DSF production to achieve disease resistance in these various varieties. Not only will this provide us evidence for the relative effectiveness of DSF production as a disease control strategy in the different grape varieties, but it will allow us to identify the most highly resistant variety for a given variety. Our goal is still to produce enough self-rooted plants of the most resistant lines for field testing, as well as to generate grafted plants, with these plants serving as rootstocks for field testing that will begin in 2018, although the delays in assessments of portions of the plants due to the greenhouse malfunction as noted above may delay the introduction of some of the plants until late 2018. The grafting process will add an additional three months to the process of generating plants for use in field studies, but we hope to be able to complete this for these grafted plants before the end of 2018.

Field tests will be initiated beginning in 2018 with the various grape variety/genetic construct combinations discussed above. Given the difficulty of producing chloroplast-targeted rpfF constructs of certain of the varieties, it is however unlikely that they will be available for planting in 2018. We will continue to evaluate such transformed lines as success in their transformation is achieved at the UC Davis Plant Transformation Facility.

**Table 2.**

Variety	Untargeted RpfF	Gene Introduced Chloroplast-targeted RpfF	Untransformed Plants
Thompson Seedless	+	+	+
Richter 110	+	+	+
Paulsen 1103	+	+	+
101-14	+	+	+
Freedom	+		+

These transgenic grape varieties will be tested as both own-rooted plants as well as rootstocks to which the susceptible grape variety Cabernet Sauvignon will be grafted. Thus, a maximum of 14 different treatments will assess each grape variety/gene construct on own-rooted plants. Additional (up to 14) treatments will evaluate each grape variety/gene construct as a rootstock onto which Cabernet Sauvignon will be grafted as a scion.

Twelve plants of each treatment will be established in a randomized complete block design with four blocks of three plants each for each treatment that will be inoculated with *Xf* after establishment. In addition, four plants in each treatment (one plant per block) will be left un-inoculated with *Xf* as a control to observe plant development and yield to determine whether DSF production had any effect on plant development under field conditions. No such effects have been observed in field studies conducted to date or in greenhouse studies. Half of the plants will be own-rooted plants and the other half will be grafted plants with a normal Cabernet Sauvignon scion. Half of the plants will be inoculated with *Xf*. Twelve of the plants from each treatment will be inoculated by needle puncture with drops of *Xf* of about  $10^9$  cells/ml, as in previous studies. Disease symptoms in continuing studies

will be measured bi-weekly starting at eight weeks after inoculation (inoculation will be done about May 1). Leaves exhibiting scorching symptoms characteristic of Pierce's disease will be counted on each occasion, and the number of infected leaves for each vine noted as in our other studies. An additional 0 to 5 rating scale will also be applied which accounts for both the number of vines on a plant that are symptomatic as well as the degree of symptoms on a given plant. This scale will be most important in the third year of the study (two years after inoculation) when spread through the plant will be assessed. Analysis of variance (ANOVA) will be employed to determine differences in severity of disease (quantified as the number of infected leaves per vine) that are associated with each treatment. As noted above, the majority of the plants are anticipated to be available for planting by early 2018, and inoculation and disease assessment will be initiated only in 2019.

### **Objective 2. Assess Population Size of *Xf* in Transgenic Plants**

To ensure that the symptoms of Pierce's disease in objective 1 above are associated with *Xf* infection and to document the limited extent of excess colonization in transgenic DSF-producing vines inoculated with *Xf* compared to that of the corresponding non-transgenic vines, five petioles from each inoculated vine will be harvested (at approximately 40 cm intervals, depending on the length of the vine for a given variety) at monthly intervals starting eight weeks after inoculation. Petioles will be surface sterilized and then macerated and appropriate dilutions of the macerate applied to periwinkle wilt gelrite (PWG) plates containing the fungicide natamycin. Colonies characteristic for *Xf* will then be counted and the population size of *Xf* determined. While this method is a bit more work than the polymerase chain reaction (PCR) method, it provides a more sensitive assay method and avoids some issues with false negative discovery rates associated with field sampling of grape tissues. ANOVA will be employed to determine differences in population size of *Xf* (quantified as log cells/petiole) that are associated with treatment. The non-parametric Sign test will also be performed to determine differences in the incidence with which any detectable *Xf* occurs in these petioles at a given sampling distance from the point of inoculation. This strategy will quantify disease to test the assumption that many petioles, especially on DSF-producing plants and at the distal ends of vines, will be free of any detectable cells of *Xf*. As only a few plants are available to establish in the field plot in 2017, and most will be available only by early 2018, inoculation and disease assessment will be initiated only in 2019.

### **CONCLUSIONS**

Since we have shown that DSF accumulation within plants is a major signal used by *Xf* to change its gene expression patterns, and since DSF-mediated changes all lead to a reduction in virulence in this pathogen, we have shown proof of principle that disease control can be achieved by a process of "pathogen confusion." These field trials are direct demonstration projects to test the field efficacy of plants producing DSF to alter pathogen behavior in a way that symptom development is minimized. Results from earlier field trials in which only a limited number of grape varieties were evaluated in Solano County and Riverside County provided solid evidence that pathogen confusion can confer high levels of disease control, both to plants artificially inoculated (Solano County) and especially to plants infected naturally with infested sharpshooter vectors (Riverside County). The earlier work therefore has provided solid evidence that this strategy is a useful one for managing Pierces disease. The current ongoing studies therefore are designed primarily to evaluate the robustness and general applicability of this strategy of disease control in a wide variety of grape varieties.

### **FUNDING AGENCIES**

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

# MAPPING PIERCE'S DISEASE AND VECTOR POPULATIONS IN THE SOUTHERN SAN JOAQUIN VALLEY AND DEVELOPING A DYNAMIC MODEL TO ASSESS MANAGEMENT STRATEGIES

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

## ABSTRACT / LAYPERSON SUMMARY

The resurgence of Pierce's disease in table grapes in the southern San Joaquin Valley over the last four to five years highlighted the need for the area-wide glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) control program to remain responsive to changing conditions in the region. GWSS development is driven by heat availability, and because of variations in the timing and quantity of warming available in different seasons, the timing and number of GWSS generations in a season can change. The annual seasonal cycle of warming and cooling gives a level of predictability to the timing of GWSS generations each year, but prolonged divergence of weather from the typical climatic averages can lead to a sufficiently large shift away from typical behavior, to cause mistiming between standard pesticide application dates and the generations of GWSS they are supposed to target. As has happened with the most recent outbreak of Pierce's disease, the industry can always respond to that type of issue retroactively and incorporate lessons learned from outbreaks into disease management plans for the future. Incorporating new information from experience into disease management plans is a good idea, but it involves learning the lesson the hard way. The aim of the current project is to give the industry tools to have some look-ahead capacity so that potentially beneficial changes to the Pierce's disease and GWSS management actions for the southern San Joaquin Valley can be investigated in advance.

## INTRODUCTION

Reports of increasing incidence of Pierce's disease in the southern San Joaquin Valley in recent years have prompted concern among growers. Well-established, and previously successful, area-wide management practices of glass-winged sharpshooters (*Homalodisca vitripennis*; GWSS) do not appear to be controlling the disease. Understanding how and why transmission by GWSS of the causative pathogen, *Xylella fastidiosa* (*Xf*), is changing over time and space is essential in order to efficiently and effectively interrupt transmission.

Controlling Pierce's disease hinges on controlling GWSS. GWSS and grapes are not, however, a closed system. Citrus and grapes both act as GWSS hosts and as *Xf* reservoirs, although citrus does not manifest disease. Further, windbreaks are believed to provide havens for GWSS. These three groups exist in close proximity in the General Beale area outside of Bakersfield in Kern County. This enclosed, well-described area presents a unique opportunity to elucidate population-level *Xf* transmission dynamics in a multi-use scenario. Findings will benefit not only local growers but may be generalized to make evidence-based recommendations in other California vineyards that are adjacent to citrus, windbreaks, or other potential GWSS and/or *Xf* harbors. Identifying a spatial risk gradient regarding proximity to citrus (even assuming citrus growers were taking GWSS control measures) in particular would be of immediate use to growers.

Pierce's disease incidence is believed to be increasing despite orchestrated area-wide management of GWSS, as mentioned above. It may not be realistic, however, to expect static management tactics to consistently return positive results in a dynamic system. For example, environmental changes in degree days may affect GWSS development and activity in ways that permit the insect to evade set spray schedules. A dynamic response to a dynamic system requires that we: (1) identify and define observable processes, and (2) use those observations as building blocks to predict what might happen in the system under further changes. To that end, the overarching goal of this research project is to identify time-varying spatial patterns of Pierce's disease incidence and GWSS abundance in the context of General Beale, and to incorporate these findings into a dynamic model that can be used to evaluate prospective disease incidence.

## **OBJECTIVES**

1. Compare spatio-temporal patterns of Pierce's disease-affected grapevines and GWSS populations in the southern San Joaquin Valley.
  - a. Analyze historical data for Pierce's disease and GWSS from the southern San Joaquin Valley to identify persistent areas of high risk of high vector pressure combined with frequent incidence of disease inoculum.
  - b. Generate risk maps for Pierce's disease spread risk based on data analysis and transfer the information to the industry.
2. Develop a dynamic simulation model of GWSS and Pierce's disease levels across the southern San Joaquin Valley to evaluate prospects for disease management under changing conditions.
  - a. Analyze the relationship between long-term GWSS populations and degree-day availability to determine correlation between GWSS population size and incidence, and heat, to assess the need for safeguarding against calendar-based treatments missing GWSS generations.
  - b. Summarize available information in a simulation model to allow industry to do scenario analysis looking at prospects for sustainable Pierce's disease and GWSS control in the future.

## **RESULTS AND DISCUSSION**

An initial analysis of the historical GWSS count database and the UC Cooperative Extension annual Pierce's disease survey was completed. The analysis of the GWSS data confirmed anecdotal reports that there are relatively stable hotspots of GWSS in Kern County bordering the northern side of the Tehachapi range (see **Figure 1**).

The qualitative results obtained from mapping out GWSS populations will be investigated further by quantitative analyses in the coming year.

Analysis of the annual Pierce's disease survey data revealed several issues with the data in relation to their usefulness for detailed analysis, but these same issues have been important in helping to devise better sampling plans for coming years. Because the Pierce's disease survey has always been to some extent a sample of opportunity, there is a wide variation in the time of year that blocks are surveyed. In addition, in the past, the UC Cooperative Extension surveyors purposely only surveyed a portion of each block, to encourage growers to complete the survey themselves. As a consequence there is a large sampling error in the Pierce's disease estimates for individual blocks. While the survey, as conducted previously, served a useful purpose in giving table grape growers a reasonable snapshot of Pierce's disease intensity each year, for more detailed analysis a more standardized sampling approach is needed. The main findings of our results were reported to the directors of the Consolidated Central Valley Table Grape Pest and Disease Control District and we have discussed changes to the sampling protocol with David Haviland of UC Cooperative Extension so that the data collected in future years will be more standardized and more useful for risk analysis.

## **CONCLUSIONS**

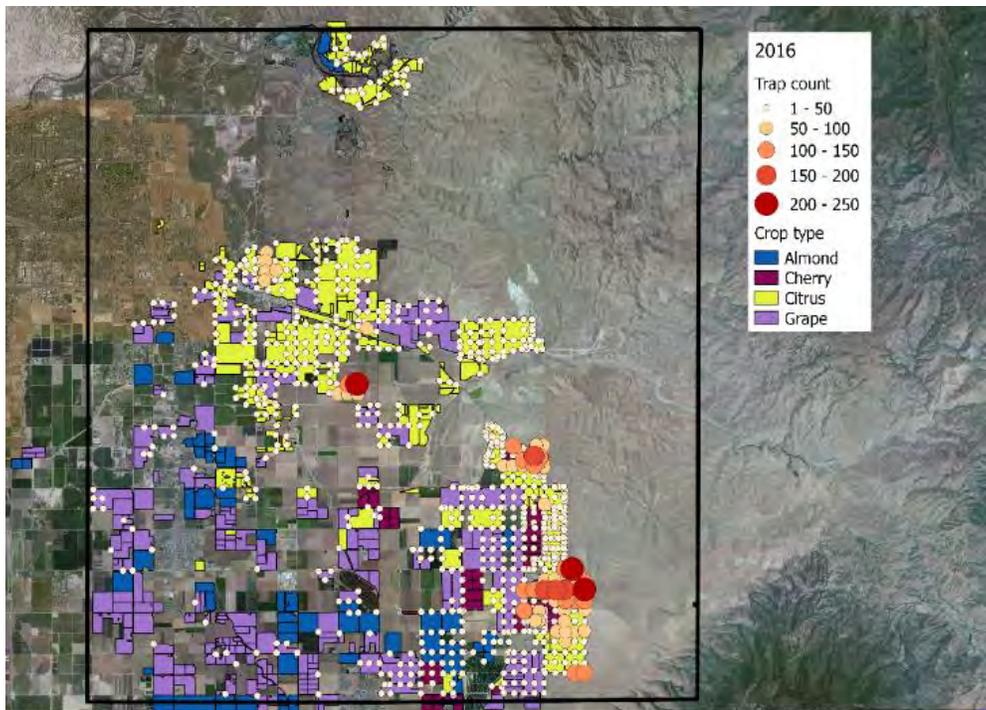
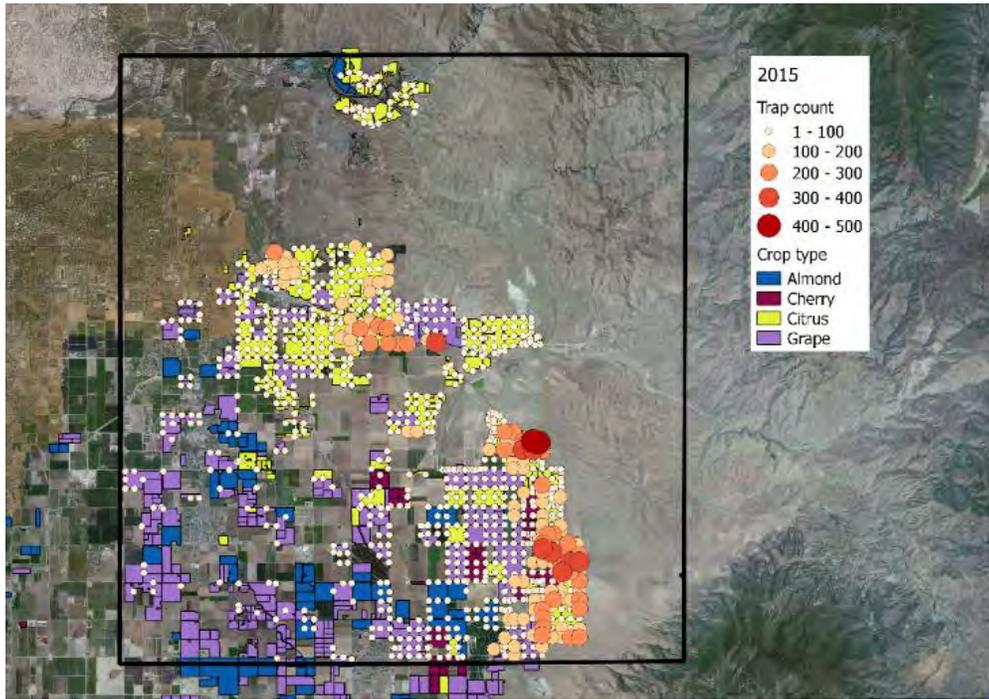
There are no conclusions at this time.

## **REFERENCES CITED**

Daugherty M. 2015. The Riverside County glassy-winged sharpshooter program in the Temecula Valley. *Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes*. December 2015. California Department of Food and Agriculture, Sacramento, CA, pp. 36-39.

## **FUNDING AGENCIES**

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



**Figure 1.** An example of stability of GWSS population hotspots in time. Two localized hotspots on the eastern side of the Kern County citrus area show persistently high GWSS counts over two seasons [2015 (top) and 2016 (bottom)].

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

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**Reporting Period:** The results reported here are from work conducted August 1, 2017 to October 30, 2017.

## ABSTRACT / LAYPERSON SUMMARY

The bacterium *Xylella fastidiosa* (*Xf*) is the cause of Pierce's disease in grapes and is a major threat to fruit, nut, olive, and coffee groves. The most damaging effect of Pierce's disease other than death of the vine is the reduction of production and shriveling of fruits. Obvious symptoms in grapevine are characteristic bands/rings of anthocyanin (red pigment) accumulation in distal zones adjacent to necrotic leaf blades. Anthocyanins can reduce insect feeding, and induction in vegetative tissues may serve as antagonists to feeding by the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) and to colonization by *Xf*. The etiology of pleiotropic Pierce's disease symptoms such as 'matchstick petioles' and 'green cane islands' is not understood. In this context it is noted that grapevine red blotch and leafroll-associated viruses cause similar pleiotropic symptoms because their genomes encode small RNA suppressor proteins evolved to disrupt host microRNA (miRNA) biogenesis and/or activity. Prior work by Leonardo De La Fuente showed that *Xf* infection causes a significant decrease in leaf elemental phosphorus (P) content, but the bioavailable form of P (e.g. phosphoproteins, lipids, nucleic acids, subcellular compartmentation, etc.) underlying this phenomenon is unknown. The myriad host responses to *Xf* are hypothesized to be due to deranged host inorganic phosphate ( $P_i$ )-regulated miRNA activities (both  $P_i$  and miRNAs are diffusible signals in plants). The data generated in two years of support is compelling and supports our testable model of phosphate-regulated miRNAs synergizing with *MIR828/TAS4* to regulate anthocyanin levels. Deep sequencing of miRNAs and their targets in *Xf*-infected leaves, petioles, and cane bark is ongoing to comprehensively understand gene functions in etiology of Pierce's disease. A clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 genome-editing approach is underway to directly test the model by disrupting host *MIR828* or four downstream effector genes for anthocyanin regulation to determine their roles in susceptibility to *Xf*, and whether they function to impact GWSS feeding preferences. We are also testing a corollary of the working hypothesis: Whether a durable, affordable, and environmentally sound 'safener/protectant' analogue of  $P_i$  (phosphite; reduced  $P_i$ ), which alters host and/or microbe phosphate homeostasis, can impact *Xf* growth and host Pierce's disease etiology. This aspect could result in development of a novel management tool for Pierce's disease complementary to the primary high-priority genome editing approach to engineer Pierce's disease resistance. Genome editing is akin to breeding in that it can produce non-"genetically modified organism" (GMO) grapevines and rootstocks after outcrossing the transgene locus. These proof-in-principle experiments could result in a new paradigm for Pierce's disease management with potential translational benefits for other crops.

## INTRODUCTION

Our working model of Pierce's disease etiology postulates miR828 and evolutionarily-related *Trans-Acting Small-interfering locus4* (*TAS4*) activities silence MYELOBLASTOSIS (MYB) transcription factor targets *VvMYBA6/A7* and other homologous *MYB* expression in response to *Xylella fastidiosa* (*Xf*) infection, mediated through inorganic phosphate ( $P_i$ ) and plant stress hormone abscisic acid (ABA) signaling crosstalk. We are currently testing the *Xf* infection/spread hypothesis directly by "knocking out" the key hypothesized genes using a new genome editing technology: Clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) [1, 2]

that was mentioned in research priorities developed by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Research Scientific Advisory Panel. A direct test of the model in grapevine by genome editing of the positive and negative anthocyanin effector loci is well grounded now, based on our deep sequencing evidence for miR828/*TAS4* roles in Pierce's disease.

We are taking a complementary "overexpression" approach to the long-term grapevine MYB target gene knockout/editing approach to test the anthocyanins-as-*Xf*-effectors hypothesis. The surrogate tobacco *Xf* infection system developed by De La Fuente [3] can quickly assess susceptibility to *Xf* infection of a transgenic tobacco line [4] (Myb237) that over-expresses the *Arabidopsis* orthologue of VvMYBA6/A7: *PRODUCTION OF ANTHOCYANIN PIGMENT2/MYB90*. We have generated strong data-driven evidence from our mRNA-Seq, sRNA-Seq and degradome datasets from *Xf*-infected grape and tobacco materials, quantitation of cane xylem sap and leaf P<sub>i</sub> in Pierce's disease infected field materials, and disease severity correlations with molecular phenotypes from greenhouse *Xf* challenge experiments. Results support a refined model that *Xf* is using host small RNAs as a 'trojan horse' that could serve as a paradigm to understand not only P<sub>i</sub> (and miRNAs) as diffusible signals for synthesis of host polyphenolic anti-bacterial metabolites in Pierce's disease etiology, but also the pleiotropic traits of "green islands" and "matchstick petioles," among others. Our results to date for *Xf* differentially regulated miRNAs in tobacco are completely novel, and what emerges is a highly correlated network of miRNA/phased small-interfering RNA-producing and *TAS* noncoding loci known to function in plant immunity across plant taxa.

We summarize in **Table 1** a chronological list of prior efforts and conclusions drawn from experiments documented in progress reports from July 2015 to July 2017. These studies have leveraged a systems approach, building on the miRNA candidate leads to discover etiological effectors/reporters of Pierce's disease and network analyses of gene interactions affecting primary and secondary metabolism. A direct test of the model in grapevine (objective 1) by genome editing of the positive and negative effector loci is well grounded now, based on our deep sequencing evidence for miR828/*TAS4* roles in Pierce's disease.

**Table 1.** Timeline of project activities and results since inception (July 2015), reported previously.

Report Venue	Activity	Experimental Results
Dec. 2015 <i>Research Progress Reports</i> ^	<ul style="list-style-type: none"> <li>- Methods development for quantitation of anthocyanins.</li> <li>- Collect field samples from GA and Temecula, CA.</li> </ul>	<ul style="list-style-type: none"> <li>- Engineered five binary T-DNA <i>Agrobacterium</i> CRISPR vectors [1]; phytoene desaturase extra target vectors.</li> </ul>
Mar. 2016 progress report*	<ul style="list-style-type: none"> <li>- Initiate grapevine transformations.</li> <li>- Characterize expression of <i>TAS4</i> in transgenic tobacco over-expressing AtMYB90 in response to <i>Xf</i> infection; correlate with disease symptom severity and <i>Xf</i> titre.</li> <li>- Spectroscopic quantitation of anthocyanins in Pierce's disease grapevines from GA and CA fields.</li> <li>- Initiate grapevine and tobacco small RNA libraries.</li> </ul>	<ul style="list-style-type: none"> <li>- Transformation problem noted; solved later by using different <i>Agrobacterium</i> strain.</li> <li>- Homozygous tobacco MYB90 over-expression line more susceptible to <i>Xf</i>; correlated with <i>TAS4</i> induction by RNA blot.</li> </ul>
Jul. 2016 progress report*	<ul style="list-style-type: none"> <li>- Repeat grapevine transformations.</li> <li>- Showed by immunoblot binary T-DNA CRISPR vector effector Cas9 expressed in <i>N. benthamiana</i>.</li> <li>- Transformed tobacco with CRISPR vectors.</li> <li>- Initiate RNA-seq libraries of grapevine.</li> </ul>	<ul style="list-style-type: none"> <li>- Small RNA libraries show strong (~5-fold) induction of <i>TAS4</i> by <i>Xf</i> infection of grapevine and tobacco; induction degree correlates with phenotypic severity of symptoms in tobacco genotypes.</li> </ul>
Jul. 2016 one year project renewal	<ul style="list-style-type: none"> <li>- Added objective 3: xylem sap and leaf P<sub>i</sub> quantitation; phosphite effects on <i>Xf</i>.</li> <li>- Co-PI De La Fuente opts for Cooperator role.</li> </ul>	

Report Venue	Activity	Experimental Results
Dec. 2016 <i>Research Symposium Proceedings</i> <sup>^</sup>	<ul style="list-style-type: none"> <li>- Develop polyacrylamide gel electrophoresis (PAGE) heteroduplex genotyping assay.</li> <li>- Repeat tobacco <i>Xf</i> challenge experiment.</li> <li>- DESeq2 statistical analysis of differential miRNA expression by <i>Xf</i> on 2015 CA libraries.</li> <li>- Complete RNA-Seq libraries and initiate degradome libraries on 2015 CA samples.</li> <li>- <i>In vivo</i> nuclear magnetic resonance spectroscopy of subcellular [Pi] on leaf 2016 samples from Temecula, CA.</li> <li>- Collected xylem sap from Napa vineyard severely stunted 'sucker' rootstock 2016 samples; quantified P<sub>i</sub>, sulfate, and nitrate by ion chromatography-flame ionization detection.</li> <li>- Methods development for anthocyanin quantitation by high performance liquid chromatography-mass spectrometry/photodiode array detection.</li> </ul>	<ul style="list-style-type: none"> <li>- Tobacco vector transformations showed issue, but restriction-mapped vectors showed no re-arrangements; concluded the <i>Agro</i> strain suspect. RNA blot evidence for miR828 up-regulation by <i>Xf</i> infection in CA samples. AtMYB75 and SPX DOMAIN (positive regulator of P<sub>i</sub> starvation) strongly down-regulated by <i>Xf</i> infection in <i>Arabidopsis</i> [5]. <i>TAS4c</i> and disease resistance leucine-rich-repeat receptors differential expression by <i>Xf</i> provides evidence as causal effectors. Preliminary results of rootstock-derived <i>Xf</i>-infected cane P<sub>i</sub> show significant differences from control.</li> </ul>
Dec. 2016 Research Symposium	<ul style="list-style-type: none"> <li>- Oral and poster presentations.</li> </ul>	<ul style="list-style-type: none"> <li>- Southern blot of <i>Agrobacterium</i> and <i>E. coli</i> CRISPR vectors show no host re-arrangements.</li> </ul>
Mar. 2017 progress report*	<ul style="list-style-type: none"> <li>- Completed degradome libraries 2015 CA samples.</li> <li>- Second attempt at grapevine rootstock 101-14 transformation initiated Feb. 2017.</li> <li>- Qualify disease symptoms and quantify anthocyanins as significantly different in transgenic tobacco MYB90 repeat experiment.</li> <li>- Statistical analyses of differential expression of miRNAs and phasiRNAs in replicate transgenic MYB90 tobacco <i>Xf</i> challenge experiments.</li> <li>- Statistical analyses of <i>Xf</i> infection effects in 2015 CA samples by deep sequencing of small RNA and mRNA libraries confirms prior observation [6] in grapevine (eight weeks post-<i>Xf</i> infection) for down regulation of target phosphate transporter <i>VvPHT2;1</i> and homologs, shown here inversely correlated with effector miR399 induction (which is phosphate-regulated). Similar results for phosphate-regulated miR827 and two <i>SPX</i> targets.</li> </ul>	<ul style="list-style-type: none"> <li>- MAPMAN analysis of small RNA-Seq and mRNA-Seq CA 2015 libraries show inverse correlation between small RNAs and expression of template biotic stress genes, signaling receptor kinases (including candidate PdR1 locus <i>VIT_14s0171g00180</i>)<sup>7</sup>, pathogenesis-related proteins and Pentatricopeptide repeat proteins, very strongly supporting the working model that <i>Xf</i> infection results in compelling differential expression of mRNAs AND their derived phasiRNAs for ontology bins known to control pathogen resistance. RNA blot shows AtMYB90 overexpression in tobacco induces the endogenous negative siRNA regulator NtTAS4-3'D4(-) and its trigger miR828, supporting deep conservation of autoregulatory loop [8] and Pierce's disease model. RNA blot analysis of transgenic tobacco corroborates statistical analysis of differential expression by deep sequencing that <i>Xf</i> suppresses (down-regulates) MYB90 → TAS autoregulation activity and Nta-MIR828ab and <i>TAS4ab</i>, strongly supporting model. Successful production of transgenic tobacco harboring grapevine CRISPR vectors, demonstrating <i>Agrobacterium</i> host strain likely responsible for initial grapevine transformation problem. Repeat experiment of tobacco MYB90 challenge with <i>Xf</i> successful.</li> </ul>

Report Venue	Activity	Experimental Results
Jul. 2017 progress report*	<ul style="list-style-type: none"> <li>- No cost extension granted until 12/31/2017.</li> <li>- New Pierce's disease field samples collected from Temecula (high quality, fully expanded leaves and canes) and St. Helena. CA.</li> <li>- Attempt to verify tobacco genome editing using grapevine synthetic guide vectors (long shot, due to low homology).</li> <li>- Quantification of <i>Xf</i> titres by quantitative reverse transcription polymerase chain reaction (qRT-PCR) for repeat tobacco <i>Xf</i> challenge experiment shows experiment successful, validating prior results.</li> <li>- Further statistical analyses with DESeq2 [9], ShortStack [10], and PhaseTank [11] of tobacco 2015 and 2016 libraries. Many novel miRNA candidates revealed.</li> <li>- Sleuth/kallisto [12] statistical analysis of 2015 CA mRNA-Seq libraries for stress- and auxin-inducible miR156, miR398, miR167, and miR393 grapevine targets reveal top leads for significantly down-regulated effectors of <i>Xf</i> etiology upstream of miR828 and other P<sub>i</sub>-regulated miR399 and miR827.</li> <li>- Initiate replicate small RNA and degradome libraries from 2017 Temecula field Pierce's disease samples and tobacco transgenic <i>Xf</i> challenge experiments for more statistical power.</li> <li>- Quantify P<sub>i</sub>, sulfate, and nitrate in 2017 stunted rootstock 'sucker' Napa Pierce's disease samples and 2017 fully expanded Pierce's disease Temecula scion samples by ion chromatography-flame ionization detection [13].</li> <li>- Quantify by <sup>31</sup>P nuclear magnetic resonance the Aug. 2016 leaf Pierce's disease samples.</li> <li>- Quantify by mass spectrometry anthocyanins in 2017 Temecula xylem sap and leaves by visible wavelength spectroscopy.</li> <li>- Visit De La Fuente lab to learn best practices re: <i>Xf</i> microbiology. Initiate plate growth <i>Xf</i> assays for phosphite.</li> </ul>	<ul style="list-style-type: none"> <li>- Grapevine somatic embryo regeneration proceeding well; some concern for MYBA6 transgenic regeneration. Principal Component Analysis of technical and biological replicate small RNA libraries made from 2015 and 2016 tobacco <i>Xf</i> challenge experiments demonstrated that biological variables of genotype and condition were reproducible. Statistically significant mis-regulated miRNAs in replicate <i>Xf</i> challenged transgenic tobacco libraries further documented; Nta-miR399, miR828, and <i>TAS4ab</i> changes correlate (down in <i>Xf</i>) with prior RNA blot and preliminary statistical results, showing <i>MIR828/TAS4</i> autoregulatory loop effects. Nta-miR827 and miR156 up-regulated, consistent with working model where SPL targets of miR156 down regulate anthocyanin biosynthesis in <i>Arabidopsis</i><sup>[14]</sup>, providing a direct link/mechanism for how VvMYBA6/7 and other miR828 MYB targets in grapevine are deranged by <i>Xf</i> infection resulting in anthocyanin accumulation.</li> <li>- Results of P<sub>i</sub> quantitation by two methods of fully expanded leaves and canes in 2016 and 2017 Temecula Pierce's disease samples support hypothesis that <i>Xf</i> infection results in significantly lower [P<sub>i</sub>] (about 60% decrease) in host leaf and xylem sap. Correlates with elevated anthocyanins quantified in Pierce's disease xylem sap by mass spectrometry and leaves by spectrophotometry. Further substantiated by prior results for other grape cultivars [15,16], supporting working model.</li> </ul>
<p>^ available at <a href="https://www.cdfa.ca.gov/pdcp/Research.html">https://www.cdfa.ca.gov/pdcp/Research.html</a>  * available at <a href="http://www.piercesdisease.org/reports">http://www.piercesdisease.org/reports</a></p>		

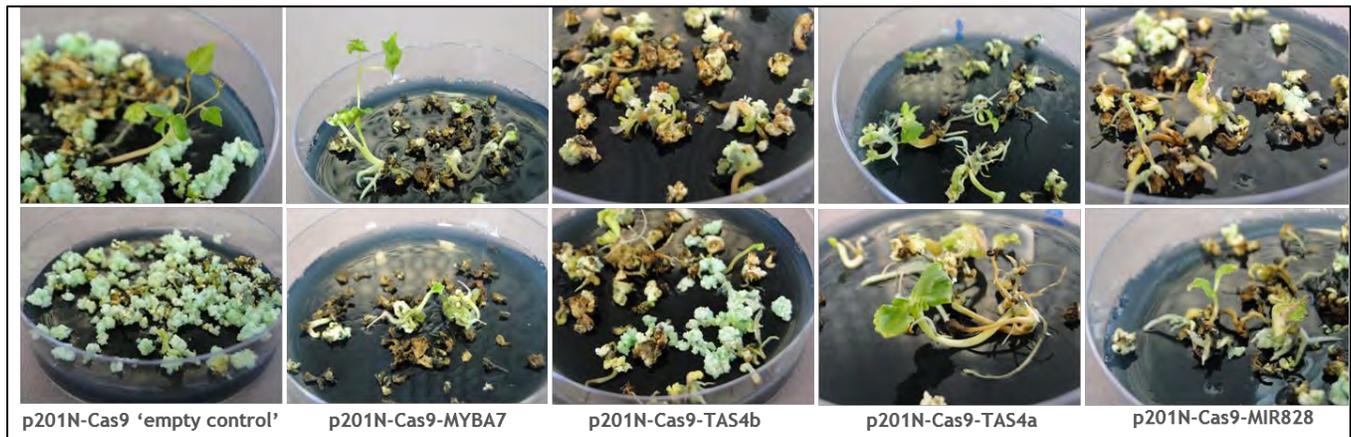
## OBJECTIVES

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

## RESULTS AND DISCUSSION

### Objective 1. Test the miR828, *TAS4*, and Target MYBA6/7 Functions in Pierce's Disease Etiology and *Xf* Infection and Spreading By Genome Editing Using CRISPR/Cas9 Transgenic Technology

Ongoing regeneration of somatic embryos from rootstock 101-14 grape transformations with five CRISPR binary T-DNA vectors (plus empty vector control) in the lab of David Tricoli were previously responding as expected, as documented in the July 2017 interim progress report, with the caveat that the MYBA6 experiment was showing higher necrosis than others, which were characteristically normal. **Figure 1** shows the current status of these materials. p201N-Cas9-MybA6 was re-transformed in August 2017 because resistant embryos were not recovered. A few plants may be delivered after working through the amounts of attrition observed.



**Figure 1.** Progress of regeneration of grapevine transformants of p201-N-Cas9 vector constructs harbored in Cooperator-sourced EHA105 *Agrobacterium* strain, initiated February 2017.

Validation of editing events going forward will be by PCR cloning and sequencing of target genes, and polyacrylamide gel electrophoresis-based genotyping [17].

### Objective 2. Characterize Tissue-Specific Expression Patterns of *TAS4* and *MIR828* Primary Transcripts, siRNAs, and *MYB* Targets in Response to *Xf* Infections in the Field

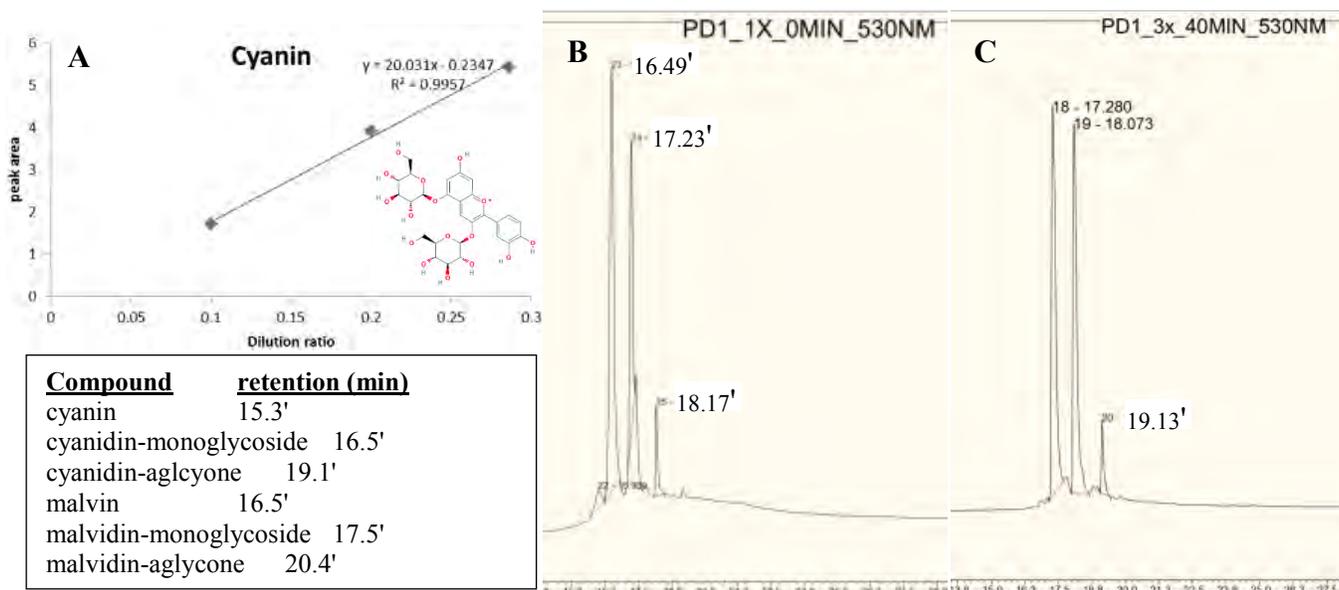
We are in the process of completing Illumina libraries for complete sets of biological replicates for small RNAs, stranded mRNAs, and degradome from the 2017 Calle Contento Temecula field leaf samples, and the 2016 replicated greenhouse *Xf* tobacco MYB90 overexpression experiment. In addition, we are preparing indexed libraries for 'green island' cane bark and 'matchstick petiole' samples from the 2017 Temecula field expedition for discovery of differential miRNA expressions associated with diagnostic yet pleiotropic Pierce's disease traits hypothesized to be due to deranged small RNA activities. We will submit them to the UC Riverside Institute for Integrative Genome Biology for two runs of HighSeq500 (400 m reads per run) in the next few weeks. There are eight small RNA libraries to be pooled and indexed with 18 degradome samples, and 12 stranded mRNA-Seq libraries sequenced separately. This level of complexity will result in ~12 million reads per small RNA library, and ~25 million reads per transcriptome library. All workflow processes and yields have been verified now as optimal/appropriate through the data analysis and genome annotation stages. Thus the statistical power from multiple replicates across years will allow defensible claims at the publication stage, which will commence when the sequencing is complete in the next couple of months.

### Objective 3. Characterize the Changes in (a) Xylem Sap and Leaf Pi, and (b) Polyphenolic Levels of *Xf*-Infected Canes and Leaves, and (c) Test the P<sub>i</sub> Analogue Phosphite on Tobacco in the Greenhouse and *Xf* Growth *In Vitro* as a Durable, Affordable, and Environmentally Sound Protectant/Safener for Pierce's Disease

#### (a) Xylem Sap [Pi]

In May 2017 the Principal Investigator collected Pierce's disease samples from Malbec rootstock sucker canes from Napa County Phelps vineyard (1109 Silverado Trail South, River Ranch Farm Workers Housing, St. Helena, CA) and healthy control scion canes under the supervision of UC Cooperative Extension agent Monica Cooper,

and Merlot variety Pierce's disease and control samples in June 2017 from the Calle Contento vineyard in Temecula, CA. The Merlot variety leaves and canes from Temecula Pierce's disease symptomatic scion samples were not developmentally stunted, allowing appropriate side-by-side controlled genotype and developmental state comparisons. We reported in the July 2017 interim progress report the results from both <sup>31</sup>P nuclear magnetic resonance from 2016 Temecula leaf samples and ion chromatography of 2017 Temecula xylem sap samples that support the working hypothesis that Pierce's disease infected canes and leaves have significantly lower Pi (~60%) concentrations than healthy controls. We plan to collect more material in 2018 to further substantiate and verify our results.



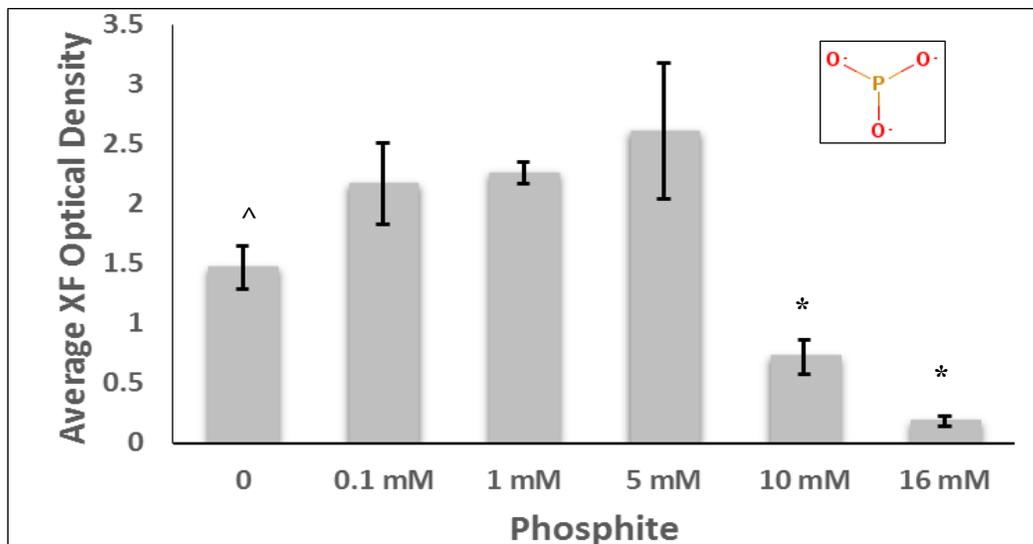
**Figure 2.** High performance reverse phase liquid chromatography for quantitation of anthocyanins cyanin and malvin and aglycone species in leaf samples. (A) Standard curve for cyanin. Structure inset. (B) Chromatogram of unhydrolyzed Temecula 2017 Pierce's disease leaf sample extract, showing major peaks of malvin and/or cyanidin-monoglycoside (retention times ~16.5'), possibly malvidin-monoglycoside (~17.23') and uncharacterized anthocyanin (18.17'). (C) Chromatogram of acid hydrolyzed Pierce's disease extract supports cyanin identification (peak 22 in panel B) by detecting aglycone species (19.13'), and peak 19 possibly as monoglycoside (see panel B, peak 25).

### (b) Polyphenolics in *Xf*-Infected Canes and Leaves

We reported in the July 2017 interim progress report preliminary results for mass spectrometric quantification of cyanin and malvin in xylem sap from the Temecula June 2017 field samples, and anthocyanins in leaves. We are in the process of quantifying *Xf* titers in concordant petioles samples from these leaf and cane samples by real time PCR. The results directly support the hypothesis that *Xf* infection results in accumulation of anthocyanins in xylem sap and leaves. Similar results have been reported for procyanidins and other polyphenolics in xylem sap two months post-*Xf* infection in Thompson Seedless and several winegrape cultivars [15,16]. Phenolic levels in Merlot xylem sap correlate with Pierce's disease severity compared to other cultivars [18]. Taken together, these results support our working hypothesis that the xylem sap anthocyanins and other polyphenolics are important for Pierce's disease disease progression.

In an effort to characterize the anthocyanin complexity in 2017 Temecula leaf samples we have conducted pilot experiments to develop quantitative high performance liquid chromatography-spectroscopic methods for malvin (a di-O-methylated anthocyanidin [less polar]) and cyanin, and their hydrophobic aglycones malvidin and cyanidin generated after acid + heat hydrolysis. We employed an Acclaim Pepmap RSLC 75  $\mu$ m x 15 cm nanoViper C18 2  $\mu$ m reverse phase column coupled to a photodiode array detector (530 nm) with 95% water:formic acid as stationary phase and 100% acetonitrile as mobile phase, linear gradient from 5-100% mobile in 40'. **Figure 2A** shows a standard curve derived for cyanin, and chromatogram traces of unhydrolyzed

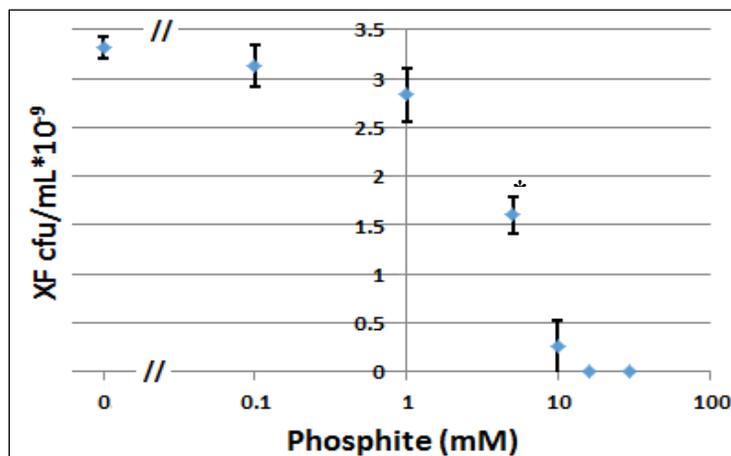
(Figure 2B) and mono-/di-aglycone (hydrolyzed, Figure 2C) Pierce's disease leaf samples. There are other abundant peaks eluting at later times (18.07'), which are likely other anthocyanins but some peaks (e.g. ~19.1') are concordant with single- and/or double-aglycones of cyanin and malvin, based on hydrolysis timecourse experiments with standards (data not shown). We are in the process of quantifying the anthocyanin species in Pierce's disease xylem sap.



**Figure 3.** Physiological concentrations of phosphite (structure inset) inhibit plate growth of *Xf*. Asterisk (\*) indicates significantly different than 0-5 mM treatments,  $P < 0.004$  (Student's two-sided t test, equal variance assumed). ^: not significantly different than 0.1-5 mM treatments. Error bars are s.e.m. ( $n = 3$ , except 0 and 0.1 mM treatments,  $n = 2$ ).

### (c) $P_i$ Analogue Phosphite as Effector of *Xf* Growth and Safener of Disease Symptoms

Figure 3 reports results of a baseline study on *Xf* growth on PD2 potato starch plates [20] ( $P_i$  component omitted and 2 g/L potato starch substituted for bovine serum albumin) as a function of physiological concentrations of phosphite added to *Xf* minimal growth medium. This experiment has been repeated at lower growth densities and including standard medium  $P_i$  concentration (16 mM) to facilitate more quantitative and physiologically relevant results normalized to colony-forming units. Figure 4 reports convincing evidence that phosphite can function as an active competitor of physiological concentrations of  $P_i$  influencing *Xf* plate growth, with a  $LD_{50} \sim 5$  mM. (application concentration for lethal dose). Future work will focus on testing phosphite as safener for tobacco plants challenged with *Xf* in the greenhouse.



**Figure 4.** Phosphite has an  $LD_{50}$  of  $\sim 5$  mM for plate growth of *Xf*. Error bars are s.e.m. ( $n = 7-9$ ). Asterisk (\*) indicates significantly different than zero phosphite control,  $P < 10^{-6}$  (Student's two-sided t-test, equal variance assumed).

## CONCLUSIONS

We are on track to achieve our objectives within the timeframe of two years' funding (plus six month no cost extension). We have generated compelling evidence supporting our working model for *MIR828/TAS4* genes, identified new lead target genes, and presented evidence that phosphite impacts *Xf* growth. This latter result underscores the practical value of the project to develop a durable management tool while generating new knowledge about Pierce's disease etiology and engineered resistance.

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

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**Reporting Period:** The results reported here are from work conducted July 2012 to October 2017.

## ABSTRACT

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

## LAYPERSON SUMMARY

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

## INTRODUCTION

*Xylella fastidiosa* (*Xf*) is a Gram negative, xylem-limited, insect-vectored bacterium and is the causal agent of Pierce's disease of grapevine (Hopkins and Purcell 2002). Pierce's disease is endemic to California but the recent introduction of a more effective vector, the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) to southern California shifted the epidemiology of Pierce's disease from a monocyclic to a polycyclic disease. This led to a Pierce's disease epidemic with severe economic consequences for the southern California grape industry. GWSS has move to the San Joaquin Valley and has impacted table grape production and it now threatens to become established in the heart of the winegrape production area, including Napa and Sonoma Counties. Current Pierce's disease management guidelines largely rely on vector control through the use of insecticides.

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

## OBJECTIVES

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

## RESULTS AND DISCUSSION

### Objective 1. Evaluate a Single Organism-Based Approach for Pierce's Disease Management

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



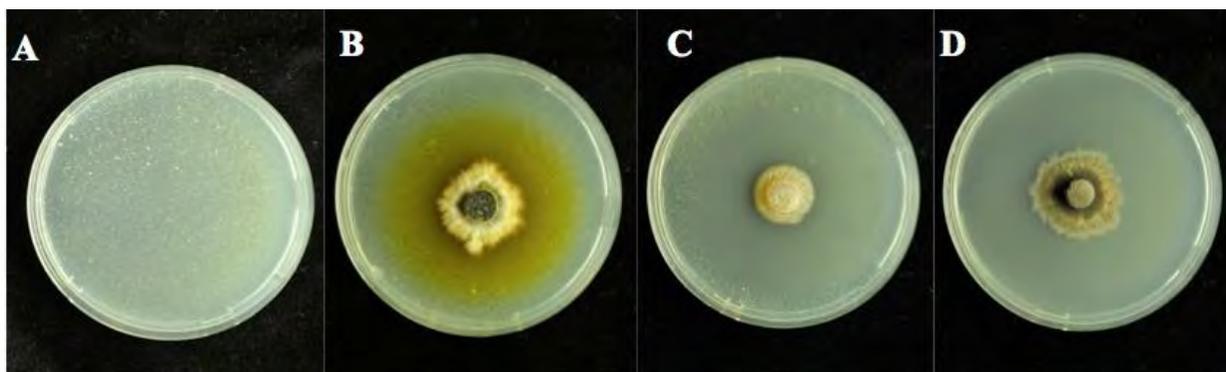
**Figure 1.** Pierce's disease symptomatic (red arrow) and Pierce's disease escaped (blue arrow) grapevines in a vineyard located close to a riparian area in the Napa Valley, California.

Using an Illumina-based culture-independent approach we were able to identify *Achromobacter* sp. and *Pseudomonas* sp. as the two most abundant bacteria inhabiting grapevine xylem that correlated negatively with *Xf* titer (Table 1; Deyett et al. 2017). In other words, those two bacteria were present in higher abundance in Pierce's disease escaped grapevines than in Pierce's disease symptomatic grapevines, suggesting that those may be good biological control agent candidates. In addition, using a culture-dependent approach we isolated eight fungi and one bacterium that showed *Xf*-growth inhibition in our *in vitro*-bioassay (Figure 2; Rolshausen et al. 2013). Interestingly, the bacterium isolated was identified as *Achromobacter* sp. We further evaluated those fungi and *Achromobacter* sp. in *in planta* bioassays and demonstrated that *Cryptococcus* sp. was the best biological control

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

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

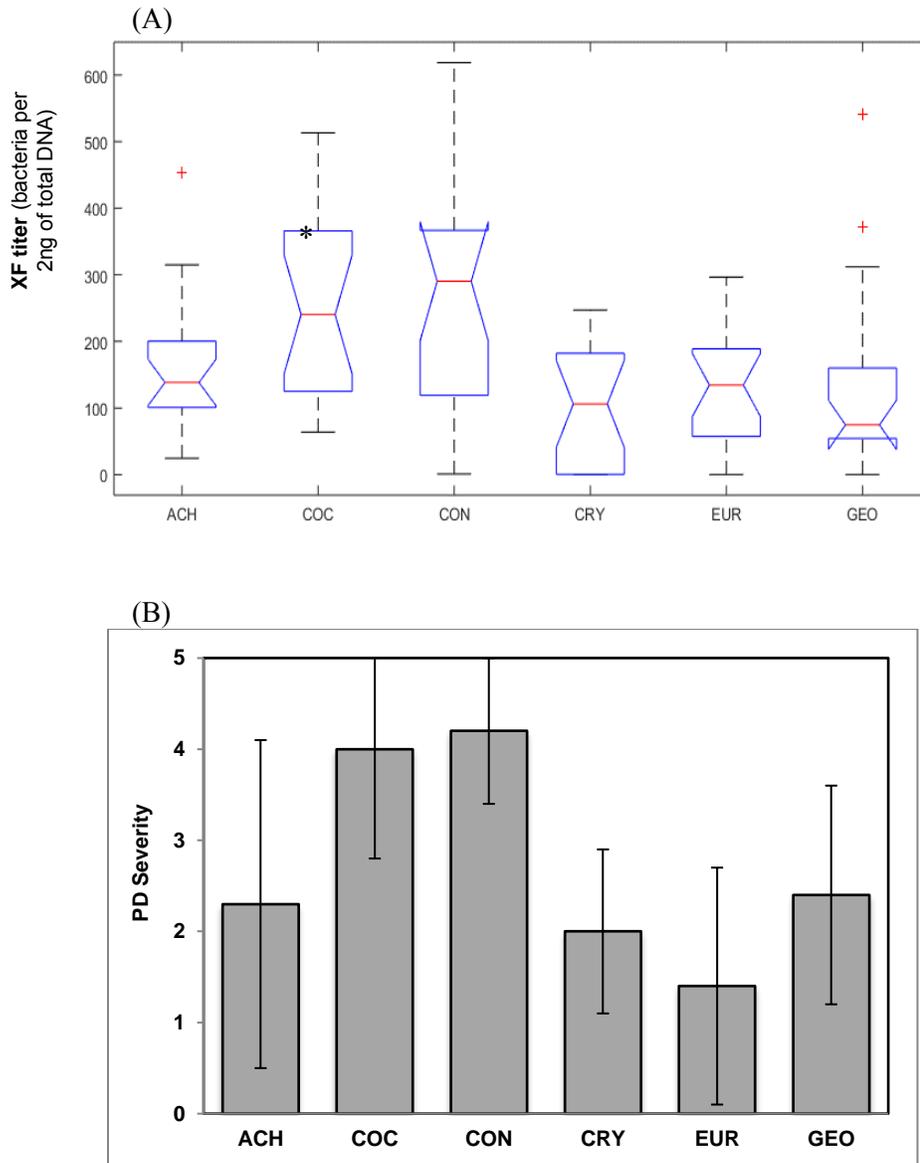
OTU	P	FDR Corrected	r	Abundance %
<i>Pseudomonas</i> sp.	0.000	0.00	-0.84	82.2
<i>Achromobacter</i> sp.	0.043	0.043	-0.25	3.9



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



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



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

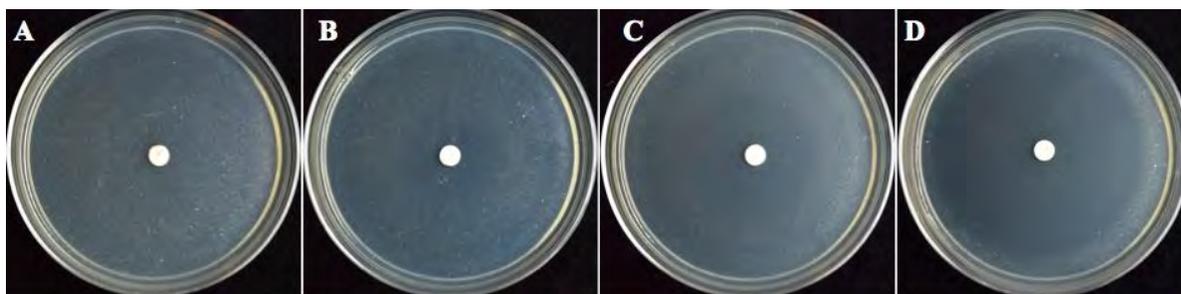
*Cryptococcus* is a yeast commonly associated with plants and is also a known biological control agent of other plant pathogens (Schisler et al. 2014, Ulises Bautista-Rosales et al. 2014). Our Illumina sequencing results confirmed its presence in grapevine xylem, although its abundance was low (below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

## Objective 2. Evaluate Natural Products and Derivatives for Their Potential as Curative Treatments for Vines Already Infected with Pierce's Disease

The goal of this objective is to identify fungal natural products produced by endophytes that can be used as curative treatments for control of Pierce's disease. We previously identified eight fungal specimens inhabiting grapevine tissues (xylem sap, shoot, petioles, and spur) that were able to inhibit *Xf* in a bioassay. Thus far, we have purified and characterized the chemical structure of three molecules (radicinin, alteichin, and cytochalasin) that are active against *Xf* growth *in vitro*. Radicinin is produced by *Cochliobolus* sp., alteichin is produced by *Ulocladium*, and cytochalasin is produced by *Dreschlera* sp. However, cytochalasin showed to be toxic to mammals so we decided to discontinue this research axis. In addition, we pursued our efforts for the bioassay-guided isolation of natural products from the remaining fungi able to inhibit *Xf* in our lab bioassay, including *Cryptococcus* sp., *Eurotium* sp., and *Geomyces* sp.

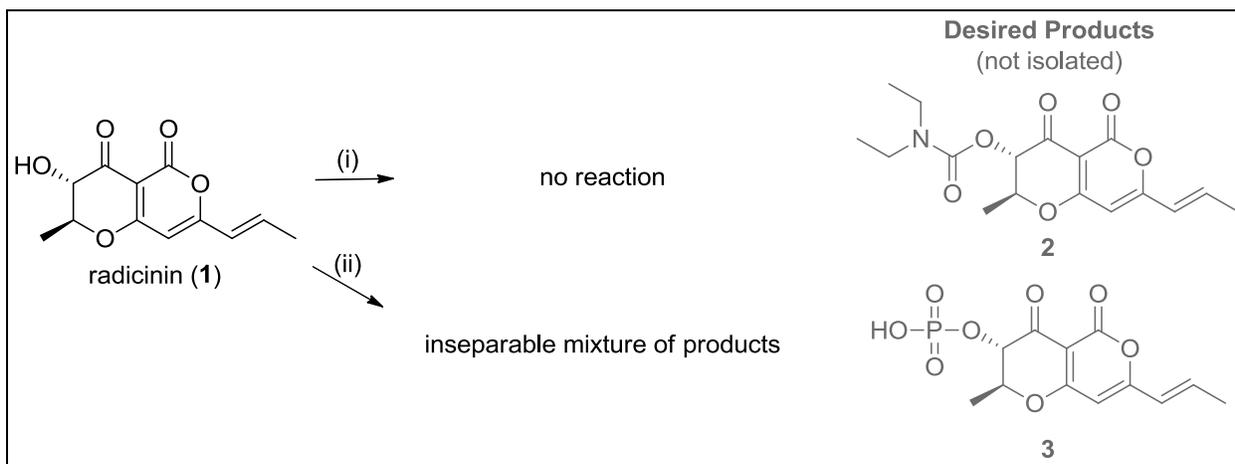
### *Cochliobolus* Natural Product

Radicinin showed great potential *in vitro* (Aldrich et al. 2015). Hence, in an *in vitro* dose response assay, where *Xf* cells are submitted to an increasing concentration of a fungal molecule, radicinin was able to inhibit *Xf* growth (Figure 5). We have now developed a more efficient procedure for isolating radicinin from *Cochliobolus* sp. This is a critical step, as it will allow us to produce substantial amounts of derivatives and further test them *in planta*. Radicinin is not commercially available, and we had been employing a multistep isolation procedure involving liquid-liquid extraction of *Cochliobolus* cultures followed by an expensive and time-consuming chromatography step to obtain pure radicinin for all our studies to date. Recently we developed a procedure for purifying radicinin by recrystallization instead of chromatography. In this way we were able to increase our yield of radicinin from 60.5 mg/liter of culture to 150 mg/liter of culture. This procedure also makes scaling up of the isolation for commercial use much more practical. In addition, the radicinin obtained by this new procedure is significantly purer, as observed by nuclear magnetic resonance spectroscopy.



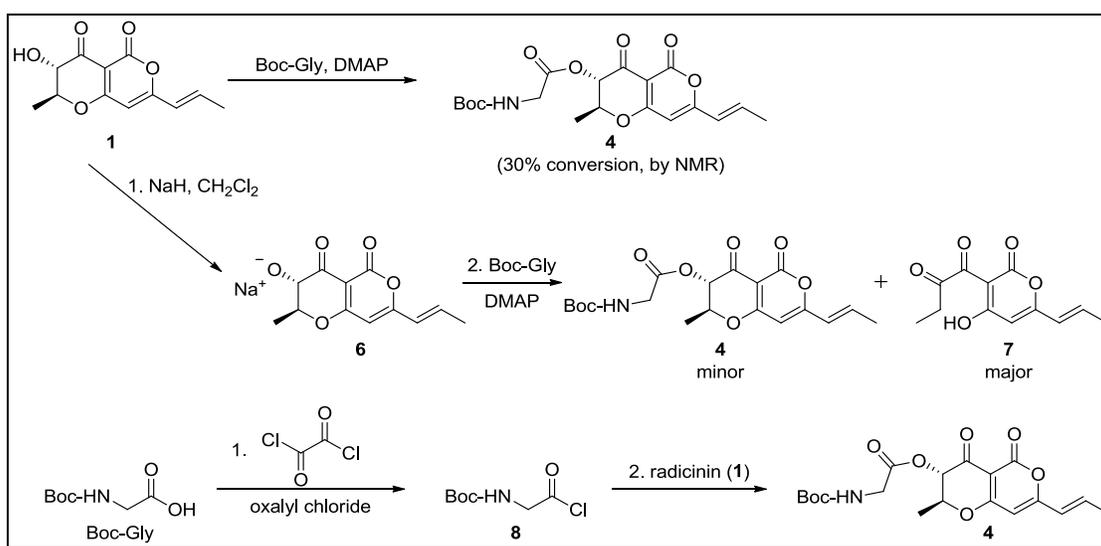
**Figure 5.** Dose response assay to evaluate *in vitro* *Xf* inhibition at increasing concentration of radicinin, a natural compound produced by *Cochliobolus* sp. (A) 0 µg molecule radicinin (control); (B) 50 µg molecule radicinin; (C) 100 µg molecule radicinin; (D) 250 µg molecule radicinin (Aldrich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

Now that we have figured out how to scale up radicinin production and purification, the next step was to prepare water-soluble semisynthetic derivatives of radicinin to facilitate testing *in planta*. We determined the solubility of radicinin in water to be 0.15 mg/mL, which is considered very slightly soluble. We have shown that acetylradicinin, which was modified at the hydroxyl group of radicinin, retains its anti-*Xf* activity (Aldrich et al. 2015). This result suggests that modification of this position may provide a viable strategy for increasing the water-solubility of radicinin without loss of activity. Adding ionizable groups is a commonly employed strategy for improving the water-solubility of bioactive molecules (Kumar and Singh 2013), so we had proposed to add two such groups at the hydroxyl position of radicinin (Scheme 1). The carbamate (2) is weakly basic and should form a water-soluble salt in low pH solutions, while the phosphate (3) is acidic and should form a water-soluble salt at high pH. Both carbamates and organophosphates are commonly found in pesticides, so we had good reason to believe that one or both of these compounds would be able to move into the xylem of grapevines. However, attempts to prepare the weakly basic carbamate and the acidic phosphate were unsuccessful. Specifically, the reaction with diethylcarbamoyl chloride (i) did not go to completion, while the phosphate reaction (ii) gave a mixture of products that we were unable to purify.

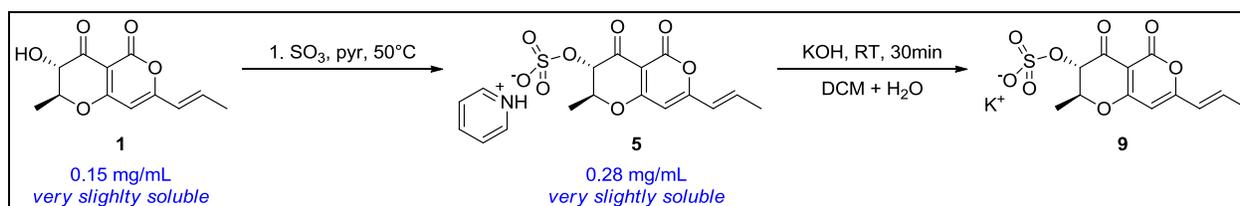


**Scheme 1.** *Xf*-inhibitory natural product radicinin (1), and semisynthetic derivatives (2-4). Reagents: (i) N,N-diethylcarbamoyl chloride, triethylamine (Vougogiannopoulou et al. 2008). (ii) 1. Cl<sub>3</sub>CCN, 2. (n-Bu)<sub>4</sub>NH<sub>2</sub>PO<sub>4</sub>, CH<sub>3</sub>CN, 3. DOWEX 50WX8, NH<sub>4</sub>HCO<sub>3</sub>.

We then attempted to make two alternate ionizable radicinin derivatives: a glycine-derivative (4, **Scheme 2**), and radicinin pyridinium sulfate (5, **Scheme 3**). The failure of reactions to form either 2 or 4 suggested that the alcohol group of radicinin is much less nucleophilic than we originally expected. We attempted to increase the nucleophilicity of this group by first deprotonating with sodium hydride to give an alkoxide (6, **Scheme 2**). We isolated 6 and found it to be more than a thousand-fold more water-soluble than radicinin, at 218 mg/mL (which is considered freely soluble). However, the high pH of the alkoxide solution leads us to be concerned about possible nonspecific toxicity. We also doubt that this high water solubility would be maintained in a cellular environment, which is buffered at neutral pH. Despite the increased nucleophilicity of 6 we never observed any formation of carbamate 2 and observed only minimal formation of the boc-glycine derivative 4. Under the reaction conditions to form 4 radicinin appeared to undergo tautomerization and ring-opening to give isomer 7 (**Scheme 2**). We successfully prepared a sulfate of radicinin, as the pyridinium salt 5. Salt 5 maintained its activity against *Xf* in our disc assay (**Figure 2**). This reaction proceeded to completion and the product proved easy to isolate. Unfortunately the water solubility of 5 was only about twice that of radicinin: 0.28 mg/mL, lower than we had hoped. Recently we were able to successfully replace the pyridinium counterion with potassium to give salt 9 (**Scheme 2**), which we hope will be more water soluble than 5, while retaining activity.



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



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

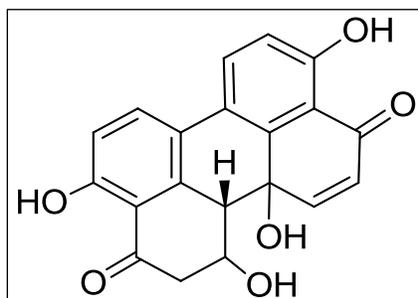
After a series of mostly unsuccessful attempts at preparing water-soluble radicinin derivatives we decided to explore another strategy for getting radicinin into grapevines, namely, using surfactants. We tested the solubility of radicinin in a variety of organic solvents that are compatible with agriculture,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

### ***Cryptococcus* Natural Product**

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

### ***Ulocladium* Natural Product**

We previously observed a compound in the ethyl acetate extract of *Ulocladium* sp. which high-resolution mass spectrometry revealed to have a molecular formula of C<sub>10</sub>H<sub>8</sub>Cl<sub>2</sub>O<sub>4</sub>. This compound has consistently been found in the active fractions from repeated fermentations and separations of *Ulocladium*. In an effort to produce enough of this compound we fermented 5.5 L of *Ulocladium* sp. and fractionated the organic extract by silica gel chromatography. This yielded 23.4 mg of a semi-purified fraction containing the compound of interest. This was enough material to permit collection of two-dimensional nuclear magnetic resonance spectral data (including gdqCOSY, gHMBC, HSQC, and NOESY experiments). We identified the active molecule as alteichin (**Figure 6**).



**Figure 6.** *Xf*-inhibitory natural product alteichin produced by *Ulocladium*

## Geomyces Natural Product

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

## CONCLUSIONS

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

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

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## ABSTRACT

*Xylella fastidiosa* (*Xf*) is a gram-negative, fastidious xylem-limited bacterium that causes Pierce's disease of grapevine. Lipopolysaccharide (LPS) covers the majority of the cell surface of Gram-negative bacteria and is a well-described pathogen-associated molecular pattern (PAMP) that can elicit host basal defense responses in plants. To understand the portions of the LPS molecule that mediate host-pathogen interactions during the *Xf* infection process in grapes we performed transcriptome profiling and histological analysis of grapevines inoculated with either *Xf* containing a wild-type LPS molecule, or a *wzy* mutant that possesses an LPS with a truncated O antigen. From these data we deduce that the outermost exposed portion of the O-antigen serves to shield the bacterium from initial recognition by the grapevine defense system, and this camouflaging strategy allows for successful infection. Furthermore, we investigated defense priming of grapevine by pre-treating plants with the *Xf* LPS PAMP and then challenging with live *Xf* cells. Pierce's disease symptoms are significantly less severe when grapevines are pre-treated with LPS, showing that the LPS molecule can prime defenses against *Xf*. Finally, we have solved the chemical structure of the *Xf* wild-type O antigen and describe the main linear  $\alpha$ 1-2 linked rhamnan.

## LAYPERSON SUMMARY

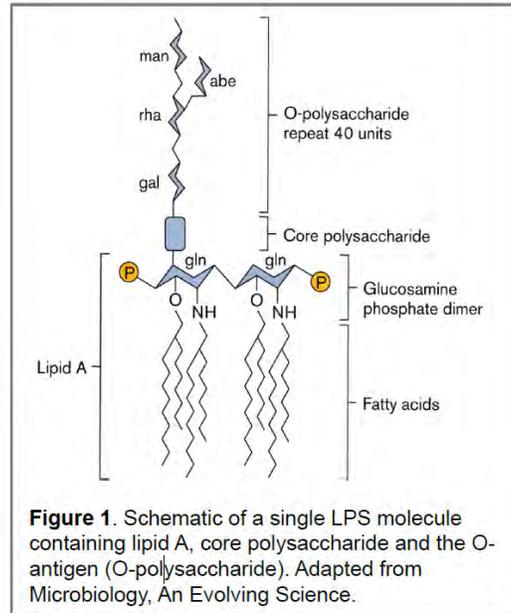
Successful plant pathogens must overcome plant immune responses to establish themselves and cause disease. Although there has been extensive research identifying factors inherent to the bacterium that allow it to be pathogenic in grapes, the mechanisms utilized by this pathogen to combat the plant immune responses have remained largely obscure. We demonstrate that *Xylella fastidiosa* covers its own surface with an abundant sugar to shield itself from the grapevine immune system, effectively delaying recognition long enough for the bacteria to circumvent the plant's defenses and establish itself in the plant. These results provide unique insight into the molecular mechanisms underlying this host-pathogen interaction.

## INTRODUCTION

*Xylella fastidiosa* (*Xf*), a Gram-negative fastidious bacterium, is the causal agent of Pierce's disease of grapevine (*Vitis vinifera*) and several other economically important diseases (Chatterjee et al. 2008, Varela 2001). *Xf* is limited to the xylem tissue of the plant host and is transmitted by xylem-feeding insects, mainly sharpshooters. Extensive xylem vessel blockage occurs in infected vines (Sun et al. 2013), and symptoms include leaf scorch, raisining of berries, stunting, and vine death. Pierce's disease has devastated some viticultural areas in California, implicating it as a major threat to the industry.

We have demonstrated that lipopolysaccharide (LPS) is a major virulence factor for *Xf*. LPS comprises approximately 75% of the Gram-negative bacterial cell surface, making it the most dominant macromolecule displayed on the cell surface (Caroff and Karibian 2003, Foppen et al. 2010, Madigan 2012). LPS is a tripartite glycolipid that is generally comprised of a highly-conserved lipid A, an oligosaccharide core, and a variable O-antigen polysaccharide (Whitfield 1995) (**Figure 1**). We demonstrated that compositional alterations to the outermost portion of the LPS, the O antigen, significantly affected the adhesive properties of *Xf*, consequently affecting biofilm formation and virulence (Clifford et al. 2013). Depletion of the 2-linked rhamnose in the O antigen locks *Xf* in the initial surface attachment phase and prevents biofilm maturation (Clifford et al. 2013). In addition, we demonstrated that truncation of the LPS molecule severely compromises insect acquisition of *Xf* (Rapicavoli et al. 2015). We coupled these studies with quantification of the electrostatic properties of the sharpshooter foregut to better understand the interface between the *Xf* cell and the insect. This project tested our additional hypothesis that the *Xf* LPS molecule acts as a pathogen-associated molecular pattern (PAMP), and the

long chain O-antigen serves to shield *Xf* from host recognition, thereby modulating the host's perception of *Xf* infection (Rapicavoli et al., *under review*).



Contrary to the role of LPS in promoting bacterial survival *in planta*, the immune systems of plants have also evolved to recognize the LPS structure and mount a basal defense response to counteract bacterial invasion (Dow et al. 2000, Newman et al. 2000). LPS is considered a PAMP. PAMPs, also known as microbe-associated molecular patterns (MAMPs), are conserved molecular signatures that are often structural components of the pathogen (i.e. LPS, flagellin, fungal chitin, etc.). These PAMPs are recognized by the host as "non-self" and can be potent elicitors of basal defense responses. This line of defense against invading pathogens is referred to as PAMP-triggered immunity (PTI) and represents the initial layer of defense against pathogen ingress (Nicaise et al. 2009). PTI is well studied in both mammalian and plant hosts. However, little is known about the mechanisms involved in perception of LPS in grapevine, particularly the *Xf* LPS PAMP. *Xf* is introduced by its insect vector directly into the xylem, a non-living tissue, which cannot mount a defense response on its own. However, in other systems profound changes do occur in the adjacent living parenchyma cells upon infection, suggesting that these cells communicate with the xylem and can recognize the presence of a pathogen (Hilaire et al. 2001). The plant immune system can recognize several regions of the LPS structure, including the conserved lipid A and core polysaccharide components (Newman et al. 2007, Silipo et al. 2005). Bacteria can also circumvent the host's immune system by altering the structure of their LPS molecule. Clearly, *Xf* has evolved a mechanism to circumvent the host basal defense response as it successfully colonizes and causes serious disease in grapevine. Our working hypothesis is that during the compatible interaction between *Xf* and a susceptible grapevine host the bacterium's long chain, rhamnose-rich O-antigen shields the conserved lipid A and core-oligosaccharide regions of the LPS molecule from being recognized by the grapevine immune system, providing an opportunity for it to subvert basal defense responses and establish itself in the host.

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

To better understand the contribution of LPS to the dynamics of the infection process we have completed the global RNA-seq-based transcriptome profiling facet of this project, where we sequenced the transcriptomes of grapevines treated with wild-type, *wzy* mutant cells, or 1x phosphate buffered saline (PBS) buffer. PTI usually causes major transcriptional reprogramming of the plant cells within hours after perception (Dow et al. 2000, Tao et al. 2003), so our initial experiments were targeted toward early time points during the infection process (0, 8, and 24 hours post-inoculation). Thus far the RNA-seq data demonstrate that the grapevine is activating defense responses that are distinct to each treatment and time point (**Figure 2A**). For example, enrichment analysis of *wzy*-responsive genes at eight hours post-inoculation identified predominant biological processes associated with cellular responses to biotic stimulus and oxidative stress (**Figure 2B**). This included a significant increase in the production of thioredoxins, glutaredoxins, and other ROS-scavenging enzymes involved in antioxidant defense. In addition, there was high expression of genes involved in the production of phytoalexins (e.g. stilbene synthase), antimicrobial peptides (e.g. thaumatin), and pathogenesis-related genes. In contrast, wild-type responsive genes at this time point were enriched primarily in responses to abiotic or general stresses (i.e. drought, oxidative, temperature, and wounding stresses) and were not directly related to immune responses (**Figure 2B**). Notably, by 24 hours post-inoculation, overall transcriptional profiles of both *wzy* and wild-type inoculated vines shifted dramatically. Grape genes in *wzy* mutant-inoculated vines were no longer enriched for immune-specific responses, and we speculate that this is due to the effective O-antigen-modulated oxidative burst. In contrast, genes of wild-type-inoculated plants were strongly enriched for immune responses (**Figure 2C**). We hypothesize that at eight hours the high molecular weight O antigen is still effectively shielding wild-type cells, therefore causing a delay in plant immune recognition. However, by 24 hours post-inoculation, the production of ethylene-induced plant cell wall modifications, compounded by progressing bacterial colonization and the potential release of damage-associated molecular patterns (DAMPs) via bacterial enzymatic degradation of plant cell walls, has triggered grapevine immune responses, and the plant is now fighting an active infection. This indicates that the O antigen does, indeed, serve to shield the cells from host recognition, allowing them to establish an infection (Rapicavoli et al., *under review*).

## OBJECTIVES

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

## RESULTS AND DISCUSSION

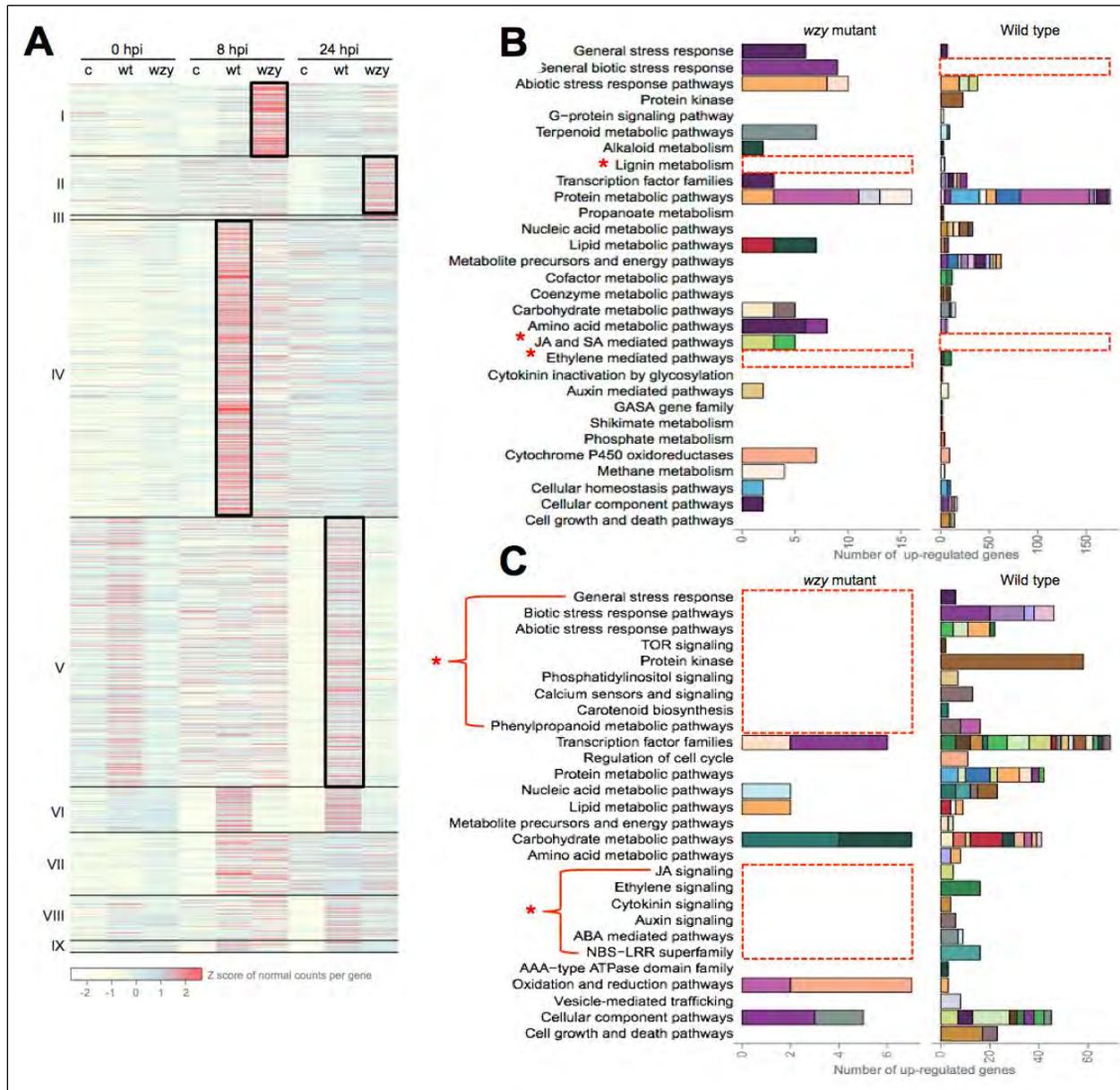
### Objective 1. Examination of the Temporal Response to *Xf* Lipopolysaccharide

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

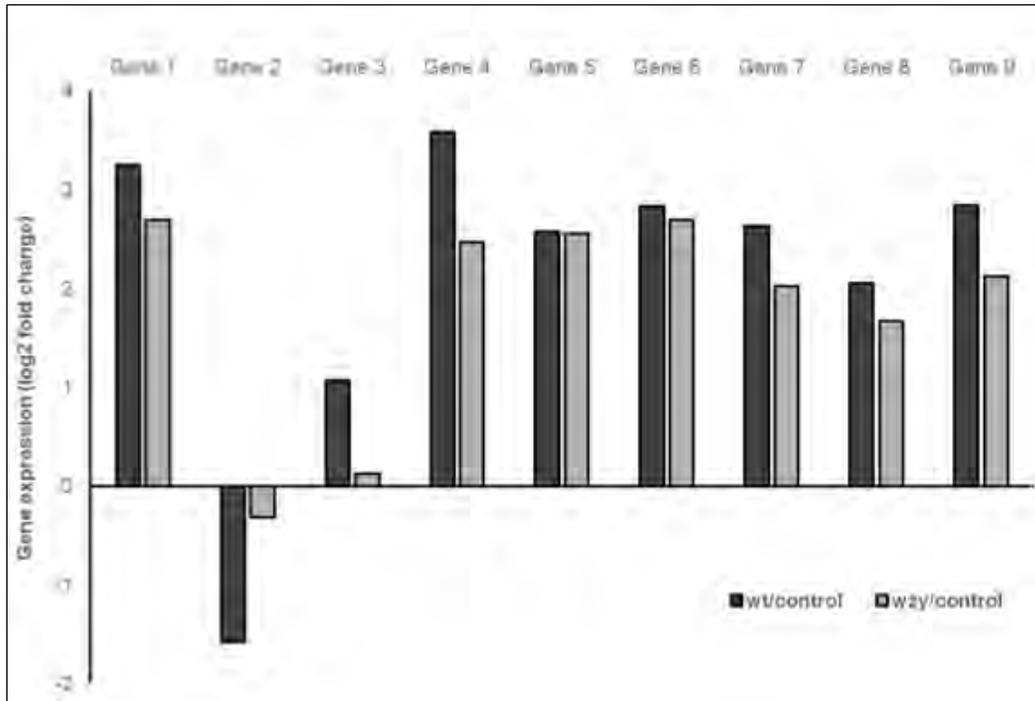
### Objective 1a. Relative Expression of Early Response Genes in LPS Treated Plants

To validate and further support our findings in our RNA-seq data from grapevine responses to early infections by *wzy* mutant and wild-type *Xf* cells (**Figure 3**), we examined expression fold-changes (log<sub>2</sub>) of early response genes observed in grapevines treated with two μg of wild-type or *wzy* mutant LPS (lipid A-core exposed in both types of LPS) or diH<sub>2</sub>O at 24 hours post-inoculation. We chose nine genes that were enriched during early

infection in grapevines treated with *wzy* mutant and wild-type cells to perform quantitative reverse transcription polymerase chain reaction on grapevines treated with wild-type or *wzy* mutant LPS at 24 hours post-inoculation. Eight of nine genes were up-regulated in both wild-type and *wzy* mutant LPS treatments. Interestingly, grapevines responded similarly to wild-type and *wzy* LPS. Our results validate our previous RNA-seq data and support our hypothesis that the highly-conserved lipid A and the oligosaccharide core but not the O antigen act as a PAMP to elicit early plant immunity (**Figure 3**).



**Figure 2.** Grapevine responses to early infections by *wzy* mutant and wild-type *Xf*. (A) Up-regulated grape genes ( $P < 0.05$ ) in response to *wzy* mutant or wild-type bacteria at 8 and 24 hours post-inoculation (hpi) when compared to the wounded control (c). Genes are classified into nine groups (I - IX) based on their expression pattern. The colors in the heat map represent the Z score of the normal counts per gene, and black boxes represent gene groups in each treatment that exhibited the most pronounced differences in expression at each time point. (B) Enriched grape functional pathways ( $P < 0.05$ ) among genes up-regulated during *wzy* (Group I) or wild-type (Group IV) infections at eight hpi. (C) Enriched grape functional subcategories ( $P < 0.05$ ) among genes up-regulated during *wzy* (Group II) or wild-type (Group V) infections at 24 hpi. Colored stacked bars represent individual pathways. Red boxes highlight functions of interest (\*) that are enriched in one treatment, but not enriched in the other at each time point.



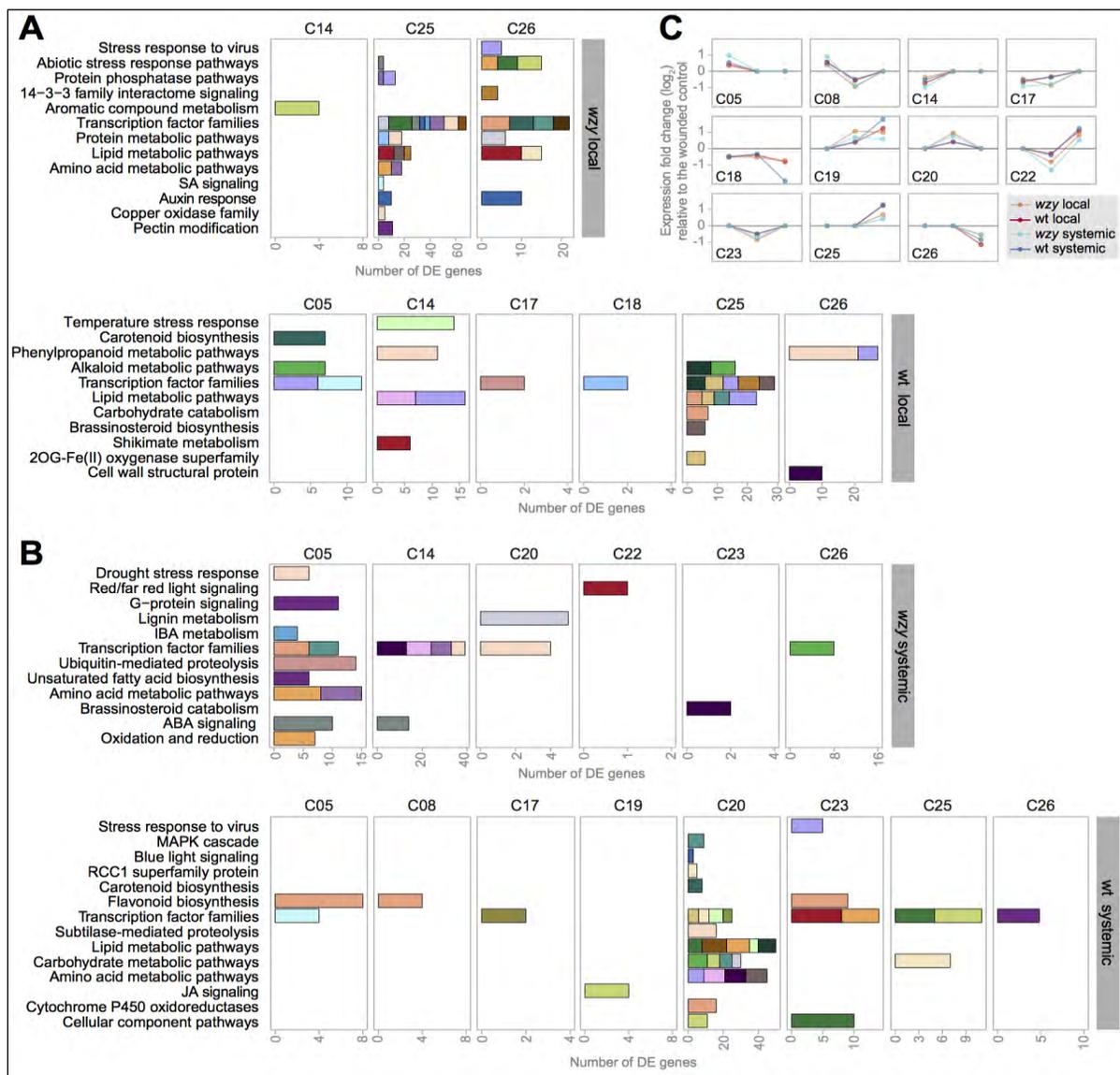
**Figure 3. Expression fold-changes of early response genes in LPS treated plants.** Expression fold-changes of early response genes observed in *V. vinifera* Cabernet Sauvignon grapevines treated with wild-type or *wzy* mutant LPS or diH<sub>2</sub>O. Genes 1-9 correspond to: *VIT\_11s0052g01780* (1-deoxy-D-xylulose-5-phosphate synthase), *VIT\_00s0253g00040* (monocopper oxidase), *VIT\_08s0040g02200* (peroxidase ATP2a), *VIT\_01s0127g00400* (polygalacturonase), *VIT\_14s0060g00480* (S-adenosylmethionine synthetase 1), *VIT\_13s0067g02360* (peroxidase, class III), *VIT\_11s0052g01650* (pathogenesis-related protein 1 precursor), *VIT\_04s0008g00420* (clavata 1 receptor kinase), and *VIT\_11s0052g01150* (nicotianamine synthase), respectively.

### Objective 1b. Transcriptome Profiling

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

In the summer of 2015, individual vines were inoculated with either wild-type *Xf*, the *wzy* mutant, or with 1x PBS buffer (Clifford et al. 2013). We inoculated three vines for each treatment. The cells were delivered mechanically by inoculating a 40  $\mu$ l drop of a 10<sup>8</sup> colony-forming unit/ml bacterial cell suspension into the main stem near the base of the plant. Petioles were harvested at two different locations on the plant: at the point of inoculation (local), and five nodes above the point of inoculation (systemic). We harvested at four different time points post-inoculation: time 0 = petiole harvested just before pre-treatment, 48 hours, one week, and four weeks post-inoculation. All harvested petioles were immediately frozen in liquid nitrogen, prior to RNA extraction. RNA was extracted from the harvested petioles and sequencing libraries were generated from the polyadenylated plant messenger RNA and sequenced using the Illumina HiSeq 2000 platform. Transcript expression levels were determined by alignment of the sequencing reads using the spliced transcripts alignment to a reference (STAR) aligner (Dobin et al. 2013) onto the PN40024 grape genome reference. Unmapped reads were *de novo* assembled using Trinity (Grabherr et al. 2011) to identify transcripts that were not present in the reference genome. Statistical inference using DESeq2 (Anders and Huber 2010) was applied to determine with confidence the subset of genes that were up- or down-regulated by LPS treatment (Cantu et al. 2011b). Grape genes with significant differential expression were grouped into 26 clusters according to their patterns of expression across time points (**Figure 4**). Local tissue of *wzy*-infected plants induced genes enriched in cell wall metabolism pathways,

specifically pectin modification, at four weeks post-inoculation (**Figure 4A**). This is a stark contrast with wild-type-inoculated vines, in which these pathways were up-regulated as early as eight hours post-inoculation. This likely explains why this pathway is not enriched in local tissue of wild-type inoculated vines at these later time points. The induction of salicylic acid (SA)-mediated signaling pathways in *wzy*-inoculated vines was further supported by the presence of four genes, including two enhanced disease susceptibility 1 (EDS1) genes, *VIT\_17s0000g07370* and *VIT\_17s0000g07420*. EDS genes are known defense genes associated with the SA pathway and have been implicated in grapevine defenses against powdery mildew. The consistent enrichment and up-regulation of SA-associated genes (and thus, the maintenance of the signal), including the presence of PR-1 and other SA-responsive genes at eight hours post-inoculation, strongly suggests that the plant is preventing the development of infections by *wzy* cells via an SA-dependent pathway. In wild-type vines, consistent enrichment of jasmonic acid (JA)-associated genes was further supported by the presence of nine genes functioning in the metabolism of alpha-linolenic acid, which serves as an important precursor in the biosynthesis of JA (**Figure 4A**).



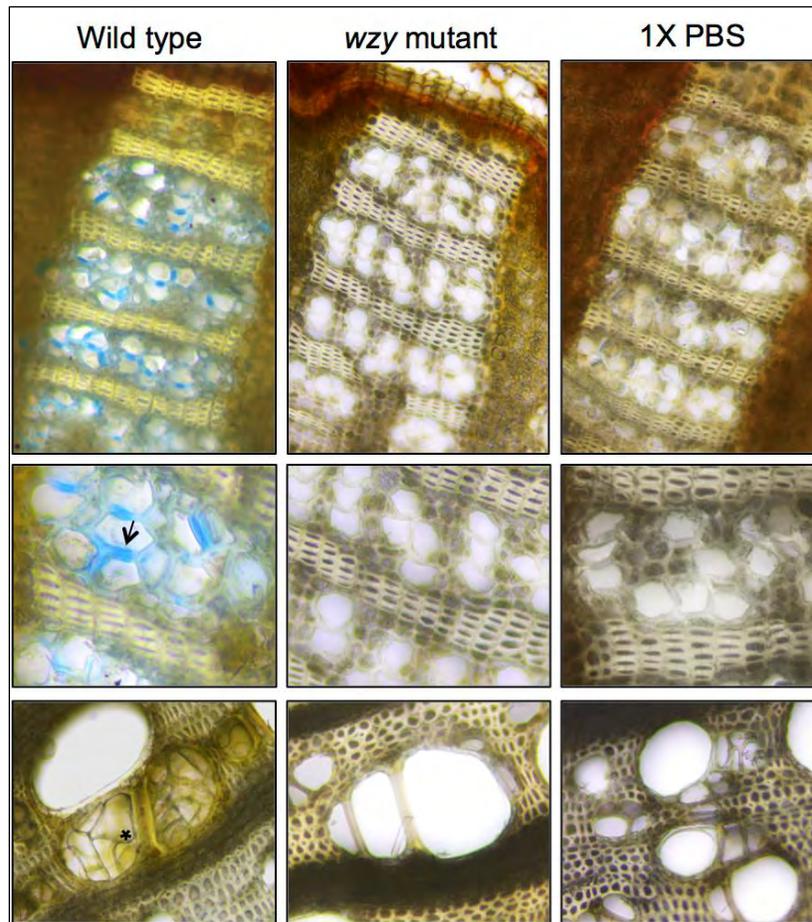
**Figure 4.** Transcriptomic analysis of late grapevine responses to *Xf* wild-type and *wzy* mutant strains in local and systemic tissue. Enriched grape functional pathways ( $P < 0.05$ ) in differentially expressed (DE) gene clusters representing local (A) or systemic (B) responses to *Xf* inoculation.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

Enrichment analyses of *wzy*-responsive genes in systemic tissue included drought stress response pathways, namely genes enriched in abscisic acid signaling (seen at 48 hours post-inoculation) (**Figure 4B**). Subsequently at one week post-inoculation, the enrichment of lignin metabolism genes is likely part of the vine's stepwise response to this abiotic stress. This is in contrast with wild-type inoculated vines in which these pathways were enriched at eight hours post-inoculation. Enrichment analysis of wild-type responsive genes in systemic tissue included regulation and signaling pathways, including mitogen-activated protein kinase (MAPK) and guanine nucleotide-binding (G) protein signaling (**Figure 4B**). Furthermore, genes enriched in ethylene responsive factor (ERF) transcription factors were up-regulated at four weeks post-inoculation, demonstrating that activation of ethylene-mediated signaling is perpetuated during the infection process. Notably, beginning at one week, genes enriched in JA-mediated signaling pathways were up-regulated in systemic tissue, and expression continued to increase at four weeks post-inoculation. This consistent enrichment and up-regulation provides further support for the role of JA in grapevine responses to wild-type *Xf*. Our findings establish that this phytohormone pathway is initiated within the first 24 hours post-inoculation, and the signal is consistently maintained in both local and systemic tissue. A total of seven genes enriched in callose biosynthesis were up-regulated at four weeks post-inoculation, in response to wild-type cells, which is over half of the total callose-related genes in the genome. The consistent up-regulation of these genes (beginning at 24 hours post-inoculation) establishes this structural barrier as an important plant defense response to *Xf* infection.

We hypothesize that the intense *wzy*-induced oxidative burst during the first 24 hours post-inoculation, in combination with other pathogenesis-related responses, had a profound antimicrobial effect on invading *wzy* cells. These responses likely eliminated a large majority of *wzy* mutant populations, and the plant no longer sensed these cells as a biotic threat. In contrast, following recognition of wild-type *Xf* cells at 24 hours post-inoculation, grapevines began responding to an active threat and initiated defense responses, such as the production of phytoalexins and other antimicrobial compounds. Furthermore, these vines were actively trying to prevent systemic spread of the pathogen through the production of structural barriers, such as tyloses and callose.

### **Objective 1c. Histological Examination of Grapevines Inoculated with *Xf* Wild-Type or the O Antigen Mutant**

We performed histological examination of stem tissue in grapevines inoculated with *Xf* wild-type or *wzy* mutant or 1x PBS control to corroborate the enrichment of plant cell wall metabolic pathways seen in the transcriptomic data. Vascular occlusions are commonly produced by plants in response to infection with vascular pathogens. Tyloses are outgrowths of the xylem parenchyma cell into the vessel lumen, and are abundant in Pierce's disease-susceptible grapevines. In fact, in susceptible grape genotypes tyloses can occur in over 60% of the vessels in a transverse section of vascular tissue (Sun et al. 2013). Tylose formation is considered a late response to *Xf*. Thus, we examined tylose formation in grapevines at 18 weeks post-inoculation with wild-type or *wzy* mutant *Xf* cells, compared with 1x PBS control vines. *Wzy* mutant-inoculated vines rated a 2 or below, representing a few leaves exhibiting marginal necrosis; wild type-inoculated vines rated over 3, representing over half of the vine exhibiting foliar necrosis; and 1x PBS controls rated 0, showing no Pierce's disease symptoms. We observed pronounced differences in the abundance of tyloses in response to wild-type vs *wzy* mutant-inoculated plants. In wild-type inoculated vines tyloses were present in nearly all xylem vessels, and vessels were often completely occluded with multiple tyloses. In contrast, *wzy* mutant-inoculated vines contained very few tyloses. In the case where a tylose was present, it was often one large tylose that only partially occluded the vessel (data not shown). All control vines, inoculated with 1x PBS, were free of occlusions. In addition to tyloses, the plant vascular tissue can initiate additional reinforcement of the cell walls to limit bacterial growth in infected plants. This includes callose and suberin deposition. Light microscopy of infected stems revealed widespread deposition of callose in the phloem tissue of *Xf* wild-type infected plants (**Figure 5, arrow**), suggesting communication between the xylem and phloem regarding the presence of *Xf*. This is the first evidence of callose production in grapevine in response to *Xf*. In addition, we also provide the first evidence of a pronounced deposition of suberin, associated specifically with tylose-occluded vessels (**Figure 5\***). In contrast, *wzy* mutant-infected plants showed little to no evidence of either callose or suberin in the vascular tissue, and these plants looked similar to 1x PBS control plants.



**Figure 5.** Callose and suberin deposition in Pierce's disease infected grapevines. Images represent grapevines at 18 weeks post-inoculation, treated with wild-type *Xf* cells, *wzy* mutant cells, or 1x PBS buffer. Wild-type inoculated plants exhibited widespread callose deposition in the phloem tissue (appears as blue color, indicated by arrow). In addition, there was pronounced deposition of suberin in xylem vessels (indicated by gold color), especially in vessels with multiple tyloses (\*). No callose or suberin was present in the stems of 1x PBS-inoculated vines.

### Objective 1d. Global sRNA Profiling

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

### Construction and Sequencing of sRNA Libraries

We have isolated sRNAs from the petioles harvested from the same plants that were inoculated in objective 1a, using an optimized Trizole extraction protocol that allows for isolation of mRNA as well as of sRNAs, for RNA-seq and small RNA-seq analyses, respectively (Cantu et al. 2010). sRNA libraries were produced using the TruSeq Small RNA Sample Preparation Kit and subjected to multiplex sequencing using an Illumina HiSeq2500 platform. Adapters were trimmed using CLC Genomics Workbench. Approximately 116 million RNA reads with length ranging from 18 to 26 nucleotides (nt) were obtained. In all samples, reads showed a similar and expected pattern of size distribution with peaks at 21 and 24 nt. These reads corresponded to an average of one million of unique small RNA sequences per sample. Protein coding gene targets in the *V. vinifera* PN40024 genome could

be identified unambiguously for 20% of the small RNA sequences. An average of 4,557 gene targets per sample were identified. The small RNA sequences included 134 of the known *Vitis* microRNAs. As recently reported by Kullán et al. (2015 <http://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-015-1610-5>), the vvi-miR166 family was the most abundant, representing about 94% of the total expression counts. Further work will be carried out to identify small RNAs that accumulate differentially in plants inoculated with the different *Xf* strains.

### **Objective 2. Examination of *Xf* Lipopolysaccharide-Mediated Defense Priming in Grapevine**

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

#### **Objective 2a. Temporal Persistence of LPS-Mediated Defense Priming**

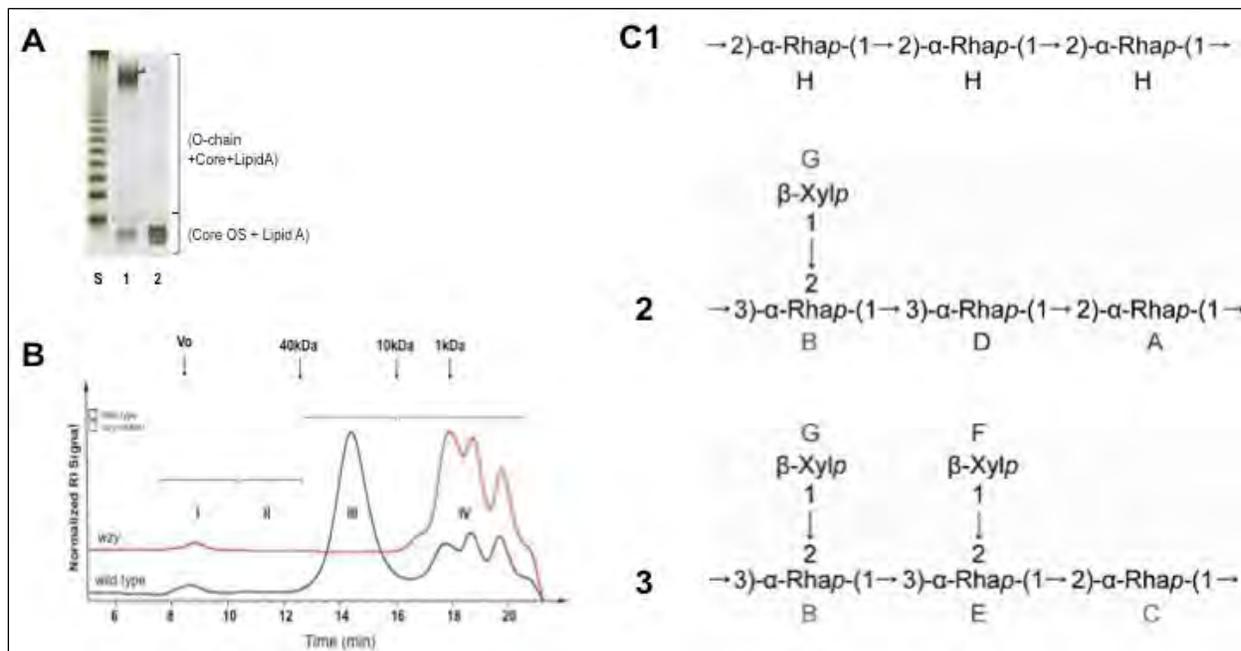
In the summer of 2015, we inoculated 20 grapevines/treatment/time point with 50 µg/ml of either wild-type or *wzy* mutant LPS re-suspended in diH<sub>2</sub>O. Vines inoculated with diH<sub>2</sub>O alone served as the negative controls for the experiment. Based on our previous greenhouse trials, we have found that 50 µg/ml is a suitable concentration to elicit an oxidative burst and to potentiate defense priming in grapevines. This is also in agreement with studies performed in *A. thaliana* (Zeidler et al. 2004). Thus, we used the same LPS concentration for this objective. The LPS was delivered by needle-inoculating a 40 µl drop of the LPS preparation into the main stem at the base of the plant. We then challenged 15 of the vines for each treatment by inoculating a 40 µl of a 10<sup>8</sup> colony-forming unit/ml suspension of live wild-type *Xf* cells in 1x PBS at either four hours, 24 hours, 48 hours, one week, or four weeks post-LPS treatment. The remaining five vines/treatment/time point were inoculated with 1x PBS to serve as negative controls. We included the additional later time points (48 hours, one week, and four weeks) because we also wanted to establish the duration of the priming effect following treatment with LPS. These inoculations were performed using the pin-prick method as previously described (Hill and Purcell 1995). The live wild-type cells were inoculated near the point of the original LPS inoculation. Plants were visually examined for Pierce's disease symptom development throughout the infection process and rated on an arbitrary disease rating scale of 0-5 where 0 = healthy and 5 = dead or dying (Guilhabert and Kirkpatrick 2005). Data was consistent with the previous year for the four and 24 hour time points, but we did not see significant attenuation of Pierce's disease symptoms in the remaining later points. This indicates that the primed state may be transient, and it is possible that these plants may need repeated applications of LPS throughout the trial to help maintain the primed state.

### **Objective 3. Linking *Xf* Lipopolysaccharide Structure to Function**

We have obtained structural data for both wild-type and the truncated *wzy* mutant LPS, particularly the structure of O-chain by using gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) spectroscopy. These experiments were conducted in close collaboration with the Complex Carbohydrate Research Center (CCRC) at the University of Georgia, Athens, GA. Through glycosyl composition analysis [trimethylsilyl methyl glycosides (TMS); alditol acetates (AA)] (York 1985) of the LPS and composition and linkage analysis [partially methylated alditol acetates (PMAA)] (Ciucanu and Kerek 1984) of O-specific polysaccharide, the CCRC has confirmed that the *Xf* wild-type high molecular weight O-antigen is comprised primarily of 2-linked rhamnose, verifying previously reported *Xf* LPS compositions (Clifford et al. 2013). We have also confirmed that the *wzy* mutant LPS is lacking the high molecular weight O antigen present in wild-type cells and appears to be capped with a single rhamnose residue (**Figure 6A**). The CCRC has recently completed extensive isolation and purification of core and O-chain polysaccharides. Knowledge of the structure of the LPS is critical to understanding which portions contain the elicitor activity. The carbohydrate portion of LPS (core + O-chain) was released from lipid A by mild acid hydrolysis, and the O-chain was purified by size exclusion and other chromatography techniques. A structure of the polymer was determined via NMR spectroscopy and mass spectrometry, and absolute configuration of sugars (d-, l-) in the polymer was determined by GC-MS (Gerwig et al. 1978).

To describe structural properties of O antigen in wild-type and *wzy* mutant LPS, the polysaccharide moiety (O antigen + core) was liberated from LPS (lipid A) and resolved based on molecular size. Comparative analysis of size-exclusion chromatography (SEC) profiles indicated different distributions of polysaccharides in both strains. In the wild-type strain, most of the polysaccharide (40.8% total column load) was eluted in Fraction III (average

molecular mass of approximately 10-20 kDa) and a remainder (24.8% of total column load) in Fraction IV (**Figure 6B**). In contrast, most of the *wzy* polysaccharide (55.0% total PS column load) was eluted in Fraction IV (average molecular mass below 10 kDa), which was only present in low quantity in the wild-type parent. This fraction likely represented different molecular size forms of core oligosaccharide or truncated core-O antigen polysaccharide. Fraction I that was eluted in void (Vo) column was due to traces of unhydrolyzed, intact LPS. Monosaccharide analysis, including the determination of absolute configurations of O antigen polysaccharides from the wild-type strain (SEC fraction III), confirmed the presence of L-rhamnose and D-xylose in an 8:1 molar ratio. Based on methylation analysis and 1D/2D NMR data, we present the first evidence that the major polysaccharide present in *Xf* wild-type O antigen is a linear  $\alpha$ 1-2 linked rhamnan (**Figure 6 C1**). We also have evidence that *Xf* wild-type cells maintain a heterogeneous population of O polysaccharides. Combining all analytical data, a repeat unit of the second polymer consists of  $\alpha$ -L-rhamnan backbone substituted with either two or one  $\beta$ -D-Xyl residues (**Figure 6 C2, C3**). Additional analysis will need to be conducted to determine if these substitutions are autonomous LPS molecules on the cell surface or if they are linked to the same core oligosaccharide as the primary linear  $\alpha$ 1-2 linked rhamnan structure.



**Figure 6.** LPS composition and structure analysis. (A) DOC-PAGE analysis of LPS isolated from *Xf* wild-type and *wzy* mutant. Lane S = *Salmonella enterica* s. *Typhimurium*, S-type LPS; Lane 1 = wild-type; Lane 2 = *wzy* mutant. Red arrow indicates the presence of high molecular weight O antigen that is not observed in the *wzy* mutant LPS. (B) SEC chromatograms of polysaccharides liberated from LPS of *Xf* wild-type (black) and *wzy* mutant (red). Standard dextrans of 40,000, 10,000, and 1,000 Da were used for calibration of the Superose 12. (C1) The structure of *Xf* wild-type O antigen polymer is composed primarily of a linear  $\alpha$ 1-2 linked rhamnan. A repeat unit of the second polymer consists of  $\alpha$ -L-rhamnan backbone substituted with either one (C2) or two (C3)  $\beta$ -D-Xyl residues. O antigen from the *wzy* mutant is predicted to contain a single rhamnose residue.

## CONCLUSIONS

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

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

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

**ABSTRACT**

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

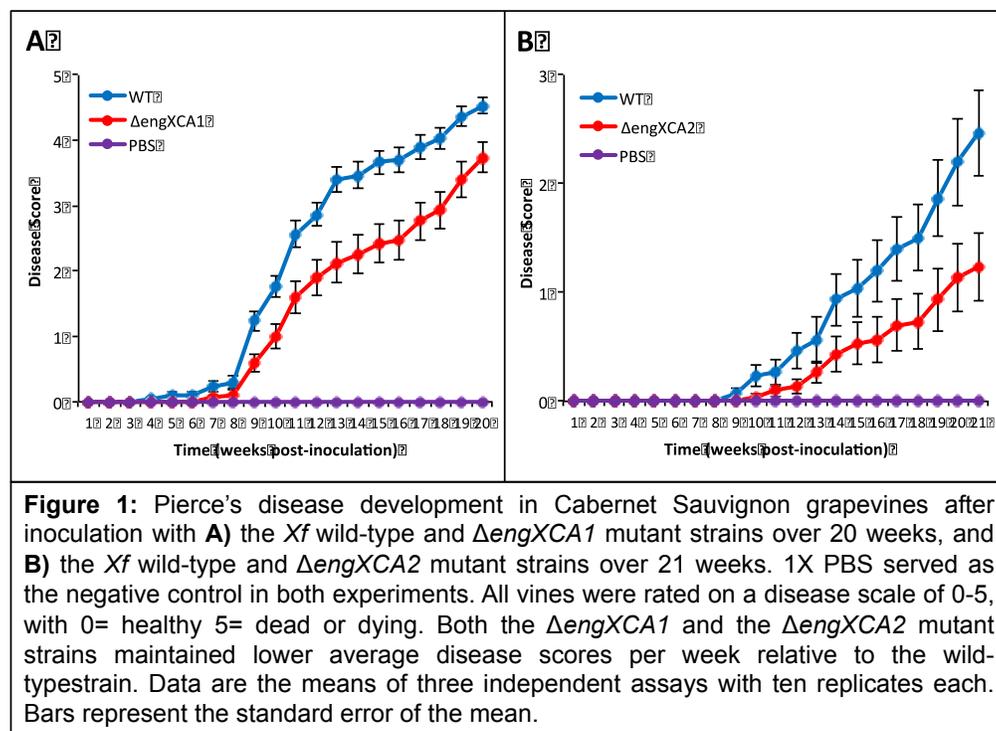
**LAYPERSON SUMMARY**

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

**INTRODUCTION**

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

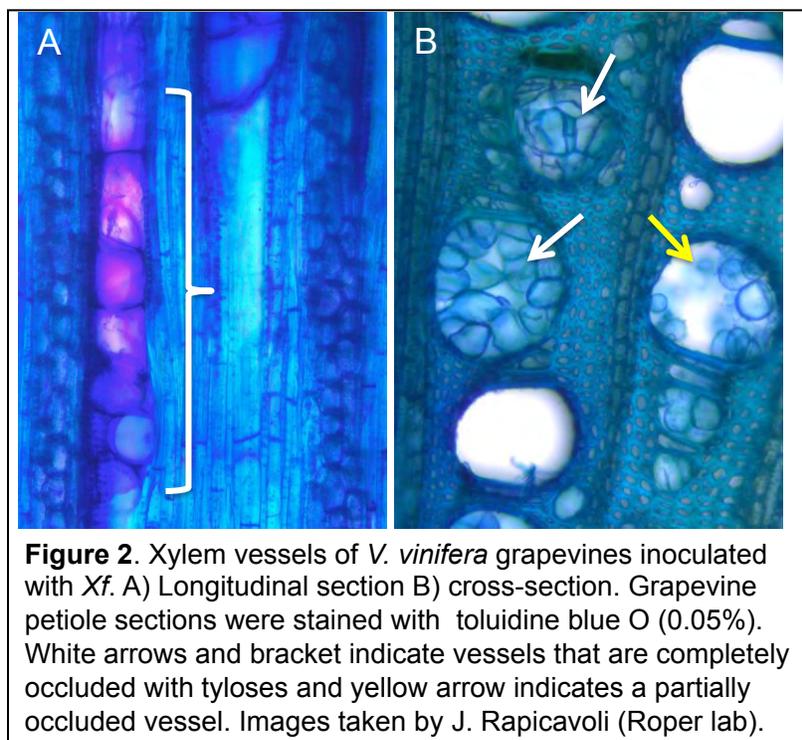
pectin and hemicellulose (Buchanan 2000). The pore sizes within that meshwork range from 5 to 20 nM, which will not allow passive passage of *Xf* cells whose size is 250-500 x 1,000-4,000 nM (Perez-Donoso et al. 2010, Mollenhauer & Hopkins 1974). Based on functional genomics and *in planta* experimental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



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

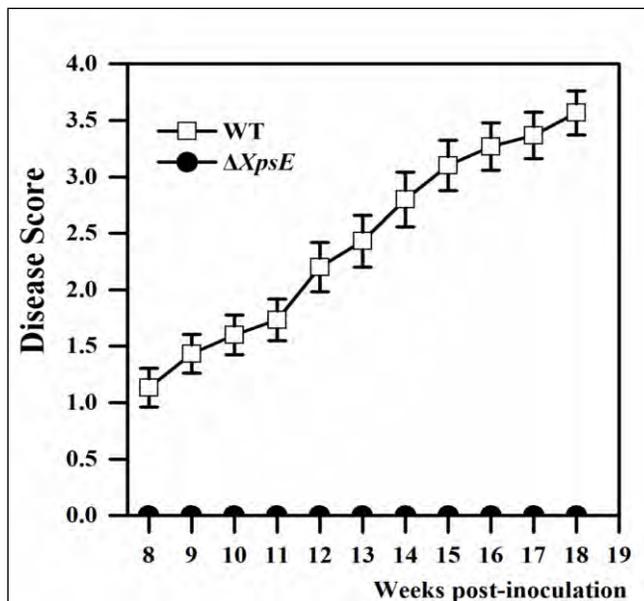
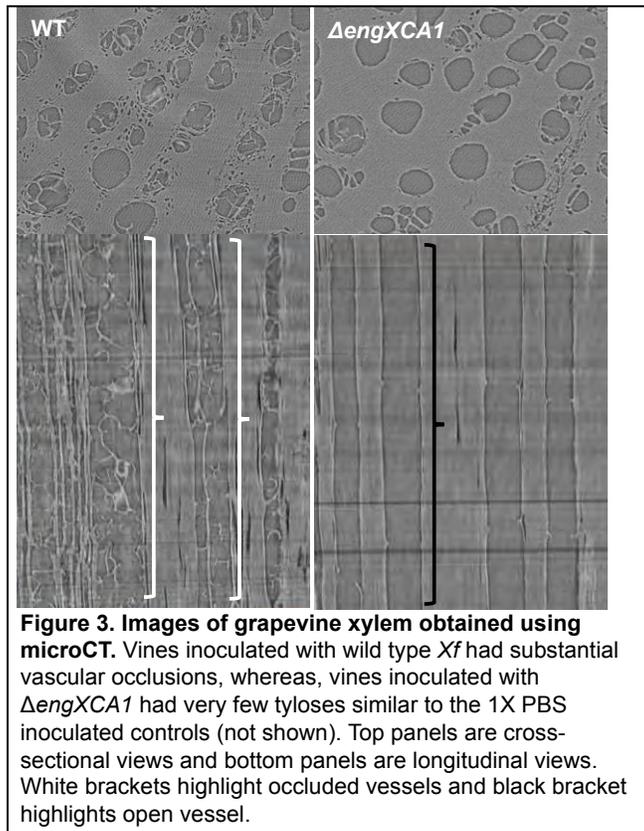
Tyloses are outgrowths of parenchyma cells that emerge through vessel-parenchyma pits into vessel lumen, and are common in a wide range of species (Bonsen and Kučera 1990, Esau 1977, Tyree and Zimmermann 2002). Tyloses impede fluid penetration (Parameswaran et al. 1985) and induce a permanent state of reduced hydraulic

conductivity, and are triggered by abiotic and biotic stresses, such as pathogen infection (Aleemullah and Walsh 1996, Collins et al. 2009, Dimond 1955, Parke et al. 2007). Tylose formation is the predominant vascular occlusion associated with *Xf* infection (**Figure 2**), and excessive tylose development has been linked to the extreme susceptibility of *V. vinifera* winegrapes to Pierce's disease (Fritschi et al. 2008, Sun et al. 2013). Importantly, rates of tylose development in *V. arizonica*, a resistant species, are much lower than those in *V. vinifera*, which may reflect differing innate immune responses to the presence of *Xf* in the xylem. To our knowledge, no one has looked at the molecular mechanisms underlying the differences in response to *Xf* among different *V. vinifera* cultivars. Thus, we propose to better understand this difference in cultivar response to *Xf* in the context of host cell wall degradation and the elicitation of specific defense responses that lead to tylose formation in grapevines. Interestingly, a preliminary analysis of tylose formation in Cabernet Sauvignon vines inoculated with the  $\Delta engXCA1$  mutant using a high resolution microCT technique (a kind of computerized axial tomography scan) by the McElrone laboratory determined that these vines exhibited fewer tyloses than those inoculated with wild-type *Xf* (**Figure 3**). Therefore, our hypothesis is that enzymatic degradation of the plant cell wall by *Xf* CWDEs is generating cell wall fragments that elicit damage-associated molecular pattern signaling defense pathways, which leads to downstream tylose production and Pierce's disease symptom development in certain grape cultivars.



Given that *Xf* CWDEs are important for the degradation of pit membranes (thus allowing systemic colonization) and their potential role in inducing tylose formation, it is imperative that these virulence factors are targeted for inhibition. However, inhibiting each CWDE individually as a commercial strategy for controlling *Xf* is difficult. Interestingly, these CWDEs are predicted (using SignalP software) to be secreted via the Type II secretion system (T2SS). The T2SS is a molecular nanomachine that transports pre-folded proteins from the periplasm across a dedicated channel in the outer membrane (Cianciotto 2005, Korotkov et al. 2012). The T2SS systems of many plant and animal pathogens are either known or predicted to secrete proteins, namely polymer degrading enzymes, which are involved in nutrient acquisition (Jha et al. 2005). The *Xf* CWDEs being studied in this project are predicted (using SignalP software) to be secreted through the T2SS. Proteins destined for secretion by the T2SS are first delivered to the periplasm via the Sec or Tat-dependent secretion pathway where they are folded (Slonczewski 2014). *Xf* appears to only possess the Sec-dependent secretion pathway. Because of our interest in host CWDEs and their mechanism of secretion, we created a mutation in the *xpsE* gene which encodes the putative ATPase that powers the T2SS. Grapevines inoculated with the *xpsE* mutant never developed Pierce's

disease symptoms and remained healthy, a phenotype similar to the grapevine response to the *Xf pglA* mutant (Figure 4). We hypothesize that this is due to the pathogen's inability to secrete the CWDEs necessary for xylem colonization.



**Figure 4.** The *Xf* T2SS is necessary for Pierce's disease development in grapevine. The  $\Delta xpsE$  mutant does not incite Pierce's disease symptoms in *V. vinifera* grapevines. Disease severity was based on a visual disease scale from 0 (no disease) to 5 (dead). Vines inoculated with 1x phosphate buffered saline (PBS) did not develop Pierce's disease symptoms.

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

## OBJECTIVES

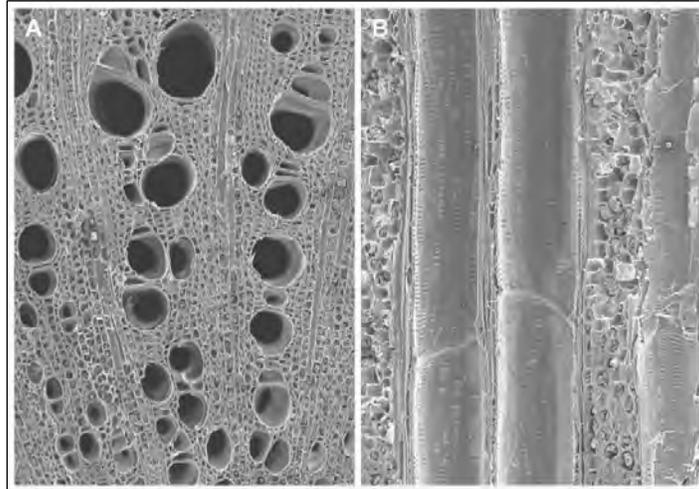
1. Qualitative analysis of the effect of cell wall degradation on the grapevine response to *Xf*.
2. Quantitative analysis of plant defense pathways induced by *Xf* cell wall degrading enzyme activity: Biochemical and transcriptional studies.
3. Inhibition of the Type II secretion system using natural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

## RESULTS AND DISCUSSION

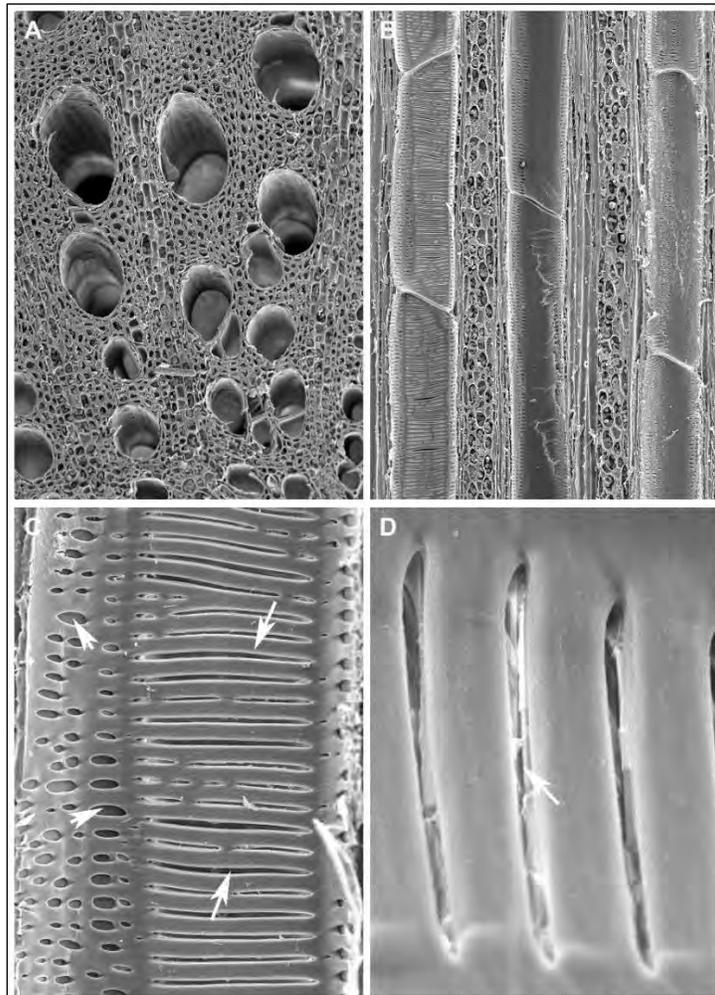
We are examining the effects that different *Xf* CWDE mutants [ $\Delta engXCA1$ ,  $\Delta engXCA2$ , *egl* (all EGases and EGase/expansin hybrid) and *pglA* (a polygalacturonase)] have on integrity and carbohydrate composition of grapevine pit membranes using both microscopic and immunological techniques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

Wild-type *Xf*,  $\Delta engXCA1$ ,  $\Delta engXCA2$ , and  $\Delta pglA$  mutants have been used to inoculate Cabernet Sauvignon and Chardonnay grapevines in the greenhouse. PBS-inoculated vines were used as negative controls. Each *Xf* strain was inoculated into 27 plants and Pierce's disease symptoms were rated each week using the 0 to 5 Pierce's disease rating index (Guilhabert and Kirkpatrick 2005). Vine tissue samples from 2016 and 2017 have been collected for each of the three experiments: Stem and petiole tissue for RNAseq, stem tissue for microCT analysis, and stem explants for electron microscopy analysis. Samples from three biological replications (consisting of three technical replications) per treatment have been collected at three time points covering early infection, mid-infection, and late infection based on the Pierce's disease rating index (early infection = 1-2, mid-infection = 2-3, late infection = 3-4).

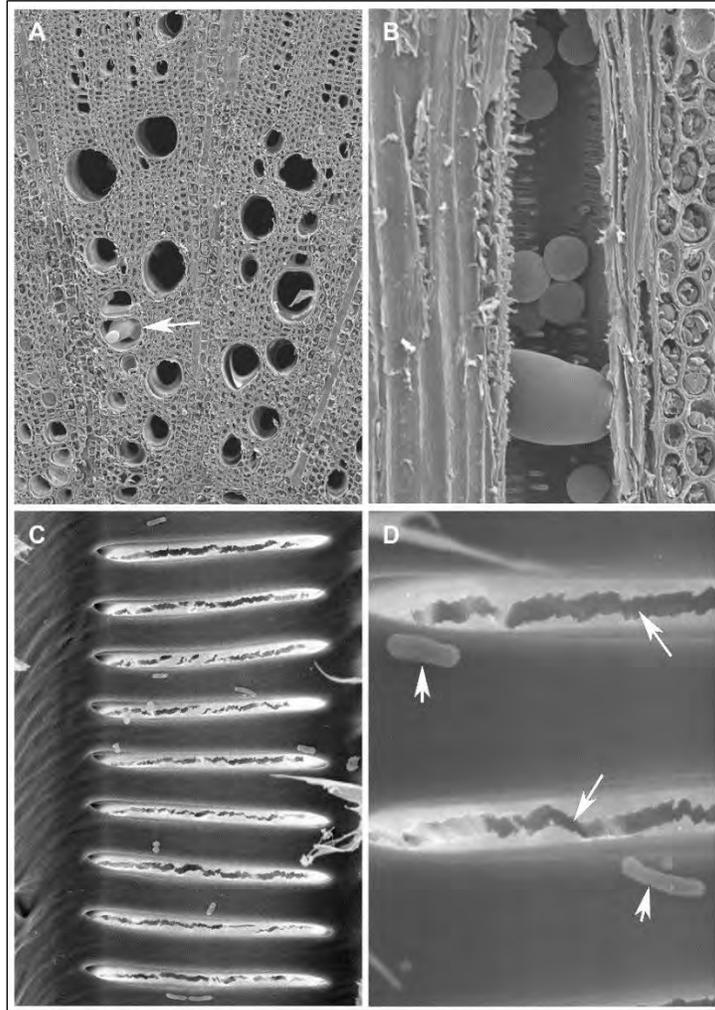
Early time point samples from Chardonnay (2016) were analyzed using scanning electron microscopy to study vascular occlusion, pit membrane integrity, and presence/absence of *Xf* in the xylem tissue after inoculation with wild-type *Xf*, the PBS negative control, the  $\Delta engXCA1$  mutant, or the  $\Delta pglA$  mutant. Our results indicate that no vascular occlusions have been observed in the vines inoculated with PBS (**Figure 5**), wild-type (Temecula 1, **Figures 6A and 6B**), and  $\Delta pglA$  (**Figure 8A**) *Xf*, respectively. Tyloses were found in very few vessels of the vine inoculated with  $\Delta engXCA1$  *Xf* (**Figures 7A and 7B**), but were at the early developmental stages and did not occlude the vessels where they occurred. *Xf* cells were not observed in all the vines except those inoculated with  $\Delta engXCA1$  *Xf* (**Figure 7D**). Vessel-parenchyma pit membranes were intact in the vines with the four different inoculums (**Figures 5B and 6C**). Some broken (degraded) intervessel pit membranes were observed in the vine inoculated with either wild-type (**Figures 6C and 6D**) or  $\Delta engXCA1$  *Xf* (**Figures 7C and 7D**), but were rare or absent in the vine inoculated with either PBS or  $\Delta pglA$  *Xf* (**Figure 8C**). This electron microscopy data is only a small subset of the dataset, as the rest of the samples are still being analyzed.



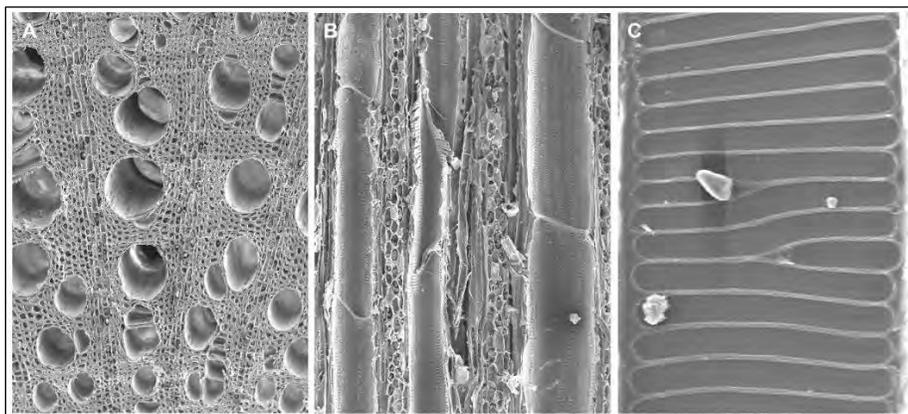
**Figure 5.** Vessel structural features in a Chardonnay stem inoculated with PBS buffer only. (A) Transverse section of secondary xylem showing absence of vascular occlusion in the vessels. (B) Tangential longitudinal section of secondary xylem, showing three transected vessels that have intact vessel-parenchyma pit membranes and do not contain vascular occlusions.



**Figure 6.** Vessel structural features in a Chardonnay stem inoculated with wild-type *Xf* (Temecula 1). (A) Transverse section of secondary xylem showing absence of vascular occlusion in the vessels. (B) Tangential longitudinal section of secondary xylem. Vessels do not contain vascular occlusions. (C) A transected vessel, showing oval vessel-parenchyma pit pairs and intact pit membranes (short arrows) and scalariform intervessel pit pairs (long arrows). (D) Broken intervessel pit membranes.

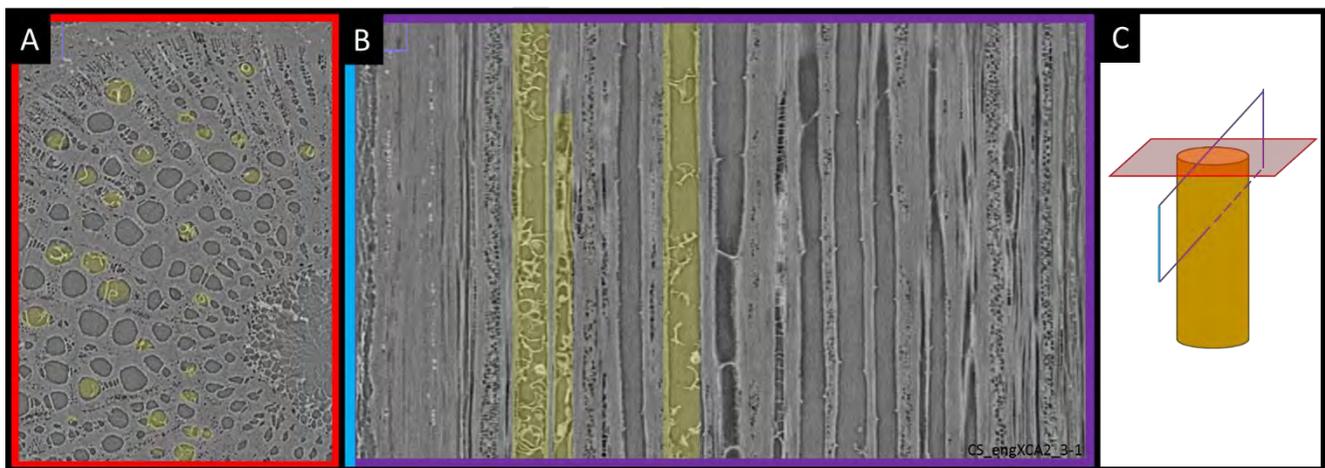


**Figure 7.** Vessel structural features in a Chardonnay stem inoculated with *ΔengXCA1 Xf*. (A) Transverse section of secondary xylem. Most vessels have empty lumens but few vessels are filled with tyloses (arrow). (B) Tangential longitudinal section of secondary xylem, showing a transected vessel with developing tyloses inside. (C) Scalariform intervessel pit pairs in a vessel lateral wall. (D) Enlargement of several intervessel pit pairs in a vessel lateral wall. Broken intervessel pit membranes (long arrows) are seen from a pit aperture and *Xf* cells (short arrows) are present on the lateral wall.



**Figure 8.** Vessel structural features in a Chardonnay stem inoculated with *ΔpglA Xf*. (A) Transverse section of secondary xylem. All the vessels are free of vascular occlusions. (B) Tangential longitudinal section of secondary xylem, showing several transected vessels without vascular occlusion. (C) Surface view of a vessel's lateral wall. Whole intervessel pit membranes are visible after removal of secondary wall borders of intervessel pits. Intervessel pit membranes are intact and are horizontally elongated, and they have a ladder-like arrangement along the vessel axial direction.

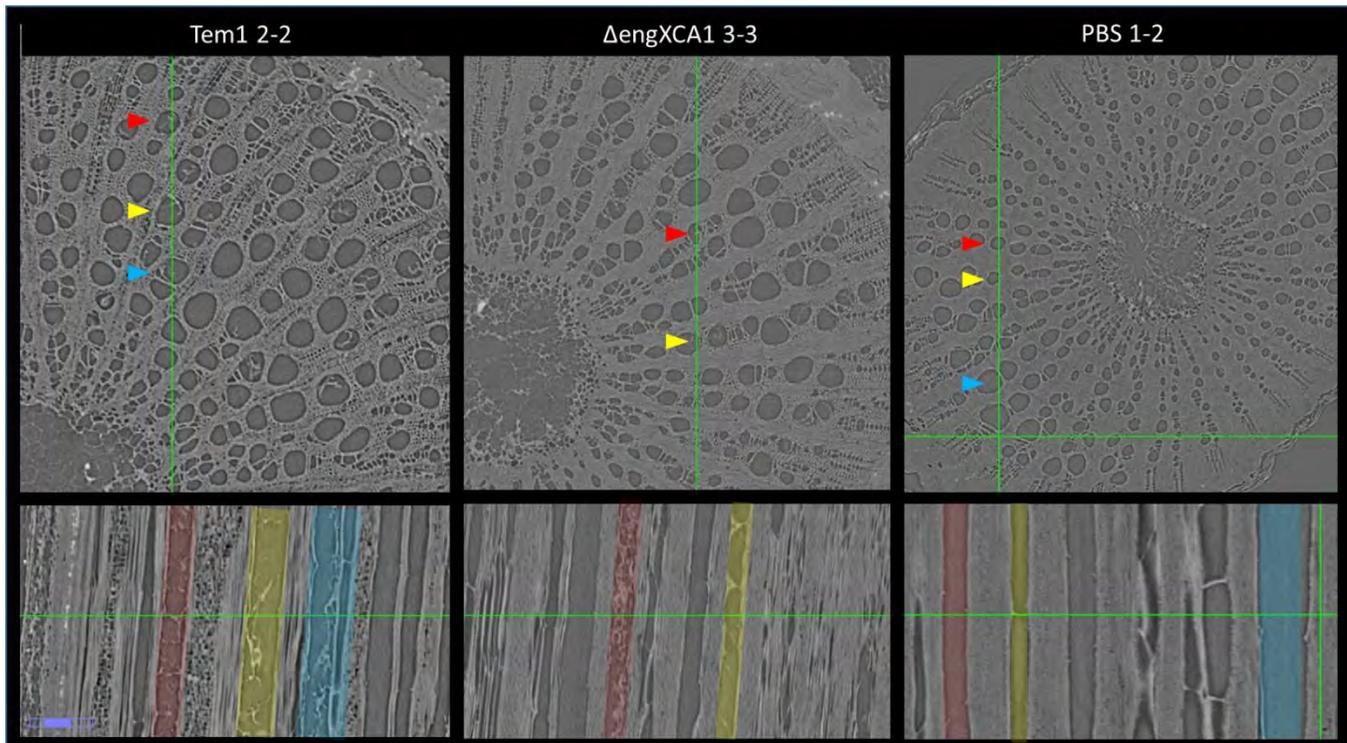
In addition to samples imaged via electron microscopy, samples from the early and middle time points in both Chardonnay and Cabernet Sauvignon have also been analyzed by microCT. This technique is particularly resource-intensive, and thus, imaging all nine samples per treatment was not feasible. Instead, three samples per treatment were chosen randomly, and singular midslice images were analyzed to determine if tyloses formed in the xylem in response to *Xf* infection. Of the analyzed stems, both Chardonnay and Cabernet Sauvignon vines (2016) inoculated with wild-type (Temecula 1) exhibited the most blocked vessels by tyloses, whereas the  $\Delta engXCA1$  and  $\Delta engXCA2$  mutants exhibited fewer tyloses (data not shown). Additionally, vines inoculated with the wild-type Fetzter strain and the  $\Delta pglA$  mutant exhibited very few tyloses, and vines inoculated with PBS (negative control) displayed no tyloses. Transverse and longitudinal images slices of the selected samples from the early time point in Chardonnay were also performed to visualize tylose formation (**Figure 9**). Several vessels from vines inoculated with wild-type (Temecula 1) displayed tyloses, while fewer vessels were occluded in vines inoculated with the  $\Delta engXCA1$  mutant (**Figure 10**). Vessels from vines inoculated with the PBS negative control were occlusion-free and displayed no tylose formation. Analysis of transverse and longitudinal images slices from the other time points in both varieties is expected soon.



**Figure 9.** Improved tyloses detection/quantification. Colored outlines in (A) (xy-axis) and (B) (yz-axis) correspond with (C) to help orient the viewer. Tyloses (highlighted in yellow) are relatively small and rare features relative to empty vessels on the xy-axis, and can easily be confused with interconnected vessels, yet appear more distinctly in the yz-axis.

### Quantitative Analysis of Plant Defense Pathways Induced By *Xf* Cell Wall Degrading Enzyme Activity: Biochemical and Transcriptional Studies

Pit membrane degradation by *Xf* CWDEs likely results in the release of small chain carbohydrates into the xylem. These oligosaccharides have been known to act as elicitors of plant immunity (i.e. damage-associated molecular patterns). It is possible that oligosaccharides released from pit membrane degradation are being recognized by associated parenchyma cells, triggering defense responses such as tylose production. To test this hypothesis, we are using RNAseq to analyze the grapevine transcriptome to determine if pit membrane degradation products act as elicitors of plant immunity and trigger tylose production. All tissue samples used for RNA extraction were collected from the same plants and time points as used for the qualitative experiments so that we can determine correlations between defense gene expression, pit membrane degradation, and/or tylose production. As these samples come from the same plants used in the qualitative experiments, all treatments, grapevine varieties, sample sizes, and time points used are the same as in the previous section. Currently, stem and petiole tissue for all treatments from each time point and variety (2016 and 2017) have been collected, and are being prepared for RNAseq analysis.



**Figure 10.** ImageJ orthogonal views of tyloses in Chardonnay (early time point) vines inoculated with wild-type (Temecula 1),  $\Delta engXCA1$ , or PBS (negative control). Colored arrows on transverse image slices (top) correspond to highlighted vessels of same color on the longitudinal image slices (bottom), cut from the vertical green line in the transverse image. Empty vessels appear dark gray, while tyloses appear as highly branched membranes within vessel elements. PBS buffer treatment exhibits no tylose formation.

## CONCLUSIONS

All samples from 2016 and 2017 have been collected and are currently being analyzed by electron microscopy, microCT, and RNAseq. As most of the data analysis is still underway, we cannot draw any definitive conclusions at this time. However, preliminary results from the 2016 samples suggest that some of the CWDEs (EngXCA1, EngXCA2, and PglA) have an effect on host tylose production post-infection. Additionally, we speculate that the differences in tylose production between vines inoculated with the mutant strains and vines inoculated with the wild-type strain (Temecula 1) become more apparent as disease progresses. Once our analysis is completed, we hope to provide some insight into the role of host-pathogen interactions in the progression of Pierce's disease.

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# **EXPANDING THE RANGE OF GRAPE ROOTSTOCK AND SCION GENOTYPES THAT CAN BE GENETICALLY MODIFIED FOR USE IN RESEARCH AND PRODUCT DEVELOPMENT**

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**Reporting Period:** The results reported here are from work conducted January 2017 through October 2017.

## **ABSTRACT**

The UC Davis Plant Transformation Facility has previously developed a method for genetically modifying 101-14 and 1103P, two important grape rootstocks for the California grape industry. This technology allows researchers to introduce genes useful in combating Pierce's disease into the rootstocks of grape, allowing researchers to test whether a modified rootstock is capable of conferring resistance to the grafted scion. If rootstock-mediated resistance strategies are to be successfully deployed throughout California additional rootstocks will need to be modified in order to adequately address the rootstock requirements of the diverse winegrape growing regions in California. To that end, we plated anthers from grape rootstocks 110R (clone 01), 140Ru (clone 01), 3309C, Freedom (clone 1), GRN-1(clone 1.1), Harmony, MGT 420A (clone 04), and Salt Creek, as well as scion genotypes Cabernet Sauvignon (clones 07 and 08), Chardonnay (clone 04), French Colombard (clone 04), and Merlot (clone 03). Embryonic cultures have been generated from anther filaments for 110R, 140Ru, Freedom, GRN-1, Harmony, MGT 420A, Cabernet Sauvignon, Chardonnay, French Colombard, and Merlot. In addition, we have successfully established suspension and stored embryo cultures for these grape genotypes.

Transformation experiments using DsRed were initiated on stored embryo cultures in order to access the utility of our existing transformation technologies in transforming these additional genotypes. To date, we have successfully generated transgenic plants for 101-14, 110R, 1103P, Freedom, MGT 420A, and French Colombard. DsRed embryos have been generated for 140Ru, GRN-1, Harmony, and Merlot, and we are attempting to convert these embryos into whole plants. Acclimatization of grape plantlets to soil has been problematic in the past. However, by altering the soil composition used and the stage of development of the rooted plantlet we have significantly improved survival in soil. To date, we have generated over 500 transgenic grape lines for Pierce's disease researchers.

## **LAYPERSON SUMMARY**

The UC Davis Plant Transformation Facility has previously developed a method for genetically modifying 101-14 and 1103P, two important grape rootstocks for the California grape industry. This technology will allow us to introduce genes useful in combating Pierce's disease into the rootstocks of grape and allow us to test whether a modified rootstock is capable of conferring resistance to the grafted scion. This strategy is commonly referred to as rootstock-mediated resistance. If rootstock-mediated resistance strategies are to be successfully deployed throughout California additional rootstock genotypes in addition to 101-14 and 1103P will need to be modified, in order to adequately address the rootstock requirements of the diverse winegrape growing regions in California. We therefore are currently testing if our method for genetically modifying grape rootstocks can be used successfully on eight additional rootstock genotypes used in California winegrape production. These include 110R, 140Ru, 3309C, Freedom, GRN-1, Harmony, MGT 420A, and Salt Creek. Since it is not yet known if a rootstock-mediated disease resistance strategy will prove to confer durable, commercially-viable levels of resistance to the grafted scion, we are also testing our method for modifying grapes on a select group of scions, including Cabernet Sauvignon, Chardonnay, French Columbard, Merlot, Pinot Noir, and Zinfandel. We have made significant progress in establishing embryos in tissue culture for a wide range of scions and rootstocks, including genotypes 110R, 140Ru, Freedom, GRN-1, Harmony, MGT 420A, Cabernet Sauvignon, Chardonnay, French Colombard, and Merlot. We are testing our transformation strategy for its utility in genetically modifying these additional genotypes. To date, we have demonstrated that in addition to 101-14 and 1103P, the rootstocks 110R, 140Ru, Freedom, and MGT 420A and the scion genotype French Colombard can be included in the list of grape genotypes that we can successfully transform. To date, we have produced over 500 genetically modified grape plants across four different varieties to enable investigators to study strategies that may be effective against Pierce's disease. In addition to its utility in producing genetically modified grape plants for testing strategies to

combat Pierce's disease, this work has established a germ bank of cell suspension cultures and a repository of somatic embryos for rootstock and scion genotypes used in California, which can be made available to the grape research community for a wide variety of research purposes.

## INTRODUCTION

The purpose of this project is to apply the progress that has been made in grape cell biology and transformation technology of rootstock genotypes 101-14 and 1103P to additional grape rootstock genotypes in order to expand the range of genotypes amenable to transformation. The research will apply the pre-existing technical expertise developed for rootstocks 101-14 and 1103P at the UC Davis Plant Transformation Facility to additional rootstock germplasm important for the California wine industry. For this project we are testing eight additional rootstocks for their amenability to transformation, including 110R (clone 01), 140Ru (clone 01), 3309C (clone 05), Freedom (clone 1), GRN-1, Harmony, MGT 420A (clone 04), and Salt Creek (clone 8). This work will expand the range of rootstocks that can be effectively transformed, which will allow rootstock-mediated disease resistance technology to be employed across the major winegrape growing regions in California. Although a rootstock-mediated resistance strategy is the preferred mechanism for achieving resistance to Pierce's disease in grape, investing in the development of transformation technology for scions will serve an important fallback position should rootstock-mediated resistance fail to confer adequate levels of resistance to the scion and direct transformation of scion varieties be required. Therefore, in addition to testing the utility of our tissue culture and transformation protocols on eight additional grape rootstocks, we will also screen six important California scion genotypes for their amenability to transformation, including Cabernet Sauvignon (clone 07), Chardonnay (clone 04), French Colombard (clone 02), Merlot (clone 03), Pinot Noir (clone 2A), and Zinfandel (clone 01A). The results of this work will allow for the establishment of grape tissue culture and transformation technologies that can be utilized by the Pierce's disease research community. It will also establish a germplasm bank of cell suspension cultures and a repository of somatic embryos for rootstock and scion genotypes, which can be made available to the research community. We have made significant progress in establishing somatic embryos, suspension cultures, and stored embryo germplasm banks for many of the targeted genotypes. We have now successfully established suspension and stored somatic embryo cultures for grape genotypes 101-14, 110R, 140Ru, 1103P, Freedom, GRN-1, Harmony, MGT 420A, Cabernet Sauvignon, Chardonnay, French Colombard, and Merlot. Based on transformation experiments using DsRed we have produced transgenic embryos for 101-14, 110R, 140Ru, 1103P, Freedom, GRN-1, Harmony, MGT 420A, French Colombard, and Merlot. We have now demonstrated that in addition to 101-14 and 1103P we can generate transgenic plants for rootstock genotypes 110R, 140Ru, Freedom, and MGT 420A, along with the scion variety French Colombard.

## OBJECTIVES

1. Develop embryogenic cultures from anthers of eight rootstock genotypes and six scion genotypes for use in establishing embryogenic suspension cultures.
2. Develop embryogenic suspension cultures for eight rootstock genotypes and six scion genotypes, which will provide a continuous supply of somatic embryos for use transformation experiments.
3. Establish a germplasm bank of somatic embryos for seven rootstock genotypes and six scion genotypes by plating aliquots of the cell suspension culture on high osmotic medium.
4. Test transformation efficiencies of eight rootstock genotypes and six scion genotypes using our established somatic embryo transformation protocols.
5. Test direct cell suspension transformation technology on seven rootstock genotypes and six scion genotypes.
6. Secure *in vitro* shoot cultures for seven rootstock genotypes and six scion genotypes using indexed material or field material from Foundation Plant Services at UC Davis and establish bulk meristem cultures for all 13 genotypes for use in transformation.
7. Test Mezzetti et al. 2002 bulk meristem transformation system for seven rootstock genotypes and six scion genotypes as an alternate to somatic embryo transformation.

## RESULTS AND DISCUSSION

### **Objective 1. Develop Embryogenic Cultures from Anthers of Seven Rootstock Genotypes and Six Scion Genotypes for Use in Establishing Embryogenic Suspension Cultures**

This spring (April 2017) we collected anthers from genotypes for which we were not successful in generating embryos in 2015 or 2016, which include 3309C and Salt Creek. We also harvested anthers from 101-14, 110R, and 1103P since we needed to generate fresh somatic embryo cultures to replace our aging cultures for these

genotypes. The media we used included Nitsch and Nitsch minimal organics medium (1969) supplemented with 60 g/liter sucrose, 1.0 mg/liter 2,4-dichlorophenoxyacetic acid (2,4-D), and 2.0 mg/liter benzylaminopurine (BAP) (PIV), MS minimal organics medium supplemented with 20 g/liter sucrose, 1.0 mg/liter 2,4-D, and 0.2 mg/liter BAP (MSE), MS minimal organics medium supplemented with 30 g/liter sucrose, 1.0 mg/liter 2,4-D, and 1.0 mg/liter BAP (MSI), or one half strength MS minimal organics medium supplemented with 15 g/liter sucrose, 1.0 mg/liter NOA, and 0.2 mg/liter BAP (NB). This year we added Chee and Poole minimal organics medium with 30 g/liter sucrose supplemented with 2.0 mg/liter 2,4-D and 0.2 mg/liter BAP (AIM) to the list of media tested. Based on previous year's results, we plated 1103P on MSI and MSE media, 110R on NB medium, and 101-14 on PIV medium since these genotype/medium combinations resulted in the highest frequency of embryo formation in the past. Freedom and 3309C were plated on PIV and AIM media formulations, and Salt Creek was plated on all five media (**Table 1**). Flowers were harvested on April 6 and April 14. We are getting a very high percentage of embryos developing for 1103P this year, very soon after plating (**Figure 1, Table 2**). We are also seeing embryogenic callus formation for 101-14, 110R, and Freedom that will be used to establish fresh stock cultures of these genotypes. To date, no embryogenic callus has developed for 3309C or fSalt Creek.

**Table 1.** Number of flowers from which anthers were extracted for each genotype and media combination tested.

Grape Anther Culture	Number of Flowers Plated for Each Genotype on Each Medium					Total # Plated
	PIV	MSI	MSE	NB	AIM	
	2017	2017	2017	2017	2017	
1103P		325	325			650
110R	200			200		400
101-14	600					600
3309C	200				200	400
Salt Creek	100	100	100	100	100	500

**Table 2.** Number (percentage) of embryogenic callus developing for each genotype and media combination tested.

Grape Anther Culture	Number (%) of Embryogenic Callus Developing Per Flowers Plated for Each Genotype on Each Medium				
	PIV	MSI	MSE	NB	AIM
1103P		39/325	47/325		
110R	1/200			0/200	
101-14	1/600				
3309C	0/200				0/200
Salt Creek	0/100	0/100	0/100	0/100	0/100

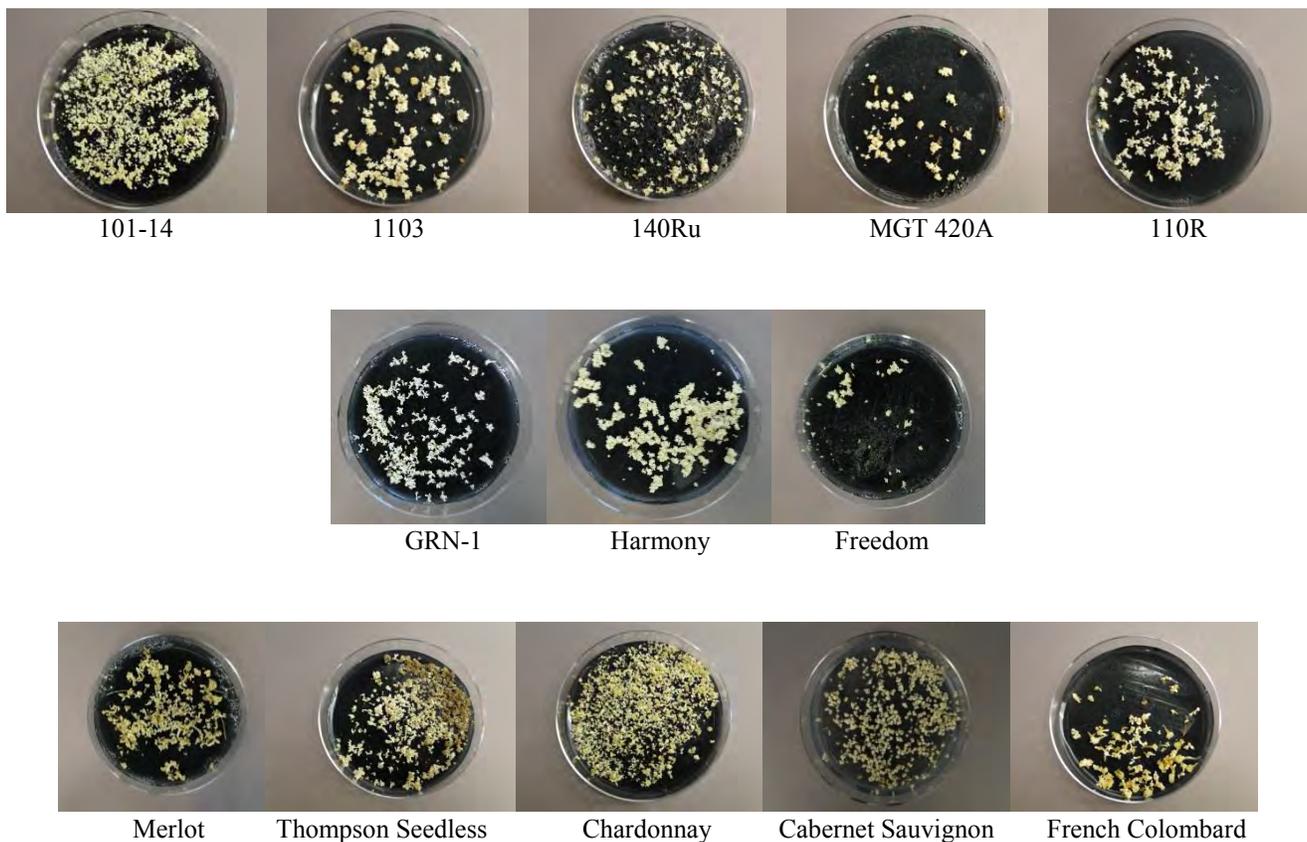
## **Objective 2. Develop Embryogenic Suspension Cultures for Seven Rootstock Genotypes and Six Scion Genotypes, Which Will Provide a Continuous Supply of Somatic Embryos for Use in Transformation Experiments**

By transferring somatic embryos into liquid culture medium composed of woody plant media (WPM) supplemented with 20 g/liter sucrose, 1 g/liter casein hydrolysate, 500 mg/liter activated charcoal, 10 mg/liter Picloram, and 2.0 mg/liter meta-topolin we have established suspensions for rootstock genotypes 101-14, 110R, 140Ru, 1103P, Freedom, GRN-1, Harmony, and MGT 420A, and scion genotypes Cabernet Sauvignon, Chardonnay, French Colombard, Merlot, and Pinot Noir. Occasionally the suspensions are sieved through a 520-micron screen to eliminate large embryos and cell clusters. Alternatively, the smaller fraction of the suspension is drawn up into a wide-bore 10 ml pipet and transferred to a new flask, leaving the larger embryos and cell aggregates behind. We have established new suspension cultures for 1103P in 2017. We also are increasing embryogenic callus of 101-14 generated from anthers collected in 2017, in order to initiate fresh suspensions later

in the year. These will replace our current suspension cultures, which were initiated from embryos produced in 2015.

**Objective 3. Establish a Germplasm Bank of Somatic Embryos for Seven Rootstock Genotypes and Six Scion Genotypes By Plating Aliquots of the Cell Suspension Culture on High Osmotic Medium**

We have established a germplasm bank of somatic embryos by plating aliquots of the suspension cultures onto agar solidified WPM supplemented with 20 g/liter sucrose, 1 g/liter casein hydrolysate, 500 mg/liter activated charcoal, 0.5 mg/liter BAP, 0.1 mg/liter NAA, 5% sorbitol, 1 mM MES, and 14 g/liter phytoagar (BN-sorb). A stored embryo germplasm bank has been established for rootstock genotypes 101-14, 110R, 140Ru, 1103P, Freedom, GRN-1, Harmony, and MGT 420A, as well as scion genotypes Cabernet Sauvignon, Chardonnay, French Colombard, and Merlot (**Figure 1**). The GRN-1 and Harmony suspension cultures have improved significantly and we can now produce high quality stored somatic embryos by plating these suspension. Although we have plated aliquots of suspension cultures of Pinot Noir on this medium, the suspensions, unlike other genotypes, do not form embryos.



**Figure 1.** Germplasm bank of embryos established from grape suspension cultures plated on sorbitol containing medium.

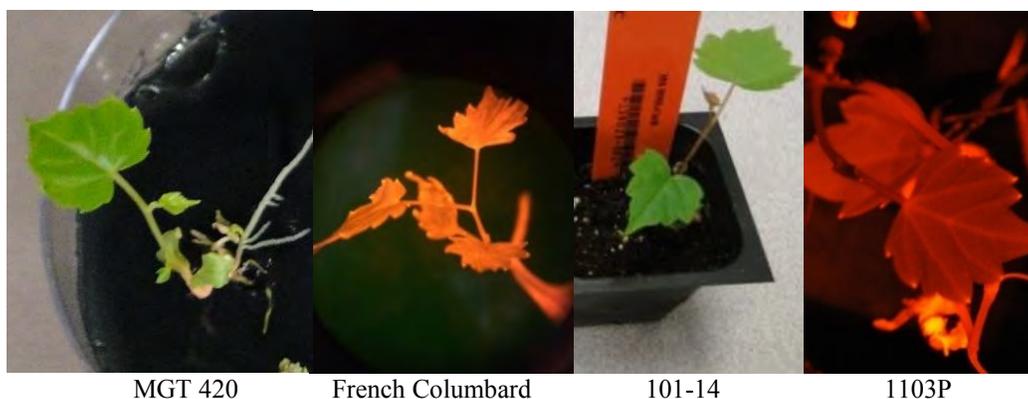
**Objective 4. Test Transformation Efficiencies of Seven Rootstock Genotypes and Six Scion Genotypes Using Our Established Somatic Embryo Transformation Protocols**

Transformation experiments were initiated using somatic embryos for rootstock genotypes 101-14, 110R, 140Ru, 1103P, Freedom, GRN-1, Harmony, and MGT 420A, and scion genotypes Cabernet Sauvignon, Chardonnay, French Colombard, and Merlot using a construct containing the DsRed fluorescent scorable marker. Thompson Seedless is being included as a positive control. DsRed expression was scored three months post-inoculation (**Table 3**) and has shown that significant numbers of transgenic somatic embryos can be generated for 101-14, 110R, 140Ru, 1103P, GRN-1, Harmony, MGT 420A, and French Colombard. Very little DsRed expression was seen in Chardonnay somatic embryos. The relative transformation efficiency based on recovery of whole plants is higher for 110R than that seen for 1103P and equal to or greater than that seen for 101-14. We have also

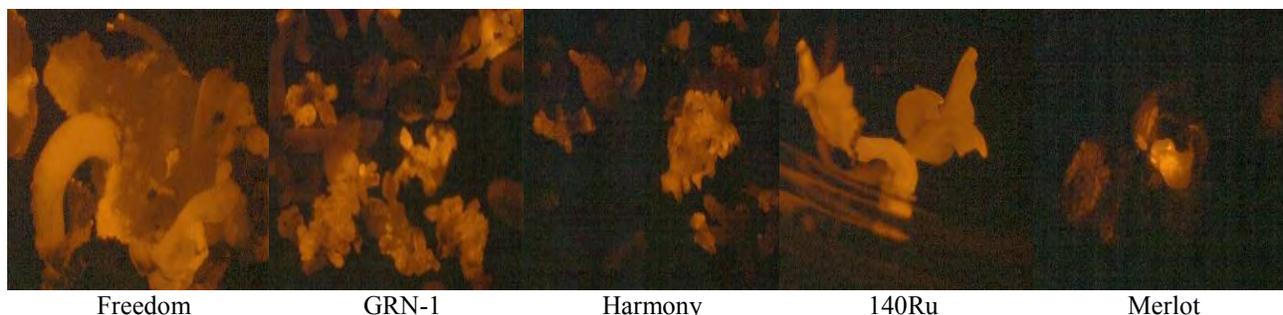
demonstrated that we can generate transgenic plants for MGT420A and French Colombard (**Figure 2**). We are in the process of determining if we can regenerate whole plants from transgenic DsRed-expressing embryos of 140Ru, GRN-1, Harmony, and Merlot. A visual examination of DsRed expression was done to determine the percentage of embryos expressing DsRed for each genotype (**Table 3**). Transformation efficiencies based on DsRed expression are very low for both Chardonnay and Merlot. Images of DsRed-expressing Freedom, GRN-1, Harmony, and Merlot are shown in **Figure 3**.

**Table 3.** Transformation experiments to assess the amenability of transformation of stored grape embryos for a range of rootstock and scion genotypes using the scorable fluorescent marker gene DsRed.

Genotype	Number of Experiments	Estimate of the % of tissue expressing DsRed
110R	5	60%
101-14	2	25%
140Ru	5	21%
MGT 40A	5	15%
1103	2	8%
TS-14	4	36%
Colombard	5	22%
Chardonnay	4	<1%
Freedom	3	25%
GRN-1	3	40%
Harmony	3	20%
Merlot	5	1.0%

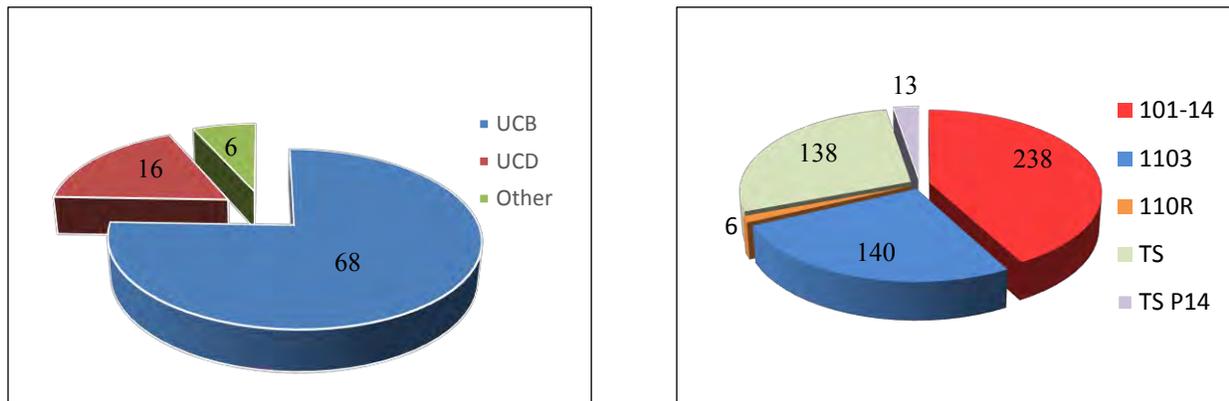


**Figure 2.** Transgenic plantlets.



**Figure 3.** Transgenic embryos expressing DsRed.

Using the stored somatic embryo-based transformation system, to date we have produced 535 genetically modified grape plants across five different genotypes using 90 constructs for principal investigators studying strategies to combat Pierce’s disease (**Figures 4 and 5**).



**Figure 4.** (Left) The number of constructs transformed into grape for testing various strategies to study Pierce’s disease. (Right) The number of transgenic grape plants produced to date for testing various strategies to study Pierce’s disease.

A summary table of our transformation progress with all the rootstock and scion genotypes is presented at the end of this report in **Table 5**.

#### Acclimation of Plants to Soil

We have improved our protocol for acclimatizing transgenic grape plants to soil. Historically, we have allowed transgenic embryos to germinate on the primary root that develops from the embryos. However, this often results in the production of callus at the shoot-root interface, and we speculated that this might be detrimental to survival of the plants during acclimatization to soil. We are now removing the shoot from the germinating embryo and re-initiate roots on the excised shoot. This has resulted in the development of a stronger root system with no associated callus tissue, as well as a healthier plant which acclimates better to soil (**Figure 5**). In addition, we were previously transferring large (six cm or larger) rooted shoots to soil. However, we have recently been rooting smaller shoots and transferring them to soil as soon as roots emerge, while the shoots are under six cm tall. This has also resulted in better survival in soil.



**Figure 5.** (Left) Transgenic grape plant from somatic embryos germinated on its own root. Note callus at the shoot/root interface. (Right) Transgenic grape shoot re-rooted as an *in vitro* cutting.

Rootstock genotypes 101-14 and especially 1103P have been difficult to acclimate to soil from tissue culture. Significant leaf necrosis develops rapidly as relative humidity is reduced from culture conditions to soil. To avoid this plants must be maintained at 100% relative humidity for a minimum of one week upon transfer to soil. To improve drainage we have modified the soil mix to include one part supersoil to two parts vermiculite. We have also employed an additional culture step prior to transplanting the plantlet to soil. We are aseptically removing the shoot tips from each transgenic plant before transfer to soil and culturing the shoot tip in fresh rooting medium in order to establish a backup clone for each transgenic plant. These backup clones can be used should the original plantlet die upon transfer to soil.

#### **Objective 5. Test Direct Cell Suspension Transformation Technology on Seven Rootstock Genotypes and Six Scion Genotypes**

We tried to leverage the progress we have made in developing high quality cell suspensions that can rapidly regenerate whole plants when plated onto agar-solidified medium by directly transforming our grape cell suspension cultures with the scorable marker gene DsRed. Ten ml of a grape cell suspension grown in liquid Pic/MT medium and containing pre-embryogenic masses or small globular embryos are collected in a 15 ml conical centrifuge tube and pelleted by centrifugation at 1,000 x G for three minutes. The cells are subjected to heat shock by placing the conical tube in a 45°C water bath for five minutes. After heat shock the supernatant is removed and replaced with five ml liquid BN medium containing 200 uM acetosyringone and the *Agrobacterium* strain and appropriate vector at an OD<sub>600</sub> of 0.1-0.2. The suspension is centrifuged at 1,000 x G for five minutes and allowed to incubate for 25 minutes at room temperature. After 25 minutes all but 0.5 ml of the supernatant is removed. The grape and *Agrobacterium* cells are then re-suspended and transferred to sterile Whatman filter paper in an empty 100 x 20 mm petri dish. Any excess fluid is carefully blotted up with a second sterile filter paper. The plates are co-cultured in the dark for two to three days at 23 °C and then transferred to selection medium consisting of WPM supplemented with 20 g/liter sucrose, 1 g/liter casein, 1 mM MES, 500 mg/liter activated charcoal, 0.5 mg/liter BAP, 0.1 mg/liter NAA, 400 mg/liter carbenicillin, 150 mg/liter timentin, 200 mg/liter kanamycin, 50 g/liter sorbitol, and 14 g/liter agar. The filter paper is transferred to fresh medium every two weeks. Within eight weeks resistant embryos develop. Developing embryos are transferred to WPM supplemented with 20 g/liter sucrose, 1 g/liter casein, 1 mM MES, 500 mg/liter activated charcoal, 0.1 mg/liter BAP, 400 mg/liter carbenicillin, 150 mg/liter timentin, 200 mg/liter kanamycin, and eight g/liter agar for germination. We tested this protocol on 101-14, 110R, 140Ru, 1103P, MGT 420A, Colombard, and Chardonnay using a construct containing the DsRed transgene. We have been able to recover transgenic plants using this protocol for 1103P and 101-14 at very low frequency. For example, only two of the twenty-one putatively transformed embryos that formed from one experiment with 101-14 germinated into plants after transfer to medium lacking sorbitol. We are observing germinating embryos of MGT 420A (**Figure 6**). However, currently the transformation frequency using this protocol is too low to be practical for routine transformations and we will not pursue this approach in the future. A summary of the experiments and the transformation frequency is given in **Table 4**.



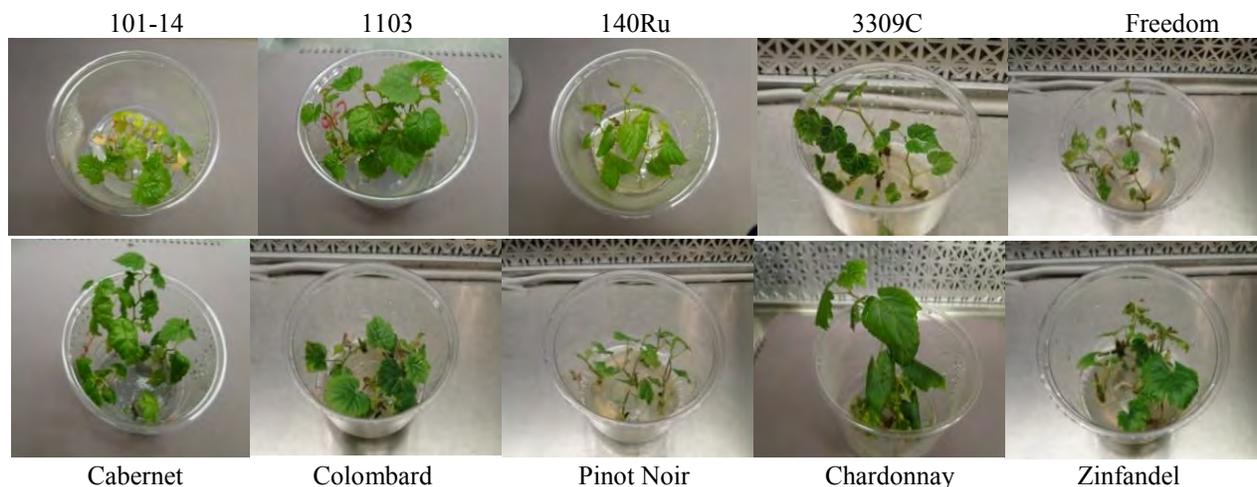
**Figure 6.** (Left) Twenty-one embryos from transformation of cell suspension cultures of 101-14 cultured on WPM supplemented with 20 g/liter sucrose, 1 g/liter casein, 1 mM MES, 500 mg/liter activated charcoal, 0.5 mg/liter BAP, 0.1 mg/liter NAA 50 g/liter sorbitol, and 14 g/liter agar and transfer to WPM supplemented with 20 g/liter sucrose, 1 g/liter casein, 1 mM MES, 500 mg/liter activated charcoal, 0.1 mg/liter BAP, and eight g/liter agar for plant regeneration. Only two of the twenty-one putatively transformed embryos on this plate germinated after transfer to medium lacking sorbitol. (Middle and Right) DsRed-expressing embryos of MGT 420A.

**Table 4.** Number of embryogenic colonies forming after inoculating approximately one to two ml of cell suspension with *Agrobacterium* and plating onto selection medium.

Genotype	Number of Experiments	# of Putative Transgenic Embryos/ml of Plated Suspension	# of Putative Transgenic Plants Produced
101-14	17	54	2
1103	20	30	2
110R	5	1	0
140Ru	2	0	0
MGT 420a	2	7	4
Colombard	2	0	0
Chardonnay	2	0	0

**Objective 6. Establish *In Vitro* Shoot Cultures for Seven Rootstock Genotypes and Six Scion Genotypes Using Indexed Material or Field Material From Foundation Plant Services at UC Davis and Establish Bulk Meristem Cultures for All 13 Genotypes for Use in Transformation**

We are maintaining disease free *in vitro* stock plants of 101-14, Cabernet Sauvignon, and Chardonnay that we received as *in vitro* cultures from Foundation Plant Services (FPS). For material that was not available through FPS, we have collected shoot tips from field material grown at FPS. This includes genotypes 110R, 140Ru, 1103P, 3309C, Freedom, MGT 420A, and Salt Creek, and scion genotypes Cabernet Sauvignon, French Colombard, Pinot Noir, and Zinfandel. We have collected shoot tips for three additional genotypes (Harmony, MGT 420A, and Merlot) with which we were not successful in establishing shoot cultures last season. Four-inch shoot tips were collected and transferred to 50 ml centrifuge tubes and surface sterilized in 0.526% sodium hypochlorite for 15 minutes, followed by three rinses in sterile distilled water. The shoot tip was cut into nodal sections and any tissue damaged by sterilization was removed. The nodal sections were transferred onto agar solidified Chee and Poole C2d *Vitis* medium containing 5 mg/liter chlorophenol red or agar solidified MS minimal organics medium supplemented with 1.0 mg/liter BAP, 0.1 mg/liter IBA, 0.1 mg/liter GA3, and 5 mg/liter chlorophenol red. Aseptic shoot cultures have been established and maintained on Chee and Poole minimal organics medium supplemented with 0.01 mg/liter IBA (**Figure 7**).

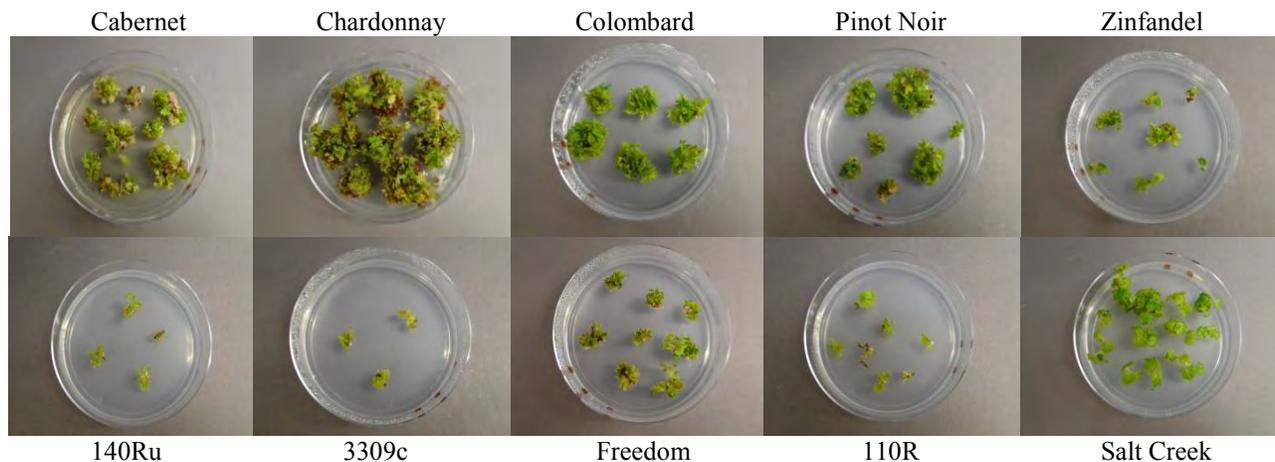


**Figure 7.** Shoot cultures established for rootstock and scion genotypes.

**Objective 7. Test Mezzetti et al. 2002 Bulk Meristem Transformation Methodology for Seven Rootstock Genotypes and Six Scion Genotypes as an Alternate to Somatic Embryo Transformation**

Shoot-tips were collected and plated onto Mezzetti medium with increasing levels of BAP in order to establish bulk meristem cultures. We have produced good quality bulk meristem cultures for scion genotypes Chardonnay,

French Colombard, Pinot Noir, and Zinfandel. However, rootstock genotypes do not readily produce bulk meristems in our hands, but instead produce elongated shoots with a significant amount of non-organized callus, making them unsuitable for bulk meristem transformation (**Figure 8**). Bulk meristems of Cabernet Sauvignon, Chardonnay, and Thompson Seedless were sliced into thin, two mm slices and inoculated with *Agrobacterium* strain EHA105 and co-cultures on Mezzetti medium supplemented with three mg/liter BAP in the dark at 23°C. After three days the thin slices were transferred to Mezzetti medium supplemented with three mg/liter BAP, 400 mg/liter carbenicillin, 150 mg/liter timentin, and 25 mg/liter kanamycin sulfate. After three weeks tissue was transferred to the same medium formulation, but the kanamycin level was increased to 50 mg/liter. After an additional three weeks the tissue was transferred to medium of the same formulation but the kanamycin level was increase to 75 mg/liter. Subsequently, tissue was subcultured every three weeks on medium containing 75 mg/liter kanamycin. Since the construct used to transform the bulk meristems contained the DsRed gene we were able to monitor transformation efficiencies in real time. To date, we have only been successful producing transgenic shoots from bulk meristems of Thompson Seedless. Twenty-four of the 75 thin sliced sections of Thompson Seedless produced DsRed sectors (**Figure 9** and **Table 5**) and three of these sectors regenerated into shoots. We were able to produce DsRed-expressing callus on Cabernet Sauvignon and Chardonnay, but none of this tissue regenerated into shoots. In our hands, the use of kanamycin at 75mg/liter appears to be suboptimal for selection. Although we did identify a limited number of DsRed shoots for Thompson Seedless, many additional shoots that developed on selection medium containing 75 mg/liter kanamycin were non-transgenic based on DsRed expression. If not for the use of the scorable marker DsRed we would not be able to distinguish the true transgenic shoots from the non-transgenic escape shoots until they were transferred to rooting medium with kanamycin. Based on the difficulty of generating bulk meristems for rootstock genotypes and the limited success we have had with transforming thin slices of bulk meristems compared to our standard somatic embryo-based transformation (see below), we are no longer pursuing this strategy. This technique may have utility for scion genotypes if somatic embryo-based transformations are unsuccessful.



**Figure 8.** Initiation of bulk meristem cultures for rootstock and scion germplasm.



**Figure 9.** DsRed-expressing shoot developing from inoculated thin slice of a Thompson Seedless bulk meristem culture. (Left) Bright field. (Right) fluorescence.

**Table 5.** Results of bulk meristem transformation using the scorable marker gene DsRed.

Genotype	Number of Experiments	Number (%) Explants Generated DsRed Callus	Number (%) Explants Generated DsRed Shoots
Cabernet Sauvignon	2	1/36 (3)	0/36 (0)
Chardonnay	2	2/38 (5)	0/38 (0)
Thompson Seedless	2	24/75 (32)	3/75 (4)

**Table 6.** Summary table providing the progress for each objective for each of the grape rootstock and scion genotypes.

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

+ Based on DsRed expression.

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

\*\* ND = not determined.

\*\*\* Not enough data has been accumulated yet to compare the relative transformation efficiencies compared to Thompson Seedless.

## FUNDING AGENCIES

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

## IDENTIFICATION OF A LOW COPY NUMBER PLASMID IN *XYLELLA FASTIDIOSA* STRAIN STAG'S LEAP

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

**ABSTRACT**

*Xylella fastidiosa* (*Xf*) causes Pierce's disease in grapevine. The Stag's Leap strain is known for its high virulence level and is a model for Pierce's disease research. Research on *Xf* has been difficult due to its nutritional fastidiousness. One difficult research issue is the low copy number plasmid. Plasmids are circular extrachromosomal genetic elements associated with bacterial environmental adaptation, including virulence. In this study, a low copy number plasmid, pXFSL21, was identified and characterized using a next-generation sequencing (NGS) approach. Plasmid pXFSL21 (21,665 bp) contains 27 annotated genes including two predicted antibiotic resistance genes, *acrA/B*, encoding a multidrug efflux pump system. Under the experimental conditions in this study, pXFSL21 likely existed as a single copy and is capable of integration into the host chromosome. These results set the base for further studies on *Xf*-host interactions.

**LAYPERSON SUMMARY**

To combat Pierce's disease of grapevine, we must first understand the causal organism *Xylella fastidiosa* (*Xf*). Despite extensive research on *Xf* in the past decade, many biological properties of the bacterium remain unclear. Plasmids are known to carry advantageous or pathogenic genes that may allow bacteria to adapt to new environments and increase the level of virulence. In *Xf*, plasmids can transfer between subspecies. For low copy number plasmids, identification through traditional DNA isolation methods can be difficult. However, next-generation-sequencing (NGS) technology has opened a new venue to resolve the problem. In this study, we applied an NGS technology (MiSeq) to identify a 21,665 bp low (single) copy plasmid, pXFSL21, from the whole genome sequencing effort of *Xf* strain Stag's Leap. The pXFSL21 plasmid has 27 predicted genes, including two antibiotic resistance genes. These results provide new information on *Xf* biology.

**INTRODUCTION**

*Xylella fastidiosa* (*Xf*) is a xylem-limited, fastidious bacterial plant pathogen that causes Pierce's disease of grape (Hopkins & Purcell 2002). In the past decade there has been considerable effort to research the bacterial pathogenicity and the genetic diversity. One contribution of genetic diversity in *Xf* is from plasmids, a group of extrachromosomal genetic elements, capable of moving between strains within and across subspecies. Plasmids carry genes that may be beneficial for bacterial survival or adaptability under new environments (Retchless et al. 2014). A plasmid may transfer through conjugation, a process known to occur in plasmids containing conjugative transfer genes (Burbank and Van Horn 2017) and/or other means. Plasmid copy number is also an interesting research topic. Chen et al. (1992) first observed and characterized a 1.3 Kb plasmid with 60 copies in *Xf* based on gel electrophoresis of total bacterial DNA. However, for low copy number plasmids, traditional DNA extraction and gel visualization methods are often not sensitive enough for identification. Recently, next-generation-sequencing (NGS) technology has been utilized to sequence the whole genome of over 30 *Xf* strains. Analyses of NGS data revealed the presence of many plasmids previously unknown, suggesting that NGS analyses could be a powerful tool for plasmid detection and research.

The *Xf* strain Stag's Leap was first isolated from grapevine in the Stag's Leap district of Napa Valley, California (Buzkan, Kocsis, and Walker 2005). This strain has been widely used as a model for Pierce's disease research due to its high virulence level and dramatic disease symptoms seen in grapevine (Burbank and Stenger 2016, Krivanek and Walker 2005). A draft whole genome sequence of Stag's Leap was obtained in 2016, in which  $6.59 \times 10^6$  paired-end reads were assembled into 15 contigs representing the 2.5 Mb genome (Chen et al. 2016). Previous attempts for plasmid detection in strain Stag's Leap using traditional methods were unsuccessful

(Hendson et al. 2001). This study continues the effort of plasmid searching in strain Stag's Leap. By utilizing NGS technology, a single copy number plasmid was identified and characterized.

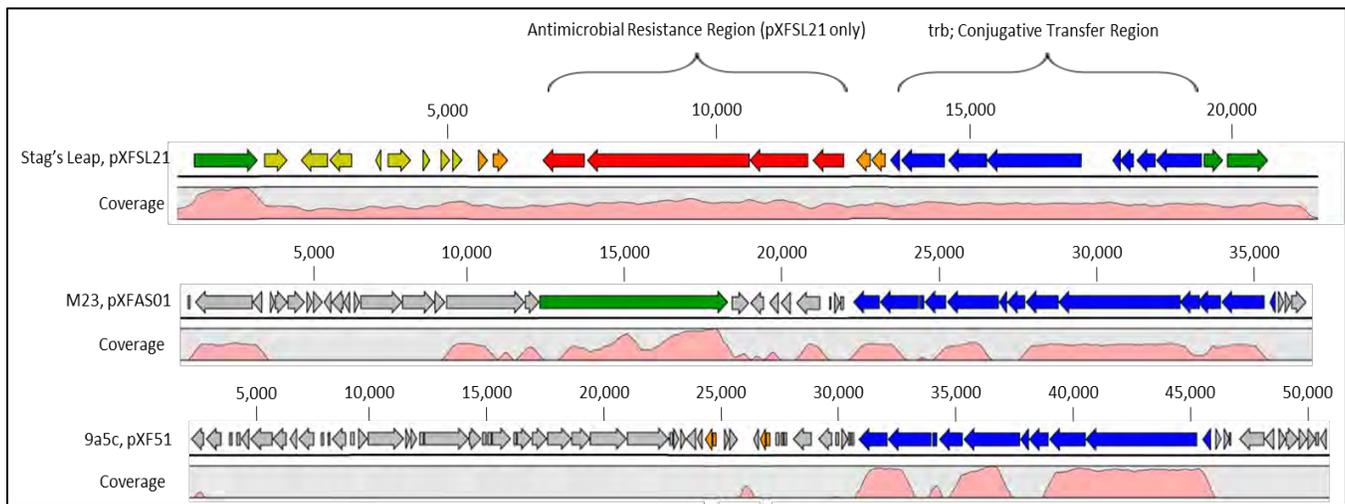
## OBJECTIVES

1. Identify plasmid in *Xf* Stag's Leap strain using NGS technology.
2. Characterize the genetic structure and content of the Stag's Leap plasmid.

## RESULTS AND DISCUSSION

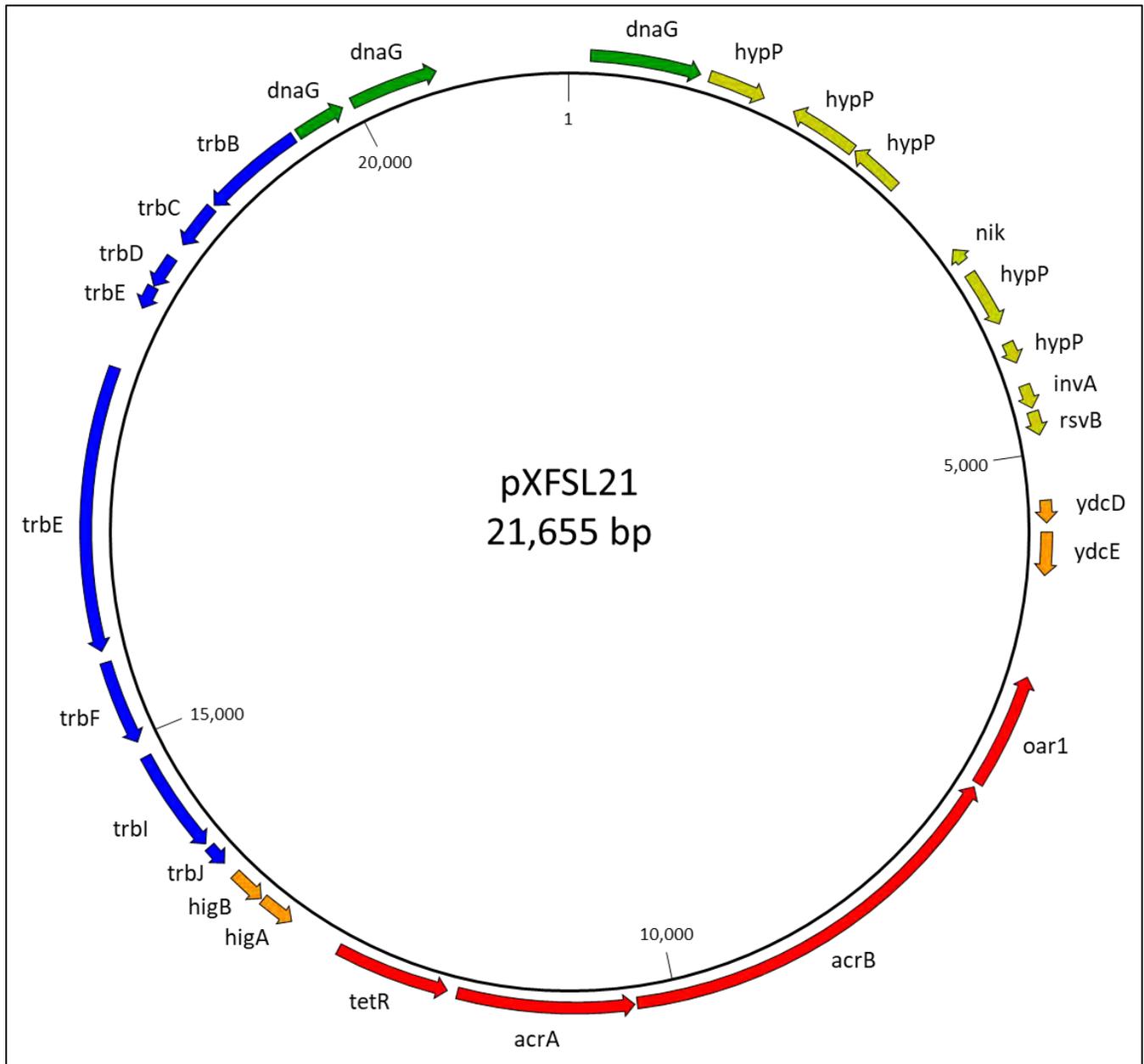
### Objective 1. Identify Plasmid in *Xf* Stag's Leap Strain Using NGS Technology

An NGS platform, Illumina MiSeq, was used to generate a total of 6,590,000 short sequence reads with 301 bp each from Stag's Leap DNA extracted from pure culture. The MiSeq reads were used to map to two published *Xf* plasmids, pXFSA01 from *Xf* strain M23 and pXF51 from *Xf* strain 9a5c. Partial coverage to pXFSA01 (47.8%) and pXF51 (15%) were observed (**Figure 1**). These suggested the possible presence of a plasmid in strain Stag's Leap.

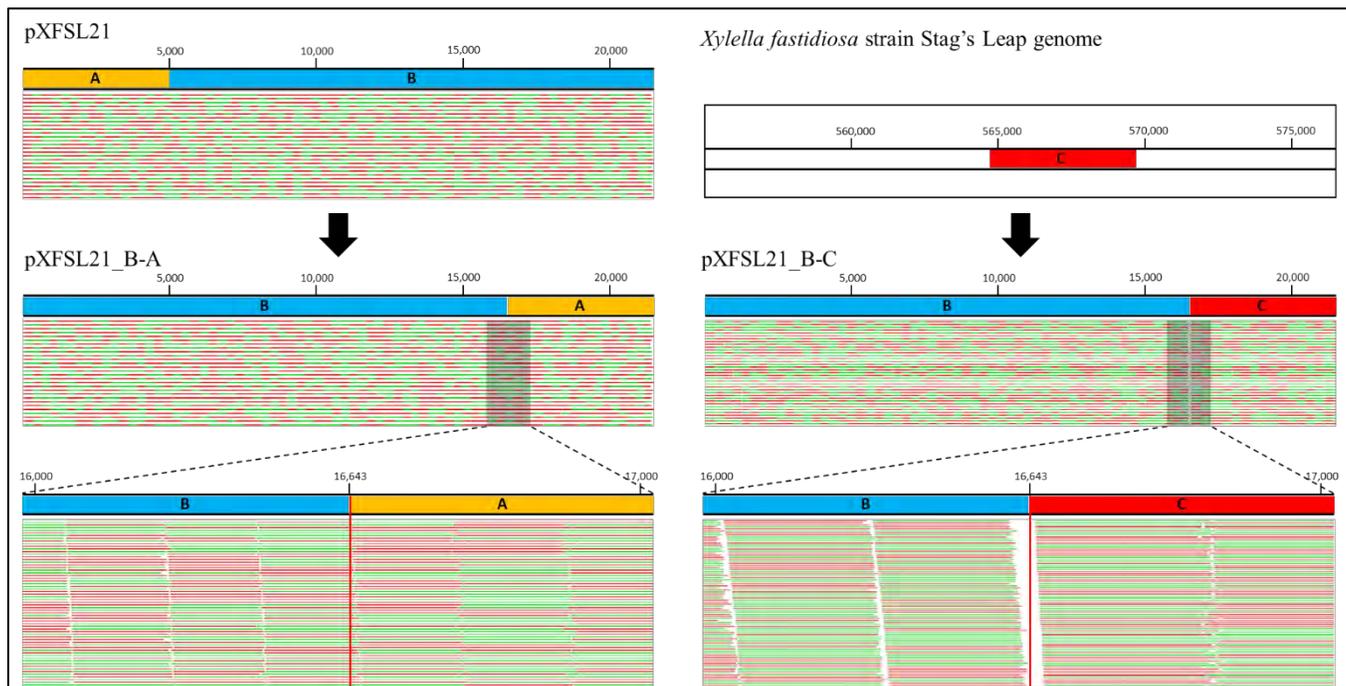


**Figure 1.** MiSeq read mapping of *Xf* strain Stag's Leap to the sequences of plasmids pXFSL21, pXFAS01, and pXF51. Annotated genes are indicated by the arrow boxes. Numbers above are nucleotide positions. Plasmid names are on the left. Coverage graphs are under each sequence (pink), scaling from 0x (bottom) to 1,614x (top). Identical genes (> 85% identity and 90% length coverage) are represented by the same color, excluding grey colored genes. The trb conjugative transfer genes are blue. Antimicrobial resistance genes are red. DNA primase genes are green. Toxin/antitoxin genes are orange.

Using *Xylella* plasmid sequences available in GenBank database to BLAST search the published Stag's Leap draft genome sequence (with 15 contigs), contig\_15 (21,665 bp) showed a bit score result > 8,000, significantly greater than that of the next contig (contig\_7) with a bit score of 2,422. Sequence extension of contig\_15 from both 3' and 5' ends using MiSeq read walking enclosed contig\_15 to a circular plasmid (**Figure 2**). However, a segment of DNA (1,200 bp) in pXFSL21 overlapped a region in contig\_2, presumably of chromosomal origin, suggesting a possible chromosomal integration site. Circularity of the plasmid was further confirmed using CLC Genomics Workbench (version 10) by manually reorganizing the linear sequence, in which 5,000 bp from the 5' end was moved to the 3' end, followed by read mapping of the MiSeq reads to show the continuous read overlap of the 5' and 3' ends (**Figure 3**). The circular contig connection was further confirmed by polymerase chain reaction (PCR) and Sanger sequencing. The identified plasmid was designated as pXFSL21 with the size of 21,665 bp (**Figure 2**).



**Figure 2.** Circular map of pXFSL21. The open reading frames are colored per presumed function: green, DNA primase; yellow, hypothetical and recombination gene region; orange, toxin/antitoxin module; red, antimicrobial resistance region; blue, conjugative transfer region (trb). Numbers indicate nucleotide position.



**Figure 3.** Circularity evaluation of pXFSL21 plasmid through MiSeq read mapping. Left: The first 5,000 bp sequence (A region) of pXFSL21 plasmid was cut and attached to the end of the pXFSL21 sequence to make the pXFSL21\_B-A sequence. Right: As a control, the sequence region from nucleotide position 564,797 to 569,797 bp (contig\_1) of the Stag's Leap strain chromosome was cut and attached to the end of the pXFSL21 sequence to make the pXFSL21\_B-C sequence. Both the pXFSL21\_B-A sequence and the pXFSL21\_B-C sequence were used as the reference for Stag's Leap MiSeq read mapping. The lower panels are magnified regions of the grey boxes above. Numbers indicate nucleotide positions. Green lines represent forward reads. Pink lines represent reverse reads. Vertical red lines indicate continuum (left) or break (right) of read mapping.

### Objective 2. Characterize the Genetic Structure and Content of the Stag's Leap Plasmid

Annotation of pXFSL21 identified 27 open reading frames (ORFs) and a GC content of 50.2%. The plasmid contains eight genes belonging to the *trb* conjugative transfer operon, two pairs of toxin-antitoxin genes, two resolvase/integrase genes, three genes annotated as DNA primase, four genes associated with a multidrug efflux pump system, possibly providing resistance to acriflavine, and five hypothetical protein genes most closely related to *Xf* through BLASTn searches (**Table 1**).

The antimicrobial resistance region located upstream of the *trb* operon contains four genes (*tetR*, *acrA*, *acrB*, and *oar1*) representing a multidrug efflux pump system, that may confer resistance to the antimicrobial agent, acriflavine. Some multiple drug resistant efflux pumps, including AcrA/B-TolC in *Escherichia coli*, contribute to the intrinsic antimicrobial resistance of the bacteria when expressed at basal levels (Kumar, Kaur, and Kumari 2012). It has been reported that *Erwinia amylovora*, an enterobacterium that causes fire blight on species of the Rosaceae family, contains the genes *AcrA/B* encoding an efflux pump capable of targeting phytoalexins (Blanco et al. 2016). These multiple drug resistant efflux pump genes in *E. amylovora* are necessary for successful colonization of the plants and for bacterial virulence (Kumar et al. 2012).

The copy number analysis between plasmid and chromosome is summarized in **Table 2**. The average nucleotide coverage, determined through MiSeq read mapping, was 727x for the pXFSL21 and 743x for the Stag's Leap chromosomal region. The plasmid/chromosome ratio was 1.02. The quantitative PCR (qPCR) analysis of plasmid and chromosomal gene copy number supports the *in silico* plasmid copy number estimation using CLC genomics workbench. Relative copy number estimates of plasmid and chromosomal located genes obtained by qPCR and measured against an internal reference gene, *GAPDH*, showed no difference in copy number, estimating the plasmid copy number to be one, equal to that of the chromosome. These results confirm that the pXFSL21 plasmid is present at a very low copy number, if not a single copy.

**Table 1.** Annotation of pXFSL21 based on RAST server and results of BLASTn searches.

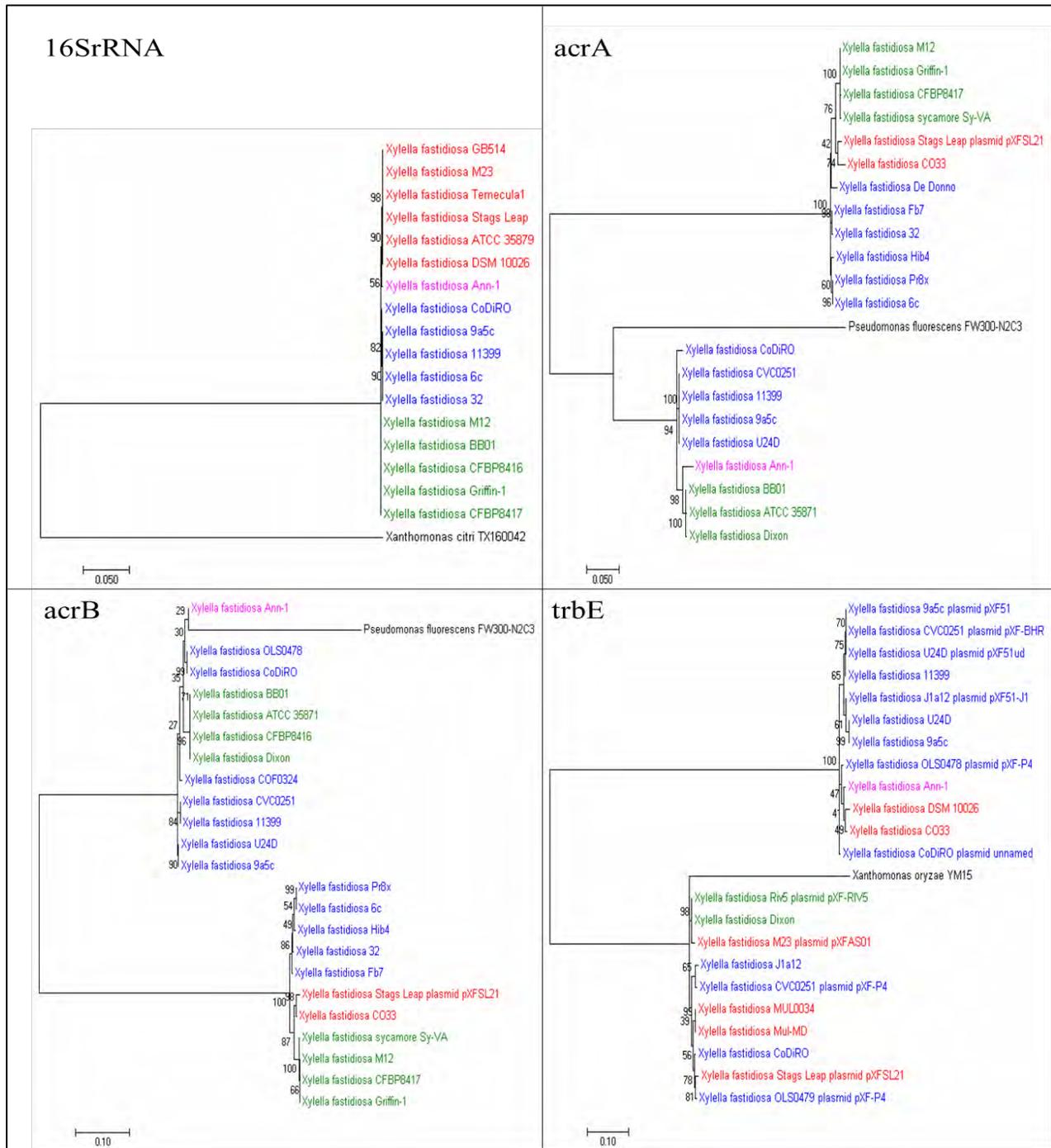
ORF coordinates	Annotation			
	Gene product	Related taxon	Accession	e value
129 – 1202	DNA primase (dnaG)	<i>X. fastidiosa</i> M23	NC_010577.1	0.0
1318 – 1773	Hypothetical protein (hypP)	<i>X. fastidiosa</i> Fb7	NZ_CP010051.1	0.0
2042 – 2596	Hypothetical protein (hypP)	<i>X. fastidiosa</i> Hib4	NZ_CP009885.1	0.0
2628 – 3092	Hypothetical protein (hypP)	<i>X. fastidiosa</i> Ann-1	NZ_CP006696.1	0.0
3571 – 3684	Nickase (nik)	<i>X. fastidiosa</i> Ann-1	NZ_CP006696.1	$7e^{-50}$
3814 – 4281	Hypothetical protein (hypP)	<i>X. fastidiosa</i> Ann-1	NZ_CP006696.1	0.0
4505 – 4657	Hypothetical protein (hypP)	<i>X. fastidiosa</i> Ann-1	NZ_CP006696.1	$3e^{-69}$
4856 – 5050	DNA invertase (invA)	<i>X. fastidiosa</i> CO33	NZ_LJZW0000000.1	$4e^{-94}$
5054 – 5437	DNA resolvase (rsvB)	<i>X. fastidiosa</i> CO33	NZ_LJZW0000000.1	$7e^{-97}$
5593 – 5775	PCD antitoxin (ydcD)	<i>X. fastidiosa</i> CO33	NZ_LJZW0000000.1	$9e^{-89}$
5884 – 6198	PCD toxin (ydcE)	<i>X. fastidiosa</i> CO33	NZ_LJZW0000000.1	$8e^{-162}$
6865 – 7671	3-oxoacyl-reductase (oar1)	<i>X. fastidiosa</i> CFPB8072	NZ_LKDK0000000.1	0.0
7717 – 10779	Acriflavine resistance protein (acrB)	<i>X. fastidiosa</i> CO33	NZ_LJZW0000000.1	0.0
10776 – 11882	Component of multidrug efflux system (acrA)	<i>X. fastidiosa</i> CFPB8072	NZ_LKDK0000000.1	0.0
11964 – 12581	Transcriptional regulator (tetR)	<i>X. fastidiosa</i> Ann-1	NZ_CP006696.1	0.0
12834 – 13124	HigA antitoxin protein (higA)	<i>X. fastidiosa</i> Ann-1	NZ_CP006696.1	$2e^{-145}$
13142 – 13420	HigB toxin protein (higB)	<i>X. fastidiosa</i> Ann-1	NZ_CP006696.1	$3e^{-136}$
13479 – 13685	Conjugative transfer protein (trbJ)	<i>X. fastidiosa</i> Ann-1	NZ_CP006696.1	$7e^{-91}$
13698 – 14546	Conjugative transfer protein (trbI)	<i>X. fastidiosa</i> Ann-1	NZ_CP006696.1	0.0
14608 – 15324	Conjugative transfer protein (trbF)	<i>X. fastidiosa</i> CO33	NZ_LJZW0000000.1	0.0
15321 – 17132	Conjugative transfer protein (trbE)	<i>X. fastidiosa</i> CFPB8073	NZ_LKES0000000.1	0.0
17714 – 17884	Conjugative transfer protein (trbE)	<i>X. fastidiosa</i> CFPB8073	NZ_LKES0000000.1	$6e^{-84}$
17872 – 18138	Conjugative transfer protein (trbD)	<i>X. fastidiosa</i> CO33	NZ_LJZW0000000.1	$3e^{-134}$
18195 – 18587	Conjugative transfer protein (trbC)	<i>X. fastidiosa</i> CFPB8073	NZ_LKES0000000.1	0.0
18600 – 19481	Conjugative transfer protein (trbB)	<i>X. fastidiosa</i> CFPB8073	NZ_LKES0000000.1	0.0
19512 – 19886	DNA primase (dnaG)	<i>X. fastidiosa</i> CFPB8073	NZ_LKES0000000.1	0.0
19966 - 20769	DNA primase (dnaG)	<i>X. fastidiosa</i> CFPB8073	NZ_LKES0000000.1	0.0

**Table 2.** Stag's Leap plasmid, pXFSL21, copy number estimation by *in silico* read mapping and *in vitro* qPCR.

Sequence	Size (bp)	<i>in silico</i>		<i>in vitro</i>	
		Average nucleotide coverage (x)	Estimated plasmid copy number	Average Ct value	Estimated plasmid copy number
pXFSL21	21,665	726.6	1.02	14.89 ± 0.02	1.0
Stag's Leap*	1,489,133	742.8	n/a	14.88 ± 0.02	n/a

\* This represents the Stag's Leap genome with the 21,665 bp plasmid sequence removed.

The evolutionary relatedness of four genes (16S rRNA in contig\_2, *acrA*, *acrB*, and *trbE* in pXFSL21) were analyzed using MEGA v.7 (**Figure 4**). Phylogenetic trees were constructed with genes having > 80% identity and > 95% coverage from BLASTn searches of the nr and WGS databases in GenBank. The trees were constructed using the maximum-likelihood method (Hall 2013). While the 16S rRNA gene tree clustered all *Xf* strains in the same group, each of the three plasmid genes clustered *Xf* strains into two groups (**Figure 4**). Further phylogenetic research is underway for this plasmid.



**Figure 4.** Molecular phylogenetic analysis by Maximum Likelihood method of the 16SrRNA gene and three pXFSL21 plasmid genes. The evolutionary history was inferred by using the Maximum Likelihood (1000 bootstraps) method based on the Tamura 3-parameter model (Tamura 1992). The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The trees are unrooted and drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA7 (Kumar, Stecher, and Tamura 2016). Colors correspond to different *Xf* subspecies: red, *fastidiosa*; fuchsia, *sandyi*; green, *multiplex*; blue, *pauca*; black, closest related taxa.

## CONCLUSIONS

Using whole genome sequence data from *Xf* strain Stag's Leap, we identified a novel, low copy number plasmid pXFSL21. The plasmid harbored predicted multidrug efflux pump genes that may confer resistance to the antimicrobial agent, acriflavine. This plasmid shares sequence similarity to many known *Xylella* plasmids, primarily in the *trb* conjugative transfer gene region, indicating the potential for this plasmid to be shared across strains and subspecies (Burbank & Van Horn 2017). pXFSL21 also contains genes not previously found in other *Xylella* plasmids, but present in the whole genome sequence of multiple subspecies. This could suggest the presence of unidentified plasmids or chromosomal integration events in these strains. Further research is necessary to understand the phylogenetic and biological function of this plasmid.

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## BREEDING PIERCE'S DISEASE RESISTANT WINEGRAPES

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### ABSTRACT

Breeding Pierce's disease resistant winegrapes continues to advance, accelerated by aggressive vine training and selection for precocious flowering, resulting in a seed-to-seed cycle of two years. To further expedite breeding progress we are using marker-assisted selection for the Pierce's disease resistance genes to select resistant progeny as soon as seeds germinate. These two practices have allowed us to produce four backcross generations with elite *Vitis vinifera* winegrape cultivars in 10 years. We have screened through about 2,000 progeny from the 2009, 2010, and 2011 crosses that are 97% *V. vinifera* with the *PdR1b* resistance gene from *V. arizonica* b43-17. We select for fruit and vine quality and then move the best to greenhouse testing, where only those with the highest resistance to *Xylella fastidiosa*, after multiple greenhouse tests, are advanced to multi-vine wine testing at Davis and other test sites. The best of these have been advanced to field testing with commercial-scale wine production, the first of which was planted in Napa in June 2013. To date 19 scion and three Pierce's disease resistant rootstocks have been advanced to Foundation Plant Services at UC Davis for certification. Stacking of *PdR1b* with Pierce's disease resistance from b42-26 (an alternative form of Pierce's disease resistance controlled by multiple genes) has been advanced to the 96% *V. vinifera* level using marker-assisted selection to confirm the presence of *PdR1* as well as the recently discovered (see companion report) Pierce's disease resistance locus on chromosome 8 from b42-26, *PdR2*. Initial selections for release will begin in 2018. Other forms of *V. arizonica* are being studied and the resistance of some will be genetically mapped for future efforts to combine multiple resistance sources and ensure durable resistance. Pierce's disease resistance from *V. shuttleworthii* and BD5-117 are also being pursued, but progress is limited by their multigenic resistance and the absence of associated genetic markers. Very small scale wines from 94% and 97% *V. vinifera PdR1b* selections have been very good and have been received well at public tastings in Sacramento (California Association of Winegrape Growers), Santa Rosa (Sonoma Winegrape Commission), Napa Valley (Napa Valley Grape Growers and Winemakers Associations), Temecula (Temecula Valley Winegrape Growers and Vintners), and Healdsburg (Dry Creek Valley and Sonoma Grape Growers and Winemakers).

### LAYPERSON SUMMARY

One of the most reliable and sustainable solutions to plant pathogen problems is to create resistant plants. We use a classical plant breeding technique called backcrossing to bring Pierce's disease resistance from wild grape species into a diverse selection of elite winegrape backgrounds. To date we have identified two different chromosome regions that house very strong sources of Pierce's disease resistance from grape species native to Mexico and the southwestern United States (*Vitis arizonica*). Because we were able to locate these resistance genes/regions - *PdR1* (Krivanek et al. 2006) and *PdR2* (Riaz et al. in press) - we have been able to use marker-assisted selection to screen for DNA markers associated with both *PdR1* and *PdR2*, allowing us to select resistant progeny shortly after seeds germinate. Marker-assisted selection and aggressive training of the selected seedling vines have allowed us to produce new Pierce's disease resistant high quality winegrape selections that are more than 97% *V. vinifera* in only 10 years. We have evaluated thousands of resistant seedlings for horticultural traits and fruit quality. The best of these are advanced to greenhouse testing, where only those with the highest resistance to *Xylella fastidiosa*, after multiple greenhouse tests, are advanced to multi-vine wine testing at Davis and at Pierce's disease hot spots around California. The best of these are advanced to field plots where commercial-scale wines can be produced. We have sent 19 advanced selections to Foundation Plant Services at UC Davis over the past four winters to begin the certification and release process. Three Pierce's disease resistant rootstocks were also sent to Foundation Plant Services for certification. Other wild grape species are being studied, and the resistance of some will be genetically mapped for future efforts to combine multiple resistance sources and ensure durable Pierce's disease resistance. Very small-scale wines made from our advanced *PdR1* selections have been very good and have been received well at professional tastings throughout California.

## INTRODUCTION

We continue to make rapid progress breeding Pierce's disease resistant winegrapes. Aggressive vine training and selection for precocious flowering have allowed us to reduce the seed-to-seed cycle to two years. To further expedite breeding progress we are using marker-assisted selection for the Pierce's disease resistance 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 have screened through about 2,000 progeny from the 2009, 2010, and 2011 crosses that are 97% *V. vinifera* with the *PdR1b* resistance gene from *V. arizonica* b43-17. Seedlings from these crosses continue to fruit and others are advancing to small scale wine trials. We select for fruit and vine quality and then move the best selections 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 advanced to field testing with commercial-scale wine production, the first of which was planted in Napa in June 2013. To date, 19 scion and three Pierce's disease resistant rootstocks have been advanced to Foundation Plant Services at UC Davis for certification. Stacking of *PdR1b* with b42-26 Pierce's disease resistance has been advanced to the 96% *V. vinifera* level using marker-assisted selection to confirm the presence of *PdR1*, as well as the recently discovered (see companion report) Pierce's disease resistance locus on LG8 from b42-26, *PdR2*. Initial selections for release will begin in 2018. Five of these have been pre-released to grapevine nurseries to build up the amounts available for grafting. Greenhouse screening is still used to select for advancement of only those genotypes with the highest possible levels of Pierce's disease resistance. Other forms of *V. arizonica* are being studied, and the resistance of some will be genetically mapped for future efforts to combine multiple resistance sources and ensure durable resistance. Pierce's disease resistance from *V. shuttleworthii* and BD5-117 are also being pursued, but progress is limited by their multigenic resistance and the absence of associated genetic markers. Very small scale wines from 94% and 97% *V. vinifera PdR1b* selections have been very good and have been received well at public tastings in Sacramento (California Association of Winegrape Growers), Santa Rosa (Sonoma Winegrape Commission), Napa Valley (Napa Valley Grape Growers and Winemakers Associations), Temecula (Temecula Valley Winegrape Growers and Vintners), and Healdsburg (Dry Creek Valley and Sonoma Grape Growers and Winemakers).

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* Pierce's disease resistant material to select from. Individuals with the best fruit and vine characteristics are then tested for resistance to *Xf* under our greenhouse screen. Only those with the highest levels of resistance are advanced to small-scale winemaking trials by grafting them onto resistant rootstocks and planting six to eight vine sets on commercial spacing and trellising at Pierce's disease hot spots around California, where they continue to thrive. We have made wine from vines that are at the 94% *V. vinifera* level from the same resistance background for eight years and from the 97% *V. vinifera* level for six years. They have been very good and don't have typical hybrid flaws (blue purple color and herbaceous aromas and taste) that were prevalent in red wines from the 87% *V. vinifera* level. b43-17 is homozygous resistant to Pierce's disease. We have named its resistance region/locus *PdR1* and the two forms/alleles of that locus *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 retain a number of selections at various backcross (BC) levels with *PdR1a* in the event that there is an as yet unknown *Xf* strain-related resistance associated with the *PdR1* alleles. We also identified a Pierce's disease resistance locus from *V. arizonica* b40-14 (*PdR1c*) that maps to the same region of chromosome 14 as *PdR1* from b43-17. In the absence of an understanding of gene function and given the very disparate origins of the b43-17 and b40-14 resistance sources, differences in preliminary DNA sequence data between them, and differences in their Pierce's disease symptom expressions, we have continued to advance the *PdR1c* line as a future breeding resource. Our companion research project is pursuing the genetic basis of these differences between *PdR1b* and *PdR1c*. In 2005, we started a Pierce's disease resistant breeding line from another Mexican accession, b42-26. Markers linked to this resistance proved elusive but strong resistance was observable 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 to select for *PdR1b* and a higher than usual resistance in our greenhouse screen to move the b42-26 resistance forward. Late last year our companion project identified the location of a significant Pierce's disease resistance locus from b42-26 on chromosome 8, which we have called *PdR2*. Three years ago, in 2014, we advanced our *PdR1* x *PdR2* line to the 92% *vinifera* level and last spring made crosses to advance it to the 96% *vinifera* level. Marker-assisted selection was used to advance only genotypes with both *PdR1b* and *PdR2* for the first time on these crosses. The resistance from southeastern United States species is being advanced in other lines. However, the resistance in these latter lines is complex (controlled by multiple genes) and markers have not yet been developed to expedite breeding. The breeding effort with alternative resistance sources and the complexing of these resistances is being done to broaden *Xf* resistance and address *Xf*'s potential to overcome resistance.

## OBJECTIVES

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

## RESULTS AND DISCUSSION

To date, over 293 wild accessions have been tested for Pierce's disease resistance with the greenhouse screen, most of which were collected from the southwestern United States and Mexico. Our goal is to identify accessions with the most unique Pierce's disease resistance mechanisms. To do so we evaluate the genetic diversity of these accessions and test them for genetic markers from chromosome 14 (where *PdR1* resides) to ensure that we are choosing genetically diverse resistance sources for population development and greenhouse screening efforts. Over the last five years, 15 of the most unique accessions were used to develop F1 populations with *V. vinifera* to investigate the inheritance of Pierce's disease resistance in their F1 progeny and the degree to which they resist *Xf*. We have reported previously the surprising result from our companion Pierce's disease mapping project that most of the resistance lines we have explored from the southwestern United States have Pierce's disease resistance associated with chromosome 14, the same region as our primary resistance line *PdR1b*. From that same project we identified *PdR2* on chromosome 8 from b42-26. *PdR2* resistance, although significant, generally doesn't confer as strong a resistance as *PdR1*. Preliminary results indicate that most of the non-*PdR1* resistance sources appear to also have at least some of their resistance derived from chromosome 8. Until we better understand the nature of chromosome 8 Pierce's disease resistance and explore additional resistance loci in these lines, it is important to continue advancing multiple sources of chromosome 8 resistance.

**Table 1** gives details of crosses made this spring to finish the expansion of our mapping populations. Group 1a crosses will complete the ANU67 and most of the T 03-16 mapping populations. The b41-13 population was completed with crosses made in 2016. In Group 1b we expand the number of T03-16 progeny used in full sib F1 intercrosses in an attempt to recover the strong resistance of the parent. In Group 1c we broadened the elite *vinifera* parents used to advance the ANU67 and T03-16 lines and used a different promising F1 selection from the b41-13 line.

**Table 1.** 2017 Crosses made to finish the expansion of the new F1 Pierce’s disease mapping populations and advance breeding lines to the next backcross level: *vinifera* parents, # crosses, actual # seeds produced.

Group	Cross PDR Source	% <i>vinifera</i>	<i>vinifera</i> Parents	No. of Crosses	Act. No. of Seeds
1a	ANU67	50%	F2-35	1	68
	T 03-16	50%	Palomino	1	73
1b	T 03-16	50%	Palomino	10	717
1c	ANU67	75%	Montepulciano, Palomino, and Sauvignon Vert	3	123
	b41-13	75%	F2-35	1	1061
	T03-16	75%	F2-35, LCC	2	184

Crosses made in 2017 in **Table 2** represent our primary focus of 96% *vinifera* backcrosses to a diverse selection of elite *vinifera* wine varieties to three of our most resistant parents carrying both *PdR1b* and *PdR2*. This will expand and broaden the *vinifera* representation initiated by the seedlings planted earlier this year from crosses made in 2016. The most promising selections would then be advanced to Foundation Plant Services (FPS) for certification and eventual release as the next iteration of our Pierce’s disease resistant winegrape breeding efforts.

**Table 2.** 2017 crosses of elite *vinifera* cultivars to three resistant genotypes that have both the *PdR1b* and *PdR2* loci. Progeny will be 96% *vinifera*.

Resistant Parent	<i>vinifera</i> Parent	Act. No. of Seeds	Resistant Parent Total Est. No. of Seeds
14309-002	Alvarelhao	119	3,501
	Dolcetto	917	
	Fiano	5	
	Matero	111	
	Montepulciano	169	
	Palomino	310	
	Pedro Ximenez	222	
	Pinot Noir FPS32	156	
	Pinot Noir FPS77	320	
	Refosco	271	
	Sauvignon Vert	480	
	Touriga Nacional	421	
14309-111	Dolcetto	619	1,390
	Fiano	75	
	Matero	341	
	Montepulciano	11	
	Morrastel	82	
	Pinot Noir FPS32	34	
	Refosco	225	
	Touriga Nacional	3	
14388-029	Arneis	178	1,300
	Montepulciano	49	
	Morrastel	272	
	Pedro Ximenez	315	
	Pinot Noir FPS32	75	
	Pinot Noir FPS77	67	
	Refosco	48	
	Sauvignon Vert	296	

We also completed the final BC4 generation in the *PdR1c*, b40-14 line (**Table 3**, Cross 3a). In Crosses 3b and 3c we take two different approaches for combining *PdR1b* and b42-26 Pierce's disease resistance. In the former, we take an approach similar to that in **Table 2** but from different initial backcross generations and selections. This approach serves as insurance should we find b42-26 resistance resides significantly in genomic locations other than chromosome 8. Rather than backcrossing in the *PdR1b* x b42-26 line as in Cross 3b, the resistance line profiled in Cross 3c was backcrossed a second time to a different relatively resistant b42-26 progeny. This is with the expectation that carrying more b42-26 minor resistance factors deeper into the backcross generations may contribute a genetically wider base of Pierce's disease resistance.

The remaining crosses in **Table 3** (Crosses 3d-3h) combine Pierce's disease resistance, either from *PdR1b* alone or in combination with b42-26 resistance with various sources of powdery mildew resistance loci. We have genetic markers for powdery mildew resistance derived from *V. vinifera* (*Ren1*), *V. romanetii* (*Ren4*), *V. piasezkii* (*Ren6*), and two forms from *Muscadinia rotundifolia* (*Run1* and *Run2.1*). Some of our most advanced lines in crosses represented here should be candidates for release. In Cross 3d we have advanced single *PdR1b* Pierce's disease resistance with *Ren1* and/or *Ren4* powdery mildew resistance. Crossing to these diverse elite *vinifera* should result in a wide range of possible selections. The challenges for the rest of the **Table 3** Pierce's disease x powdery mildew (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, and they, unlike those in Cross 3d, 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 Pierce's disease resistance alone. For the first time, some of the crosses in 3e and 3f integrate powdery mildew resistance from *Ren6* from *V. piasezkii* and *Run 2.1* from *Muscadina rotundifolia* into our Pierce's disease resistant lines. Crosses in 3h are similar in result to those made last year, however, we have selected for parents with better germination and anticipate a higher percentage of progeny with desirable marker-assisted selection results. In addition to the 2017 crosses presented in **Tables 2** and **3**, we also made crosses in the b46-43 line to advance to the BC2 level using Alvarelhao and Muscat Blanc as elite *vinifera* parents with 222 seeds produced.

**Table 3.** 2017 advanced Pierce's disease (PD) and Pierce's disease x powdery mildew (PD x PM) resistant crosses with *vinifera* heritage, # crosses, and estimated # of seeds produced. *Ren1*, *Ren4*, and *Ren6* are powdery mildew resistance loci from *V. vinifera*, *V. romanetii*, and *V. piasezkii*, respectively. *Run1* and *Run 2.1* powdery mildew resistance loci are from *Muscadina rotundifolia*.

Cross PDR Type	Cross PM Type	<i>vinifera</i> Parent...Grandparents	% <i>vinifera</i>	No. of Crosses	No. of Seeds
3a. b40-14	None	Dolcetto, Fiano, Grenache Noir 224, Malvasia Bianca, Montepulciano, Morrastel, Pedro Ximenez, Touriga Nacional	97%	8	1,004
3b. <i>PdR1b</i> x <b>b42-26</b>	None	Arneis, Morrastel, Palomino, Pedro Ximenez	97%	4	235
3c. <i>PdR1b</i> x <b>b42-26</b> <sup>2</sup>	None	Arneis, Dolcetto, Malvasia Bianca, Montepulciano, Morrastel, Pedro Ximenez	93%	6	1,556
3d. <i>PdR1b</i>	<i>Ren1</i> & <i>Ren4</i>	Alvarelhao, Malvasia Bianca, Morrastel, Sauvignon Vert	98%	4	505
3e. <i>PdR1b</i> x <b>b42-26</b>	<i>Ren4</i> or <i>Ren6</i>	F2-35,...Cab, Chard, Zin	90%, 98%	4	1,404
3f. <i>PdR1b</i> x <b>PdR2</b>	<i>Ren1</i> x <i>Ren4</i> or <i>Ren1</i> x <i>Run2.1</i>	...Cab, Chard, Zin	93%, 94%	5	300
3g. <i>PdR1b</i> x <b>b42-26</b>	<i>Ren1</i> x <i>Ren4</i> or <i>Ren1</i> x <i>Run1</i>	...Cab, Chard, Zin	95%-98%	6	1,251
3h. <i>PdR1b</i> x <b>b42-26</b>	<i>Ren1</i> x <i>Ren4</i> x <i>Run1</i>	...Cab, Chard, Zin	96%	3	446

Our rapid greenhouse screen is critical to our evaluation of Pierce's disease 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 Pierce's disease greenhouse screens analyzed, initiated, and/or completed over the reporting

period. In Group 4A we tested 127 individuals from the *PdR1b* x *PdR2* stacked line (92% *vinifera* level) as well as 24 Pierce's disease x powdery mildew stacked genotypes at about the 90% *vinifera* level. This test was low in severity and was the first screen to implicate the critical temperature relationship between the first 14 days following inoculation and greenhouse screen severity. In Group 4B we tested 136 and 67 genotypes in the T03-16 and b41-13 F1 populations, respectively, as part of Pierce's disease resistance gene discovery work being done in our companion Pierce's disease resistance mapping project. In addition, we confirmed the relative resistance of the genotypes used in the 2016 Pierce's disease and Pierce's disease x powdery mildew crosses. A mechanical failure of the greenhouse heater during the first 14 days post-inoculation in 4C resulted in low greenhouse screen severity and confirmed what we suspected from 4A.

This year was also our most extensive Pierce's disease x powdery mildew screen to date, and we evaluated 98 genotypes from eight different crosses (4D). Pierce's disease resistances included *PdR1b* either alone or with b42-26 resistance and the *Ren1*, *Ren4*, and *Run1* powdery mildew resistance loci. In previous reports we have reported some negative effect on Pierce's disease resistance when Pierce's disease and powdery mildew resistance loci were combined. In this trial, the percent of highly resistant progeny ranged from 9% to 75%. Sample sizes were too small to make a definitive conclusion, but it appeared the selection of the Pierce's disease resistant parent played a more important role in the resistance of a cross progeny than whether the cross was to a powdery mildew resistant parent.

Part of Group 4D was the testing of 50 genotypes in an alternative *PdR1b* x b42-26 line at the 93% *vinifera* level. Fifty percent were promising and one was used as a parent in 2017 crosses. The main focus in 4E was to refine resistance in the b42-26 line primarily associated with chromosome 8. Similarly, in this same group, we retested eight genotypes in the b46-43 line that had anomalous greenhouse screen results relative to their chromosome 14 markers, and provided the results to our companion Pierce's disease mapping project. Promising parents for breeding in novel PdR lines including b40-14, b46-43, and ANU5 were retested, as were remnants of our BD5-117 lines (another multigenic resistance source from a Florida breeding program). One female genotype in the BD5-117 line has tested highly resistant in all three screens, offering the possibility of creating outcrosses to our other lines or crossing to one of the few other BD5-117 line highly resistant genotypes. This latter strategy, however, doesn't allow us to increase the *vinifera* level.

In addition to testing additional Pierce's disease x powdery mildew crosses in Group 4F, we tested 20 accessions of *V. berlandieri* for the first time to evaluate Pierce's disease resistance in this Texas grape species. High enzyme-linked immunosorbent assay (ELISA) results and severe Pierce's disease symptoms suggest that these aren't promising candidates for creating additional Pierce's disease resistant lines. Screening in Group 4G focused on the b47-32 *V. arizonica-monticola* line to identify if resistance is unique or segregates with either chromosome 8 or chromosome 14 markers. Thirty-seven genotypes were tested, with results provided to our companion Pierce's disease mapping project. Only one individual would be a candidate for advancing this as a new Pierce's disease resistance line. In addition, we tested 75 genotypes in the 92% *vinifera* *PdR1* x *PdR2* line to confirm previous tests and identify potential parents. Fully a third were promising, showing the benefit of stacking and careful parent selection.

Testing in Group 4H supports graduate student research in our companion mapping/genetics program looking for non-chromosome 14 Pierce's disease resistance loci in b46-43, which may have additional resistance loci. Four promising parents were identified from the 24 Pierce's disease x powdery mildew genotypes also tested. In Group 4I, we continue to test the F1 progeny of the new T03-16 and b41-13 lines to facilitate genetic mapping of their Pierce's disease resistance. We also included 33 genotypes that should complete the extensive testing of the 92% *vinifera* *PdR1b* x *PdR2* stack group and allow further evaluation of the resistance derived from combining chromosome 14 and chromosome 8 loci as well as minor resistance factors.

We continue to explore Pierce's disease resistance from *Muscadinia rotundifolia* with the testing of 54 genotypes in Group 4J. In the same group we test 75 F1 genotypes to improve the map of the b41-13 resistance source, as well as a confirmatory test of the 2017 parents. On September 28, 2017, cuttings of the first 80 genotypes in the 96% *vinifera* *PdR1* x *PdR2* stack line were taken to initiate the greenhouse screening of this next iteration of our Pierce's disease resistant candidates for release.

**Table 4.** Greenhouse Pierce's disease screens analyzed, completed and/or initiated during the reporting period. Projected dates are in italics.

Group	Test Groups	No. of Genotypes	Inoculation Date	ELISA Sample Date	PD Resistance Source(s)
4A	SRs 2014 Recomb, <i>PdR1</i> x <b>b42-26</b> Stack 2nd tests	170	8/11/2016	11/10/2016	<i>PdR1b</i> ,b42-26
4B	T 03-16,b41-13,2016 parents	259	9/13/2016	12/13/2016	b41-13, b42-26, <i>PdR1b</i> , T 03-16
4C	<i>PdR1b</i> x b42-26 stack & recent promising	115	10/11/2016	1/10/2017	<i>PdR1b</i> , b40-14, b42-26
4D	2015 PD & PD-PM Crosses	155	1/5/2017	3/23/2017	<i>PdR1b</i> , b42-26
4E	b42-26 BC1 & BC2 locus refinement, 2014 Cross highly rated; b46-43, BD5-117	262	3/14/2017	6/15/2017	b42-26, b46-43, BD5-117
4F	Addn PDxPM HW & <i>V. berlanderi</i>	113	3/30/2017	6/29//2017	<i>PdR1b</i> , b42-26, <i>berlandieri</i>
4G	b47-32 & low severity screen retests	170	5/25/2017	8/29/2017	<i>PdR1b</i> , b42-26, b47-32
4H	14-399 b46-43 BC1 Mapping	262	8/1/2017	10/31/2017	b46-43
4I	T 03-16 & b41-13 F1, <i>PdR1</i> x <b>b42-26</b> Stack	92	8/17/2017	11/16/2017	T 03-16, b41-13, <i>PdR1</i> x <i>PdR2</i>
4J	2017 Parents, Rot, b41-13 F1s	159	10/12/2017	1/11/2018	<i>PdR1b</i> , <i>PdR2</i> , <i>rotundifolia</i> , b41-13

**Tables 5a through 5c** detail the vine, fruit, and juice characteristics for the 15 Pierce's disease resistant selections used to make wine lots in 2017. 03182-084 is 75% *vinifera* with multigenic resistance from the Florida cultivar BD5-117 crossed with a pure *vinifera* Cabernet Sauvignon x Carignane genotype. 07355-075 is 94% and thirteen 97% (starting with 09311-160 and ending with 10317-035) *vinifera PdR1b* selections represented the majority of wines made. Selection 12351-03 is our most advanced *PdR1a* selection and is also 97% *vinifera* most recently crossed to a selfed Zinfandel selection 08319-62. In addition, we made wines from a number of *vinifera* controls and Blanc du Bois and Lenoir as reference Pierce's disease resistant cultivars. All were made from Davis grown fruit.

**Table 5a.** The 15 Pierce's disease resistant selections used in small scale winemaking in 2017. Background and fruit characteristics.

Genotype*	Parentage	2017 Bloom Date	2017 Harvest Date	Berry Color	Berry Size (g)	Ave Cluster Wt. (g)	Prod 1 = v low, 9 = v high
03182-084	F2-7 x BD5-117	05/16/2017	09/07/2017	B	1.8	393	6
07355-075	U0505-01 x Petite Syrah	04/30/2017	08/22/2017	B	1.9	329	7
09311-160	07371-20 x Cabernet Sauvignon	05/7/2017	08/29/2017	B	1.6	377	5
09314-102	07370-028 x Cabernet Sauvignon	05/20/2017	08/31/2017	W	1.3	388	9
09330-07	07370-039 x Zinfandel	05/30/2017	09/12/2017	B	1.7	533	8
09331-047	07355-020 x Zinfandel	05/16/2017	08/29/2017	B	1.7	402	5
09331-133	07355-020 x Zinfandel	05/14/2017	08/29/2017	B	2.2	398	6
09333-370	07355-020 x Chardonnay	05/16/2017	08/31/2017	B	1.6	497	6
09338-016	07371-20 x Cabernet Sauvignon	05/30/2017	09/05/2017	W	1.2	390	6
09356-235	07371-19 x Sylvaner	05/30/2017	09/05/2017	B	1.5	368	7

Genotype*	Parentage	2017 Bloom Date	2017 Harvest Date	Berry Color	Berry Size (g)	Ave Cluster Wt. (g)	Prod 1 = v low, 9 = v high
10302-178	07370-028 x Riesling	05/11/2017	08/15/2017	W	1.3	136	4
10302-293	07370-028 x Riesling	04/29/2017	08/15/2017	W	1.0	99	7
10302-309	07370-028 x Riesling	04/27/2017	08/15/2017	W	1.7	262	8
10317-035	07370-028 x Riesling	05/09/2017	08/15/2017	W	1.2	157	7
12351-03	08319-62 x 10312-064	05/20/2017	09/07/2017	B	1.4	266	7

\* Turquoise highlight = pre-released to nurseries in winter/spring 2017.

**Table 5b.** Juice analysis of Pierce's disease resistant selections used in small scale winemaking in 2017.

Genotype*	°Brix	TA (g/L)	pH	L-malic acid (g/L)	potassium (mg/L)	YAN (mg/L, as N)	catechin (mg/L)	tannin (mg/L)	Total anthocyanins (mg/L)
03182-084	20.9	6.1	3.50	1.7	2,190	151	45	350	558
07355-075	27.7	6.3	3.63	1.5	2,390	265	20	408	1219
09311-160	26.0	5.9	3.75	2.5	2,530	276	29	263	887
09314-102	23.3	8.6	3.68	6.6	2,840	432			
09330-07	23.6	5.7	3.73	1.9	2,540	249	26	769	1293
09331-047	27.0	5.1	3.83	1.5	2,230	300	18	470	1191
09331-133	24.8	5.2	3.66	1.6	1,960	251	10	718	929
09333-370	24.9	4.4	3.71	1.6	1,880	196	20	583	848
09338-016	23.8	5.2	3.79	2.4	2,250	280			
09356-235	25.2	5.4	3.81	2.7	2,800	278	50	375	1414
10302-178	23.4	7.3	3.48	2.2	2,200	273			
10302-293	24.5	5.6	3.53	1.1	2,050	112			
10302-309	21.6	5.7	3.40	1.4	1,680	65			
10317-035	22.3	4.9	3.56	1.4	1,730	76			
12351-03	23.5	5.4	3.60	1.4	2,140	141	14	338	323

\* Turquoise highlight = pre-released to nurseries in winter/spring 2017.

**Table 5c.** Pierce's disease resistant selections used in small scale winemaking in 2017. Berry sensory analysis.

Genotype*	Juice Hue	Juice Intensity	Juice Flavor	Skin Flavor	Skin Tannin Intensity (1 = low, 4 = high)	Seed Color (1 = gr, 4 = br)	Seed Flavor	Seed Tannin Intensity (1 = high, 4 = low)
03182-084	pink	light	strawberry, raspberry	neutral, slight hay, slightly canned	1	4	woody, nutty	4
07355-075	red-pink	medium+	fruity, berry, cherry	fruity, slight hay	2	3	woody, nutty	2
09311-160	green	pale	apple, spice	spice, red fruit, slight grass	2	4	woody, spicy, hot	3
09314-102	green	light	apple, pear	hay, straw	1	3.5	woody, spicy, hot	2
09330-07	red	medium	cherry, strawberry	jam, hay, plum	2	4	woody, bitter, salty	3

Genotype*	Juice Hue	Juice Intensity	Juice Flavor	Skin Flavor	Skin Tannin Intensity (1 = low, 4 = high)	Seed Color (1 = gr, 4 = br)	Seed Flavor	Seed Tannin Intensity (1 = high, 4 = low)
09331-047	pink-orange	medium-	cherry, berry	fruity, plum	2	4	ashy, slightly bitter	2
09331-133	red-pink	medium-	raspberry, spice	fruity, slight hay	3	4	buttery, woody	3
09333-370	pink-orange	medium-	berry, plum, spice	fruity, plum	2	4	hot, spicy, bitter	1
09338-016	green-yellow	medium-	green apple, slight spice	neutral, slight hay	1	4	woody, nutty, spicy	3
09356-235	red, tech orange	medium	Berry, plum, spice	fruity, plum	4	4	woody, nutty, spicy	1
10302-178	green-yellow	pale	green apple, slight spice	neutral, straw, vs veg?	3	4	spicy, hot, acrid, bitter	1
10302-293	green-white	very pale	pear, melon, rutabaga	neutral, melon, hay	1	4	woody, smoky	3
10302-309	yellow brown	medium	ripe apple	spicy, neutral	2	3	warm, woody, buttery	3
10317-035	green-yellow	light	pear, melon, sweet spice	veg, hay	3	3	warm, bitter	1
12351-03	orange	medium	hay, dust, chlorine	neutral, slight hay	1	4	woody, spicy	3

\* Turquoise highlight = pre-released to nurseries in winter/spring 2017.

To determine the field resistance of our various Pierce's disease resistant varieties we have established field trials for the last 16 years at Pierce's disease hotspots around California and in several southern states where Pierce's disease is endemic. Our resistant selections in all field trials continue to be free of Pierce's disease symptoms. For example, this fall we scored a trial along the Napa River with Silverado Vineyards planted in the summer of 2014. This site has extreme Pierce's disease incidence and pressure and we relied on natural infection to assess Pierce's disease resistance in the trial. Of the 79 Chardonnay control vines, 53 (67%) had obvious visual Pierce's disease symptoms in this, their fourth leaf. Most of these vines were severely stunted and dying (**Figure 1**). In contrast, none of the 193 vines of our Pierce's disease resistant selections had Pierce's disease symptoms. Shown in **Figure 2** is a typical vine of 09314-102, one of the 97% *vinifera* Pierce's disease resistant selections in the trial. ELISA results to confirm observations from both Chardonnay control and Pierce's disease resistant test vines are pending.

We continue to host wine tastings of our Pierce's disease resistant selections [almost all 97% with the exception of 07355-075 (94%)] for grower and vintner groups. Some of these tastings are at UC Davis with industry and student tasters, and others are at various industry gatherings. On August 24, 2017, wines from the 2016 vintage of three of our 97% *vinifera PdR1b* resistant selections were tasted by about 60 attendees at the North American Grape Breeders Meeting held at UC Davis. All were well received, with particular accolades given to the 09314-102 wine which many thought was a fine Pinot Blanc. On May 6, 2016, a tasting was held at UC Davis to evaluate the 2015 vintage wines from our new Pierce's disease resistant varieties. A total of 17 tasters comprised of winemakers, viticulturists, faculty, staff, and students rated the wines on a hedonic quality scale from 1 = poor to 5 = very good. All wines were produced from grapes grown in Davis. The tasters didn't assess the wines uniformly, however, no taster rated every wine as poor, and most wines were considered "very good" or nearly so by at least one taster. Considered together, all eight of the UC Davis Pierce's disease wines and the Chardonnay and Cabernet Sauvignon control wines were perceived as being of average quality. This is significant praise from a group of professionals familiar with evaluating some of the finest *vinifera* wines in the world, especially

considering that the wines were produced from grapes grown in Davis, were made at a three to five gallon scale, were less than a year old, and had no oak treatment.



**Figure 1.** Silverado Vineyards field trial shown in its fourth leaf on September 18, 2017. The obviously stunted and diseased vines in the foreground are the Chardonnay controls. Healthy 97% *vinifera* UCD test vines are in the background.



**Figure 2.** A typical healthy vine of the UCD Pierce's disease resistant selection 09314-102 shown in its fourth leaf on September 18, 2017 at Silverado Vineyards along the Napa River.

I also conducted tastings at Driftwood, west of Austin, Texas, at the American Society of Enology and Viticulture East Section meetings in Charlottesville, Virginia, and will be conducting a tasting at the January 2018 meeting of the Georgia Wine Producers in Gainesville, Georgia. There were about 125 people at the Texas tasting and I presented the five Pierce's disease resistant selections that have been pre-released (see **Table 5**). The wines were very well received and generated a lot of discussion and excitement. We have three trials with 88% and 94% *vinifera* selections in Texas (in cooperation with Jim Kamas of Texas A&M) and they presented small-scale wines from their trials. Three 88% *vinifera* selections are planted in Alabama have been expanded to 1,000 vines each. This plot is in cooperation with Randall Wilson of White Oak Cellars. The vines are thriving and commercial scale wines are being made.

New trials established this year in Pierce's disease hot spots include 400 each of the five selections on pre-release planted in Ojai with Adam Tolmach, 350 buds each of 07355-075 and 09331-047 for a field-budded vineyard with Ashley Anderson at Cain Vineyards in Napa, and 1,000 bench-grafted vines on 101-14 for an early spring 2018 Napa planting with Daniel Bosch at Constellation. We are now testing our resistant selections in multiple Napa sites, Sonoma, Temecula, Ojai, Texas (three sites), Alabama, and Florida. The resistant selections are not showing Pierce's disease symptoms and are thriving under very diverse environments and under what must be a wide diversity of *Xf* strains.

## CONCLUSIONS

We continue to make rapid progress breeding Pierce's disease resistant winegrapes through aggressive vine training, marker-assisted selection, and our rapid greenhouse screen procedures. These practices have allowed us to produce four backcross generations with elite *V. vinifera* winegrape cultivars in 10 years. We have screened

through thousands of seedlings that are 97% *V. vinifera* with the *PdR1b* resistance gene from *V. arizonica* b43-17. Seedlings from these crosses continue to crop, and others are advanced to greenhouse testing. We select for fruit and vine quality and then move the best to greenhouse testing, where only those with the highest resistance to *Xf*, after multiple greenhouse tests, are advanced to multi-vine wine testing at Davis and in Pierce's disease hot spots around California. The best of these are being planted in vineyards at 50 to 1,000 vine trials with enough fruit for commercial-scale winemaking. We have sent 19 advanced scion selections to Foundation Plant Services (FPS) over the past four winters to begin the certification and release process. Three Pierce's disease resistant rootstocks were also sent to FPS for certification. Pierce's disease resistance from *V. shuttleworthii* and BD5-117 is also being pursued, but progress and effort is limited because their resistance is controlled by multiple genes without effective resistance markers. Other forms of *V. arizonica* are being studied, and the resistance of some will be genetically mapped for future efforts to combine multiple resistance sources and ensure durable resistance. Very small-scale wines from 94% and 97% *V. vinifera PdR1b* selections have been very good and have been received well at tastings in the campus winery and at public tastings throughout California, 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 October 2016 to October 2017.

### ABSTRACT

The aims of this project are to identify new sources of Pierce's disease resistance, genetically map their resistance loci, and enable the development of DNA markers that can be used in marker-assisted selection to expedite our breeding program. This project is also physically mapping and cloning candidate Pierce's disease resistance genes from grape, with native promoters, to understand how the genes function. We continue to achieve success on all fronts. A wide range of southwestern United States and northern Mexico *Vitis* species accessions was screened and 14 resistant accessions were selected to develop breeding populations. We employed a limited mapping strategy to verify that the new resistances were genetically different from the previously identified locus *PdR1* by ensuring the new resistances were from different genomic regions. This approach identified three new resistant accessions, T03-17, b41-13, and ANU67, all of which have Pierce's disease resistance on different chromosomes. We also identified a second resistance locus *PdR2* on chromosome 8 in the resistant *V. arizonica* accession b42-26. The identification of this new locus and the new sources of resistant germplasm bring us much closer to stacking resistance from multiple backgrounds so that we can broaden resistance, making it more durable. We continue to develop and expand breeding populations from new promising resistant lines. Physical maps were completed for b43-17 (*PdR1a* and *PdR1b*) and for the *PdR1c* locus (from *V. arizonica* b40-14). Upstream and downstream sequences, as well as the gene sequences of two candidate genes, open reading frame (ORF)14 and ORF18 from *PdR1b*, were verified, and constructs were developed with native promoters. We generated and maintain embryogenic callus of *V. vinifera* cvs. Chardonnay and Thompson Seedless and *V. rupestris* St. George for use in verifying that the loci we map do control Pierce's disease resistance. Transgenic lines with both candidate genes, and their native promoters, were developed, propagated, and are now being greenhouse screened to validate their function. These efforts will help us characterize and validate candidate resistance genes by complementation and allow us to better understand how they function. Such efforts could also lead to genetically engineering *V. vinifera* cultivars with grape Pierce's disease resistance genes. A large-scale, multiple time point gene expression project was completed in the greenhouse and RNA extractions were finished for over 400 samples. Quantitative polymerase chain reaction experiments were used to test the expression of the two candidate resistance genes. The molecular genetic tools developed in this project are used to expedite our Pierce's disease resistant winegrape breeding program and are crucial for its success.

### LAYPERSON SUMMARY

Our main focus is to identify and genetically characterize unique Pierce's disease resistance sources from southwestern United states and Mexican *Vitis* species collections. In order to carry out the task we create genetic maps that associate regions of chromosomes with Pierce's disease resistance. These regions (markers) are used to expedite screening for resistance, since they can be used to test seedlings for resistance as soon as they sprout. Markers developed from different sources of resistance allow us to combine multiple resistance forms and therefore produce offspring with more durable resistance. These markers also allow us to identify resistance genes and engineer them into susceptible grapes, which we are doing to better understand the genes and the resistance.

## INTRODUCTION

This project continues to provide molecular support to the Pierce's disease resistant grape breeding project ("Breeding Pierce's Disease Resistant Winegrapes") by acquiring and testing a wide range of resistant germplasm, tagging resistance regions with markers by genetic mapping, and functionally characterizing the resistance genes from different backgrounds. To meet five key objectives of the program we have surveyed over 250 accessions of *Vitis* species growing in the southern United States and Mexico in an effort to identify new Pierce's disease resistant accessions. Analysis using population genetics tools allowed us to better understand gene flow among resistant species and their taxonomic and evolutionary relationships. Twenty resistant accessions were identified from screening of more than 250 accessions of germplasm collected from Mexico and the southwestern United States. Markers were used to determine the diversity and relationships of these accessions to each other. Small breeding populations were developed and more than 700 seedlings were marker tested to ensure correct parentage and identity. We used a limited mapping strategy by utilizing markers from chromosome 14, in conjunction with greenhouse screen data of the small breeding populations, to determine if resistance to Pierce's disease is different from the previously identified resistance locus *PdR1*. Three new unique resistance sources (T03-16, ANU67, and b41-13) were identified as having a different resistance region than chromosome 14 (Riaz et al. submitted). More crosses were made in spring 2016 to expand these breeding populations for map-based identification of genomic regions that contribute to resistance. We are continuously developing and expanding breeding populations from new promising resistant lines.

The identification and characterization of resistance genes and their regulatory sequences will help determine the basis of resistance/susceptibility in grape germplasm. In addition, these genes and their promoters could be employed in production of 'cisgenic' plants. Cisgenesis is the transformation of a host plant with its own genes and promoters (Holmes et al. 2013). Alternatively, other well characterized *vinifera*-based promoters, either constitutive (Li et al. 2012) or activated by *Xylella fastidiosa* (*Xf*) (Gilchrist et al. 2008), could be utilized. We have completed the physical mapping for b43-17 to clone and characterize resistance genes (*PdR1a* and *PdR1b*, see earlier reports). The physical map of the *PdR1c* locus (from b40-14) is also now complete. Development of *V. vinifera* plants transformed with our Pierce's disease resistance genes and grape promoters might work more effectively and allow us to better understand the *PdR1* resistant gene's function.

Upstream and downstream sequences as well as gene sequences of two candidate genes, open reading frame (ORF)14 and ORF18 from *PdR1b*, were verified, and constructs were developed. Transformation experiments with the *PdR1* resistance gene with a native grape promoter were completed with ORF18, and transgenic lines are being developed and maintained for later resistance verification. A large-scale multiple time point gene expression project was completed in the greenhouse and RNA extractions were completed for over 400 samples. We used quantitative polymerase chain reaction to test the expression of candidate genes. Embryogenic callus cultures of *V. vinifera* cvs. Chardonnay and Thompson Seedless and *V. rupestris* St. George are being maintained. These efforts will help us to identify candidate resistance genes by complementation and better understand how they function.

## OBJECTIVES

The specific objectives of this project are:

1. Provide genetic marker testing for mapping and breeding populations produced and maintained by the Pierce's disease resistance-breeding program, including characterization of novel forms of resistance.
2. Complete a physical map of the *PdR1c* region from the b40-14 background and carry out comparative sequence analysis with b43-17 (*PdR1a* and *b*).
3. Employ whole genome sequencing (50X) of recently identified Pierce's disease resistant accessions and a susceptible reference accession, and use bioinformatics tools to identify resistance genes, perform comparative sequence analysis, and develop single nucleotide polymorphism (SNP) markers to be used for mapping.
4. Clone *PdR1* genes with native promoters.
5. Compare the Pierce's disease resistance of susceptible grapevines transformed with native vs. heterologous promoters.

## RESULTS AND DISCUSSION

### Objective 1. Provide Genetic Marker Testing for Mapping and Breeding Populations Produced and Maintained By the Pierce's Disease Resistance-Breeding Program, Including Characterization of Novel Forms of Resistance

Greenhouse testing was completed for over 250 southwestern and northern Mexico *Vitis*, which included accessions collected from multiple collection trips across the southwestern States bordering Mexico or previously collected from Mexico by Olmo. Both simple sequence repeat (SSR) and chloroplast markers were used to establish relationships with known sources of resistance currently being used in the breeding program (Riaz and Walker 2013). Small breeding populations were developed with 14 of the most promising resistant accessions by crossing to highly susceptible *V. vinifera*. In spring 2016 we extracted DNA from the 704 individuals obtained from these breeding populations that were also greenhouse screened. We carried out a limited mapping strategy by utilizing markers from chromosome 14 that are linked to the *PdR1* locus (see previous reports for details of the *PdR1* locus). This strategy allowed us to identify resistance sources whose resistance is similar to *PdR1* and sources that are different among the newly identified accessions. Twelve SSR markers that cover a 3.5 megabase region including the *PdR1* locus and genotypic data with 22 markers from 19 chromosomes was used to analyze how genetically distinct the resistant accessions were from each other. Based on the polymorphic markers for each breeding population, a genetic map was created to determine the relative marker order and then quantitative trait locus (QTL) analysis for each population was carried out. The results from this study identified nine accessions with a major resistance locus within the genetic window where the *PdR1* locus from accession b43-17 was mapped. Results were not conclusive for two accessions, A14 and b47-32, due to small population size and/or lack of polymorphic markers. The phenotypic data of three accessions, ANU67, b41-13, and T03-16, did not correlate with the resistance markers from chromosome 14. These three accessions were identified as candidates for further work to develop a framework map with larger populations to detect new unique loci for Pierce's disease resistance breeding. The small breeding populations used in this study effectively identified the presence or absence of a major resistance locus. This approach is being used to enhance the Pierce's disease resistant winegrape breeding program by rapidly identifying new resistance loci and broadening the genetic base of resistance. The major findings of this work are submitted for publication in a peer-reviewed journal.

Accession T03-16 from the Big Bend region in Texas and b41-13 from Tamaulipas state in Mexico are strong candidates that do not possess *PdR1*. These accessions have great potential for use in the Pierce's disease grapevine breeding program. In order to identify the genomic regions in these two accessions, crosses were made in spring 2016 to expand population sizes. In three backgrounds we were not able to determine if resistance is different than *PdR1* due to the small population size (**Table 1**). We plan to expand the number of individuals in those backgrounds, greenhouse test them for Pierce's disease resistance, and carry out analysis next year to determine whether they possess *PdR1*. These results will get us one step closer to finding a new mechanism of Pierce's disease resistance that we can use in our breeding program. **Table 1** presents the breeding populations that were developed with new resistance sources (for details, see previous reports). We completed propagation of four to five replicates for the subset of crosses mentioned in **Table 1**. These plants were inoculated with *Xf* in September and the results of the assay will be available in winter 2018.

We have also identified a new locus, *PdR2*, in the *V. arizonica/girdiana* b42-26 background. To create a genetic map of the F1 population 05347 (F2-35 x b42-26) we expanded the population to 352 seedling plants and tested more than 1,000 markers. The level of polymorphism in b42-26 is very low, likely because of its geographic isolation and resulting inbred genetic background. The genetic map was developed with 163 markers grouped to 17 chromosomes. Chromosomes 10 and 19 were not represented. We carried out analysis with this map and identified resistance on chromosome 8, which was also verified on the basis of linked alleles in the pBC1 and pBC2 populations. The resistance locus is called *PdR2*, and it resides between markers FAM82 and VMC 7h2. In spring 2017 we began using closely linked markers to assist the breeding program with the use of marker-assisted selection to stack the *PdR1b* and *PdR2* loci together. Additional markers from chromosomes 10 and 19 were also tested to get complete representation of the genome for the final genetic map and QTL analysis. A manuscript detailing genetic mapping in b42-26 and b40-14 is approaching publication.

This project also provides molecular support to the companion Pierce's disease resistance winegrape breeding project by marker testing seedling plants. In spring 2017 we marker tested 1,895 seedling plants, from 23 different crosses, for *PdR1* and *PdR2* loci. A total of 1,380 seedlings were tested for both loci and 515 seedlings were

tested for *PdRI* locus only. A total of 902 seedling plants from 14 different crosses were tested for veracity. In total, we extracted DNA from 2,797 seedling plants for different Pierce's disease resistance breeding projects.

**Table 1.** Resistant accessions used for the 23 breeding populations.

Resistance Source	Species Description	Populations Tested	Number of Screened Genotypes	Results of Limited Mapping Strategy*
ANU5	<i>V. girdiana</i>	12-314	60	LG14
b40-29	<i>V. arizonica</i> , brushy	12-340, 12-341, 14-367, 14-368	29	LG14
b46-43	<i>V. arizonica</i> , glabrous hybridized with <i>V. monticola</i> ?	12-305, 14-308, 14-321, 14-322, 14-324, 14-336	159	LG14
b41-13	<i>V. arizonica-mustangensis</i> and <i>champinii</i> hybrid, red stem with hairy leaves	13-355	47	Inconclusive
b47-32	<i>V. arizonica</i> glabrous with <i>monticola</i> , small clusters, red stem	13-344	13	Inconclusive
SC36	<i>V. girdiana</i>	13-348	35	LG14
T03-16	<i>V. arizonica</i> glabrous	13-336	62	Inconclusive
A14	<i>V. arizonica</i>	14-313	25	Inconclusive
A28	<i>V. arizonica</i>	14-347, 14-364	42	LG14
ANU67	<i>V. arizonica</i> glabrous	14-362	28	Inconclusive
ANU71	<i>V. arizonica-riparia</i> hybrid	14-340	30	LG14
C23-94	<i>V. arizonica</i> glabrous and brushy	14-303	44	LG14
DVIT 2236.2	<i>V. cinerea</i> like, long cordate leaves, short wide teeth, small flower cluster	14-360	30	LG14
SAZ 7	<i>V. arizonica</i>	14-363	52	LG14

\* Resistant accessions with different sources of resistance are marked as Not 14 in the last column. Accessions marked as LG14 possess the *PdRI* locus. Resistance affinity to chromosome 14 could not be determined for the accessions that are marked as Inconclusive due to small population size and less informative markers.

## Objective 2. Complete a Physical Map of the *PdRIc* Region from the b40-14 Background and Carry Out Comparative Sequence Analysis with b43-17 (*PdRIa* and *b*)

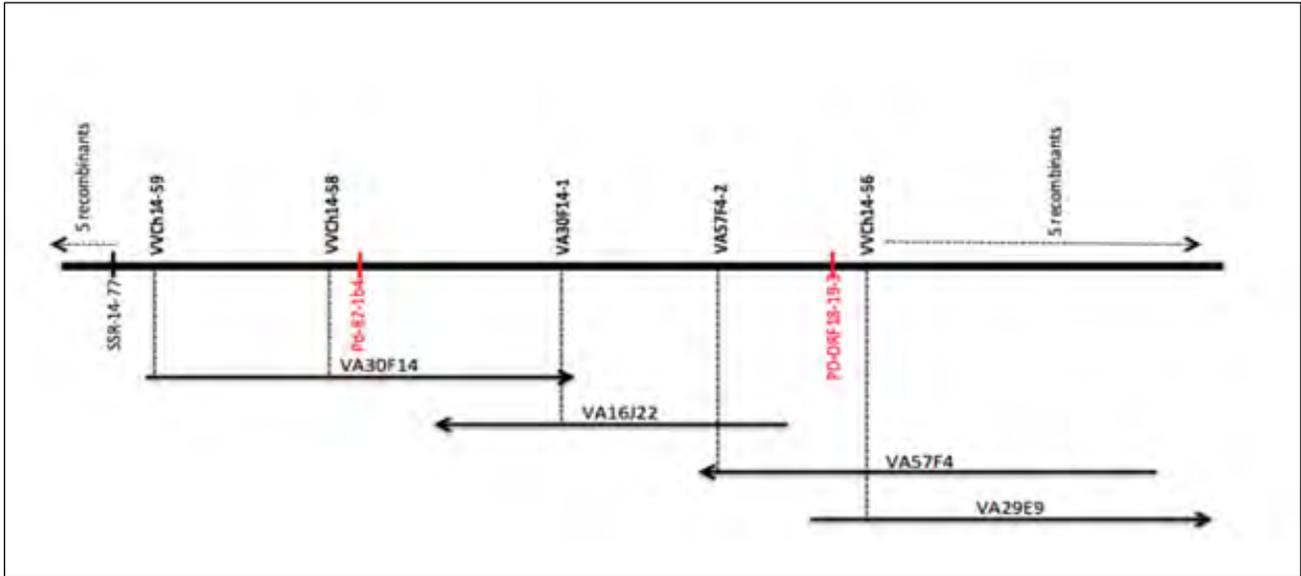
QTL analysis with the SSR-based genetic map of *V. arizonica* b40-14 identified a major Pierce's disease resistance locus, *PdRIc*, on chromosome 14 (see previous reports for details). The genomic location of the *PdRIc* locus is similar to the *PdRIa* and *PdRIb* loci. An additional 305 seedlings were marker tested to identify unique recombinants using new SSR markers developed from the b43-17 sequence to narrow the genetic mapping distance. Four recombinants were identified between chromosome 14-81 and VVIn64, and one recombinant between the chromosome 14-77 and chromosome 14-27 markers. The new markers position the *PdRIc* locus in a 325 kilobase (kb) region based on the sequence of b43-17.

A bacterial artificial chromosome (BAC) library from b40-14 genomic DNA (see details in previous reports) was screened and 30 BAC clones were identified with two probes, chromosome 14-56 and chromosome 14-58. BAC clones that represent *PdRIc* were separated from the other haplotype and four overlapping BAC clones, VA29E9, VA57F4, VA30F14, and VA16J22, were selected for sequencing. Common probes between the *PdRIc* and *PdRIb* region were used to align the sequences. The assembly of four BAC clones is presented in **Figures 1A** and **1B** that represents the sequence analysis of *PdRIb* and reference grape genome PN40024 region. A manuscript titled "The Physical Map of Pierce's Disease Resistance Locus, *PdRIc*" is in preparation.

The assembly of H43-I23 from the b43-17 BAC library that represents the *PdRIa* haplotype (F8909-17) was also completed. The length of assembled sequence was 206 kb. The ORFs of the *PdRIb* region and the BAC clone H69J14 were used to make comparisons. There was complete homology between the over-lapping BAC clone sequences that reflect two different haplotypes. The BAC clone H43I23 has ORF16 to ORF20, and all five ORFs

have identical sequences to the *PdR1b* haplotype. Based on these results we concluded that there is complete sequence homology between haplotype a and b of the *PdR1* locus. Therefore, cloning and functional characterization of genes from any one haplotype will be sufficient for future work. Complete sequence homology also reflects that the parents of b43-17 must be closely related and may have a first-degree relationship and acquired resistance from shared parents. This also explains why we observed complete homozygosity of SSR markers for the *PdR1* locus in the resistant accession b43-17.

(A)



(B)



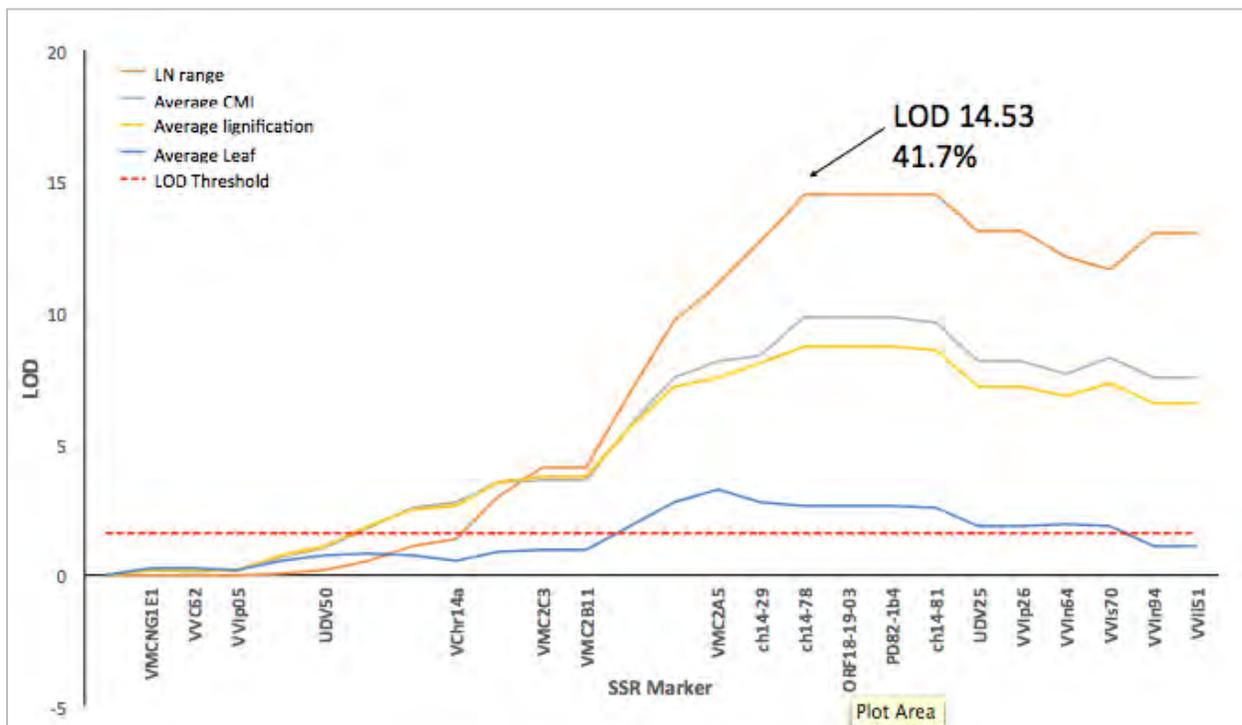
**Figure 1.** (A) BAC library was developed from genomic DNA of b40-14 and screened with probes. Four over-lapping clones were selected for sequencing the complete region. (B) The sequences of four BAC clones were assembled and full-length open reading frames were identified. Sequences were compared with the reference genome and checked for synteny in that region.

**Objective 3. Employ Whole Genome Sequencing (50X) of Recently Identified Pierce’s Disease Resistant Accessions and a Susceptible Reference Accession, and Use Bioinformatics Tools to Identify Resistance Genes, Perform Comparative Sequence Analysis, and Develop SNP Markers to Be Used for Mapping**

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

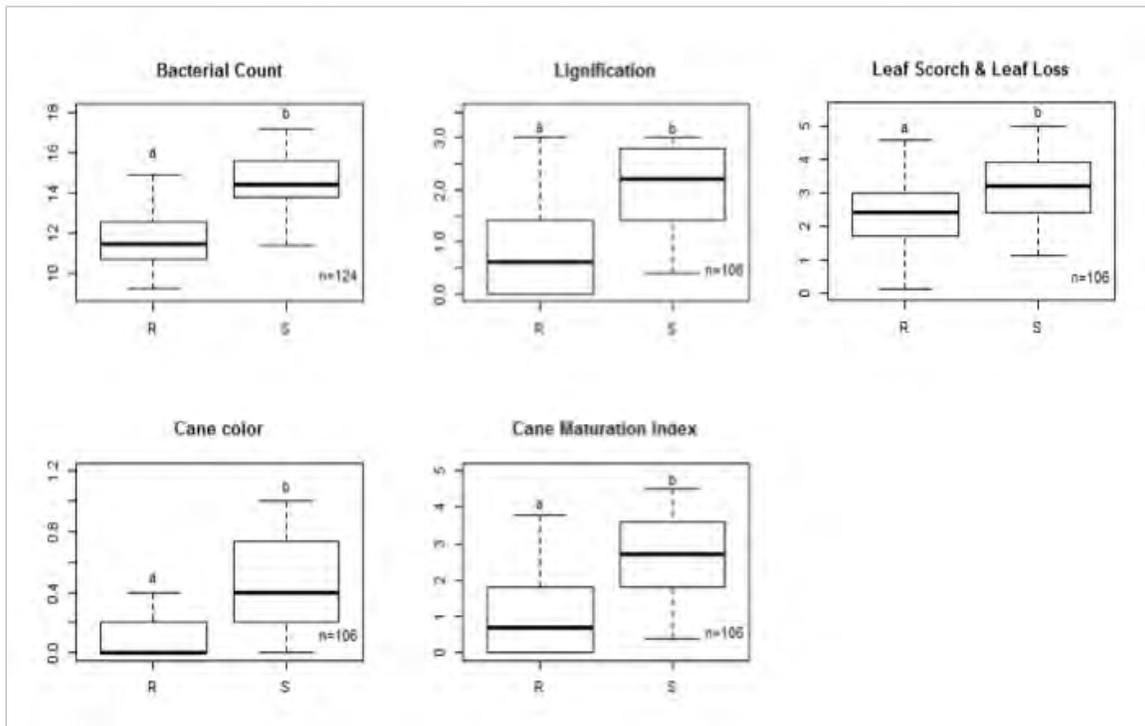
The *V. arizonica* accession b46-43 is homozygous resistant to Pierce’s disease. Multiple crosses to *V. vinifera* were made to develop BC1 populations in 2014 and 2015. Breeding populations were tested with markers to verify the integrity of the crosses. Greenhouse screening of the BC1 populations with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

We completed the map of only chromosome 14 for the BC1 mapping population (14399) and completed greenhouse screening for 121 seedling plants. QTL analysis results indicated that the identified locus explains only ~42% of the phenotypic variation, indicating that there might be another locus on a different chromosome (Figure 2).



**Figure 2.** QTL analysis results of interval mapping of the pBC1 14399 population for chromosome 14. The arrow represents the maximum logarithm-of-odds (LOD) for marker ch14-78 and the percent-explained variation for Pierce’s disease resistance. The red dotted line is LOD threshold for a significant QTL call. All mapped markers are on the x-axis.

**Figure 3** presents the correlation of different phenotypic parameters we have used to screen the pBC1 population. We are currently repeating the greenhouse screen and expanding the mapping effort to develop a framework map of all chromosomes to identify any other genomic region(s) that contribute to the resistance.



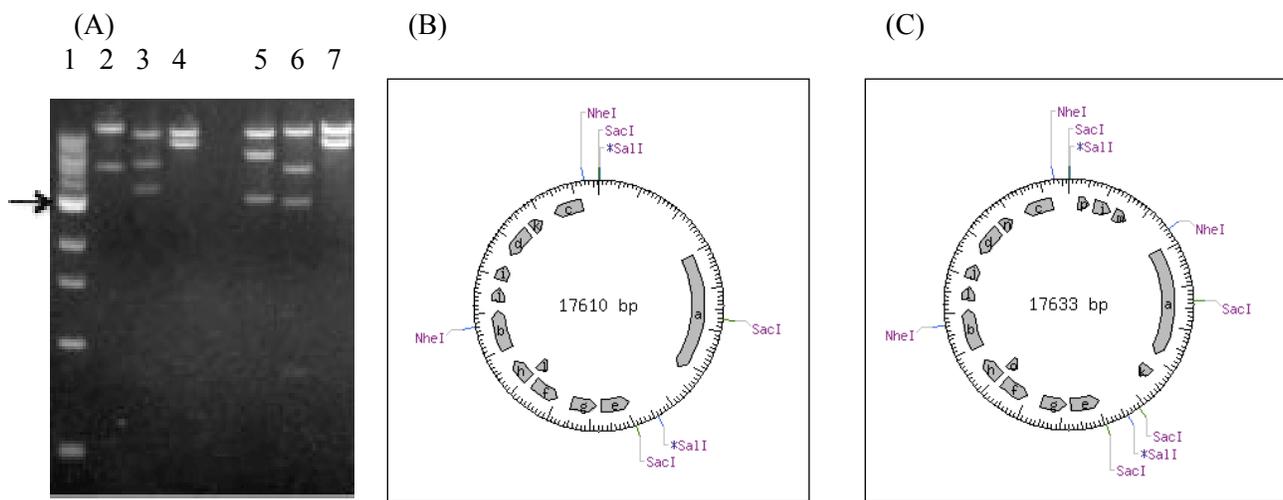
**Figure 3.** Comparison between resistant (R) and susceptible (S) genotypes in each measured phenotypic parameter. Significant differences with Tukey's test are indicated with letters a and b. The letter 'n' denotes the number of genotypes screened.

#### **Objective 4. Cloning of *PdR1* Genes with Native Promoters**

We employed PAC BIO RSII sequencing approach to sequence H69J14 and three other overlapping BAC clones containing both markers flanking the *PdR1b* resistance locus. The assembled sequence data generated a 604 kb long fragment without any gaps. 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 and non-host specific defense. With the help of molecular markers we limited the genetic region to 82 kb, with five ORFs associated with disease resistance and other plant functions described above. ORF sequences found outside the 82 kb window are also highly similar. Two ORFs, V.ari-RGA14 and V.ari-RGA18, within the resistance region boundaries, are the most likely candidates for *PdR1b*. The other three sequences, V.ari-RGA15, 16, and 17, are shorter and contain a large number of transposable elements.

Both resistance gene analogs (RGA) 14 and 18 have a very similar sequence profile except that RGA18 is 2,946 base pairs in size and lacks the first 252 base pairs of sequence that is part of RGA14. Functional analysis of the protein sequence of both RGAs revealed that RGA14 lacks a signal peptide in the initial part of the sequence. This result was verified using 3' rapid amplification of cDNA ends (RACE) to specifically amplify RNA from grapevines transformed with V.ari-RGA14 under the 35S promoter. The results found that mature mRNA does not contain a signal peptide, necessary for proper membrane localization, at the beginning of the sequence thus leaving RGA18 as the strongest candidate. Sequence verification for RGA14 and RGA18 and flanking sequences were completed and fragments that contain the entire coding region plus ~3 kb upstream and ~1 kb downstream sequences were synthesized and cloned into pCLB2301NK at Genewiz, Inc. pCLB2301NK is an optimized vector (Feechan et al. 2013), capable of carrying large DNA sequences, thus allowing us to insert the candidate genes plus surrounding sequences.

New plasmids, called pCLB2301NK-14 and pCLB2301NK-18, were verified by restriction analysis in our lab (**Figure 4**). Besides the corresponding 7 kb fragment, containing RGA14 or RGA18, these plasmids contain a 35S:mGFP5-ER reporter cassette and a kanamycin-selectable marker gene with the nopaline synthase promoter.



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

We sequenced genotype U0505-22, which is used as a biocontrol in our greenhouse screenings. This genotype was originally selected for the presence of *PdR1b* markers in our breeding program. However, U0505-22 is susceptible to Pierce's disease despite being positive for the markers, which then offers the opportunity to explore the changes that could explain this behavior at the DNA level. Primers were designed to produce three kb fragments that include sequences upstream and downstream of RGA14 or RGA18, in order to increase the specificity of the amplification and facilitate cloning. Results obtained with U0505-22 showed the amplification of fragments of the predicted size, but with sequences that differ from RGA14 and RGA18 in several bases. On the other hand, sequencing of cDNA from b43-17, the original source of resistance, 16 days after inoculation resulted in the amplification of fragments with sequences identical to RGA14 and RGA18 with a 500 base pair deletion close to the 5' end.

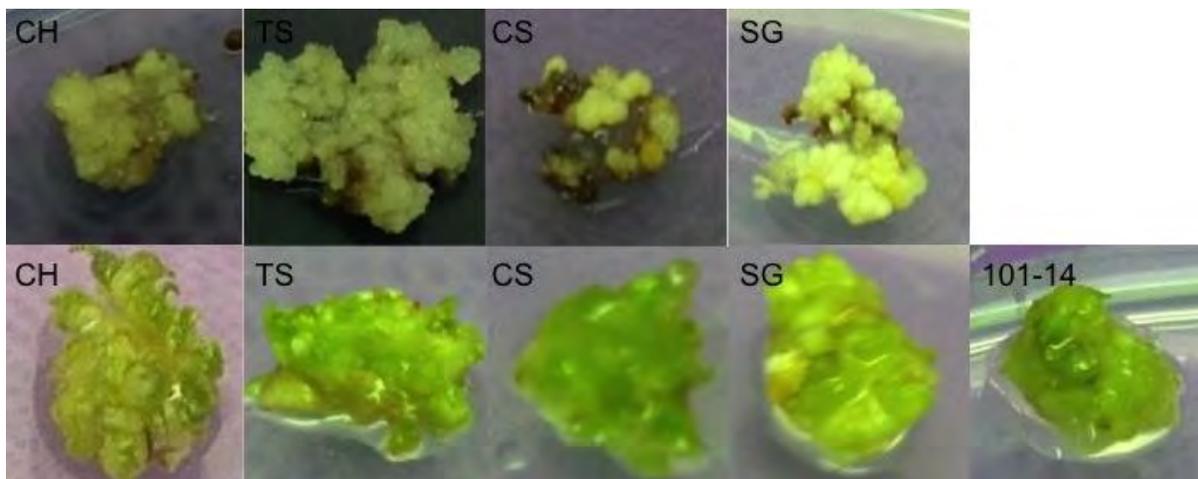
A large experiment with resistant and susceptible plants using multiple replicates and time points for control (uninoculated) and inoculated plants (see details in previous report) was completed. To date, we have completed RNA extractions from 450 samples in the above-mentioned experiment. We have also designed primers and determined primer efficiency for gene expression studies with both RGA14 and RGA18. Two different primer pairs with efficiencies of greater than 90% were selected to carry out preliminary analysis with uninoculated and inoculated samples of Chardonnay and F8909-17 (source of *PdR1*). Preliminary results with samples from six time points indicates that the expression level of both RGA14 and RGA18 in F8909-17 increases after day eight in comparison to uninoculated, which peaks at day 23 and then decreases. Uninoculated and inoculated susceptible Chardonnay did not show any expression. Gene expression and cDNA sequence analysis is underway.

#### **Objective 5. Comparing the Pierce's Disease Resistance of Plants Transformed with Native vs. Heterologous Promoters**

We have established an *Agrobacterium* mediated transformation system followed by regeneration of plants from embryogenic callus. We have streamlined the protocol and have established cultures of pre-embryogenic callus derived from anthers of *V. vinifera* Thompson Seedless, Cabernet Sauvignon, Chardonnay, and the rootstock *V. rupestris* St. George (Agüero et al. 2006). In an earlier phase of this project we transformed these varieties with

five candidate genes containing the 35S cauliflower mosaic virus promoter, the nopaline synthase terminator, and an *hptII*-selectable marker gene (see previous reports for details). We completed testing and found that the transgenic plants did not confer Pierce's disease resistance or tolerance. These results are in accordance with the latest assembly obtained using PAC BIO SRII system. They show that only one of the sequences tested, V.ari-RGA14, lays within the more refined resistance region of 82 kb. The 3'RACE technique was used to amplify RNA from V.ari-RGA14 transformed grapevines and results showed that mature mRNA does not contain the signal peptide, necessary for proper membrane localization, at the beginning of the sequence. However, this could result from a lack of effect of 35S on splicing.

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



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

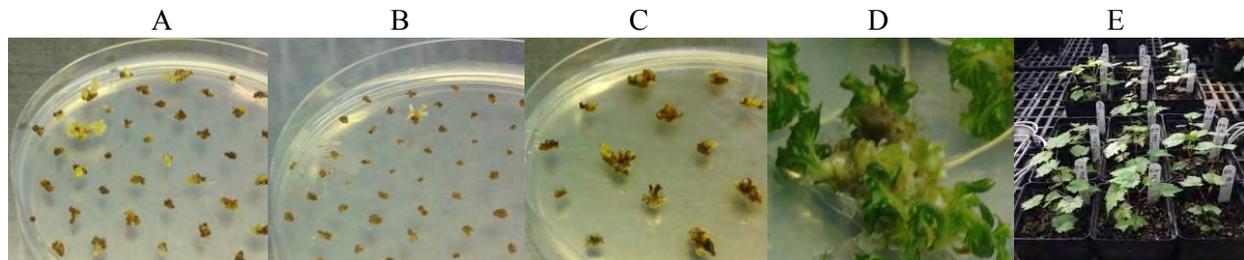
In order to include native promoters and terminators in constructs for future genetic transformations, we verified sequences upstream and downstream of V.ari-RGA14 and 18, the two most likely *PdR1b* candidates. Sequence verification was completed up to four to six kb in the upstream region and one kb in the downstream region. *In silico* analysis of the upstream regions with PlantCare, a database of plant cis-acting regulatory elements, showed that upstream sequences contain several motifs related to drought and defense responses.

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

*Agrobacterium tumefaciens* strain EHA 105 pC32 was chemically transformed with pCLB2301NK-14 or pCLB2301NK-18 and subsequently used to transform embryogenic calli of *V. vinifera* cvs. Chardonnay, Thompson Seedless, and the rootstock *V. rupestris* St. George. Transformation experiments with pCLB2301NK-18 and pCLB2301NK-14 were initiated in March and July 2016, respectively, after synthesis and cloning was completed. In addition, *Agrobacterium* was used to transform meristematic bulk of Pierce's disease susceptible

genotypes selected from the 04-191 population, which are 50% *vinifera*, 25% b43-17, and 25% *V. rupestris* A. de Serres (as in the original population used for *PdR1b* mapping). These genotypes can provide an additional genetic background for analysis of expression of *PdR1* candidate genes. Two of these genotypes, designated 29-42 and 47-50, exhibited great potential for the development of meristematic bulks (**Figure 6**) and transformation experiments with *Agrobacterium* have been initiated.

**Table 2** shows the number of independent lines regenerated up to date, while **Figure 6e** shows the most advanced cultures growing in the greenhouse. V.ari-RGA18 lines in the greenhouse were multiplied from green cuttings and were inoculated with *Xf* in August 2017. It is expected that V.ari-RGA14 lines will be tested in January 2018. Lines in the greenhouse have tested positive for the presence of transgene by polymerase chain reaction. Transgene expression will be analyzed two months after inoculation.



**Figure 6.** (A) Embryo regeneration from embryogenic callus in Thompson Seedless. (B) Embryo regeneration from embryogenic callus in St. George. (C) Shoot regeneration from meristematic bulks in St. George. (D) Meristematic bulk development in genotype 47-50 from the 04-191 population. (E) First group of independent lines inoculated with *Xf*.

**Table 2.** Number of independent lines regenerated after transformation with *Agrobacterium* carrying pCLB2301NK-18 or pCLB2301NK-14.

Genotype	No. Lines <i>In Vitro</i>	No. Lines in Greenhouse
pCLB2301NK-18		
Chardonnay	13	10
T. Seedless	30	11
St. George	4	-
pCLB2301NK-14		
Chardonnay	20	10
T. Seedless	18	10
St. George	4	-

## CONCLUSIONS

We completed greenhouse screening, marker testing, and QTL analysis of breeding populations from 15 new resistance sources including b46-43 and T03-16. We identified T03-16 and b41-13 as possessing resistance on a different region than chromosome 14. Crosses were made to expand these breeding populations for framework map development in order to identify other genomic regions of resistance. Our primary goal is to identify new sources of resistance that do not reside on chromosome 4 so we can facilitate stacking of these resistance sources with *PdR1* from b43-17, since the incorporation of multiple resistances should make resistance more durable. We have also identified a new resistance locus (*PdR2*) from the b42-26 background and closely linked markers are being used in marker-assisted selection to stack resistance loci from these different backgrounds. We have completed the genetic and physical mapping of Pierce’s disease resistance from b40-14. This resistance source maps within the *PdR1b* locus, but it may be an alternative gene within this complex replicated locus. Finally, we verified the sequence of two candidate genes from the *PdR1b* locus, completed transformations with ORF18 and ORF14, and obtained transgenic lines for complementation tests in the greenhouse, scheduled to complete in fall

2017 to spring 2018. This effort is also identifying the promoters of these genes, so that we can avoid the use of constitutive non-grape promoters like CaMV 35S.

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#### **FUNDING AGENCIES**

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

**GENETIC ANALYSIS OF PIERCE'S DISEASE RESISTANT PROGENY OF N18-6 x FLAME  
SEEDLESS GRAPEVINE BREEDING POPULATION**

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**Reporting Period:** The results reported here are from work conducted October 2016 to September 2017.

**ABSTRACT**

One hundred eighty three hybrids of advanced breeding parent N18-6 crossed with Flame Seedless (*Vitis vinifera*) were evaluated for Pierce's disease (PD) under greenhouse growing conditions. N18-6 is genetically inherited from PD resistant sources derived from the cross DC1-56 (W1521 x Aurelia) x Orlando Seedless (D4-176 x F9-68). N18-6 possesses several desirable horticultural traits such as high yield, good flesh texture, flavor, and PD resistance. It is one of the few germplasms that could survive under high disease pressure environments in Florida. Results showed that while most *Xylella fastidiosa*-infected hybrid lines developed typical PD symptoms, about 15 lines showed partial resistance with mild or no PD symptoms and significantly lower bacterial titers compared to PD-susceptible progeny. To further identify the molecular basis of PD resistance, a molecular marker assisted approach has been developed to facilitate mapping resistance loci linked to PD resistant traits. This study adds new PD resistance breeding lines for PD management.

**FUNDING AGENCIES**

Funding for this project was provided by the Consolidated Central Valley Table Grape Pest and Disease Control District.

# ASSESSING EFFECTS OF SEASONALITY ON THE EPIDEMIOLOGY OF PIERCE'S DISEASE IN THE SOUTHERN SAN JOAQUIN VALLEY

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**ABSTRACT**

The introduction of the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) to California resulted in epidemics of Pierce's disease in the Temecula Valley and the southern San Joaquin Valley in the late 1990s and early 2000s, respectively. In response, an area-wide suppression program was initiated that successfully suppressed GWSS populations from 2002-2011. Since 2011, population levels of GWSS have been high in some locations in the southern San Joaquin Valley, resulting in increased levels of Pierce's disease. A field study was initiated in the spring of 2016 to identify the time of year that GWSS are most likely to acquire and transmit *Xylella fastidiosa* (*Xf*). To accomplish this, four citrus orchards and four vineyards were sampled every three weeks beginning in April 2016. On each visit the abundance of GWSS on five to ten citrus trees or five to ten grapevines was assessed. In addition, GWSS were collected and tested for the presence of *Xf* by quantitative polymerase chain reaction (qPCR). Finally, plant samples were collected at vineyard sites from chronically infected grapevines to determine seasonal changes in *Xf* population densities in plants. In 2016 the abundance of GWSS was high at vineyard and citrus sites. Specifically, 536 sharpshooters were collected from citrus locations and 146 sharpshooters were collected from vineyards during 2016, with 74 (14%) and 28 (19%) testing positive for *Xf*. The number of *Xf*-positive sharpshooters in 2016 was low from April to late July and markedly increased in late July through early September. The timing of the increase in *Xf*-positive sharpshooters occurred simultaneously with an increase in qPCR detection in grapevines chronically infected with *Xf*. At citrus sites, the highest percentage of *Xf*-positive sharpshooters was observed in fall and winter. In 2017, the abundance of GWSS had declined at collection locations, presumably due to area-wide insecticide treatments. During 2017, 314 sharpshooters were collected from citrus sites and no sharpshooters were observed at vineyard sites. At citrus sites, 36 (11%) of sharpshooters were *Xf*-positive, with the highest percentage of *Xf*-positive sharpshooters observed in late winter and early spring. Sampling is ongoing and will end in fall of 2018.

**FUNDING AGENCIES**

Funding for this project was provided by the Consolidated Central Valley Table Grape Pest and Disease Control District, and by the USDA Agricultural Research Service, appropriated project 2034-22000-012-00D.

## CHARACTERIZATION OF *XYLELLA FASTIDIOSA* STRAINS BY USING FATTY ACID METHYL ESTER ANALYSES AND GROWTH ON PHENOLIC-AMENDED MEDIA

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**Reporting Period:** The results reported here are from work conducted May 2015 to October 2017.

### ABSTRACT

Different strains or subspecies of *Xylella fastidiosa* (*Xf*) cause different diseases when various hosts become infected, ranging from mild diseases such as bacterial leaf scorch of hardwoods to Pierce's disease of grapevines. Although strains and subspecies of *Xf* have been well characterized by genotyping, complementary studies are needed to decipher phenotypic differences among isolates. Therefore, studies were conducted to characterize the fatty acids that comprise *Xf* cell membranes, which could mediate interactions between this bacterial pathogen and its hosts. Additionally, studies to observe the ability of *Xf* to survive on media supplemented with phenolic compounds were also conducted, as xylem is rich in plant-produced phenolic compounds. So far, seven different *Xf* isolates had fatty acid profiles collected: Dixon, M12, M23, Mulberry, 5A (isolated from olive in California), Stag's Leap (SL), and Temecula. For each isolate, the twelve most abundant fatty acids were selected for further analyses. Cluster analyses (both with furthest neighbor linkage using Pearson's correlations and nearest neighbor linkage using squared Euclidean distances) and principal component analyses were conducted using fatty acid profile data to identify differences and similarities among isolates. These analyses determined that M23 and SL, both of which are ssp. *fastidiosa*, consistently grouped together; Dixon and 5A, both of which are ssp. *multiplex*, consistently grouped together; Mulberry and Temecula consistently grouped together; and M12 was separate from the other isolates. Regarding phenolic-amended growth media studies, the spp. *fastidiosa* strain SL grew better on media amended with quercetin, polydatin, and coumaric acid than standard non-amended PD3 media. However, the ssp. *multiplex* strain Dixon did not have observable changes of growth on phenolic-amended media compared with that on non-amended PD3 media. These results suggest differences between *Xf* subspecies in the ability to utilize plant phenolic compounds, albeit results from additional isolates to verify are still pending. For both the fatty acid and phenolic-amended media studies, additional strains and replication will be necessary to verify and expand results. Completion of these studies should yield greater information about phenotypic differences among *Xf* subspecies and strains.

### FUNDING AGENCIES

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## **Section 2:**

# **Glassy-winged Sharpshooter**



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

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**Reporting Period:** The results reported here are from work conducted November 2016 to October 2017.

### ABSTRACT

For over 15 years the Temecula Valley has been part of an area-wide control program for an invasive vector, the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS). The goal of this program is to limit Pierce's disease spread by suppressing vector populations in commercial citrus, an important reproductive host for this insect, before they move into vineyards. To achieve effective GWSS control, spring applications of the systemic insecticide imidacloprid to citrus have been made in years past. As part of this program there is ongoing monitoring of GWSS to ensure that its populations are being adequately suppressed. Notably, since 2013 reimbursements to citrus growers have not been made. As a result, over the past several seasons no Temecula Valley citrus acreage was treated specifically for GWSS, although it is likely that some treatments are occurring to target important citrus pests. Nearly 135 yellow sticky traps were inspected on a biweekly basis throughout 2017 to monitor GWSS in citrus, the results of which were shared regularly with local grape growers. The results over the past season show a marked increase in GWSS abundance, with a total of nearly 4,000 GWSS caught during the summer peak (July through September). This total is more than 50% higher than previous highs dating back nearly 15 years. The mechanism driving this rebound in GWSS abundance is not known. As a result, it is unclear whether this past season represents an acute spike in GWSS abundance or a more chronic resurgence.

### LAYPERSON SUMMARY

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

### INTRODUCTION

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

GWSS has the potential to develop high population densities in citrus. Fortunately, GWSS is also highly susceptible to systemic insecticides such as imidacloprid. Insecticide treatments in citrus groves, preceded and followed by trapping and visual inspections to determine the effectiveness of these treatments, have been used to manage this devastating insect vector and disease. In addition, parasitoid wasps that attack GWSS egg masses are also contributing to management in the region.

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

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

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

## OBJECTIVES

1. Monitor regularly GWSS populations in citrus groves throughout the Temecula Valley to evaluate the effectiveness of prior insecticide applications and to provide a metric of Pierce's disease risk for grapegrowers.
2. Disseminate a newsletter for stakeholders on GWSS seasonal abundance in citrus throughout the region.

Double-sided yellow-sticky cards (14 cm x 22 cm; Seabright Laboratories, Emeryville, CA) are being used to monitor for adult sharpshooters in citrus. Approximately 135 such traps have been placed in citrus groves throughout the Temecula Valley. All traps are labeled, numbered, and georeferenced with a handheld global positioning system monitor. Most traps are placed at the edge of the groves at the rate of approximately one per ten acres. Traps are attached with large binder clips to wooden stakes around the perimeter of the grove. For large groves, traps are also placed in the interior. The total number of traps depends on the size of the orchard block.

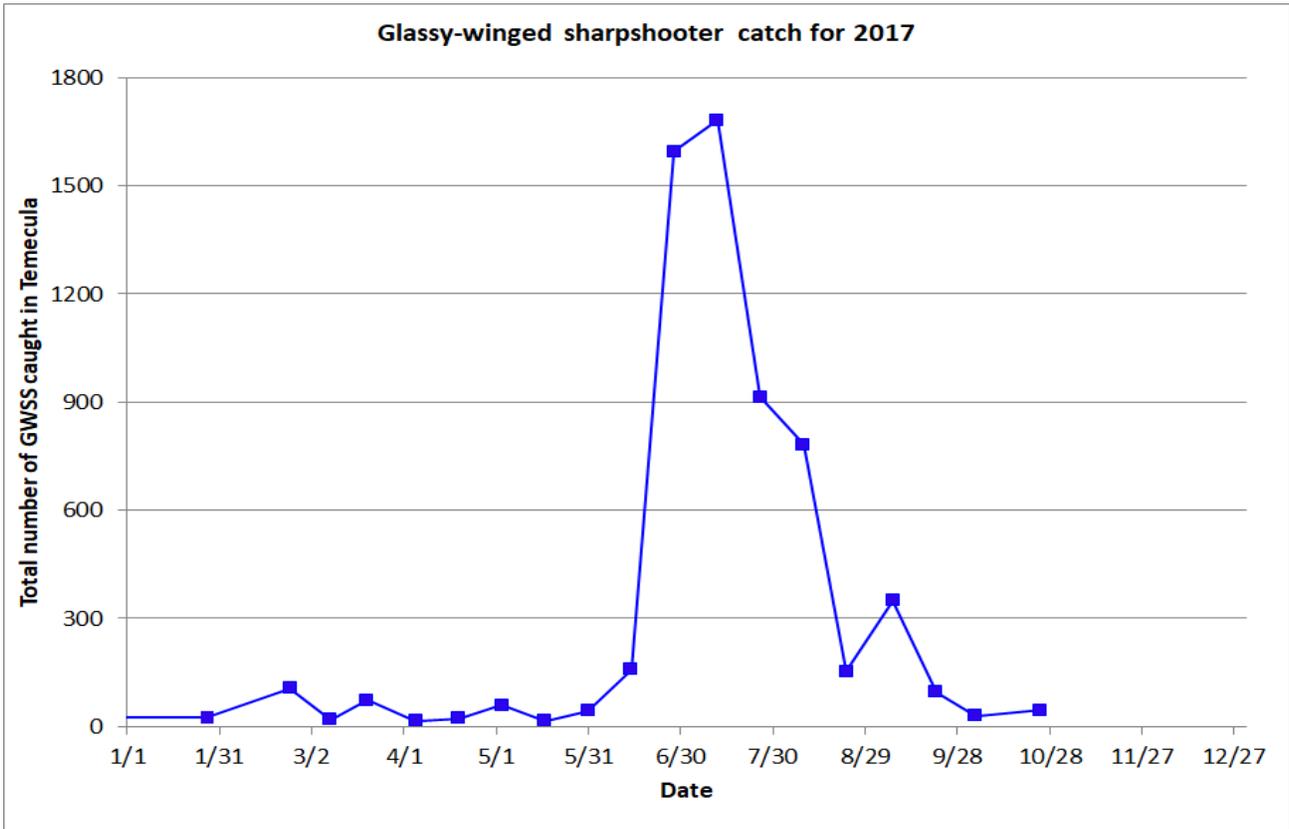
The yellow-sticky cards are collected, inspected under a dissecting microscope, and replaced every two weeks from late spring through early fall (May through October) and monthly the rest of the year. At each census the number of adult GWSS and smoke-tree sharpshooters (*Homalodisca liturata*) are recorded, along with the abundance of common generalist natural enemy taxa (i.e. lacewings, lady beetles).

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

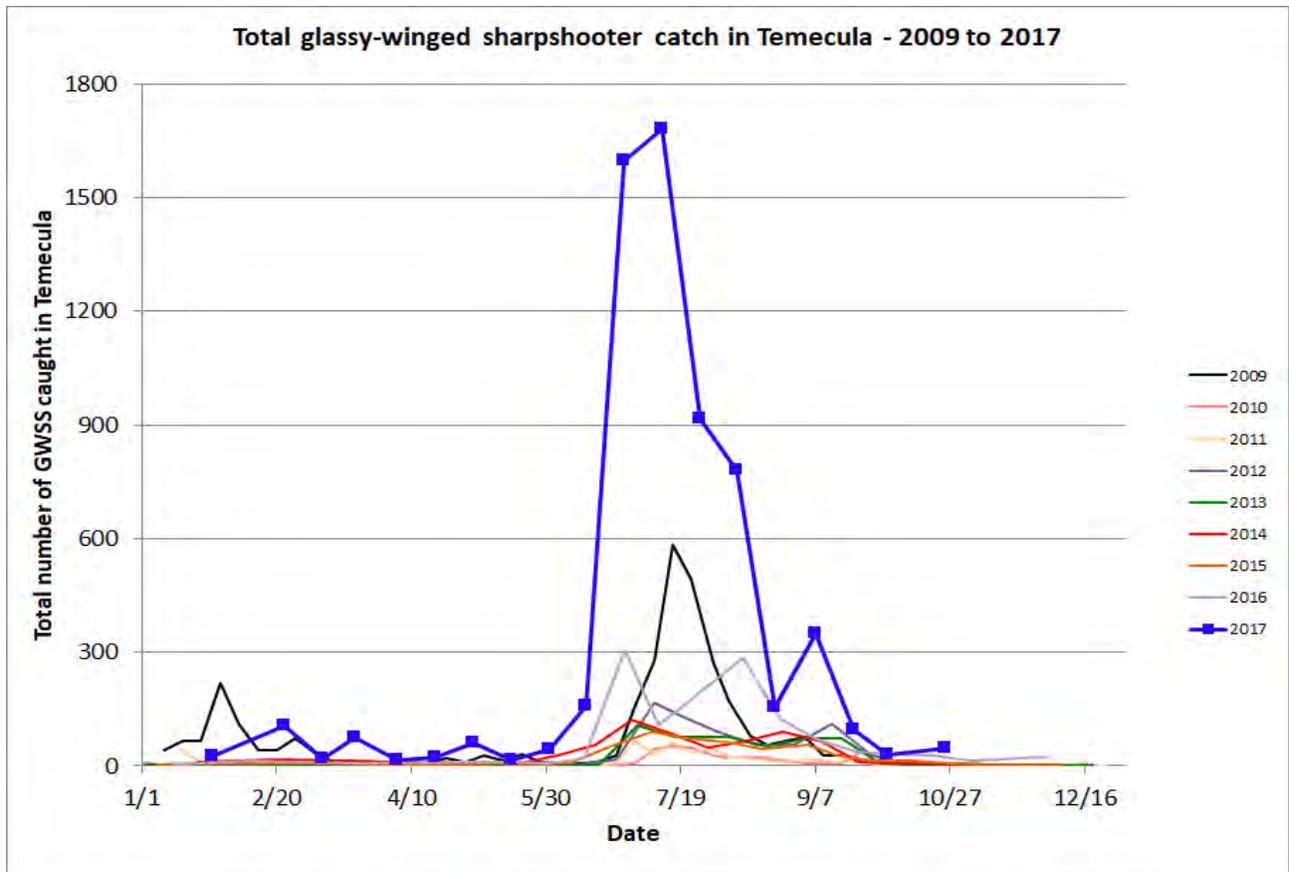
## RESULTS AND DISCUSSION

The results for 2017 are shown in **Figure 1**. This includes monthly censuses of GWSS in citrus through April, then biweekly censuses from May through October. Results for the remainder of 2017 are pending. Census results, thus far, show GWSS abundance and activity levels substantially higher than is typical for the Temecula region. GWSS catch was higher than usual for pre-season activity (February through early June) and then increased significantly in late spring and early summer, then dropped in late July through September. As of late-October, GWSS populations appear to have declined substantially.

**Figure 2** shows the GWSS catch in 2017 relative to other years. 2017 had a higher overall catch compared to recent years over most of the season. Indeed, the peak GWSS catch in 2017 was more than 50% higher than prior year peaks dating back to at least 2003.



**Figure 1.** Seasonal total GWSS catch in 2017 for approximately 135 traps throughout the Temecula Valley.



**Figure 2.** Seasonal total GWSS catch in the Temecula Valley in 2017 compared to the previous eight years.

## **CONCLUSIONS**

The observed trapping results for GWSS in the Temecula Valley represent a sizeable increase in GWSS activity after approximately a decade of modest GWSS populations and low Pierce's disease pressure. As a result of this observed increase in GWSS activity the researchers ramped up extension efforts, including holding workshops for vineyard managers and a small winegrowers group on Pierce's disease identification and disease management. Temecula Valley grape growers were cautioned to remain vigilant and consider alternative steps to managing Pierce's disease pressure in their vineyards, particularly if this season ends up being the beginning of a sustained resurgence in GWSS populations.

## **FUNDING AGENCIES**

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## **ACKNOWLEDGEMENTS**

We would like to thank the Temecula Valley citrus growers and grape growers for their continued cooperation in making this work possible.

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

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**Reporting Period:** The results reported here are from work conducted July 2016 through October 2017.

### ABSTRACT

Monitoring for resistance to insecticides continued in 2017 with a series of insecticide bioassays conducted on the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) in Kern County. Two organic and two conventionally-treated citrus sites were chosen for monthly monitoring from July through October based on high densities of GWSS determined in the CDFR trapping program. These sites were located in two different regions of the county, Edison Highway and Highway 65. Bioassays were conducted solely with imidacloprid as its use in citrus and grapes remains high, and previous studies have found seasonal increases in GWSS resistance to this material. This trend of increasing resistance over the season continued again this year. In the conventional plots, GWSS pesticide resistance increased from July through September, and in organic plots resistance increased from July through October. For the September bioassays, resistance was significantly higher in treated citrus than in organic citrus with LC50s of 51.5 and 7.7, respectively. To address additional objectives, spray records and GWSS trap counts from previous years are being input into our geographic information system for evaluation of historical GWSS population dynamics. Insecticide treatment records and GWSS numbers from 2015, 2016, and 2017 have been recorded. Our current bioassay studies combined with historical spray records and trap counts will contribute to a better understanding of insecticide use for the management of GWSS.

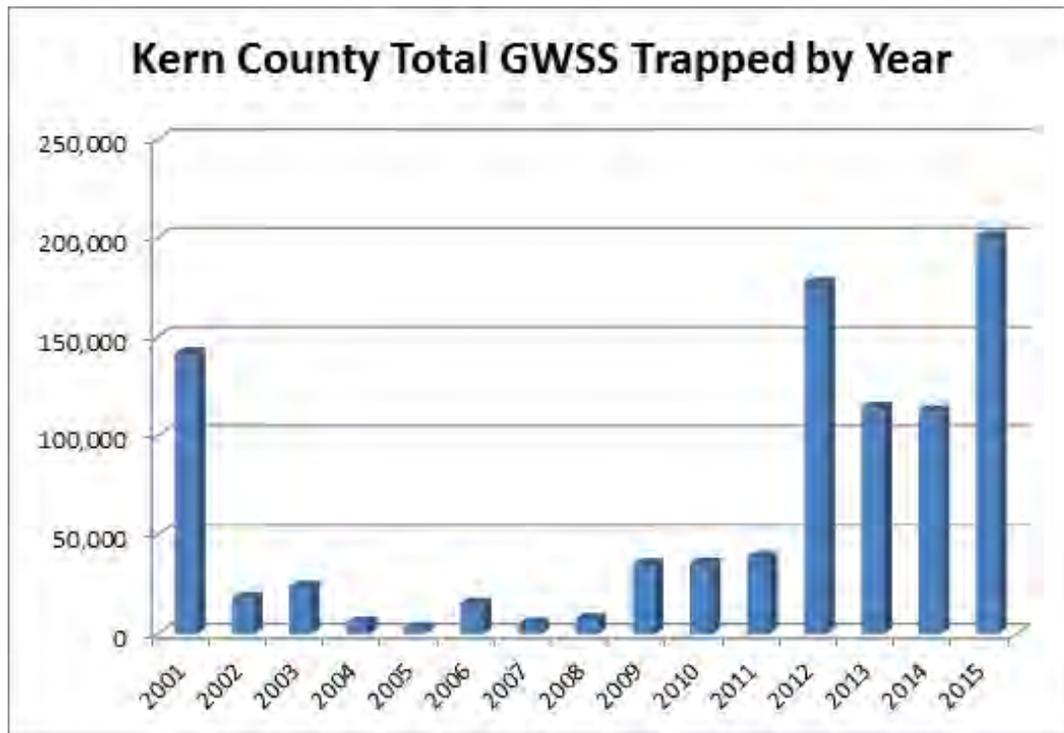
### LAYPERSON SUMMARY

By reducing the number of glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) vectors in the field, insecticides are key to the management of Pierce's disease. High numbers of GWSS in California from 2012-2015, despite continued monitoring and treatment, suggested a change in the pest's susceptibility to commonly used products. Research in our lab during 2015 demonstrated high levels of resistance to insecticides in GWSS in Kern County, with declining susceptibility as the season progressed. Fortunately, there was no further reduction in susceptibility in 2016, but the levels of susceptibility were still much lower than in 2000-2001 when the area-wide GWSS program was initiated. Further work in 2017 focused solely on imidacloprid, the insecticide that has been used most frequently in citrus and grapes. Selecting citrus blocks near conventional (insecticide-treated) groves and organic groves for GWSS sampling, we found an increase in resistance for both field types as the season progressed and a much higher level of resistance in the conventional plots compared to the organic plots. These data suggest that local use of imidacloprid contributes to an increase in imidacloprid resistance in Kern County.

### INTRODUCTION

Chemical management of glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) populations within citrus orchards and vineyards in Kern County is informed by the CDFR Pierce's Disease and Glassy-winged Sharpshooter Area-wide Management Program. From its initiation in 2001, this program managed to dramatically reduce and then maintain low numbers of GWSS within Kern County fields through 2008, and Pierce's disease incidence in grapes remained low. In 2009, GWSS numbers increased and eventually lead to extremely high densities in 2012, alarming the industry and leading experts to hypothesize that insecticide resistance had developed in Kern County GWSS populations. Despite continued insecticide usage, high densities of GWSS from

2012-2015 existed and the numbers in 2012 and 2015 surpassed the 2001 density (**Figure 1**). At the same time, surveys of Pierce's disease infected vines indicated an increase in disease incidence in the General Beale region of Kern County (Haviland 2015).



**Figure 1.** Total number of GWSS caught on CDFA traps in Kern Co. from 2001-2015 (from Haviland 2015).

The systemic neonicotinoid insecticide, imidacloprid, has been used preferentially for GWSS suppression over the course of the management program. In addition, other insect pests in grapes (Daane et al. 2006) and citrus (Grafton-Cardwell et al. 2008) have been treated with this material. With the selection pressure that has resulted from the use of imidacloprid across citrus and grape acreages over the past 16 years there is reason to believe that the resurgence of GWSS is related to imidacloprid resistance. Resistance to imidacloprid has been documented for numerous insects (Liu et al. 2005, Nauen and Denholm 2005, Karunker et al. 2008, Fewkes 1969). Although Rosenheim et al. (1996) argues that sap feeding insects might be less prone to resistance than leaf-chewing insects, the possibility of pesticide resistance development remains in any organism that is subjected to a specific mortality factor over time. Pesticides are an integral part of the citrus and grape pest management, and understanding the levels of resistance to insecticides is critical to the future selection of materials that are used to manage GWSS and Pierce's disease.

This project was initiated in July 2015. In that year we used laboratory bioassays on field-collected GWSS to evaluate eight commonly used compounds. These studies showed that GWSS were much less susceptible to the tested insecticides than they were in 2001 and 2002 (Prabhaker et al. 2006), when the area-wide management program was initiated (Perring et al. 2015). For some insecticides, the studies showed LC50 values to be much higher in 2015, an indication of resistance in the populations. These results were similar to those obtained by Redak et al. (2015) in the same geographic region.

In the same study we documented variation in the relative toxicities at different times and locations throughout the 2015 season (Perring et al. 2015). In particular, there was a 79-fold increase in the LC50 for imidacloprid from the first bioassay of the season to the last, and there were differences in susceptibility of sharpshooters collected from different fields and geographic areas. This study suggested that toxicity was related to factors in the local context.

The research was continued in 2016 and, despite low numbers of sharpshooters, we evaluated two pyrethroids and three neonicotinoids on two dates from table grapes and one date from citrus. The data from 2016 showed similar resistance levels to those from 2015 for all five chemicals (Perring et al. 2016). Even so, resistance levels in 2015 and 2016 were higher than in 2001-2002, indicating a declining susceptibility over the years. Since GWSS numbers were limited in 2017, and considering that imidacloprid has been used extensively in citrus (Grafton-Cardwell et al. 2008) and grapes (Daane et al. 2006), our 2017 bioassays were focused on imidacloprid testing.

## **OBJECTIVES**

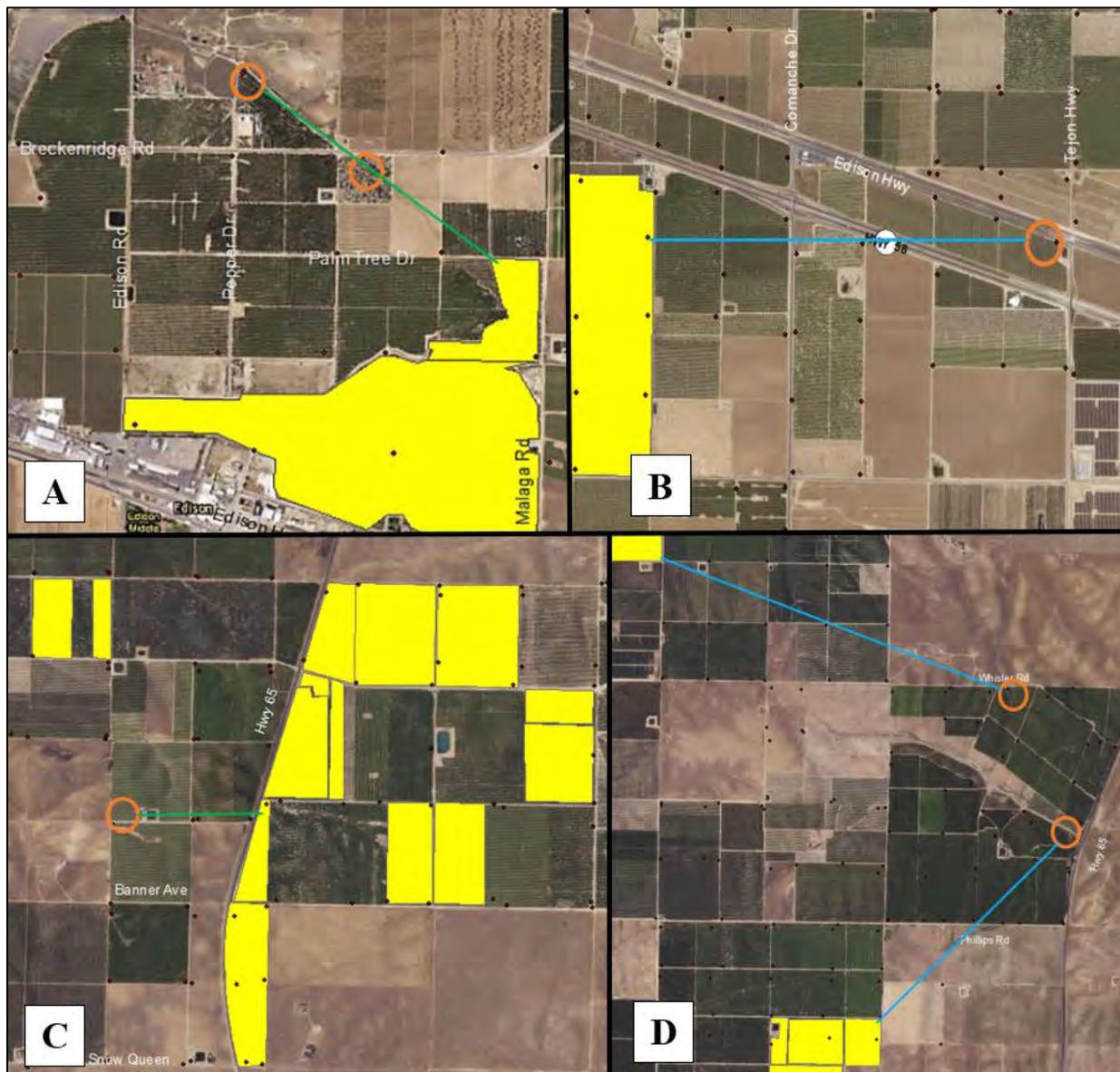
1. Conduct laboratory bioassays on field-collected GWSS from Kern County to document the levels of resistance at the beginning of the 2016 and 2017 field seasons, and to document changes in susceptibility as each season progresses.
2. Document differences in insecticide susceptibility in GWSS collected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

## **RESULTS AND DISCUSSION**

### **Objectives 1 and 2**

In 2017, we conducted bioassays on GWSS collected in citrus on July 24, August 8, August 29, September 12, and October 9. All collections were made in citrus fields because we observed consistently higher GWSS counts in citrus than in grapes this year. Collections were made from four sites in Kern County throughout the season. Two treated and two non-treated (organic) sites were chosen from two different zones of the Kern County area-wide trapping map (**Figure 2**). The 2017 spray records were placed into our GIS, and sites were selected based on two primary criteria: (1) proximity to recent imidacloprid-treated regions; and (2) GWSS population densities. Treated areas were considered those in which imidacloprid was applied in the 2017 growing season within 0.75 miles of the collection site. Organic sites were defined as those in which imidacloprid had not been applied this season within at least one mile of the collection site. The four sites were spread throughout Kern County with the treated 1 (T1) and organic 1 (O1) sites located in the Edison region in Zone 3 and the treated 2 (T2) and organic 2 (O2) sites occurring along Highway 65 in Zone 1 (**Figure 2**). The CDFA GWSS trap counts were used to determine the sites from which to collect GWSS on each collection date. Totals of 750, 600, 100, 420, and 510 GWSS were collected on each aforementioned date, respectively.

Bioassays conducted on organic versus treated sites throughout the 2017 season demonstrated different levels of resistance as the season progressed. The results from the two organic sites were combined, as were the bioassays from the two treated sites. This enabled us to assess the overall resistance rates at organic sites versus treated sites throughout the season (**Table 1**). The August 8 and August 29 bioassay results also were combined for our treated sites because of the low GWSS population densities at Site T1 on August 29. For organic and treated locations we observed an increase in resistance levels as the season progressed. In the organic locations this increase was not statistically different than earlier in the season in July as indicated by overlapping 95% confidence intervals. However, in the treated locations there was a significant increase in resistance, approximately 35-fold, from July to September. Comparing the organic versus treated September bioassays, resistance is significantly higher in treated sites than in organic sites. Unfortunately, there were not enough GWSS available at sites T1 or T2 in October, so we could not evaluate if the resistance levels increased in treated sites past September.



**Figure 2.** Four Kern County locations chosen for GWSS collection and imidacloprid bioassays. (A) Treated Site 1 (T1), (B) Organic Site 1 (O1), (C) Treated Site 2 (T2), and (D) Organic Site (O2). Citrus or grapes treated with imidacloprid in 2017 are represented by the yellow areas. Orange circles indicate collection sites. Green lines represent distances between collection sites and treated areas that are less than 0.75 miles. Blue lines represent distances between collection sites and treated areas of one mile or more.

**Table 1.** Probit statistics for imidacloprid tested against GWSS adults from organic and treated sites in Kern County from July to October 2017.

Site	Date	Mortality Over 2017 Season		
		LC50 ( $\mu\text{g/ml}$ )	95% C.I.	Slope ( $\pm$ SE)
Organic	July 24	1.253	0.367 – 5.354	1.253 (0.187)
	August 8	0.845	0.163 – 3.133	1.003 (0.090)
	September 12	7.724	3.421 – 20.062	1.031 (0.167)
	October 9	8.710	2.932 – 27.277	0.894 (0.093)
Treated	July 24	1.429	0.720 – 2.360	1.678 (0.333)
	August 8 & 29	0.335	0.014 – 1.271	1.028 (0.204)
	September 12	51.525	21.334 – 204.985	0.534 (0.111)

To get an estimate of the overall levels of imidacloprid resistance in Kern County over the 2017 season, we combined the mortalities observed at all sites per collection date. Again, data from August 8 were combined with those from August 29. This analysis demonstrated an overall increase in GWSS resistance to imidacloprid as the season progressed (**Table 2**). With overlapping 95% confidence intervals, the LC50 values for July and August levels were not statistically different from each other. However, from August to mid-September there was a significant increase in LC50s. In October, the LC50 decreased but was not statistically different than in September, nor than earlier in the season. This is likely because only the O2 (organic site) was sampled on this day, due to a lack of GWSS at the treated sites. As we showed in **Table 1**, LC50s the month before were significantly lower in organic orchards than in treated orchards, which we hypothesize would have been the case for October as well. If a treated location could have been tested in October, the overall LC50 value likely would have been higher. This trend of increasing resistance to imidacloprid over a season is consistent with our 2015 bioassays (Perring et al. 2016). The results of our bioassays over the past three years suggest that resistance increases within each season, although it appears to revert back to a susceptible state at the beginning of the next growing season. Overall, there was little difference in GWSS susceptibility to imidacloprid this year versus the previous two years, but susceptibility remains lower than levels determined in 2001-2002.

**Table 2.** Probit statistics for imidacloprid tested against GWSS adults on five dates from July to October 2017.

Date	LC50 (µg/ml)	95% C.I.	Slope (± SE)
July 24	1.710	0.613 – 3.480	1.362 (0.155)
August 8 & 29	0.684	0.037 – 3.614	1.003 (0.084)
September 12	22.122	12.046 – 40.887	1.068 (0.188)
October 9	8.710	2.932 – 27.277	0.894 (0.093)

### Objectives 3 and 4

Our GIS now has the crop coverages from Kern County, and we are creating attribute layers for the neonicotinoid sprays for each year since the area-wide program was initiated. To date we have 2015, 2016, and 2017 data in the GIS, and we continue to work on previous years. At the same time we are working to input the GWSS trap data from the past 16 years. This has turned out to be more difficult than we anticipated because the trap data from the thousands of traps that have been counted every two weeks do not reside in a GIS database format. Thus, we are determining the best way to analyze the data so that we can gain an understanding of how spray sites may have impacted subsequent number of GWSS near those sites.

### CONCLUSIONS

GWSS resistance to imidacloprid appears to build within one season, particularly within regions immediately surrounding areas that are treated with imidacloprid. While we have observed this trend in previous years, further monitoring should be conducted over the next couple years to provide a complete understanding of how resistance to imidacloprid varies geographically and temporally. This year, Kern County pesticide use records aided our evaluations of imidacloprid resistance in organic versus conventionally treated citrus orchards. Pairing our annual bioassay results with historical analyses of pesticide use patterns will provide essential information for understanding the basis of GWSS resurgence in Kern County.

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

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**Reporting Period:** The results reported here are from work conducted October 1, 2016 to October 1, 2017.

**ABSTRACT**

Having confirmed in 2016 that glassy-winged sharpshooters (*Homalodisca vitripennis*; GWSS) in the General Beale Road citrus-growing area were exhibiting high levels of imidacloprid resistance, our focus in 2017 was to broaden the geographical range of our resistance monitoring program, and to determine levels of cross-resistance to the neonicotinoid insecticide acetamiprid and the pyrethroid fenpropathrin. In 2017, we established toxicological profiles for a population of GWSS collected from an organic citrus grove in the Temecula Valley in Riverside County, where there were extraordinarily high numbers of insects during the summer. The Temecula insects exhibited a slight shift in toxicological response to imidacloprid compared with our historical (2003) data for Riverside County, but were similar in response to the Tulare 2016 population that also originated from organic citrus. The Temecula and Tulare populations represent the most susceptible insects that we have encountered during our recent monitoring. GWSS numbers at our General Beale Road collection sites were lower in 2017 due to enhanced control efforts using pyrethroids, but the CDFA GWSS mapping database alerted us to other sites within the region where we could monitor for resistance. Resistance to imidacloprid was also expressed in these populations, and the insects were cross resistant to acetamiprid but not to fenpropathrin. Based on our current data, the GWSS insects that are expressing resistance to imidacloprid are not showing high levels of cross resistance to fenpropathrin. The lack of cross resistance accounts for the continued effectiveness of the pyrethroids in the management of field populations of GWSS.

We are using biochemical and molecular techniques to investigate putative resistance mechanisms to the neonicotinoid, pyrethroid, and organophosphate (OP) insecticide classes. Thus far we have not identified any acetylcholinesterase (AChE) insensitivity, indicating that there is no target site resistance to OPs (or carbamates, which share the same AChE target site as OPs). Esterase levels in susceptible and resistant populations are also very homogeneous, confirming that elevated esterase levels are unlikely to play a significant role in conferring imidacloprid resistance. The similarity in esterase levels between populations also concurs with the similarity of responses to fenpropathrin in bioassays. The genomics data have thus far not identified any specific markers for resistance that could be utilized for field monitoring, but we are continuing to evaluate RNAseq data for susceptible and resistant populations to determine the likely involvement of cytochrome P450s in conferring resistance to imidacloprid. As part of that effort we have also collected GWSS insects from nursery locations, so that we can compare cDNA sequence data for sodium channel (pyrethroid target site) and nicotinic acetylcholine receptor (neonicotinoid) genes in insects from broad geographical and host plant ranges to determine whether mutations known to confer insecticide resistance in other arthropod species occur in GWSS.

## LAYPERSON SUMMARY

The goal of this research is to investigate the potential for the development of insecticide resistance in glassy-winged sharpshooters (*Homalodisca vitripennis*) to chemicals in the carbamate, pyrethroid, and neonicotinoid classes of insecticides, and to determine mechanisms where differences in susceptibility between populations are identified. Additionally, we wish to simultaneously evaluate the development of resistance in various populations of these insects that have been undergoing different levels of chemical control in grapes, citrus, commercial nursery, and urban environments. Using topical application bioassays we have now detected substantial differences in response to imidacloprid (neonicotinoid) between populations collected from citrus groves in Kern, Tulare, and Riverside Counties. Our data suggest that imidacloprid resistance confers strong cross resistance to acetamiprid (neonicotinoid) and mild cross resistance to fenpropathrin (pyrethroid). At this time the imidacloprid resistance appears to be directly related to usage, with the highest levels of resistance occurring in populations receiving conventional insecticide treatments and no resistance in those under organic management.

## INTRODUCTION

Systemic imidacloprid treatments have been the mainstay of glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) management in citrus, grapes, and commercial nursery operations. The treatments in citrus groves are generally applied post-bloom to suppress the newly emerging spring populations. The use of winter or early spring foliar treatments of pyrethroid or carbamate treatments were introduced to the management program to suppress overwintering adults and reduce the first early season cohort of egg-laying adults. The combination of early season foliar treatments combined with the more persistent systemic treatments has effectively managed GWSS populations in Kern County for many years.

In Kern County, GWSS populations have been monitored since the area-wide treatment program was instigated by the CDFA following an upsurge in GWSS numbers and an increase in the incidence of Pierce's disease. The data shows an interesting pattern of sustained suppression of GWSS populations throughout most of the 2000s, following the implementation of the area-wide treatment program, until 2009 when numbers began to increase again, culminating in a dramatic flare-up in numbers in 2012. In 2012, a single foliar treatment with either Lannate® (methomyl: carbamate insecticide class), Assail® (acetamiprid: neonicotinoid insecticide class) or Baythroid® (cyfluthrin: pyrethroid insecticide class) was applied in groves in late March, while systemic treatments with imidacloprid (neonicotinoid insecticide class) were applied mid-March to early April. The application of systemic imidacloprid during 2012 mirrored the strategy used in 2001 when the imidacloprid treatments were highly effective in suppressing the GWSS populations. Despite the additional foliar treatments in 2012, the insecticide treatments failed to suppress the insect population to a level that had occurred previously. There were concerns that in the two years prior to 2012 there was a steady increase in total GWSS numbers, an early indication that the predominant control strategy might be failing. The consequence of the increase in GWSS populations has been an increase in the incidence of Pierce's disease. In the Temecula area this worrisome increase in GWSS has not occurred; however, the selection pressure in this area remains high as similar management approaches are in use here as in Kern County.

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

The focus of this study is to investigate the role of insecticide resistance as a contributing factor to the increased numbers of GWSS that have been recorded since 2009 in commercial citrus and grapes in Kern County. Although the primary focus of our research to date has been in Kern County, we will broaden the scope of our investigations to include populations from agricultural, nursery, and urban settings. This broader approach will result in a more comprehensive report on the overall resistance status of GWSS within southern California and will contribute to more effective resistance management plans.

## OBJECTIVES

1. For commonly used pyrethroid, carbamate, and neonicotinoid insecticides, determine LC<sub>50</sub> data for current GWSS populations and compare the response to baseline susceptibility levels generated in previous studies.
2. Define diagnostic concentrations of insecticides that can be used to identify increased tolerance to insecticides in insects sampled from other locations (where numbers are relatively low).
3. Monitor populations for known molecular markers of resistance to pyrethroids.
4. Monitor populations for target-site insecticide resistance, by testing enzymatic activity against carbamates using the acetylcholinesterase (AChE) biochemical assay.
5. Monitor populations for broad-spectrum metabolic resistance, by comparing esterase levels in current populations of GWSS to baseline susceptibility levels we previously recorded.
6. Develop assays for additional resistance mechanisms not previously characterized in GWSS.

## RESULTS AND DISCUSSION

### Imidacloprid Bioassays

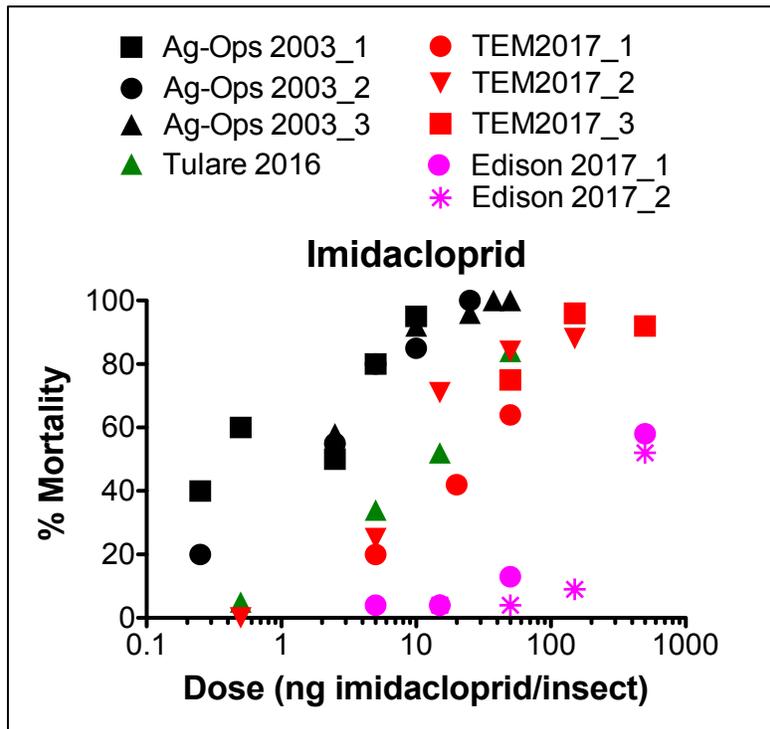
During 2017, an extensive bioassay program was undertaken that evaluated the responses of different Central Valley and southern California GWSS populations to imidacloprid, acetamiprid, and fenpropathrin. The data generated from topical application bioassays were compared with similar bioassays from studies conducted in 2003 with Riverside County populations, and from data generated during our resistance monitoring effort in 2016. The 2003 data serve as a useful historical reference against which current populations can be compared. In bioassays, insecticide is topically applied to the abdomen of adult GWSS and mortality is assessed at 24 hours and 48 hours post-treatment (Byrne and Toscano 2005). Although imidacloprid is used systemically under field conditions to target GWSS feeding on citrus and other host plants, topical application of insecticide to individual insects ensures that the insect receives a uniform dose and eliminates any behavioral factors that might occur when the insect encounters the insecticide (either through direct contact or during feeding). Imidacloprid is one of the most important insecticides used for the control of GWSS, and this insecticide has been shown to elicit anti-feedant effects in several pest species (Nauen et al. 1998).

In 2016, we were unable to generate full dose-response lines in bioassays with Temecula Valley GWSS due to the low numbers of insects available (Redak et al. 2016). The only data we were able to obtain was with discriminating doses of imidacloprid, and they indicated that the Temecula insects were susceptible to imidacloprid. In 2017, the extremely high numbers of GWSS in the region facilitated full evaluations of imidacloprid and other insecticides in bioassays.

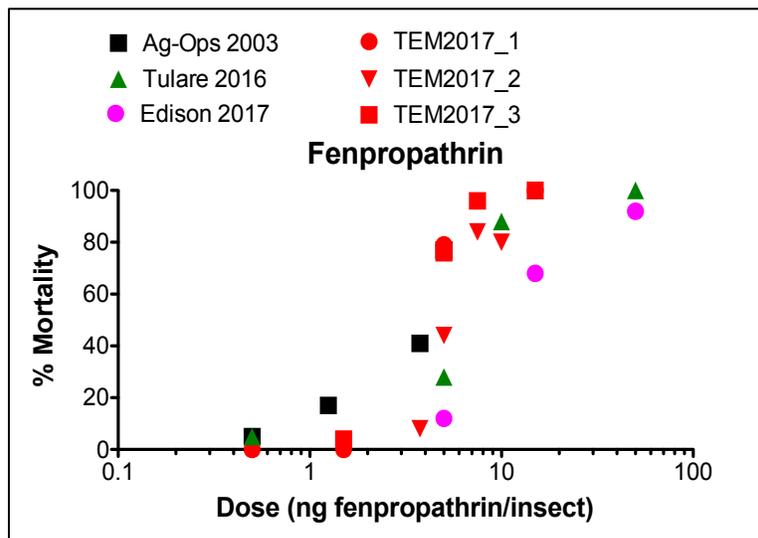
The response of the Temecula population (TEM 2017) to imidacloprid mirrored that of the Tulare 2016 population, with a noticeable shift in response compared with the Ag-Ops 2003 data. The location of the TEM2017 population was well removed from the site where the 2016 insects were collected, so the data suggest some degree of variation in Temecula Valley populations in their response to imidacloprid (**Figure 1**). In the Central Valley's Edison 2017 population, insects exhibited strong resistance to imidacloprid. Interestingly, the 500 ng/insect dose did elicit a response in this population, whereas in the GBR 2016 population that was collected from the General Beale Road area, there was no effect. Again, these data demonstrate the variable expression levels of resistance in GWSS populations.

### Pyrethroid Bioassays

In bioassays with the pyrethroid fenpropathrin, the response of TEM2017 was similar to Ag-Ops 2003 and Tulare 2016, indicating that these three populations were highly susceptible to the insecticide (**Figure 2**). In contrast, the insects collected from conventionally managed citrus (Edison 2017) exhibited slight tolerance to the pyrethroid. Although the marginal shift in response does not seem to have compromised the efficacy of the insecticide under field conditions, where it has been effectively used to suppress GWSS populations, it is imperative to continue monitoring for resistance to this insecticide to ensure that it remains an effective chemistry for use by growers in the region. An effective rotational strategy will help achieve this goal.



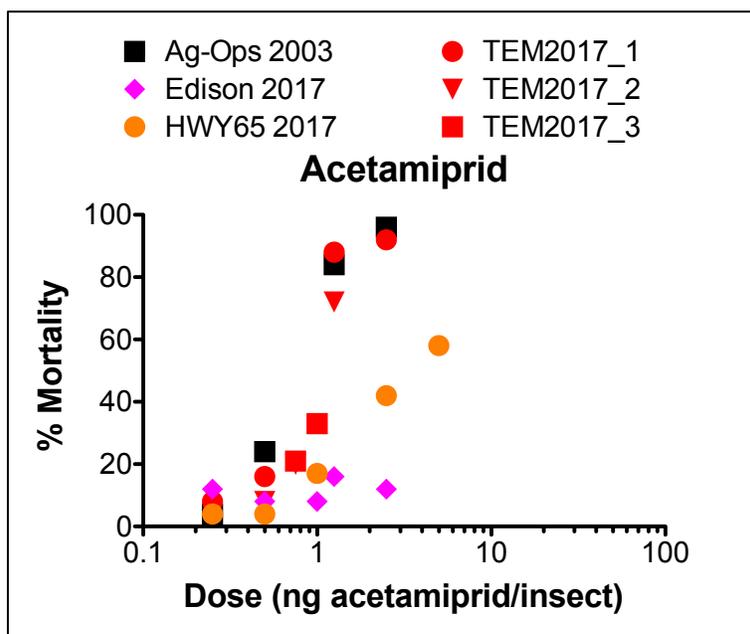
**Figure 1.** Dose response of GWSS adults to imidacloprid applied topically to the abdomen. Mortality was assessed at 48 hours post-treatment. Data for Ag-Ops (black symbols) were generated in 2003 and are included for comparison. Tulare 2016 (green symbols) was collected from an organic grove in Tulare County during the 2016 monitoring program. The Edison 2017 population (pink symbols) originated from conventionally managed groves west of Bakersfield in Kern County. TEM2017 (red symbols) was collected from an organic grove in Temecula Valley. For the latter, three separate collections of insects were evaluated by bioassay to generate the full dose-response line.



**Figure 2.** Toxicological response of GWSS adults to the pyrethroid fenpropathrin applied topically to the abdomen. Mortality was assessed at 48 hours post-treatment. Data for Ag-Ops (black symbols) were generated in 2003 and are included for comparison. Tulare 2016 (green symbols) was collected from an organic grove in Tulare County and tested during the 2016 monitoring program. The Edison 2017 population (pink symbols) originated from conventionally managed groves west of Bakersfield in Kern County. TEM2017 (red symbols) was collected from an organic grove in Temecula Valley. For the latter, three separate collections of insects were evaluated by bioassay.

### Acetamiprid Bioassays

Acetamiprid is a neonicotinoid insecticide and belongs to the same insecticide class as imidacloprid. Acetamiprid is used exclusively as a foliar treatment, in contrast to imidacloprid which is most commonly used as a systemic treatment. In 2017 we were interested in determining whether resistance to imidacloprid conferred cross-resistance to acetamiprid, as this is an important consideration when developing resistance management strategies and in evaluating resistance mechanisms. The TEM2017 population exhibited a similar dose-response to acetamiprid as Ag-Ops 2003 (**Figure 3**), indicating that it was fully susceptible to the insecticide. The response of HWY65 2017 and Edison 2017, both Central Valley populations, indicated some degree of resistance. We were unable to generate full dose-response lines for the Edison 2017 population due to dwindling insect numbers late in September, but the shift in response at the doses tested is a clear indication of cross-resistance likely caused by the widespread use of imidacloprid in the region. The response of the HWY65 2017 was intermediate between those of the Ag-Ops/TEM and Edison 2017 populations, and this is likely a reflection of the mixed management systems that occur in the area, located north of Bakersfield, where the HWY65 insects originated.



**Figure 3.** Toxicological response of GWSS adults to the neonicotinoid acetamiprid applied topically to the abdomen. Mortality was assessed at 48 hours post-treatment. Data for Ag-Ops (black symbols) were generated in 2003 and are included for comparison. The Edison 2017 population (pink symbols) originated from conventionally managed groves west of Bakersfield in Kern County. TEM2017 (red symbols) was collected from an organic grove in Temecula Valley, Riverside County. The HWY65 2017 insects (orange symbols) were collected from a grove north of Bakersfield in Kern County.

### Genetic Analyses

Based on the study of the aphid *Myzus persicae*, the mutation R81T in the loop D region of the nicotinic acetylcholine receptor beta subunit is associated with resistance to neonicotinoid insecticides. In sequence analysis of GWSS from Riverside and Kern Counties, the R to T mutation was not detected (**Figure 4**).

Species	Amino acid number of the insect nicotinic acetylcholine receptors				
	79	80	81	82	83
Drosophila melanogaster	W	L	R	Q	E
Anopheles gambiae	W	L	R	Q	E
Bemisia tabaci	W	L	R	Q	E
Locusta migratoria	W	L	R	Q	E
Heliothis virescens	W	L	R	Q	E
Myzus persicae	W	L	T	Q	E
Homalodisca vitripennis (HWY65)	W	L	R	Q	E
Homalodisca vitripennis (Tulare)	W	L	R	Q	E
Homalodisca vitripennis (GBR)	W	L	R	Q	E
Homalodisca vitripennis (Riverside)	W	L	R	Q	E

**Figure 4.** Comparison of amino acid sequence data for loop D of the nACh receptor between GWSS populations and several insect species, including *Myzus persicae* which expresses target site resistance to neonicotinoids conferred by the R81T mutation.

The classic leucine to phenylalanine (L to F) mutation in the domain II region of the sodium channel gene that confers *kdr* resistance in houseflies and other species was not detected in the HWY65 or GBR 2016 populations, despite the expression of resistance in bioassays. Also, the L to F mutation was not detected in insects tested from Riverside County (**Figure 5**). We are currently evaluating several synonymous and non-synonymous mutations that have been found in individuals from these populations to determine whether they play a significant role in conferring resistance.

Md	CMYVGDVSCIPFFLATVVIGNL	VVLNLF	LALLSNFGSSLS	SAPTADND	TNKIAEAFNRIARFKNWVKR
Md-R	CMYVGDVSCIPFFLATVVIGNF	VVLNLF	LALLSNFGSSLS	SAPTADND	TNKIAEAFNRIARFKNWVKR
HWY65	CMYVGDVSCIPFFLATVVIGNL	NVLNLF	LALLSNFGSSLS	SAPAADNETNKIAEAFDRIGRFS	SAWVKR
Tulare	CMYVGDVSCIPFFLATVVIGNL	NVLNLF	LALLSNFGSSLS	SAPAADNETNKIAEAFDRIGRFS	SAWVKR
GBR	CMYVGDVSCIPFFLATVVIGNL	NVLNLF	LALLSNFGSSLS	SAPAADNETNKIAEAFDRIGRFS	SAWVKR
Riverside	CMYVGDVSCIPFFLATVVIGNL	NVLNLF	LALLSNFGSSLS	SAPAADNETNKIAEAFDRIGRFS	SAWVKR
Corona	CMYVGDVSCIPFFLATVVIGNL	NVLNLF	LALLSNFGSSLS	SAPAADNETNKIAEAFDRIGRFS	SAWVKR
	*****	*****	***	*****	** ** *
HWY65	SRNSTSTVTHQFP	PKHYRDFDT	FVSP	TLLRVVRVAKVGRVL	GYEPVSSTLWVRQREEYC
Tulare	SRNSTSTVTHQFP	PKHYRDFDT	FVSP	TLLRVVRVAKVGRVL	GYEPVSSTLSRQREEYC
GBR	SRNSTSTVTHQFP	PKHYRDFDT	FVSP	TLLRVVRVAKVGRVL	GYEPVSSTLSRQREEYC
Riverside	SRNSTSTVTHQFP	PKHYRDFDT	FVSP	TLLRVVRVAKVGRVL	GYEPVSSTLSRQREEYC
Corona	SRNSTSTVTHQFP	PKHYRDFDT	FVSP	TLLRVVRVAKVGRVL	GYEPVSSTLWVRQREEYC
	*****	*****	*****	*****	*****

Md: *Musca domestica*  
Md-R *Musca domestica* pyrethroid resistance strain

**Figure 5.** Comparison of amino acid sequence data in domain II of the sodium channel between GWSS populations and several insect species, including *Musca domestica* which expresses target site resistance to pyrethroids conferred by the L1014F mutation. Although the L1014F mutation was not detected in GWSS, additional mutations (highlighted in red) were identified, and the significance of these to pyrethroid resistance has yet to be evaluated.

## CONCLUSIONS

We have confirmed the variable levels of resistance to imidacloprid in Central Valley populations of the GWSS. The dramatic shift in susceptibility is based on a comparison with bioassay data generated in 2003 for a population in Riverside County that we regard as a reliable reference susceptible, and a comparison with 2016 bioassay data for a population collected from an organic grove in Tulare County. Of major concern is the cross resistance between imidacloprid and acetamiprid. The presence of cross resistance to acetamiprid should preclude the use of this insecticide as an alternative management option for insects where imidacloprid resistance has been identified. In addition to imidacloprid resistance, we have also identified low levels of resistance to the pyrethroid fenpropathrin. The pyrethroids appear to work effectively against imidacloprid-resistant GWSS. However, continued monitoring for pyrethroid resistance should be a high priority if this important insecticide class is to remain effective. Finally, based on our bioassay data, resistance does not appear to have been a contributing factor to the high numbers of GWSS recorded during the 2017 season in the Temecula Valley.

The genomic work is becoming increasingly important as a tool for identifying resistance mechanisms. In particular, we are confident that the RNA-seq analysis of populations expressing different levels of resistance to imidacloprid, acetamiprid, and fenpropathrin will identify specific enzymes that are involved in conferring resistance.

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## FUNDING AGENCIES

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

# A WAVEFORM LIBRARY FOR SHARPSHOOTERS AND PRELIMINARY EFFECTS OF APPLIED VOLTAGE ON BEHAVIORS CONTROLLING *XYLELLA FASTIDIOSA* INOCULATION

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**Reporting Period:** The results reported here are from work conducted May 2016 to September 2017.

**ABSTRACT**

Electropenetrography (EPG) is the most rigorous, quantifiable means of observing and measuring sharpshooter feeding, and has recently been shown to reveal the mechanism and real-time tracking of *Xylella fastidiosa* (*Xf*) inoculation by sharpshooter vectors. In EPG, a small signal is applied to the plant; when a gold wire-tethered insect inserts its mouthparts into the plant, a circuit is closed and variable-voltage waveforms are displayed on a computer. These waveforms represent electrical conductivity of fluids flowing through the mouthparts. Over the 50 years since its invention, EPG has undergone three major electronic transformations. The newest, third generation of electropenetrograph, the AC-DC EPG monitor, offers the researcher a selection of settings so that recordings can be tailored to the precise characteristics of the insect being studied. Any of six different amplifier sensitivity (input impedance) levels ( $10^6$ ,  $10^7$ ,  $10^8$ ,  $10^9$ ,  $10^{10}$  and  $10^{13}$  Ohms) can be chosen, combined with either alternating current (AC) or direct current (DC) applied voltage to the plant. Different types of information about feeding can be revealed by each type of setting. Waveforms gradually “morph” into one another with each setting, with varying proportions of information from either of two electrical origins: biopotentials (which indicate membrane breakages, or direction of fluid flow) or electrical resistance (which indicate valve and pump movements, or salivation). Thus, it is possible to develop a library of waveform appearances for each of the 12 settings. A waveform library allows a researcher to identify, for any insect species, the most informative setting(s), revealing the largest number of waveforms from a balance of electrical origins.

Waveforms of the blue-green sharpshooter (*Graphocephala atropunctata*) have been previously published using the first generation, AC EPG monitor (with fixed AC voltage at  $10^6$  Ohms), but not updated with the new AC-DC monitor. The present study recorded blue-green sharpshooter feeding using all 12 amplifier settings with varying applied voltage levels to develop the first, complete waveform library for any sharpshooter species. DC applied signals, especially at higher applied voltages, apparently prevented the insects from initiating stylet probing, until the signal was switched to AC or the insects were very hungry. On the other hand, high AC voltages seemed to cause insects to probe longer. Thus, quantitative testing of effects of AC versus DC, low versus high voltages was begun. The first of two, 2 x 2 factorial tests was completed. This test compared feeding of eight blue-green sharpshooters per treatment, exposed to AC applied signals at low voltage (50 mV) or high voltage (200 mV) at input impedances of  $10^7$  or  $10^9$  Ohms; a future test will use the same experimental design but with DC applied signals. Waveforms from the AC experiment were recorded, measured, and statistically analyzed via mixed model analysis of variance (ANOVA) and least significant difference (LSD) pairwise comparisons. Results showed that AC voltages are relatively benign, with few significant differences. Interestingly, the differences found were in the X wave, the biopotential-dominated waveform representing salivation and egestion of mixed fluids from the anterior foregut. X waves are repetitively performed after first penetration of a xylem cell. The X wave represents the inoculation behavior for *Xf*, whereby dislodged bacterial cells are injected into xylem shortly before onset of sustained ingestion. High voltage (200 mV) caused longer overall performance of X waves. Thus, experiments using EPG to study the *Xf* inoculation behavior should use low AC voltages. Results from the library and AC quantitative studies identified the best settings to use for future EPG studies of blue-green sharpshooter. Low AC voltages (<50 mV) at  $10^8$  (for host plant resistance studies emphasizing pathway waveforms) or  $10^9$  Ohms (for transmission studies emphasizing the X wave).

**FUNDING AGENCIES**

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**PRELIMINARY FINDINGS SUGGEST THAT  
VECTOR FEEDING BEHAVIORS CONTROLLING INOCULATION OF *XYLELLA FASTIDIOSA* ARE  
PERFORMED LESS ON *VITIS ARIZONICA* THAN ON *V. VINIFERA* CHARDONNAY**

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**ABSTRACT**

To date, the most successful example of classical grapevine breeding for resistance to *Xylella fastidiosa* (*Xf*) is the *PdRI* gene, which mediates resistance to *Xf* multiplication and spread in the host grapevine. During 15 years of breeding studies at the University of California, Davis by A. Walker, *PdRI* was introgressed into *Vitis vinifera* cultivated genotypes from wild grape species such as *V. arizonica*. During all of these breeding studies, no effort was made to determine whether resistance of *PdRI* or its parent wild *Vitis* species affects the feeding of vectors, or their transmission (i.e, acquisition, retention, and inoculation) of *Xf* during feeding. Use of electropenetrography (EPG) to study sharpshooter vector feeding in relation to *Xf* transmission has recently facilitated discovery that the EPG X wave represents the *Xf* inoculation behavior. That is, the X wave represents a mixture of plant fluid and insect saliva being taken up into the insect's mouth (buccal) cavity, swished around, and spit back out (thereby injecting it and any suspended *Xf* loosened from the cuticle of the mouth cavity) into a xylem cell. With this knowledge, it is now possible to determine whether wild grapes or their *PdRI*-containing offspring might be resistant to vector behaviors that control *Xf* inoculation, in addition to bacterial multiplication and spread. A quantitative EPG study was performed to test this hypothesis. Stylet probing behaviors of 80 blue-green sharpshooters (*Graphocephala atropunctata*) were EPG-recorded for about 10 hours each; 20 insects on each of four treatments of a 2 x 2 factorial experimental design. Host plants were either *V. arizonica* or *V. vinifera* Chardonnay. Sharpshooters had putatively acquired *Xf* (via a 4-day acquisition access period [AAP] on symptomatic leaves of Chardonnay grapevines previously mechanically inoculated with *Xf* strain 'Stags Leap') or had not acquired *Xf* (although allowed a 24-hour acclimation period on healthy Chardonnay grapevines, to reduce variability in feeding by grape-naïve, basil-reared insects). Of the 20 insects recorded per treatment, waveforms from six per treatment have now been completely measured and analyzed using mixed model analysis of variance (ANOVA) and least significant difference (LSD) pairwise comparisons via SAS. It should be noted that quantitative polymerase chain reaction (qPCR) verification of *Xf* infection of symptomatic leaves used for the AAP has not yet been performed. Nonetheless, tentative conclusions based on statistically significant differences ( $\alpha = 0.05$ ) among treatments for these 24 insects are quite suggestive. Overall, inoculative sharpshooters feeding on both host genotypes spent more than twice as much time performing X wave behaviors as did clean insects. This finding supports that *Xf* biofilm formation in the mouth cavity causes inoculative insects to more actively taste and swish fluids around in their mouth cavities (to remove clogging deposits of biofilm) than do clean insects. One X wave component, C1, is the most important for *Xf* inoculation, because it represents discharging egestion, the direct expulsion of fluid (probably containing saliva-loosened bacteria) from the mouth cavity. C1 was performed two to seven times more often by inoculative insects on *V. arizonica*, compared with all other insects. However, each C1 event was four to 13 times shorter, compared with those of all other insects, causing the overall duration of C1 to be shorter. These preliminary findings suggest that, despite unusually frequent and repeated attempts to spit up bacteria, inoculative sharpshooters on *V. arizonica* are prevented from doing so for the typical long durations seen on Chardonnay. Some structural (narrow cell diameter?), physical (low xylem tension?), or chemical feature of the xylem cells may present an impediment to fluid injection. If analysis of feeding by all 80 insects continues to support these findings, then EPG can be used to identify previously unknown mechanisms of resistance to *Xf* inoculation by its sharpshooter vectors. Such evidence would support use of EPG to identify novel resistance traits to pyramid with the *PdRI* traits, for more durable field resistance to *Xf* in the future.

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## PLAYBACK OF NATURAL VIBRATIONAL SIGNALS IN VINEYARD TRELLIS FOR MATING DISRUPTION OF GLASSY-WINGED SHARPSHOOTER

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### ABSTRACT

The glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) is a vector of *Xylella fastidiosa*, an important bacterial pathogen of several crops in the Americas and Europe. Mating communication of this and many other cicadellid pests involves the exchange of substrate-borne vibrational signals. Exploitation of vibrational signals to interfere with GWSS communication and suppress populations could prove to be a useful tool, but knowledge of the mating behavior was insufficient to initiate development of control methods. In this 2.5-year study, different GWSS communication signals were identified and described, candidate disruptive signals (natural and synthetic) were designed and tested in the laboratory via playback to individuals and male-female pairs, and efficacy of candidate signals in disrupting GWSS mating were validated under field conditions via playback of signals through wires used in vineyard trellises. Data support application of vibrational mating disruption as a novel method to control GWSS populations.

### FUNDING AGENCIES

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**Section 3:**

**Other Pests & Diseases  
of Winegrapes**



# SURVEY AND ANALYSIS OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUS-3 GENETIC VARIANTS AND APPLICATION TOWARDS IMPROVED REVERSE TRANSCRIPTION QUANTITATIVE POLYMERASE CHAIN REACTION ASSAY DESIGN

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## ABSTRACT

This project involves the use of high throughput sequencing (HTS) to identify and characterize additional genetic variants of grapevine leafroll-associated virus 3 (GLRaV-3), construct a representative library of GLRaV-3 genome sequences, and apply this information to the design of a reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay that will detect all known GLRaV-3 variants. Previous attempts to design a sensitive and robust GLRaV-3 RT-qPCR have been unsuccessful because GLRaV-3 is genetically highly diverse (eight distinct subclades) and not all GLRaV-3 isolates have been fully sequenced. To streamline the HTS analysis, a newly developed enzyme-linked immunosorbent assay (ELISA) test will be used for prescreening symptomatic vines from different locations for new potentially divergent GLRaV-3 variants in the fall when the virus titer is highest.

## LAYPERSON SUMMARY

Using sensitive polymerase chain reaction (PCR)-based methods to reliably detect grapevine leafroll-associated virus 3 (GLRaV-3), the most important virus associated with grapevine leafroll disease (GLD), requires the identification of diverse isolates and the acquisition of sequence data that can be used to inform assay design. High throughput sequencing (HTS) analysis can efficiently characterize all viruses present in infected grapevines. In this project, we use prescreening [enzyme-linked immunosorbent assay (ELISA) test] of select populations followed by HTS to identify additional genetically diverse GLRaV-3 isolates, generate a representative collection, and use this information to design a highly reliable reverse transcription quantitative PCR (RT-qPCR) assay.

## INTRODUCTION

Grapevine leafroll-associated virus 3 (GLRaV-3; genus *Ampelovirus*, family *Closteroviridae*) is the most important virus pathogen of grapevine, causing issues in wine, juice, table grape, and rootstock cultivars (Burger et al. 2017). The long-distance spread of GLRaV-3, caused by the movement of infected vines, can be controlled

effectively if clean stock is made available to growers. The economic benefits from the provision of GLRaV-3 certified virus-free planting stock is valued at \$53.5 million annually for the north coast of California alone (Fuller et al. 2013). However, the control and management of GLRaV-3 in planting stock depends on accurate identification of the virus.

To date, designing a sensitive and robust GLRaV-3 reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay has been complicated by the fact that GLRaV-3 is genetically highly diverse. Recent studies based on genome-wide phylogenetic analyses demonstrated that the species can be divided into eight distinct subclades (Groups I-VIII; Maree et al. 2015). Assay design has also been hindered by incomplete sequence data in the GenBank. No complete genome sequences exist for group IV and V isolates (Maree et al. 2013) and the partial sequence data available for New Zealand variants (Chooi, et al. 2013a, 2013b) was not included in the most recently designed GLRaV-3 assays by Bester et al. (2014), which opens the possibility of missing such isolates employing the current standard detection test. We hypothesize that additional diverse isolates exist and propose that a more complete characterization of GLRaV-3 diversity is a prerequisite for the design of a reliable RT-qPCR assay that detects all known variants.

Multiple studies have demonstrated that high throughput sequencing (HTS) is a very useful new research tool for detecting viruses present in grapevines independent of high sequence identity (reviewed in Hadidi et al. 2016). In this project we prescreen different grapevine populations via enzyme-linked immunosorbent assay (ELISA), and later analyze select vines using HTS. The GLRaV-3 antibody for the ELISA, developed by Adib Rowhani (Cooperator), has been able to detect new variants in preliminary studies. While potentially not as sensitive as RT-qPCR, it will be used in this project, along with our current GLRaV-3 RT-qPCR assay, to prescreen for divergent isolates.

## **OBJECTIVES**

The overall goal of this research project is to design a reliable and robust RT-qPCR assay that detects all known variants of GLRaV-3. The specific objectives are:

1. Screen select grapevine populations for new variants of GLRaV-3.
2. Incorporate new genetic data into a more complete characterization of genetic variation across the GLRaV-3 genome to inform assay design.
3. Construct improved assays utilizing multiple primer sets for detecting all existing GLRaV-3 variants.
4. Empirically test and validate proposed assay designs using GLRaV-3 positive controls.
5. Disseminate research progress and results.

## **RESULTS AND DISCUSSION**

In order to accomplish objective 1 we contacted several collaborators from main grape-growing areas of California to identify vineyards with observably high grapevine leafroll disease (GLD) symptoms. Hence, we visited 16 potential fields in the Central Sierra region and Napa County.

In collaboration with Hans J. Maree (Cooperator) we imported nine grapevine selections (cuttings) from South Africa, which represent the different GLRaV-3 groups present in that country. Such plants are currently being propagated at Foundation Plant Services, University of California, Davis. Additionally we received a selection from Australia, a plant infected with GLRaV-3 that shows mild leafroll symptoms. We have been also in contact with Karmun Chooi from the New Zealand Institute for Plant and Food Research Limited, where several new GLRaV-3 isolates have been reported recently. We are planning to exchange both plant material and our GLRaV-3 antibodies. Similar collaboration involving exchange of plant material is in progress with Sebastian Gomez Talquenca from the Instituto Nacional de Tecnología Agropecuaria in Argentina.

Finally, we are currently scouting for symptomatic plants at the UC Davis Virus Collection (DVC) and the USDA National Clonal Germplasm Repository (NCGR), Davis. The NCGR contains about 5,900 living grapevine selections collected from around the world, which represents a wide geographical distribution.

We plan to start collecting and testing by both ELISA and RT-qPCR, samples from symptomatic vines from the NCGR, DVC, and any sample collected from a commercial vineyard and sent by cooperators from select California regions or from abroad.

## CONCLUSIONS

There are no conclusions at this stage of the project.

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

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## ABSTRACT

Although there is a substantial body of research concerning how plants defend and respond to virus infection, there is limited characterization of the molecular determinants of grapevine leafroll-associated virus (GLRaV) susceptibility, responses, and symptoms specifically. GLRaVs cause an array of symptoms that include impaired ripening. This report summarizes current work undertaken to characterize the effects of different GLRaVs, combinations of GLRaVs, and given different rootstocks, on gene expression, metabolite accumulation, and hormones during grape berry ripening. Preliminary analyses, consistent with earlier studies, indicate that the effect of virus infection on total soluble solids was related to the combination of viruses present and the rootstock. The transcriptomic and metabolite experiments undertaken as part of this study are ongoing.

## LAYPERSON SUMMARY

Grapevine leafroll-associated viruses (GLRaVs) are the most widespread and economically damaging viruses affecting viticulture (Goheen, Hewitt, and Alley 1959, Maree et al. 2013, Naidu, Maree, and Burger 2015). GLRaVs are sometimes present as mixed infections with other viruses (Fuchs, Martinson, Loeb, and Hoch 2009, Prosser, Goszczynski, and Meng 2007). The severity of GLRaV symptoms is influenced by host genotype (Guidoni, Mannini, Ferrandino, Argamante, and Di Stefano 2000), which virus or combination of viruses is present, scion-rootstock pairings (Golino, Sim, and Rowhani 2003, Lee and Martin 2009), and environmental factors (Cui et al. 2017). The effects of GLRaVs can include poor color development in red grapes, non-uniform or delayed ripening, reduced sugar content in berries, curling leaves, reddening or chlorotic interveinal areas, and high crop loss (Atallah, Gomez, Fuchs, and Martinson 2012, Guidoni et al. 2000, Vega, Gutiérrez, Peña-Neira, Cramer, and Arce-Johnson 2011). The purpose of these experiments is to determine the effects of individual and dual GLRaV infections on ripening in Cabernet Franc vines grafted to different rootstocks. This information can be used to develop vineyard management strategies to improve berry quality despite viral infection. Our core hypotheses are that (1) GLRaVs disrupt berry development and the accumulation of flavor and aroma metabolites by altering hormone networks, and (2) the differences in symptoms associated with different GLRaVs are due to non-uniform impacts on some metabolite and gene regulatory pathways.

## INTRODUCTION

Grapevine leafroll-associated viruses (GLRaVs) are the most consequential viruses affecting grapevine (Atallah et al. 2012, Maree et al. 2013, Naidu et al. 2015). Plants' responses to viruses generally include a multitude of changes in metabolism, gene expression, and gene regulation (Alazem and Lin 2014, Bester, Burger, and Maree 2016, Blanco-Ulate et al. 2017, Moon and Park 2016). In berries, GLRaV infection has been associated with depressed or asynchronous ripening and affects the accumulation of diverse metabolites including sugars, tannins, pigments, and acids (Alabi et al. 2016, Lee and Martin 2009, Lee and Schreiner 2010, Vega et al. 2011). There is a growing body of knowledge concerning the molecular and hormonal controls of plant virus responses generally (Alazem and Lin 2014). However, there remains a gap in knowledge concerning the specific regulation of the response to GLRaVs and which pathways determine the GLRaV symptoms and their severity. This study is using

RNA sequencing and metabolite profiling to explore the effects of individual and mixed infections of GLRaVs on ripening and to identify which pathways are involved in responses and symptoms.

The rootstocks, scions, and infections used in this study were selected to improve the likelihood of generating commercially transferable knowledge. The vineyard used for this study consists of Cabernet Franc grapevines grafted to different rootstocks and carrying commercially consequential GLRaVs. Cabernet Franc was used because it produces clear symptoms to GLRaVs. Among the treatments established in the vineyard, vines carrying GLRaV-1, GLRaV-3, GLRaV-5, GLRaV-1 + GLRaV-2, and GLRaV-1 + GLRaV-3 were included in this study because these infections are associated with a range of symptoms and symptom severities. Among the rootstock-scion pairings planted in the experimental vineyard, Cabernet Franc grafted to Kober 5BB and MGT 101-14 rootstocks were used because these rootstocks are commonly used in California. GLRaV infections (or their lack of in control vines) as well as the specific strains involved were confirmed by molecular testing at Foundation Plant Services (FPS; University of California, Davis) prior to sampling. Berries were collected at four distinct developmental stages (pre-véraison, véraison, post-véraison, and harvest) from Cabernet Franc grapevines grafted to MGT 101-14 and Kober 5BB rootstocks. Twenty berries were picked from each of six vines at each sampling date and from each viral treatment. Berries were sampled evenly throughout the plant. Following their sampling, berries were crushed and their total soluble solids (TSS) were measured. The preparation of RNA sequencing libraries is underway. The RNA-sequencing data to be generated will provide a quantitative, comprehensive view of the changes in gene expression due to GLRaVs associated with primary and secondary berry metabolism. Changes in the expression of hormone biosynthesis and signaling genes may reveal mechanisms that underlie impaired berry metabolism. The same samples used for RNA sequencing will be also used for further metabolite measurements: hormones, sugars, organic and amino acids, flavonoids, tannins, terpenoids, and anthocyanins. This will enable an association between changes in gene expression and metabolite abundance.

## OBJECTIVES

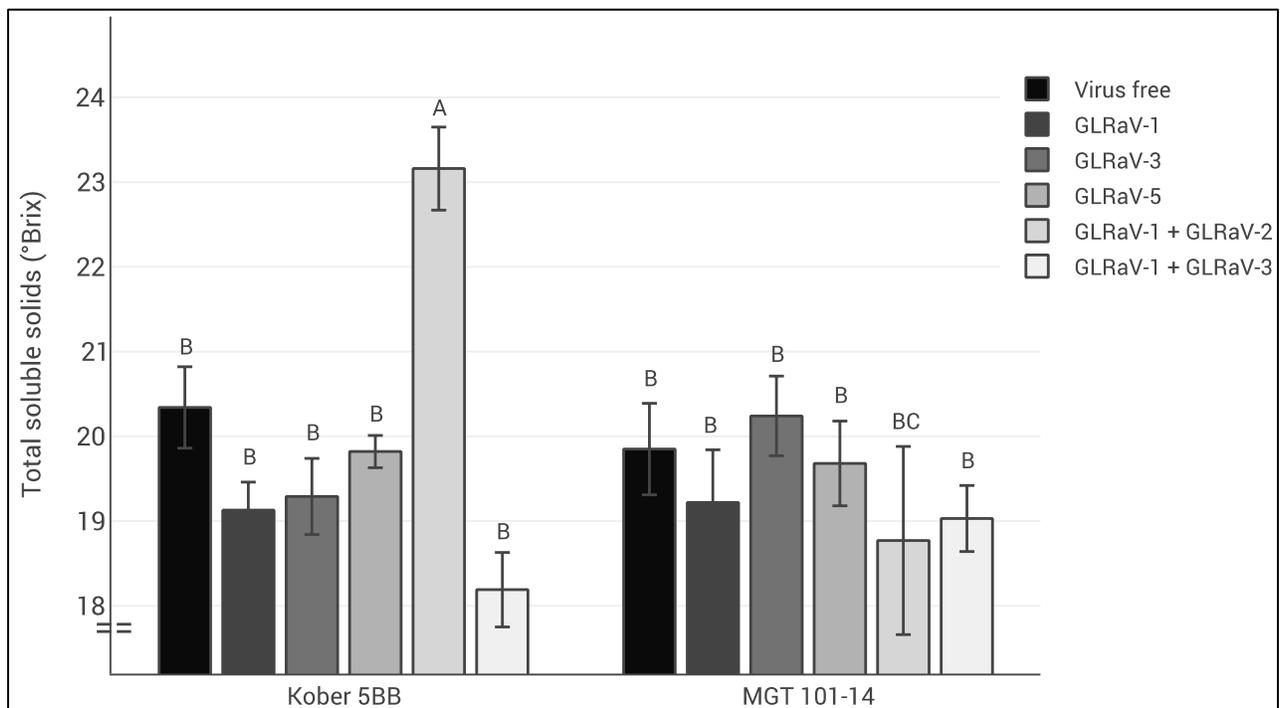
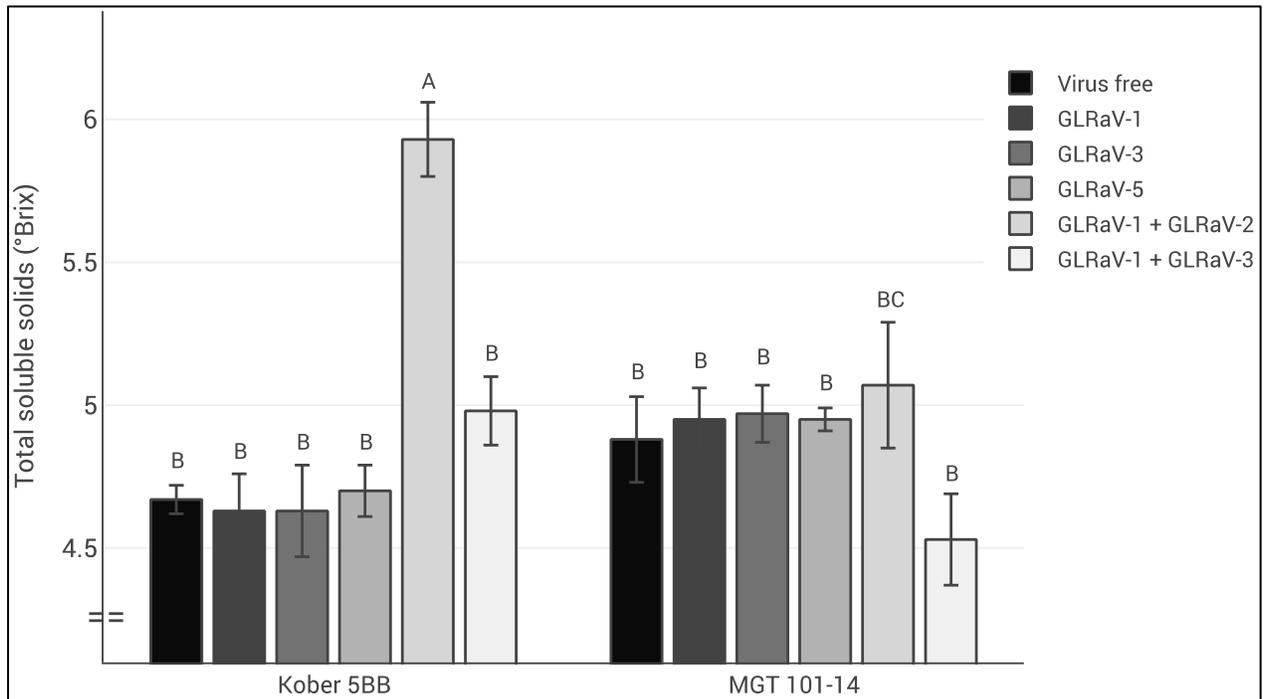
1. Profile transcriptome changes caused by individual and dual GLRaV- infections during fruit development.
2. Identify the metabolic pathways altered by GLRaV infection that explain changes in fruit composition.
3. Determine whether infection(s) are associated with changes in the dynamics of ripening-associated hormones and other metabolites.

## RESULTS AND DISCUSSION

Differences in TSS were observed both three weeks before and two weeks after véraison that were dependent on the combination of infections and rootstock. TSS in berries from both sampling dates were significantly higher in plants grafted to Kober 5BB with GLRaV-1 + GLRaV-2 dual infections than in healthy, single infection, and GLRaV-1 + GLRaV-3 dual-infection plants on the same rootstock (**Figure 1**). In plants with the GLRaV-1 + GLRaV-2 dual infection, TSS were significantly higher in plants grafted on Kober 5BB than on MGT 101-14 (Tukey HSD test,  $p$ -value < 0.05). Though surprising, this might be associated with visibly poorer fruitset on these plants (**Figure 2**). Furthermore, the fruits on these plants were visibly beginning to desiccate by harvest. Berries from plants grafted on Kober 5BB and with GLRaV-1, GLRaV-3, and GLRaV-1+GLRaV-3 dual infections appeared to have lower TSS than healthy plants on the same rootstock, though these differences were not significant. Differences in TSS relative to healthy plants and between treatments were not observed among berries from plants grafted to MGT 101-14 rootstock. These results suggest that rootstock may impact the severity of disease symptoms, though how is unclear. Further, it appears that different leafroll viruses or different combinations of viruses disparately impact ripening. Why this occurs is also not clear, but its investigation is ongoing.

## CONCLUSIONS

The results presented show differential impact of virus combination on the accumulation of total soluble solids and berries. In conjunction with the forthcoming RNA sequencing and metabolite analyses, the data generated may be used in the future to develop strategies to mitigate the detrimental effects of these viruses on ripening.



**Figure 1.** The effects of single and dual leafroll-associated virus infections on berry TSS three weeks before (**top**) and two weeks after (**bottom**) véraison. TSS is reported for fruits from vines grafted to two different rootstocks, Kober 5BB and MGT 101-14.



**Figure 2.** Photographs of grapevines and berries. Plants are grafted to Kober 5BB. Top two, Healthy; Bottom two, GLRaV-1 + GLRaV-2. The top-most photo is rotated counterclockwise.

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# EVALUATION OF COMMERCIAL ANT BAITS AS A COMPONENT OF AN INTEGRATED PEST MANAGEMENT PROGRAM FOR VINE MEALYBUG

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## ABSTRACT

Vine mealybug (*Planococcus ficus*) is a destructive phloem-feeding pest in California vineyards. Vine mealybug can reach very large population densities; feeding activity can debilitate vines while excrement and the associated sooty mold can contaminate clusters, making them unsuitable for harvest. Vine mealybug's cryptic habits -- populations are typically found under the bark -- complicate management, particularly with contact insecticides. An integrated pest management program that relies on several tactics (insecticides, mating disruption, and biological control) can provide sustainable control of vine mealybug populations. Argentine ants (*Linepithema humile*) may disrupt integrated pest management programs by interfering with the activity of biological control agents. Baits are an effective means to control ant populations and minimize their disruptions. We evaluated broadcast applications of a commercial ant bait and an experimental ant bait in northern California vineyards and measured the effects on Argentine ant populations. Pre- and post-application, Argentine ant populations were measured indirectly via feeding activity, assessed as the number of ants present on cotton balls (Fisher Scientific) soaked in 25% sucrose solution. Both baits reduced feeding activity, although the effect was more sustained in the experimental bait treatment, suggesting the potential of this bait to provide long-term control of Argentine ants in coastal California vineyards.

## LAYPERSON SUMMARY

Vine mealybug (*Planococcus ficus*) is a destructive pest in California vineyards; it contaminates fruit and reduces vine health and productivity. Grape growers may use multiple tactics (integrated pest management) including insecticides, mating disruption, and biological control to achieve control of vine mealybug populations. Argentine ants (*Linepithema humile*) are invasive insects common in coastal California vineyards. Ants disrupt integrated pest management programs for vine mealybug because they interfere with the activity of a small parasitic wasp that attacks vine mealybugs. Ant baits are an effective approach to manage ant populations while minimizing impacts on non-target organisms. We are investigating the potential of commercial and experimental baits to control Argentine ants in vineyards. Both baits reduced ant activity in the treated areas, although the effect was more sustained with the experimental bait, suggesting its potential as a component of sustainable vine mealybug management in coastal California vineyards.

## INTRODUCTION

Vine mealybug (*Planococcus ficus*) is a destructive vineyard pest that contaminates fruit, debilitates vines, and vectors plant pathogens such as *grapevine leafroll-associated virus-3* (Daane et al. 2012). First reported from vines in the Coachella Valley (Gill, 1994), vine mealybug soon spread throughout California, likely on infested nursery stock (Haviland et al. 2005). It is currently found in most California grape-growing regions (Godfrey et al. 2002, Daane et al. 2004a, 2004b) and has the potential to spread throughout the western United States.

Management of vine mealybug populations can prove challenging and often requires the use of multiple tactics, including biological control, mating disruption, and insecticides (Daane et al. 2008). Management can be particularly complicated in coastal winegrape growing regions where vine mealybug populations are tended by Argentine ants (*Linepithema humile*). In the presence of tending ants biological control of mealybugs can be significantly interrupted, resulting in large vine mealybug populations that may be more easily spread to new areas. These populations also contaminate the fruit, causing yield losses and decreased fruit quality. In vineyards where Argentine ants are prevalent, management of ant populations is a critical part of an integrated pest management program for vine mealybug and necessary for containment of insect populations (Nyamukondiwa and Addison, 2011, Mgochecki and Addison, 2009).

Liquid ant baits adapted from the urban environment (Klotz et al. 2002) for use in vineyards (Cooper et al. 2008) significantly reduce mealybug populations in vineyards by contributing to increases in biological control (Daane et al. 2007). The costs associated with the manufacture, deployment, and maintenance of bait stations have been prohibitive to widespread adoption of Argentine ant management in vineyards, despite the benefits that could result from such programs (Nelson and Daane, 2007). There is continued interest among coastal grape growers in the development of a simpler and more economical bait program that could be widely implemented. Baits formulated as granular products or polyacrylamide gels that can be broadcast with a fertilizer spreader could be distributed more quickly and frequently over a large area, and would not require the manufacture and maintenance of bait stations. The sustained use of the granular or polyacrylamide baits could lead to longer-term containment and control of Argentine ant populations (Boser et al. 2014, Krushelnycky et al. 2004). We are evaluating granular and polyacrylamide ant baits that can be broadcast to reduce populations of Argentine ant. Ant control would in turn contribute to the sustainable control of vine mealybug populations. In the absence of an economical bait program, ant suppression must be achieved with the broad-spectrum insecticide chlorpyrifos that can affect water quality, disrupt populations of beneficial insects, and pose vertebrate health risks.

## OBJECTIVES

The broad goal of this research is to increase the efficacy and adoption of integrated pest management programs for vine mealybug, a destructive pest of grapevines in California. Our specific objective is:

1. Evaluate the efficacy of two bait formulations to reduce Argentine ant populations as part of an integrated pest management program for vine mealybug.

## RESULTS AND DISCUSSION

### 2015 Field Season

#### Granular Bait Trial

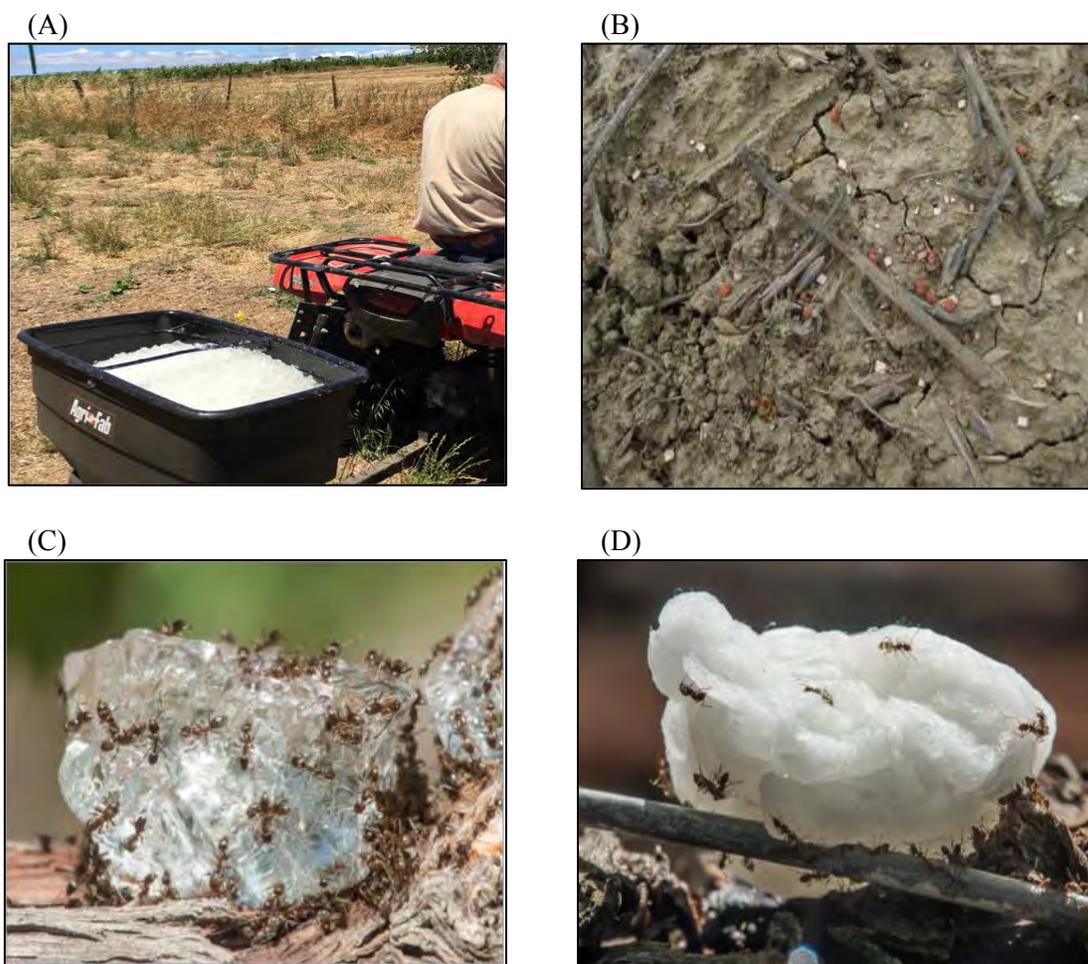
In 2015 our experiment was established in two vineyard blocks in Napa, California [Carneros American Viticultural Area (AVA)]. Both blocks were planted in 1999 and are a mix of Chardonnay clones [17 on Robert Young and six on SO4 rootstock (*Vitis berlandieri* x *V. riparia*)]. We used a randomized complete block design and established six, 6-row replicates of each treatment. The treatments were three commercial granular bait products (**Table 1**).

**Table 1.** Ant bait products applied in trial blocks in a Napa County vineyard.

Treatment	Active Ingredient (Concentration)	Rate Per Acre	Bait Applications (2015)
Altrevin	metaflumizone (0.063%)	1.5 lb.	March 14 & 15; April 15 & 16; June 15 & 16
Altrevin & powdered sugar	metaflumizone (0.063%)	1.5 lb.	
Extinguish	hydramethylnon (0.365%) & methoprene (0.25%)	1.5 lb.	
Seducer	Spinosad (0.07%)	20 lb.	
Untreated	none	none	none

In March, April, and June 2015 the cooperating vineyard manager applied the bait in the vine row with a modified broadcast spreader mounted on an all-terrain vehicle (ATV). Because Altrevin and Extinguish are formulated with a protein attractant specifically for control of red imported fire ant (*Solenopsis invicta*), we included one Altrevin treatment in which the bait was coated with powdered sugar before application to make it more attractive to Argentine ants. The spinosad bait, Seducer, is formulated with a carbohydrate attractant (sugar) specifically to target the Argentine ant (**Figure 1B**). Additionally, Seducer has been approved for use in organic vineyards. Since there are a limited number of insecticides approved for vine mealybug management in organic vineyards, ant bait can be an essential component of an integrated pest management program in these vineyards.

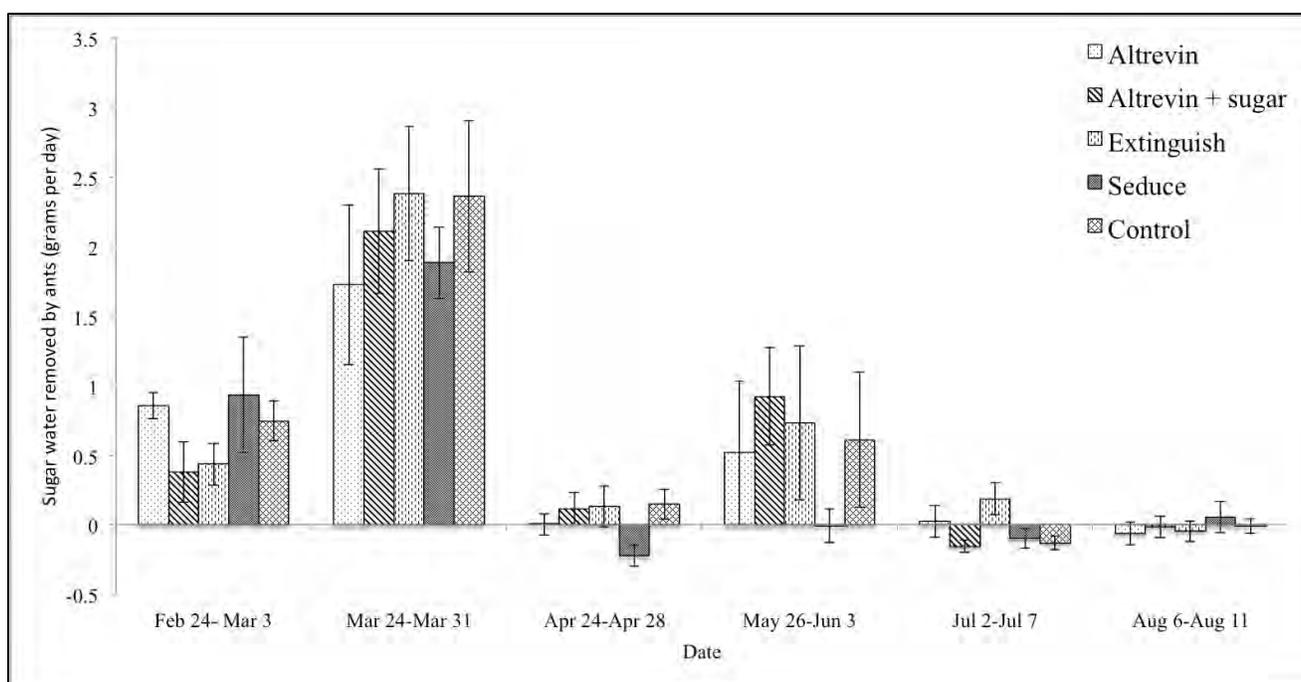
Ant densities were determined indirectly as a measure of feeding activity, assessed as the amount of nontoxic sucrose water removed from 50-milliliter (ml) polypropylene centrifuge tubes (Corning Inc., Corning, NY) tied to the vine trunk (Klotz et al. 2002, Daane et al. 2008a) in the center two rows of each plot. The 50-ml tubes are henceforth referred to as monitoring tubes. A two centimeter (cm) hole was drilled in the cap and a square of permeable plastic mesh (Weedblock, Easy Gardener Inc., Waco, TX) was placed between the cap and the filled tube, covering the hole. The mesh is fine enough to retain the liquid when the tube is inverted but coarse enough to allow ants to remove the liquid on contact. A second lid was fixed to the original lid and covered with a permanent mesh to discourage feeding by honeybees and wasps. Before the tubes were deployed in the vineyard each tube was filled to 45 ml with 25% sucrose water and the weight of each tube was recorded. Tubes were inverted on a vine trunk for four to seven days depending on ant activity at a density of 12 tubes per plot, or a total of 72 tubes per treatment. At the end of the monitoring period the tubes were brought back to the laboratory and the new weights were recorded. One additional monitoring tube per plot was attached to an ant-excluded bamboo stake to measure the amount of water lost to evaporation; this amount was averaged across all plots and used to adjust the final weight.



**Figure 1.** (A) Broadcast spreader with polyacrylamide bait mounted on ATV; (B) Seduce bait (reddish pellets) under the vine row; (C) Argentine ants feeding on polyacrylamide bait; (D) Argentine ants feeding on cotton ball used for monitoring ant activity. Photo credits: (A) C. Bianucci; (B) M. Cooper, UC Cooperative Extension (UCCE); (C) & (D): M. Hobbs, UCCE.

Ant feeding activity is reported as grams (g) of sugar water removed from monitoring tubes per day (**Figure 2**). During the February and March monitoring periods (pre-treatment and 10 days after the first treatment, respectively) ant feeding activity was not significantly different across all treatments. This is not surprising since we blocked for consistent ant populations prior to treatment; also, and most importantly, baits have delayed

toxicity and would not be expected to control populations so quickly (10 days) after application. During the April 24 to 28, 2015 monitoring period, feeding activity was significantly reduced in the Seduce bait treatment (Tukey's pairwise comparison,  $p = 0.0099$ ); this is roughly six weeks after the first bait application and one week after the second. From May 26 to June 3, 2015, feeding activity in the Seduce treatment ( $-0.007 \pm 0.12$  g per day) was reduced compared to other treatments (0.52 to 0.92  $\pm$  0.35 to 0.55 g per day), although the difference was not statistically significant due to the high variability in ant feeding -- particularly in the Altrevin and untreated blocks. During the July and August monitoring periods, ant feeding was low to none in all treatments. In other ant bait trials we have detected similar feeding lulls at our monitoring tubes during the summer (Daane et al. 2006, 2008a). We did not see any differences in population suppression between the powdered sugar-coated bait and those protein-based baits without powdered sugar. Since the sugar is not an inert ingredient of the bait, it may not adhere well to the bait. It could have been removed during the application process or not durable in the field. At this point there does not appear to be a measurable improvement in bait performance through the addition of the powdered sugar under these conditions. Also, adverse effects were noticed as the sugar heated (and melted) in the spreader, thereby clogging the mechanisms of the spreader that impacted application efficiency and necessitating additional disassembly / cleaning time. Overall, the Seduce bait was the easiest to apply. We attributed this to weight and consistency of the bait as well as application rates (higher rates made the applied bait more visible, and therefore easier to calibrate the spreader and adjust drive speeds).



**Figure 2.** Average sucrose water removed (grams per day) from monitoring tubes by Argentine ants during six monitoring periods in a Chardonnay vineyard (Carneros AVA) in 2015. Results are reported for each bait treatment and the untreated control. During the April 24 to 28, 2015 monitoring period feeding activity was significantly reduced in the Seduce bait treatment (Tukey's pairwise comparison,  $p = 0.0099$ ). On all other dates, there were no significant differences among treatments.

## **2016 Field Season**

### **Granular Bait Trial**

Based on the results of our 2015 trials we eliminated both Altrevin and Extinguish ant baits from our 2016 trials, focusing solely on Seduce (0.07% spinosad), the product that was most efficacious in preliminary trials. We selected five experimental blocks (Oakville and Rutherford appellations of Napa Valley AVA), and established split-plot design (bait and untreated) in all blocks. In two of those blocks (designated I1 and I2) Seduce ant bait was applied at a rate of 20 lbs per acre on April 15 and 16, 2016. In the remaining three blocks (designated T1, T2, and F1) Seduce ant bait was applied at a rate of 28 lbs per acre (slightly higher than the target rate due to

challenges with calibration and the spreader equipment) on May 19 and 20, 2016. A second application at the rate of 20 lbs per acre was applied in blocks T1, T2, and F1 on June 25 and 27, 2016. The spreader equipment was the same as that used in the 2015 trial. The cooperating vineyard managers made all the bait applications.

We monitored ant activity pre- and post-application using cotton balls (Fisher Scientific) soaked in 25% sucrose solution (**Figure 1C**). Ant activity was measured once every two weeks. Forty-five or fifty vines per treatment per block were selected as monitoring vines. One saturated cotton ball was deployed on each monitoring vine, either on the ground (early season) or on the vine (after fruit set), depending on where the ants were predicted to be most active. After 2.5 to 3 hours cotton balls were retrieved from each monitoring vine and ant activity on the cotton balls was assessed using a 0 to 3 scale, where ‘0’ equals no ants, ‘1’ equals the presence of 1 to 10 ants, a value of ‘2’ is assigned to cotton balls with 11 to 50 ants, and a rating of ‘3’ is assigned for the presence of greater than 50 ants.

Due to some challenges with site selection the first bait applications in blocks T1, T2, and F1 occurred later (May 19 and May 20, 2016) than would be desired to optimize results. In blocks I1 and I2 bait applications were initiated early in the growing season (April 15 and 16, 2016) and within 14 days of when ants were detected and temperatures were adequate for foraging to occur. We tested for significant differences between baits and control at each sampling date using Mann-Whitney U tests (**Table 2**). Our analyses suggest that (1) the dry bait treatment at sites I1 and I2 was only significantly different from control on one date after treatment. Given that ant levels were near zero pre-treatment, it seems unlikely this was due to the treatment. (2) Dry bait in block F1 was no different from control until after the second treatment. After the second treatment ant levels were significantly lower than the control but were not statistically different on the last sampling date (October 7). (3) Dry bait in blocks T1 and T2 was significantly lower than control at every sampling date. As ants were significantly lower than control pre-treatment and actually increased after the first treatment, there is no convincing evidence that the bait had an effect. In conclusion, this study did not generate convincing evidence that dry bait (Seduce) reduced ant levels in two vineyards (I and T). At vineyard F, the dry bait treatment was lower than control after the second treatment but ant levels were not actually reduced until October when they also had decreased in the control. At best there was a very limited effect of dry bait in only one vineyard in this study. These results are not encouraging with regards to the efficacy of Seduce ant bait for controlling Argentine ants in commercial vineyards. Future studies should evaluate a higher product rate and / or more applications to determine whether improved control can be achieved. More applications were not explored during the current study as the cooperating vineyard managers did not find this to be an economically attractive strategy.

**Table 2.** Results of Mann-U tests comparing dry bait vs. control for each sampling date.

Sampling Trial	Blocks I1/I2			Block F1			Blocks T1, T2	
	Trial Date	<i>p</i> value		Trial Date	<i>p</i> value		Trial Date	<i>p</i> value
1	8-Mar	.55	1	6-May	.19	1	6-May	<b>.01*</b>
2	23-Mar	.56		TREATMENT 20-MAY			TREATMENT 19-MAY	
3	6-Apr	.45	2	27-May	.70	2	27-May	<b>&lt;.01*</b>
	TREATMENT 15, 16-APR		3	10-Jun	.86	3	10-Jun	<b>&lt;.01*</b>
3	19-Apr	1.0		TREATMENT 20-JUN			TREATMENT 25-JUN	
4	6-May	<b>&lt;.01*</b>	4	24-Jun	<b>&lt;.01*</b>	4	24-Jun	<b>&lt;.01*</b>
5	3-Jun	.53	5	8-Jul	<b>&lt;.01*</b>	5	8-Jul	<b>&lt;.01*</b>
6	10-Jun	.23	6	22-Jul	<b>&lt;.01*</b>	6	22-Jul	<b>&lt;.01*</b>
7	24-Jun	.02	7	7-Oct	.82	7	7-Oct	<b>&lt;.01*</b>
8	8-Jul	.97						
9	22-Jul	.68						
10	11-Oct	.04						

### Polyacrylamide Gel Bait Trial

Based on a pilot study that eliminated >99% of ants from treated plots in the California Channel Islands (Boser et al. 2014) and a preliminary vineyard study conducted by the Principal Investigators in 2015 (unpublished data), we are evaluating the efficacy of a polyacrylamide gel bait formulation in vineyards. We established three experimental blocks (split-plot design: treated and untreated treatments); two of these blocks (designated C1 and C2) are located in the Carneros appellation (Napa Valley AVA) and one (designated M1) is located in the St. Helena appellation. Blocks C1 and C2 are populated with the invasive vine mealybug; block M1 is populated with the native grape mealybug (*Pseudococcus maritimus*). In addition to the economic damage sustained by vine mealybug populations, the spread of grapevine leafroll-associated virus 3 (GLRaV-3) is a major concern in all of these blocks.

The bait solution consists of 0.0006% thiamethoxam (Platinum insecticide, Syngenta US) in 25% sucrose solution, deployed at a rate of 10 gal per acre in polyacrylamide Water Storing Crystals (MiracleGro®) (**Figure 1C**). These crystals absorb water and water-soluble chemicals, and when hydrated present a thin layer of liquid bait solution on the surface for 24 to 72 hours following application. To allow sufficient time for the crystals to absorb the bait solution they were added to the mixture 24 hours prior to the application. The hydrated crystals were deployed using an 85 lb tow spreader (Agri-Fab, model #45-0315) pulled with an all-terrain vehicle (ATV) (**Figure 1A**). Bait applications were initiated once foraging ants were detected at sugar-soaked cotton balls. The cooperating vineyard manager made the bait applications on March 16 and April 14, 2016 in blocks C1 and C2, and on April 15 and May 26, 2016 in block M1. Because block M1 is in a more northerly location within Napa County, ants did not become active until later in the season [ant foraging is reduced below 60° F (15° C)]. Ant monitoring pre- and post-application followed the method described previously, using cotton balls soaked with a 25% sucrose solution (**Figure 1D**).

We tested for significant differences between baits and control at each sampling date using Mann-Whitney U tests (**Table 3**). In summary, pre-treatment ant ratings were no different between the bait and control vines at either vineyard. After the first treatment the bait treatment had significantly fewer ants (near zero) than the control in vineyard M1; this continued throughout the season until the final sampling date on October 7, 2016. In the C1 and C2 blocks there were significantly fewer ants on the first sampling date following the first treatment. From one month after the second bait application until the end of the season (October 11, 2016) ant populations in the baited blocks in vineyards C1 and C2 remained significantly lower than in the untreated control. In summary, our trials indicate that the polyacrylamide bait laced with thiamethoxam nearly eliminated ants for 1.5 months and provided sustained control of ants for up to six months after the second bait treatment.

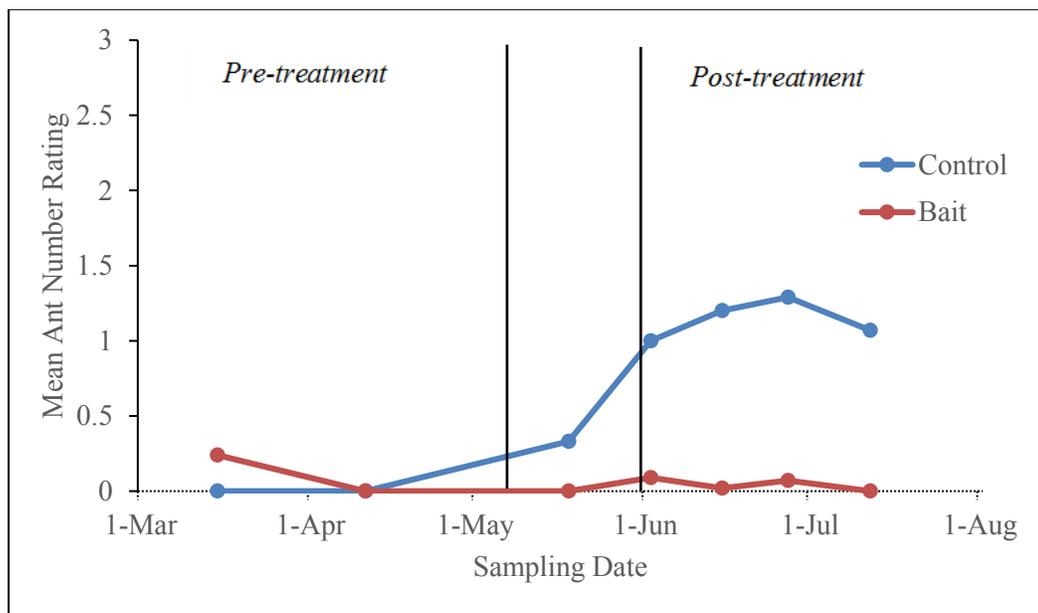
**Table 3.** Results of Mann-U tests comparing polyacrylamide bait vs. control for each sampling date.

Sampling Trial	Blocks C1, C2			Block M1	
	Trial Date	<i>p</i> value		Trial Date	<i>p</i> value
1	26-Feb	.29	1	8-Mar	1.0
2	8-Mar	.16	2	23-Mar	.06
TREATMENT 16 <sup>th</sup> MAR			3	6-Apr	.49
3	23-Mar	<.01*	TREATMENT 15 <sup>th</sup> APR		
4	15-Apr	1.0	4	19-Apr	<.01*
TREATMENT 15 <sup>th</sup> APR			5	6-May	<.01*
5	28-Apr	1.0	TREATMENT 25 <sup>th</sup> MAY		
6	11-May	<.01*	6	3-Jun	<.01*
7	30-May	<.01*	7	10-Jun	<.01*
8	14-Jun	<.01*	8	27-Jun	<.01*
9	30-Jun	<.01*	9	8-Jul	<.01*
10	11-Jul	<.01*	10	27-Jul	<.01*
11	25-Jul	<.01*	11	7-Oct	.026
12	11-Oct	<.01*			

## 2017 Field Season

### Polyacrylamide Bait Trial

These trials are a continuation of our 2016 trials with polyacrylamide crystals laced with six ppm thiamethoxam (Platinum insecticide, Syngenta US). We are also evaluating bait laced with boric acid (0.5%) as an option for organic growers. The bait was mixed and applied as described previously (**Figure 1C**). Because ants re-invaded the treated areas in 2016 (split-plot design), in 2017 we designated entire blocks as either treated or untreated and paired the treated and untreated blocks. We have two pairs of blocks in the Carneros region and two pairs in the Oakville region. We also continued our trial in one split-plot block in St. Helena. The Carneros blocks were treated with thiamethoxam-laced bait on May 3 and June 9, 2017, the Oakville blocks were treated with boric acid-laced bait on May 4 and May 26-31, 2017, and the St. Helena block was treated with thiamethoxam-laced bait on May 5 and June 10, 2017. Ant monitoring pre- and post-application followed the method described previously, using cotton balls soaked with a 25% sucrose solution (**Figure 1D**).



**Figure 3.** Mean ant level rating for St. Helena vineyard block. Ants were rated on a 0 to 3 scale, where a value of ‘0’ was assigned to cotton balls with no ants, a value of ‘1’ assigned for the presence of 1 to 10 ants, a value of ‘2’ assigned for 11 to 50 ants, and a value of ‘3’ assigned for greater than 50 ants. Vertical lines represent dates of two bait applications.

The polyacrylamide gel bait reduced ants at all sites; however, the extremely variable ant populations in the untreated blocks made it more challenging to attribute an explanation to the effects than in previous years. In the treated blocks in Oakville, ant numbers decreased significantly only after the second treatment (within two weeks), where they remained close to (or at) zero for up to two months. This appeared to show an effect of the bait treatment. However, the control did not show much variation in ant numbers, remaining just above zero for the study period and ending at the same level as the bait blocks (despite some significant fluctuation). In the first Carneros site ant numbers in the bait block were significantly reduced after the first treatment and remained at zero until the end of the study period, two months after the second treatment. Ants in the control block were low generally with little increase (if any) from March to August. However, they were almost always significantly higher than the control block after the first treatment. In the second Carneros site, ant numbers in the control block were low but there was an overall small increase from March to August. In comparison, ant numbers were initially higher in the bait block but crashed to zero immediately after the first treatment, only rising to match the control two months after the second treatment. This provides evidence that the thiamethoxam-laced polyacrylamide bait reduced ants. At the St. Helena site ant numbers in the control block started at zero in March and increased over the season until August (**Figure 3**). In comparison, ant populations in the bait block were low /

zero pre-treatment and continued at this low level for the entire study period, becoming significantly lower than the control within two weeks of the first bait application. This is evidence that the wet bait suppressed ant numbers from first treatment, although it should be noted that a decline in ant numbers due to the bait was not measured because of the lack of ants pre-treatment.

## CONCLUSIONS

We evaluated two baits (one commercial and one experimental product) to reduce Argentine ant populations in a coastal California vineyard. Because Argentine ants disrupt biological control of vine mealybug by interfering with the activity of predators and parasitoids, control of Argentine ants can be an essential component of integrated pest management programs for vine mealybug. Handling and distribution of baits that can be broadcast is simpler and more efficient than liquid baits that must be contained within bait stations. Additionally, Argentine ant nests are typically multiple and widely dispersed throughout agricultural ecosystems in the spring, summer, and fall (Markin, 1970) so multiple point-sources make bait more accessible to all nests within an infested area (Boser et al. 2014). Our results suggest that an experimental bait treatment (0.0006% thiamethoxam in polyacrylamide crystals) has the potential to reduce populations of Argentine ant, whereas a commercially available spinosad-laced bait (Seduce) was less effective in our trials. Future studies may explore the use of multiple applications or higher rates of Seduce to obtain adequate control of Argentine ant populations. Two applications of a thiamethoxam-laced polyacrylamide bait reduced ant populations for two to six months after treatment. Because ants may reinvade from untreated areas, large-scale, regional treatments such as those conducted in the California Channel Islands (Boser et al. 2014)) could be more successful and future studies should concentrate in this area.

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**IMPROVING VINE MEALYBUG WINTER AND SPRING CONTROLS:  
I. BIOASSAYS. II. USING HIGH PRESSURE LIQUID CHROMATOGRAPHY TO FOLLOW  
INSECTICIDE MOVEMENT IN THE VINE**

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**Reporting Period:** The results reported here are from work conducted March 2017 to September 2017.

**ABSTRACT**

The vine mealybug (*Planococcus ficus*) has become one of the more important insect pests of California vineyards, threatening economic production and sustainable practices in this multi-billion-dollar state industry. This work has begun to better understand and optimize registered insecticides used to control the vine mealybug in the winter and spring periods, when the mealybug population is located primarily under the bark on the trunk and cordons. In the initial work we selected vineyards in three regions and have taken spring through fall samples. We applied treatments of Movento and monitored commercial spray applications in vineyards for different commodities (e.g. wine vs. table grapes) and with various management practices (e.g. trellis systems). We monitored mealybug densities but found little difference among the plots, in part because of the low mealybug populations. We collected approximately 6,000 tissue samples at vineyards being used for the field bioassays, as well as from vineyards with unusual vine mealybug densities, or where we can manipulate spray application to test movement of key metabolites of Movento. For analyses, we have developed protocols for tissue analysis using high pressure liquid chromatography, and verified that the procedure is accurate.

**LAYPERSON SUMMARY**

The vine mealybug (*Planococcus ficus*) has become one of the most important insect pests of California vineyards. Researchers, pest control advisors, and farmers have developed relatively good controls that target exposed vine mealybugs (those on the leaves or canes). However, controlling the more protected mealybug population found under the bark of the trunk or on the roots has been more difficult. Our objectives are to improve pre- or post-harvest controls that target the winter-spring vine mealybug population and to better determine the spring emergence of vine mealybug crawlers to better time foliar applications. In 2016 research focused on bioassays (e.g. the number of live or dead mealybugs) and the movement of Movento (or, more correctly, its metabolites) in the vine, using high pressure liquid chromatography methodology.

**INTRODUCTION**

The vine mealybug (*Planococcus ficus*) has become one of the most important insect pests of California vineyards, threatening economic production and sustainable practices in this multi-billion-dollar commodity. Insecticides are the primary control tool for vine mealybug (Prabhaker et al. 2012, Daane et al. 2013, Bentley et al. 2014), especially when grapevine leafroll diseases (GLDs) are a concern (Daane et al. 2013). Researchers, pest control advisors, and farmers have developed relatively good controls that target exposed vine mealybugs (those on the leaves or canes). However, controlling the more protected mealybug population found under the bark of the trunk or on the roots has been more difficult. The vine mealybug population is primarily on the trunk and upper root zone near the soil line during the winter and early spring (Daane et al. 2013). This population has a refuge from natural enemies (Gutierrez et al. 2008) and can be the most difficult to control even with systemic insecticide applications (Daane, pers. obsrv.). Moreover, mealybugs can remain on even the remnant pieces of

vine roots after vineyard removal, hosting pathogens and infesting new vines after replanting the vineyard (Bell et al. 2009).

Insecticides with systemic action are the best materials to control this protected population, but their proper use can vary among vineyards and regions. Moreover, vineyards with mealybug damage typically have large overwintering populations that are never fully regulated, and annually are the source for new generations throughout the summer that infest leaves and fruit of that vineyard and can disperse to other vineyards. Therefore, it is critical to develop better control programs for this overwintering population.

A delayed dormant (typically in February) application of chlorpyrifos (Lorsban) was the standard post-harvest or pre-season control that targeted mealybugs on the trunk and cordon (Daane et al. 2006). The best in-season insecticide for vine mealybugs that move from the trunk and cordon to the leaves, canes, and fruit has been an application of Movento (Bayer Crop Science), with the active ingredient spirotetramat, which may also help control root feeding nematodes (Mike McHenry, pers. comm.). Still, the effectiveness of any systemic material will depend on application timing, soil moisture, vine condition, age, and commodity (for example, post-harvest application timing). Our objectives are to improve controls that target the winter-spring vine mealybug population and to better determine the spring emergence of vine mealybug crawlers to better time foliar applications. Specifically, we are conducting field bioassays to determine the effect of application timing, soil moisture, vine condition, and age and commodity (for example, post-harvest application timing, wine vs. raisin management practices) on systemic insecticide effectiveness. We plan to work with all vineyard-registered insecticide materials, but this past year's work has focused on the field application bioassays and movement of Movento in the vine, timing of Applaud (buprofezin) treatments, and mating disruption.

To follow the movement of Movento, we are collecting vine samples and using high pressure liquid chromatography (HPLC) to determine amounts of different metabolites associated with Movento in different parts of the vine. For example, two of the questions we plan to address is whether spirotetramat converts to the metabolite spirotetramat-enol (which is the primary toxicant) similarly under different vines condition, such as nutrient status or cultivar, and where on the vine the metabolites move to and in what concentration are the metabolites found on different vine sections, such as the leaves versus the roots. We will also use our protocols to help confirm the presence of spirotetramat metabolites in the root system, in support of Andreas Westphal's proposal.

## **OBJECTIVES**

This project seeks to develop better controls for the overwintering vine mealybug population found primarily under the bark of the trunk or on the roots at the soil line.

1. Bioassay
  - a. Investigate population dynamics and controls for overwintering vine mealybug.
  - b. Determine the temperature relationship of vine mealybug and grape mealybug to better predict spring emergence and spray timing.
2. Using HPLC to follow the movement of Movento in the vine.
  - a. Improve the protocols to determine levels of spirotetramat and its first metabolite, the enol form, in vine tissue samples.
  - b. Investigate the dissipation and transformation mechanisms of the active ingredient of the pesticide Movento after application.

## **RESULTS AND DISCUSSION**

### **Objective 1. Bioassay**

#### **Objective 1a. Insecticide Controls for Vine Mealybug**

##### **Movento Applied in Different Regions**

We used bioassays (visual counts of mealybugs) to look at control effectiveness across vineyards in different regions and with different management practices or vine structures. Commercial vineyards were selected in the central San Joaquin Valley (Fresno County), with four vineyard blocks near Fresno (one Thompson Seedless raisin grapes, one Crimson Seedless table grapes, and two Thompson Seedless table grapes); the Lodi-Woodbridge winegrape region (San Joaquin County), with three vineyards near Lodi (one Cabernet Sauvignon, one Pinot Noir, one Chardonnay); and the North Coast winegrape region (Napa County), with two vineyards at a

site in the Carneros region of Napa (one Pinot Noir, one Chardonnay). We are also sampling numerous ‘experimental’ vineyard blocks at the Kearney Agricultural Research and Extension Center that represent wine and table grape blocks undergoing studies for nitrogen, irrigation, and winegrape cultivars. At each site we have counted mealybug densities on the vine, measured cluster damage, and taken vine fresh tissue samples before and after Movento applications (sections from the leaf, cane, and trunk) (**Figure 1**). Together, the treated vineyards include several factors that could be affecting the pesticide efficiency, such as the age of vineyards, irrigation type, commodity (table, raisin, and wine grapes), the presence of a girdle, and geographical area.



**Figure 1.** Sampling different vine sections (leaves and petioles, low and high trunk sections, and roots) using both (A) timed (one minute) visual counts for the bioassay, and (B) taking leaf or bark chip samples for HPLC analyses.

The areas of the vine searched change with the seasonal movement of the mealybug population (i.e. during the winter the roots and lower trunk sections are the most likely regions to find vine mealybug). The pre-treatment mealybug density was then used to block treatments against density, because vineyard mealybug populations can be clumped. In 2016, the visual count of mealybugs took place from April to October. This allows us to monitor mealybug populations at different phenological stages of the crop. We monitored when the grape clusters were not ready to be harvested, when they were ready to be harvested, and after they were harvested.

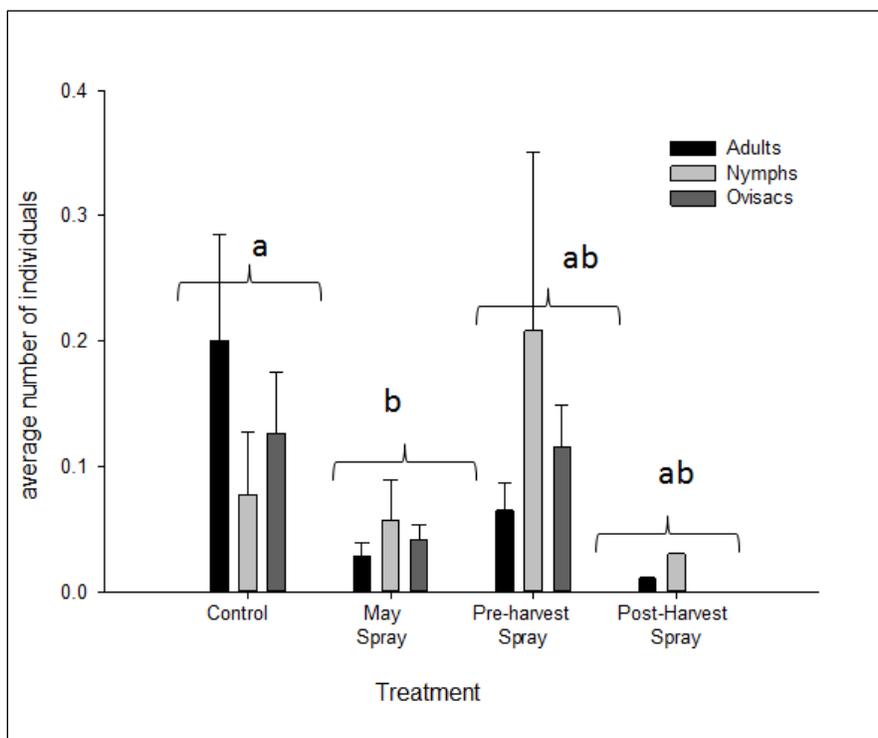
We applied the insecticide Movento as a single insecticide treatment at different application timings, as measured by calendar date as well as by weeks before or after harvest (Movento has a seven-day pre-harvest interval). We applied Movento at the label rate and determined the percentage kill of mealybugs on different sections of the vine during the summer and fall (completed), and will continue this in the coming spring (**Figure 2**).



**Figure 2.** Applying insecticides.

Results from the studied commercial fields found overall mealybug density to be low, making treatment comparisons difficult throughout all the sampling areas and spray treatments. Spray treatments did not affect mealybug density or percentage mealybug life stage at any of the vineyard sites sampled in either Napa Valley or the Lodi Woodbridge region (winegrapes). In most of these sites we found it difficult to make comparisons among bioassay treatments, including the control treatments, because the levels of mealybugs were too low.

To account for this we pooled data across all sites sampled in the Central Valley. Using this analysis, we showed that the mid-May and post-harvest (the previous year) application of Movento lowered mealybug numbers more than the control or pre-harvest applications ( $F = 3.816$ ,  $df = 3,4280$ ,  $P = 0.009$ ; **Figure 3**). These results are similar to previously published results, where April to May is the best time period to apply Movento. Our tests of a pre-harvest application did not show any impact the following year.

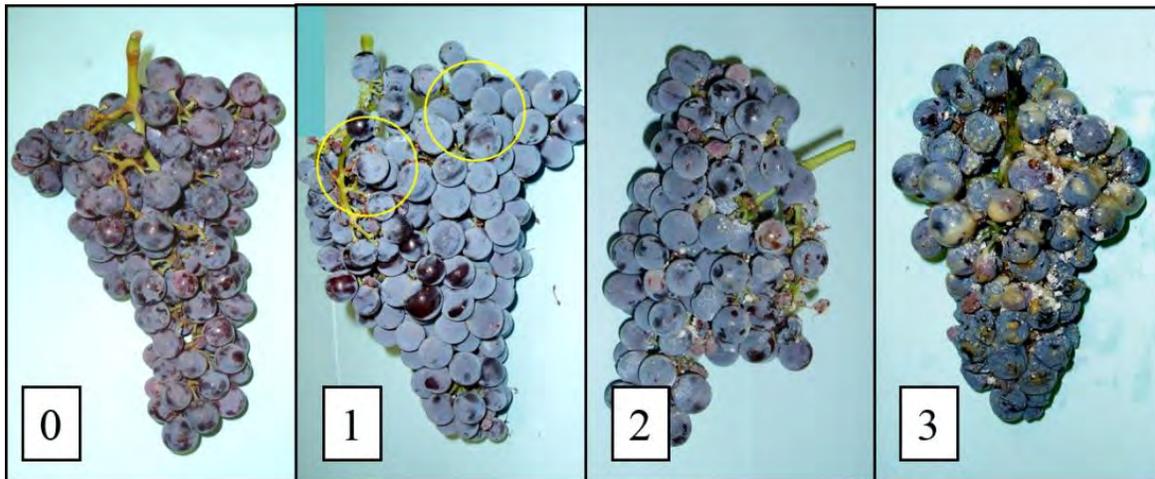


**Figure 3.** Average number of nymphs, adults, and ovisacs on vines treated in mid-to-late May (farmer standard treatment), pre-harvest and post-harvest, and a no-spray control.

We also measured economic damage on five clusters on each vine using a scale of 0 to 3, with 0 = no mealybug damage, 1 = honeydew present but the bunch is salvageable, 2 = honeydew and mealybugs present but at least part of the bunch is salvageable, and 3 = a total loss (**Figure 4**). The economic damage of clusters took place from June through harvest in 2016 (we did not take similar measurement in 2017 because of the low mealybug densities).

Results of cluster damage were similar to those of mealybug density. Data from winegrapes in Napa Valley and Lodi Woodbridge showed no difference among treatments using mid-May, July, or pre-harvest Movento applications. However, mealybug densities were too low to make any strong statements. Note that all of the selected vineyards had mealybug populations that were considered to be economically damaging to the vineyard managers when the study began.

There were higher mealybug densities at some sites in the Fresno area, where we found the May application of Movento had less fruit damage compared to untreated, mid-July (pre-harvest), and post-harvest (the previous season) spray treatments (Chi Square = 65,659,  $P < 0.001$ ).



**Figure 4.** A visual rating of 0 to 3 cluster economic damage for mealybug infestation in the fruit clusters.

In our two-year field bioassay studies, the low number of mealybugs found at the monitored sites and the low cluster damage recorded was a frustration with these trials. We suspect that the mealybug's clumped distribution on the host plant necessitated a great number of samples to get an accurate estimate of population response, but there was also a repeated issue of grower overspray on the control plots that we suspect happened at some sites.

#### **Delayed Dormant Comparison**

In a second trial, we used a 25-year-old raisin field (cv. Thompson Seedless) in the Fresno area to compare different spring applications with the May application of Movento (**Table 1**). Applaud (buprofezin, Nichino) is an insect growth regulator that is typically applied in season against early stage mealybugs. In this trial we tested Applaud as an alternative delayed-dormant spray to Lorsban-4E (chlorpyrifos, Dow Chemical). The insecticides were applied at different rates and timings (**Table 1**). Note that the insecticides have different modes of action, such that we expected combinations to provide additive control (Movento is classified in group 23, Applaud is group 16, and Lorsban is group 1B by the Insecticide Resistance Action Committee, IRAC). A standardized application method was used for each material so that surfactant and application rates would not be an influence. At each site there were 15 replicates (individual vines) per treatment per vineyard, arranged in a complete randomized design.

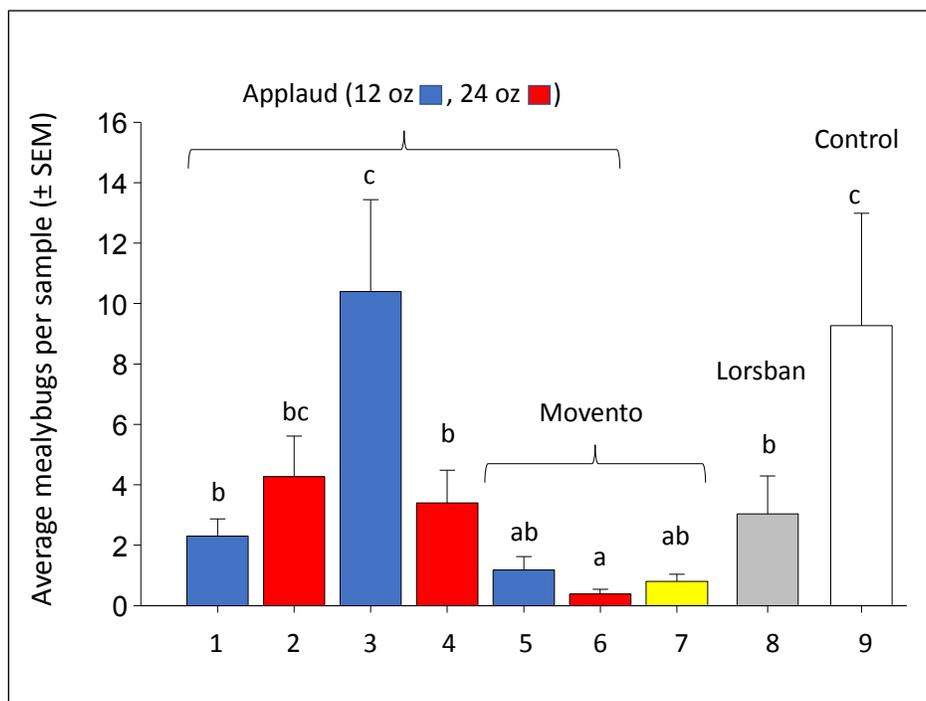
**Table 1.** Schedule of spray treatments investigating novel insecticide combinations for a delayed dormant to spring application to control overwintering mealybugs. In all trials, Movento was applied at the full label rate (for a single application) of eight ounces per acre.

<b>Spray Treatment</b>	<b>Insecticide, Application Rate, and Application Timing</b>
1	Applaud, 12 fl oz, 1 March 2017
2	Applaud, 24 fl oz, 1 March 2017
3	Applaud, 12 fl oz, 22 March 2017
4	Applaud, 24 fl oz, 22 March 2017
5	Applaud, 12 fl oz, 22 March 2017 AND Movento, 4 May 2017
6	Applaud, 24 fl oz, 22 March 2017 AND Movento, 4 May 2017
7	Movento 8 fl oz, 4 May 2017
8	Lorsban 4E, 4 pts, 1 March 2017
9	Untreated control

Results from the delayed dormant spray trial comparing Applaud applied at different times (and with or without a Movento spray in May) with the standard Lorsban delayed dormant treatment showed significant effect on the numbers of individuals found per vine sample ( $F = 6.258$ ;  $df = 8,531$ ;  $P < 0.001$ ; **Figure 5**). There was no

difference between Applaud applied at 12 ounces as a late dormant (22 March) and the control (treatments 3 vs. 8). However, Applaud applied 1 March (treatments 1 and 2) was similar to the Lorsban treatment (8). As described above, Applaud applied just three weeks later (22 March) was similar to the control at the 12 ounces per acre rate, but lower at the off-label 24 ounce rate.

The three Movento treatments had the lowest counts, and the Movento treatments that included Applaud at the 24 ounce rate as a delayed dormant had the lowest counts (**Figure 5**).



**Figure 5.** Average number of mealybugs on vines treated with different pesticides (**Table 1**) at different rates and timings (samples were taken during a timed count).

### Objective 2. Using HPLC to Follow the Movement of Systemic Insecticides in the Vine

Data were presented in the previous report and the latest data have not yet been analyzed.

### Objective 3. Temperature Development of Vine Mealybug

#### Insect and Vine Cultures

All experiments were conducted with vine mealybugs obtained from insectary cultures, originally established with mealybugs collected in vineyards located near Sanger, CA (Fresno County) and maintained at the University of California Kearney Agricultural Research and Extension Center near Parlier, CA (Fresno County). Mealybugs were reared on butternut squash (*Cucurbita moschata*) which was cleaned in a 0.5% bleach solution to reduce mold growth and then triple rinsed. Each squash was inoculated with 5 to 10 gravid female mealybugs, which resulted in an initial infestation level of 600 to 1,000 mealybugs. Cultures were held at  $22 \pm 2^\circ\text{C}$ , with 12:12 light:dark photoperiod.

The grape plants (*Vitis vinifera*) were two year old Thompson Seedless, originally obtained from cuttings from vines at the UC Kearney Research and Extension Center. Cuttings were rooted in 3.8 liter pots filled with a sandy loam soil and watered and fertilized throughout the experiment as needed.

#### Temperature-Dependent Development

The effect of constant rearing temperatures on vine mealybug development time was determined at 12, 16.5, 19, 23, 26, 30 and  $34^\circ\text{C}$ . Temperature cabinets maintained temperatures at  $\pm 1.5^\circ\text{C}$ , as recorded by HOBO data recorders (Onset, Bourne, MA) placed in each cabinet. There was a light:dark regime of 16:8, with grow lights

used to maintain vine health. Humidity was not controlled, and ranged between 60 to 90%. To begin each trial, seven to ten adult vine mealybug females which were beginning to produce ovisacs were placed on each vine, which was then held at 25°C for a 24 hour inoculation period, after which the vines were checked for freshly deposited eggs still in the ovisac, and the adults and excess eggs were removed. In this manner each plant was inoculated with 30 to 120 eggs. Barriers of petroleum jelly were added at the base of the vine to restrict mealybug movement off the vine.

Inoculated plants were then randomly assigned to temperature treatments. Thereafter, plants were checked every one to two days for mealybug development and survival. After two weeks this period was extended to three to six days, depending on the development rate at each temperature. Mealybug density was recorded by the following developmental stages: Egg, first instar, second instar, third instar female (pre-oviposition), third instar male (prepupa), adult female (producing an ovisac), male pupa, ovisac with eggs, and adult male (male pupa with an emergence hole).

Towards the end of each generation adult females were individually numbered for future identification (after the ovisac deposition begins, there is very little movement of adult females) and to record eggs per individual female. For each ovisac deposited eggs were collected on each observation date and placed in a gelatin capsule, which was then returned to the respective temperature treatment for 30 days or until egg hatch was complete. After this period egg production and the proportion of hatched eggs were recorded for each female.

### Statistical Analyses

Results are presented as means per temperature treatment ( $\pm$  standard error of the mean; SEM). Development times were estimated as the number of days spent in each life stage, based on peak densities for each life stage. As will be discussed later, individual development times were not collected because there was too much movement on the vine and the individual mealybugs could not be marked. Mortality rates are the number of individuals entering each development stage divided by the number of individuals dying in each stage. Adult male and female stages were excluded from calculations of the mortality rates as these stages concluded the lifecycle. Fecundity rates are the number of eggs produced per female, captured and isolated at the end of the development period for each tested temperature. Egg viability rates are the number of hatched eggs divided by the total (hatched and unhatched) number of eggs per female. For all analyses, mealybugs lost due to escape or injury were omitted.

### Results for Temperature-Dependent Development

Vine mealybugs completed development from egg to adult (with ovisac) at temperatures from 16.5–30.0°C, but failed to complete development at the lowest (12°C) or highest (34°C) temperatures tested (**Figure 6**). The estimated development times from egg to adult, based on the production of adults with ovisacs, were fit to the nonlinear model. There are a number of nonlinear models commonly used to describe temperature development (reviewed in Roy et al. 2002). We selected the Brière et al. (1999) temperature development rate model, which provided lower, optimal and upper temperature thresholds and is described as:

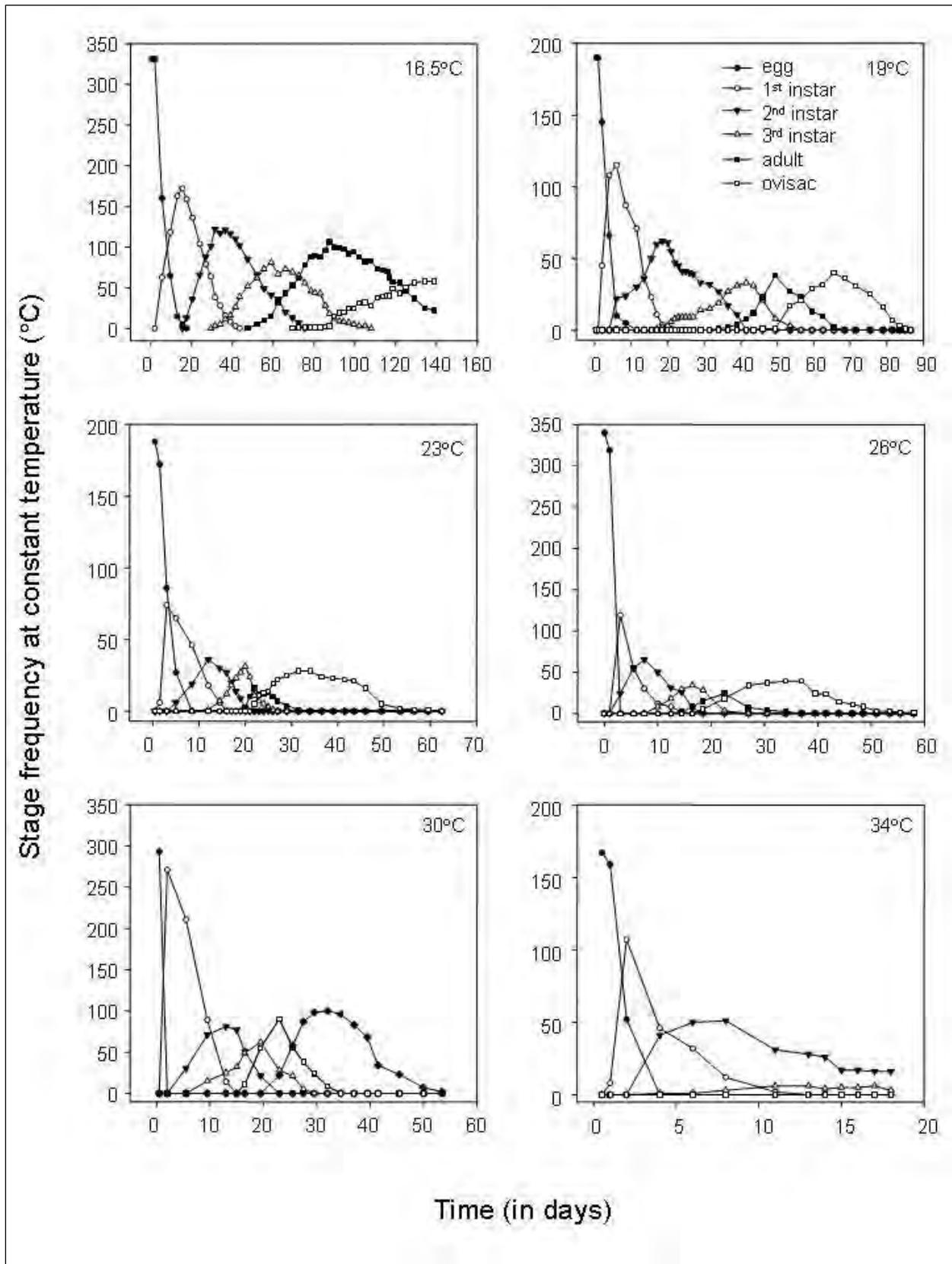
$$r(T) = aT(T - T_O)(T_L - T)^{1/b}$$

where  $T$  is the rearing temperature (°C),  $T_O$  is the lower temperature threshold,  $T_L$  is the lethal (upper) temperature threshold, and  $a$  and  $b$  are empirical constants. The optimum temperature ( $T_{opt}$ ) is calculated as:

$$T_{opt} = \frac{2bT_L + (b+1)T_O + \sqrt{4b^2T_L^2 + (b+1)^2T_O^2 - 4b^2T_OT_L}}{4b+2}$$

where  $T_L$ ,  $T_O$ ,  $a$ , and  $b$ , are obtained from equation 1.

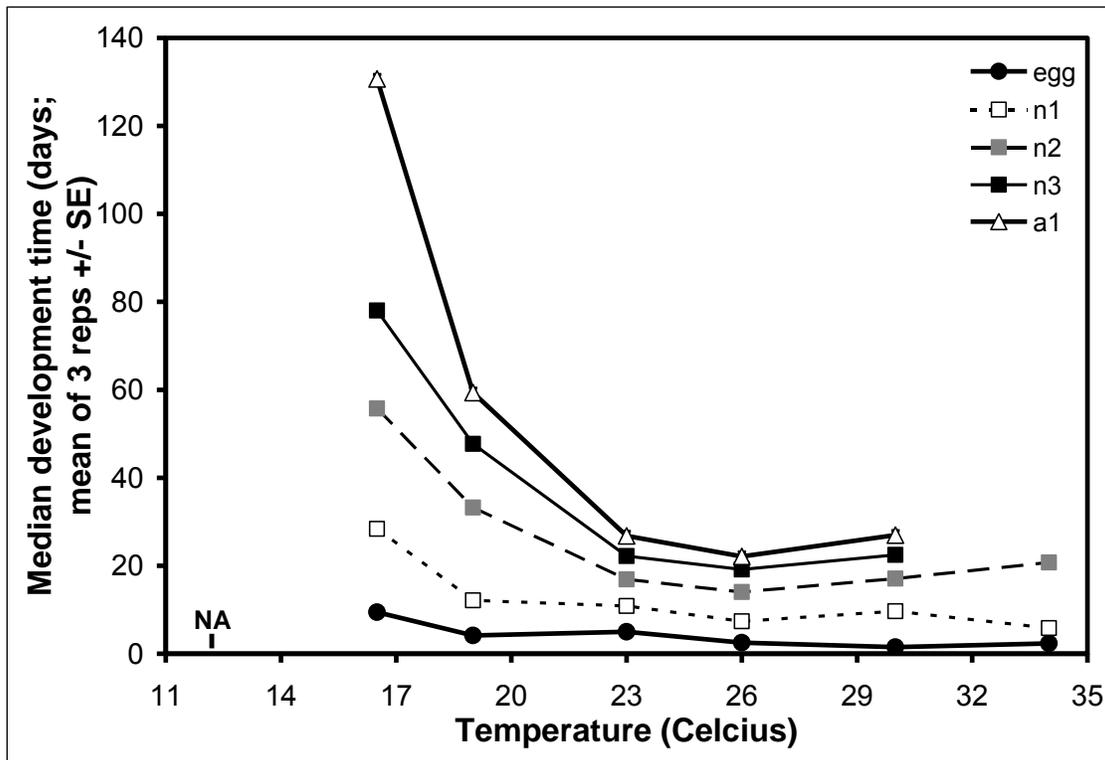
The low threshold temperature was also determined using simple linear regression ( $r(T) = \alpha T + \beta$ ) with data from temperature treatments 16.5 to 23°C, which most closely resembles a straight line. The development rate is a linear function of temperature, and  $\alpha$  and  $\beta$  are regression parameters fitted to the data. The low development threshold is calculated as  $T_L = -\alpha/\beta$ , and the thermal constant ( $k$ ) from birth to adult, in required degree-days, is calculated as  $k = 1/\beta$  (Liu and Meng 1999).



**Figure 6.** Development for each life stage of vine mealybug at six constant temperatures.

Results show development times decreased as temperatures increased (**Figure 7**), ranging from about 140 days at 16.5°C to about 25 days at 30°C (**Figure 7**). The estimated lower and upper temperature thresholds were 14.55°C and 35.41°C, respectively, while the optimum developmental temperature was 26.93°C. Using linear regression

with mid-range temperatures (19 to 30°C) a lower temperature threshold of 14.6°C was estimated ( $y = 0.00362x - 0.053$ ;  $F_{1,2} = 156.84$ ;  $P < 0.0507$ ;  $R^2 = 0.987$ ). The thermal constant is 276.31 degree-days.



**Figure 7.** Vine mealybug stage development times at different temperatures (development time defined as the number of days required for 50% of the population to move beyond a given stage). N1, N2, N3, and A1 refer to first, second, and third instar nymphs and pre-reproductive adults, respectively. Most error bars are obscured by symbols. Estimates were not possible for some stages at some temperatures (12, 30, and 34°C).

### Reproductive Parameters

The net reproduction rate ( $R_o$ ) was greater than zero at all temperatures that permitted complete development, indicating positive population growth. The maximum  $R_o$  (433.34) was obtained from data collected at 26°C. The lowest estimated  $R_o$  (82.61) occurred at 16.5°C. The female:male ratio of offspring, which impacts  $R_o$ , also varied among temperatures, ranging from 10.25:1 at 19°C to 5.10:1 at 16.5°C.

Mean generation times ( $T$ ) values estimated for each of the trialed temperatures decreased with increasing temperatures, with a gradual decrease in mean generation time as temperatures increased between 16 and 30°C. The shortest generation time ( $T$ ) was also recorded at this temperature. This decrease was more pronounced between 16, 19, and 23°C, and reached a plateau between 23 and 30°C. The largest  $T$ -value was recorded at 16.5°C. These values decreased to 32.19 at 26°C, after which there was a slight increase.

Intrinsic rate of natural increase ( $r_m$ ) values were positive at temperatures ranging from 16.5 to 30°C, indicating positive population growth. The lowest estimated  $r_m$  value was 0.037 at 16.5°C; the highest was 0.26 at 26°C. At 30°C the  $r_m$  values dropped to 0.195. The fitted model was  $y = (0.000000161) \times x(x-34.04632) \times ((15.8684)-x) \times \exp(1/0.151912)$  ( $F_{1,4} = 41.76$ ;  $P = 0.11$ ;  $R^2 = 0.9864$ ; **Figure 6**). Using these  $r_m$  values, the lower, upper, and optimal temperatures for population increase are estimated at 15.87, 34.05, and 26.47°C, respectively.

### Fecundity and Egg Viability

Across all temperatures at which ovisacs were produced (16.5 to 30°C) average life time egg production was  $220.8 \pm 15.5$  eggs per female. Temperature influenced egg production, which ranged from a maximum of  $364.4 \pm 0.8$  eggs per female at 26°C to a minimum of  $155.25 \pm 0.1$  eggs per female at 16.5°C. There was a decrease in egg production at lower and higher temperatures, indicated by a good fit ( $R^2 = 0.94$ ) to the Briere et al. (1999) model

modified for fecundity (**Figure 7**). The lower, upper, and optimal temperatures for egg laying were determined at 11.59, 34.08, and 25.22°C, respectively. Egg viability was highest at 16.5°C, similar between 19 to 26°C, and significantly lower at 30°C;  $F_{4, 2185} = 383.49$ ,  $P < 0.0001$ ).

We have worked with two entomologists who are very qualified to model data (Mark Sisterson and Mathew Daugherty). One aspect of this study that failed was our inability to track the development time of individual mealybugs. With our design, we expected more uniform development times for each life stage at each of the tested temperatures. We suspect that feeding on different parts of the vine may have added to mixed development times. The end result is that we used the “average” development based on peak population densities. This produced an informative figure showing life stage development and mortality. However, without being able to produce standard errors around each mean, we cannot complete a statistically accurate development model. For this reason we have begun a simpler temperature development trial, counting only development from egg to ovisac.

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## SEARCHING FOR POTENTIAL VECTORS OF GRAPEVINE RED BLOTCH-ASSOCIATED VIRUS

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**Reporting Period:** The results reported here are from work conducted July 2015 to October 2017.

**ABSTRACT**

Grapevine red blotch-associated virus (GRBaV) is a newly identified vineyard pathogen causing vine damage similar to other grape leafroll diseases (GLD). There has been some initial laboratory evidence that leafhoppers are a potential vector of GRBaV; however, there have been mixed reports of possible vector-borne movement in vineyards. Our goal is to identify and test potential vectors to provide concrete evidence that organisms can or cannot move GRBaV among vines. This work must be completed to develop a control program for “red blotch” and develop accurate information on the epidemiology of this newly reported pathogen. To date, we have tested leafhoppers (*E. elegantula*, *E. variabilis*, *E. ziczac*), grape whitefly (*Trialeurodes vittatas*), mealybugs (*Planococcus ficus* and *Pseudococcus maritimus*), blue-green sharpshooter (*Graphocephala atropunctata*), and foliar form grape phylloxera (*Daktulosphaira vitifoliae*). So far none of these insects have moved the pathogen from an infected plant or plant material to a clean plant in laboratory studies. More recently there has been evidence that a membracid may transmit GRBaV (Bahder et al. 2016) and transmission experiments evaluating the three-cornered alfalfa hopper (*Spissistilus festinus*) are still in progress. Our field studies have surveyed insects and potential non-crop reservoirs in vineyards with suspected movement of red blotch. None of the herbivores in this survey have tested positive for the virus responsible for red blotch, although many samples are still being tested in the laboratory. We have also conducted detailed mapping of red blotch in vineyards where movement of the virus is suspected in order to evaluate spatial trends related to virus spread. Similarly, we are also mapping GRBaV titer levels within the vine itself to help with the identification of novel vectors which may preferentially feed on regions of the vine where the virus is localized.

**LAYPERSON SUMMARY**

Grapevine red blotch-associated virus (GRBaV) is a newly identified vineyard pathogen causing vine damage similar to other grape leafroll diseases (GLD). There has been some initial laboratory evidence that leafhoppers are a potential vector of GRBaV; however, there have been mixed reports of possible vector-borne movement in vineyards and recent work at the University of California, Davis identified an insect called a ‘treehopper’ as a likely vector. Our goal is to identify and test potential vectors to provide concrete evidence that organisms can or cannot move GRBaV among vines. This work must be completed to develop a control program for “red blotch” and develop accurate information on the epidemiology of this newly reported pathogen. To date, we have tested many leafhoppers (which are common in vineyards), grape whitefly, mealybugs (which are also commonly found in vineyards), blue-green sharpshooter, and foliar form grape phylloxera. None of these insects have moved the pathogen from an infected plant or plant material to a clean plant in laboratory studies. We have begun transmission experiments evaluating a treehopper (three-cornered alfalfa hopper) to determine its efficiency. Our

field studies have surveyed insects and potential non-crop reservoirs in vineyards with suspected movement of red blotch. None of the herbivores in this survey have tested positive for the virus responsible for red blotch, although many samples are still being tested in the laboratory. We have also conducted detailed mapping of red blotch in vineyards where movement of the virus is suspected in order to evaluate spatial trends related to virus spread. Similarly, we are also mapping GRBaV titer levels within the vine itself to help with the identification of novel vectors which may preferentially feed on regions of the vine where the virus is localized.

## INTRODUCTION

In 2006 an increase in grapevine leafroll disease (GLD) and vines with “red leaf” symptoms was observed by growers in vineyards located within Napa Valley, CA. Symptoms were also observed at the Oakville Experimental Vineyard (OEV) by Jim Wolpert (UC Davis Viticulture Extension Specialist), Ed Weber (former UC Cooperative Extension Viticulture Farm Advisor), and Mike Anderson (UC Davis Staff Research Associate). Tissue samples were collected from symptomatic vines and tested by commercial laboratories and UC Davis Foundation Plant Services. Test results were most often negative for known grapevine leafroll-associated viruses (GLRaVs).

The increasing awareness of blocks containing vines with grapevine leafroll disease symptoms, primarily in Napa and Sonoma counties, but testing negative for grapevine leafroll-associated viruses resulted in a renewed focus on virus species and strains causing GLD. New GLRaV-3 strains have been discovered (e.g., Sharma et al. 2011); however, this did not fully explain all of the observed symptomatic vines. In 2010, next generation sequencing analyses identified a new pathogen (Al Rwahnih et al. 2013). Soon after a circular DNA virus, similar to members of the family Geminiviridae, was isolated (Krenz et al. 2012) and, concurrently, polymerase chain reaction (PCR) primers were developed (Al Rwahnih et al. 2013) for this pathogen now known as grapevine red blotch-associated virus (GRBaV). GRBaV has since been isolated from vines throughout North America and in Switzerland (Krenz et al. 2014), highlighting either a rapid dissemination or, more likely, its long hidden presence (e.g., misidentified as GLD).

This project focuses on possible vectors of GRBaV. Multiple viruses in the Geminiviridae are insect transmissible (Ghanim et al. 2007, Chen and Gilbertson 2009, Cilia et al. 2012), and there has been some initial evidence that leafhoppers may transmit GRBaV (Poojari et al. 2013) and better evidence that a membracid may transmit the pathogen (Bahder et al. 2016). However, there has been mixed evidence of GRBaV field spread in association with leafhoppers. Concern for the spread of GRBaV led to an off-cycle project in summer 2013, funded by the Napa County Winegrape Pest and Disease Control District to initiate appropriate scientific studies of possible insect vectors of GRBaV. The work was continued in 2014 with American Vineyard Foundation (AVF) and Napa County funds.

Our goal is to test potential vectors to provide concrete evidence that organisms can or cannot move GRBaV among vines. Determining field epidemiology of GRBaV is critical in the development of a control program – whether the pathogen is moved via infected nursery material, mechanically or, as with the focus of this study, by a vector. There are ample California vineyard sites where the pathogen is present but does not appear to have moved from infected vines over a period of many years, but in some vineyards, vine to vine movement has been recorded. This difference – whether there is no vector movement and disease presence is exclusively from infected nursery material or there is a vector – completely changes the needed control programs.

Our proposed work will screen all common vineyard arthropods, as well as the “long shots” that are potential GRBaV vectors, thereby providing the proper target for control. **Table 1** provides a partial list of the common vineyard insect species that should be screened as potential vectors of GRBaV, based on their incidence and distribution in California vineyards. Once tested, organisms are either identified as vectors or our work shows that they are either not vectors or that they are so inefficient that spray programs are not needed. This information will be disseminated to farmers, pest control advisors (PCAs), and extension personnel, thereby having a practical, direct and immediate impact on control decisions to “spray or not to spray.”

**Table 1.** Arthropods targeted for GRBaV tests.

Common name	Scientific Name	Common Distribution
western grape leafhopper	<i>Erythroneura elegantula</i>	North Coast (north of Tehachapi Mtns.)
variegated leafhopper	<i>Erythroneura variabilis</i>	Central Valley (San Joaquin Co. to So. Cal.)
Virginia creeper leafhopper	<i>Erythroneura ziczac</i>	Northern CA
potato leafhopper	<i>Empoasca</i> sp.	Sporadic vineyard populations
vine mealybug	<i>Planococcus ficus</i>	California vineyards
grape mealybug	<i>Pseudococcus maritimus</i>	North Coast and San Joaquin Valley
obscure mealybug	<i>Pseudococcus viburni</i>	Central and North Coast
blue-green sharpshooter	<i>Graphocephala atropunctata</i>	Northern CA
European fruit lecanium scale	<i>Parthenolecanium corni</i>	North Coast
grape phylloxera	<i>Daktulosphaira vitifoliae</i>	North Coast, Sacramento Delta, Foothills
grape whitefly	<i>Trialetrodes vittatas</i>	California
mites	<i>Tetranychus</i> spp.	California

## OBJECTIVES

To screen potential vectors for their ability to acquire and transmit GRBaV and, if a vector is discovered, to determine vector efficiency. Objectives for this research program are as follows:

1. Screen common vineyard insects and mites as potential vectors for GRBaV.
2. Screen uncommon organisms that feed on vines as potential vectors for GRBaV.
3. Follow disease progression in established vineyard plots to collect preliminary data on field epidemiology.

## RESULTS AND DISCUSSION

### Objective 1. Screen Common Vineyard Insects and Mites as Potential Vectors of GRBaV

#### 2013-2014: Initial Transmission Trials with Potted Vines

In 2013 and 2014, we prioritized the screening of leafhoppers (western grape leafhopper and Virginia creeper leafhopper), grape whitefly, mealybugs (vine mealybug and grape mealybug), and blue-green sharpshooter because of the published work by Poojari et al. (2013), their prevalence in California vineyards, and/or their phloem feeding (this category of viruses [Geminiviridae] are phloem-limited, although the biology and ecology of GRBaV is not fully understood).

In both years, canes were collected from Cabernet Sauvignon (clone 6) and Cabernet Franc (clone 04) vines in vineyard blocks where vines are known to have tested positive for GRBaV, and negative for all known GLRaVs and other known grapevine viruses. PCR test results for these vines were made and canes negative for all viruses except GRBaV and rupestris stem pitting (RSP; UC Berkeley and UC Davis Foundation Plant Services [FPS] test results) were transferred to UC Berkeley's Oxford Tract Greenhouse and established in pots on a mist bench. Vines were maintained in the greenhouse, strictly treated to be insect and mite-free, and isolated from other vines that may have harbored viral pathogens. As indicators for these studies, we used Cabernet Sauvignon vines propagated from material provided by FPS and maintained under similar conditions.

Initial tests were conducted using the most mobile stages of key species, including adults of the leafhopper species and the grape whitefly, and crawlers of the vine mealybug and grape phylloxera. We employed standard transmission protocols to evaluate the potential of these insects to transmit GRBaV, as has recently been done for GLRaVs (Tsai et al. 2008, Tsai et al. 2011) and Pierce's disease (Almeida and Purcell 2003a, b). We used a standard Acquisition Access Period (AAP) and Inoculation Access Period (IAP) of 120 hours (five days) each for all tested insect species except the more delicate grape whitefly, which could feed on plants for an AAP and IAP of 48 hours (two days) each. In the "controlled trials," known infected source plants or uninfected control plants in pots (one-liter size) were inoculated with 30-50 insects for the AAP, and surviving insects were then transferred to uninfected plants for the IAP. Field-collected leafhopper adults and blue-green sharpshooter adults were taken from an insectary colony and released on plants that were placed singly in 61 x 61 x 61 cm BugDorm cages. Grape whitefly adults reared from pupae were collected in Napa County vineyards and then released into nylon bags enclosing five leaves on potted grape plants. Mealybug crawlers were moved onto individual grape

leaves (three leaves per plant) using a brush, and grape leaves were then enclosed with white paper bags. Following the IAP, all vines were treated with a contact insecticide to kill any remaining insect species. All insects were collected and tested for GRBaV within 48 hours after the AAP period. Every four months thereafter, three petioles were collected from each host plant and assayed for GRBaV infection. A total of 20 test vines were inoculated for each of the above insect species in the 2014 trials.

Results from the 2013-2014 trials have not indicated that any of these insects (i.e. leafhoppers [western grape leafhopper and Virginia creeper leafhopper], grape whitefly, mealybugs [vine mealybug and grape mealybug], and blue-green sharpshooter) are capable of transmitting GRBaV to uninfected grapevines. Inoculated vines from these trials are being held for a two-year period, during which petioles are tested for GRBaV every four months and vines are visually evaluated for symptoms every fall. All insects that fed on infected plant material in these trials have tested negative as well. That said, we have recently begun to redesign our insect testing procedures to improve the sensitivity and accuracy of these laboratory tests. Insects from the 2013-2014 trials are being re-tested using new protocols that have been developed and verified.

### **2015: Improved “Bouquet” Transmission Trials**

In 2015 and 2016 protocols for these transmission experiments were modified due to concerns about (a) potentially low virus titer levels in the potted vines grown from cuttings of GRBaV-positive vines at vineyard field sites and (b) small number of insects per trial. Our concern is that candidate vector ability to transmit GRBaV is confounded by low titer levels in the GRBaV-positive vines used in previous trials and/or inadequate insect sample size.

The new approach involves using “bouquets” of mature grape leaves collected from GRBaV-positive vines at vineyard field sites that were not sprayed with insecticides. Each bouquet consists of ten mature grape leaves held in a 16 oz. plastic container that contains moist perlite. Ten leaves were collected from each of ten GRBaV-positive vines (nodes 1-5) in an established vineyard in Napa County (100 leaves total). Each bouquet consisted of one leaf from each of the ten vines, totaling ten leaves per bouquet and ten total bouquets (i.e. one bouquet per replicate). Bouquet degradation was initially evaluated by testing petioles for GRBaV 6-48 hours after collection. Results indicated no degradation of the petioles. Finally, each trial now contains at least 100 insects/replicate (when possible) and 10 replicates per treatment.

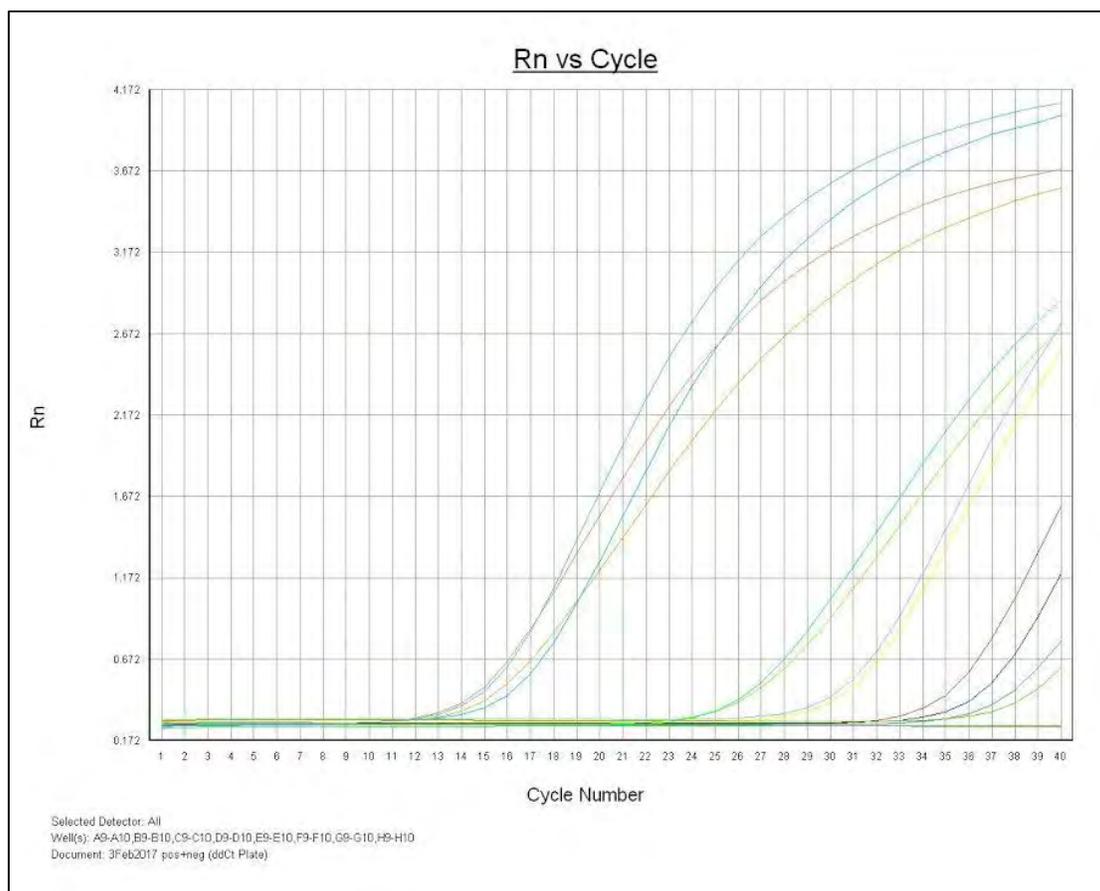
Since July 2015 we have completed trials using the bouquets with Virginia creeper leafhopper adults, vine mealybug crawlers, and foliar form grape phylloxera crawlers. Due to concerns about bouquet degradation, these experiments used an AAP of 48 hours (two days) and an IAP of 72 hours (three days). Clip-cages (7 cm diameter x 2 cm height) were used to confine 10 insects/leaf to each bouquet (100 insects/bouquet). Bouquets with insects were placed in a 61 x 61 x 61 cm BugDorm cage and there was a total of 10 replicates per treatment. After the 48 hour AAP, clean potted vines were introduced into the cages. The clip cages were then removed, thus allowing the insects to move onto the clean vine. Bouquets were also removed at this time, after ensuring that they were free of the candidate vectors. Petioles from the bouquets were then collected for GRBaV testing as well as a subsample of the candidate vectors (10-50 insects per replicate). After the 72 hour IAP, another subsample of the candidate vectors was collected for testing (10-50 insects per replicate) and the potted vines were then treated with a contact insecticide to kill any remaining insects. Three petioles were sampled from each vine (nodes 1-5) for immediate testing. Vines are now being maintained for a two-year period and petioles tested for GRBaV every four months.

Bouquet experiments with grape phylloxera were initially unsuccessful due to their rejection of the bouquet material. Following the 48 hour AAP it was observed that none of the phylloxera crawlers had settled on the leaves and instead were mostly desiccated inside the cages. As such, we reverted to the previous experimental approach utilizing potted vines that were confirmed to be GRBaV positive. This time, two-year-old GRBaV-positive vines were used in these trials to possibly provide vines having elevated virus titer levels. Negative control source vines were one year old. Vines were placed in 61 x 61 x 61 cm BugDorm cages and inoculated by pinning ten leaf discs containing a large number of galls (>15) on each vine. The galls on these discs had been cut open with a razor to encourage movement of the crawlers onto the vine. After 25 days all of the potted vines exhibited >50 galls (i.e. 25 day AAP). At this point clean vines were introduced into the cages and sub-samples of grape phylloxera adults, eggs, and crawlers were collected for testing. Acquisition and inoculation vines remained

together in the cages until the inoculation vines had >50 galls/vine, which resulted in a 38 day IAP. At this point vines were treated with both a contact and systemic insecticide. As before, vines will be held for a two-year period and tested every four months. So far, our 2015 and 2016 “bouquet” trials have shown no transmission of GRBaV by either the Virginia creeper leafhopper or vine mealybug. Similarly, the trial with foliar form grape phylloxera on two-year-old GRBaV-positive vines did not show any transmission.

### Testing Plant Material for GRBaV

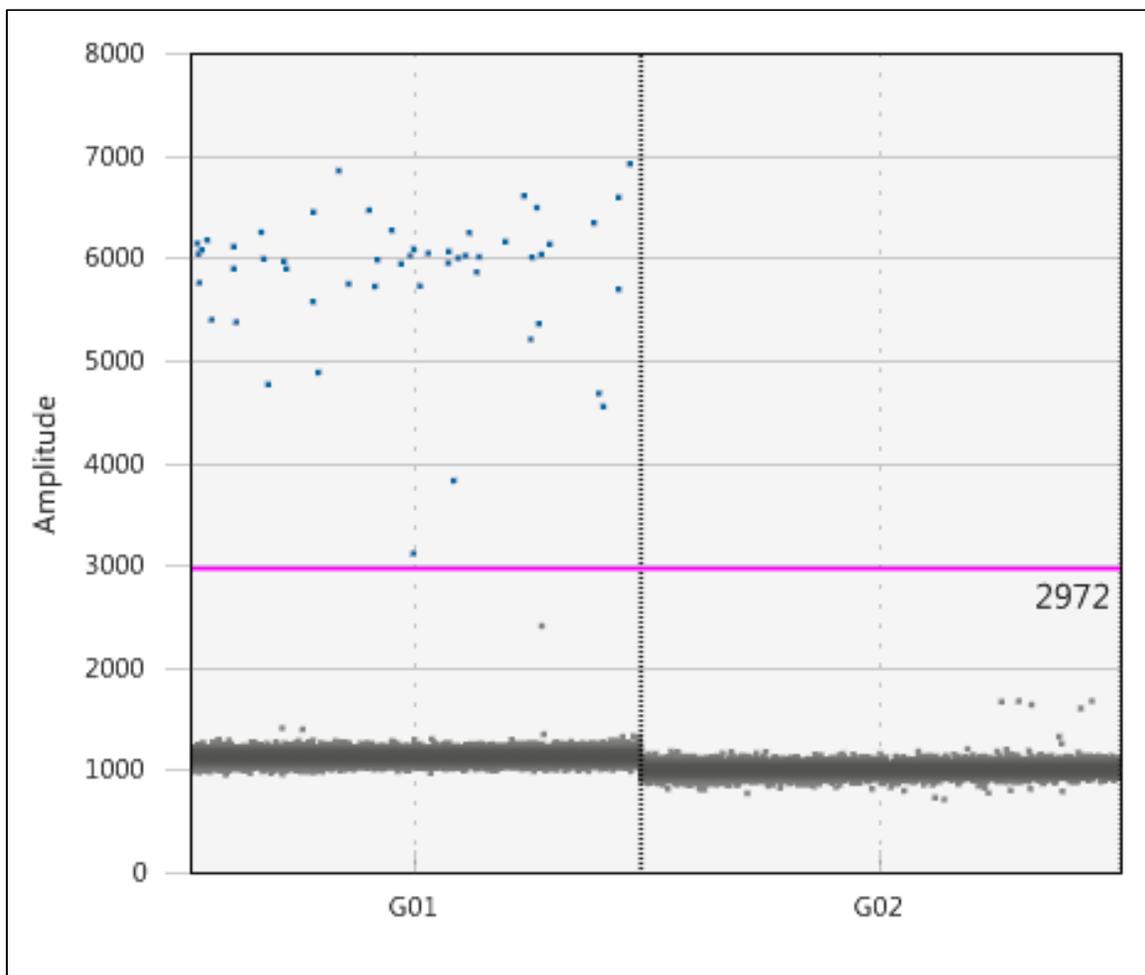
To test for the presence of GRBaV in grapevine petioles potentially infected with red blotch disease (Sharma et al. 2011), whole genomic DNA was extracted from three randomly selected petioles (nodes 1-5) from each target grapevine using the ISOLATE II Plant DNA Extraction Kit (Bioline Corp.). Briefly, 0.1 g of each petiole tissue was homogenized in Mo-Bio 2.0 ml tough tube containing a Boca chrome steel ball-bearing using a Precellys 24 Tissue Homogenizer set for two 10-second cycles at 6,500 Hz for with a 30-second intermission between cycles. DNA was then extracted following the manufacturers protocols. The presence of GRBaV clade 1 and/or clade 2 viruses were then determined using quantitative polymerase chain reaction (qPCR). Duplicate qPCR reactions were run for each petiole with both either primers specific to clade 1 or clade 2 (in total four qPCR reactions were run for each sample). Reactions were conducted on an Applied Biosystems 7500 Fast Real-Time PCR System with SDS Software used for analysis with the following reaction conditions: 12.5  $\mu$ l Promega GoTaq master mix, 2.5  $\mu$ l of 10  $\mu$ M primers (either GVGF1 and GVGR1 to test for the presence of clade 1 or GVGF2 and GVGR2 to test for the presence of clade 2), 0.25  $\mu$ l CXR reference dye, 8  $\mu$ l water, and 2  $\mu$ l of each target sample (Al Rwahnih et al. 2013). Thermocycling conditions included one cycle of 95°C for 2 minutes; forty cycles of 95°C for 15 seconds, 58°C for 1 minute; and one cycle of 72°C for 10 minutes, followed by a final dissociation cycle. Results were then analyzed by the 7500 Fast System SDS Software, accounting for the Ct values, melting temperatures, and component curves, with infected samples scored as those with positive amplification curves prior to 30 cycles (See **Figure 1** for an example). All reactions were run with positive and negative controls.



**Figure 1.** Example results from GRBaV plant petiole testing. Samples with amplification curves present prior to 30 cycles (x-axis) are scored as infected (first eight curves) and those with amplification after 30 cycles are scored as uninfected (final four curves).

### Testing Insects for GRBaV

After field collection, insects were frozen at  $-80^{\circ}\text{C}$  prior to testing for the presence of GRBaV. Whole genomic DNA was extracted from individual insects using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Corp.) based on the manufacturer's protocol. Prior to extraction, insects were homogenized using the same method as above. Recently, Bahder et al. (2016) found that digital PCR (dPCR) may be an effective tool for identifying the presence of GRBaV virus in insect vectors. The development of digital droplet PCR (ddPCR), however, now allows us to build upon the increased sensitivity of the dPCR system, with the added benefit of being able to directly quantify gene copy numbers (i.e. virus infection loads) for each insect. Therefore, we developed two directly labeled primer-probe sets that can be used to simultaneously determine whether collected insects are infected with both GRBaV clades 1 and/or clades to GRBaV and to quantify the infection. Digital droplet PCR reactions were conducted on a Bio-RAD QX200 ddPCR system with  $12.5\ \mu\text{l}$  of BioRad ddPCR 2x MasterMix (BioRad, Inc.),  $1.25\ \mu\text{l}$  of each primer-probe pair, and  $10\ \mu\text{l}$  of extracted DNA, with the following thermocycler conditions: one cycle of  $95^{\circ}\text{C}$  for 1 minute; forty cycles of  $94^{\circ}\text{C}$  for 30 seconds,  $56^{\circ}\text{C}$  for 1 minute; followed by a final hold at  $12^{\circ}\text{C}$  prior to quantification with the Bio-RAD QuantaSoft™ software. All reactions were run in duplicate, with an example of the results for infected and uninfected insects presented in **Figure 2**.



**Figure 2.** Results for ddPCR analysis of infected (left) and uninfected (right) insects. Each blue dot represents a copy of GRBaV, and the total infection level for each insect is calculated as the ratio of droplets containing amplified GRBaV and the number of droplets without amplified GRBaV. The infected sample has a GRBaV concentration of 88 copies per  $20\ \mu\text{l}$  of sample, and the uninfected sample has a GRBaV concentration of 0 copies per  $20\ \mu\text{l}$  of sample.

## **Conclusion: No Transmission Observed to Date**

We have evaluated a total of seven vector candidates, which includes grape leafhopper, Virginia creeper leafhopper, grape whitefly, grape mealybug, vine mealybug, blue-green sharpshooter, and foliar form grape phylloxera. In 2015 and 2016 we modified experimental protocols that were designed to overcome perceived limitations in previous transmission experiments from 2013-2014. This led to the re-evaluation of two candidates, Virginia creeper leafhopper and vine mealybug, as well as evaluation of a new candidate, foliar form grape phylloxera. To date, none of the candidate vectors have tested positive for GRBaV and no transmission has been observed, although testing of insect and plant material from these experiments is ongoing. Transmission vines from these experiments were most recently tested in October 2016.

## **Objective 2. Screen Uncommon Organisms that Feed on Vines as Potential Vectors for GRBaV**

### **Vineyard Insect Survey**

We used the same methodologies described for objective 1 to screen lesser known vineyard organisms or unlikely vectors. Insects were collected once per month from five established vineyards where movement of GRBaV has been observed or reported (assumed to have happened). Samples were collected from grapevines, groundcovers, and non-crop vegetation in the surrounding landscape using a combination of sweep-nets (on groundcovers, five samples per site, 30 sweeps per sample) and a D-Vac type suction sampling machine (on grapevines and non-crop vegetation), which consisted of a 25 cc gas blower/vacuum (Craftsman) fitted with a five-gallon (18.9 liter) bucket on the vacuum tube to create a 1 ft<sup>2</sup> (0.093 m<sup>2</sup>) sampling cone. Each D-Vac sample consisted of five thrusts with the D-Vac running at full speed (5 samples of grapevine per site, 5-10 samples of non-crop vegetation). All samples were held in a cooler and brought to the laboratory for immediate processing. Specimens were incapacitated using CO<sub>2</sub> gas, sorted and identified to species or genus, and then stored in 95% EtOH and stored at -80° C until testing. So far we have collected leafhoppers in the genera *Aceratagallia* sp., *Acinopterus* sp., *Alconeura* sp., *Colladonus* sp., *Empoasca* spp., *Macrosteles* sp., *Osbornellus* sp., and *Scaphytopius* spp., as well as the species *Deltocephalus fuscineruosus*, *Dikrella californica*, and *Euscelidius schenki*. Other organisms include members of the families Acanaloniidae, Cixidae, Membracidae, Miridae, Lygaeidae, Psyllidae, and Tingidae.

Many novel insects have been collected from vineyard sites where movement of GRBaV is suspected, but to date none have tested positive for GRBaV, although many specimens are still in the process of being tested, and as mentioned above, we are still in the process of refining our laboratory techniques to improve sensitivity of detection for insect material.

### **Non-Crop Plant Survey**

As a complement to the insect collection and testing, plant material was also collected from non-crop vegetation and tested for GRBaV in order to identify plant species that serve as reservoirs of GRBaV outside of the vineyard. Plant material was sampled from maple (*Acer* sp.), California buckeye (*Aesculus californica*), alder (*Alnus rhombifolia*), madrone (*Arbutus menziesii*), manzanita (*Arctostaphylos* sp.), coyotebrush (*Baccharis pilularis*), Oregon ash (*Fraxinus latifolia*), English ivy (*Hedera helix*), toyon (*Heteromeles arbutifolia*), California walnut (*Juglans californica*), wild cucumber (*Marah macrocarpa*), olive (*Olea europaea*), plum (*Prunus* sp.), coast oak (*Quercus agrifolia*), blue oak (*Q. douglasii*), valley oak (*Q. lobata*), wild rose (*Rosa californica*), blackberry (*Rubus* spp.), willow (*Salix* sp.), elderberry (*Sambucus* sp.), California bay (*Umbellularia californica*), periwinkle (*Vinca major*), and wild grape (*Vitis californica*) as well as various vineyard groundcovers and weedy vegetation (*Artemisia douglasiana*, *Avena fatua*, *A. sativa*, *Brassica* spp., *Calendula officinalis*, *Conium maculatum*, *Convolvulus arvensis*, *Foeniculum vulgare*, *Malva parviflora*, *Raphanus sativa*, *Taraxacum officinale*, *Vicia fava*, and *Vigna* sp.). To date, most of this plant material has tested negative for GRBaV, except for wild grape which has tested positive fairly consistently across multiple sites. It should be noted that “wild grape” at these sites may be a hybrid form *Vitis californica* x *V. vinifera* due to its proximity to commercial vineyards.

### **Vineyard Insect and Plant Survey: Preliminary Findings**

The insect and non-crop plant survey concluded in May 2016, marking one full year of monthly insect and plant sampling in five vineyards with suspected spread of GRBaV. As mentioned, testing of plant and insect material is ongoing, but here we present some preliminary summaries of the data based on findings to date. In our surveys, the only non-crop plant species to test positive for GRBaV has been wild grape (*V. californica* x *V. vinifera*), indicating a potential role of this plant in the spread of GRBaV into commercial vineyards. Here we present a

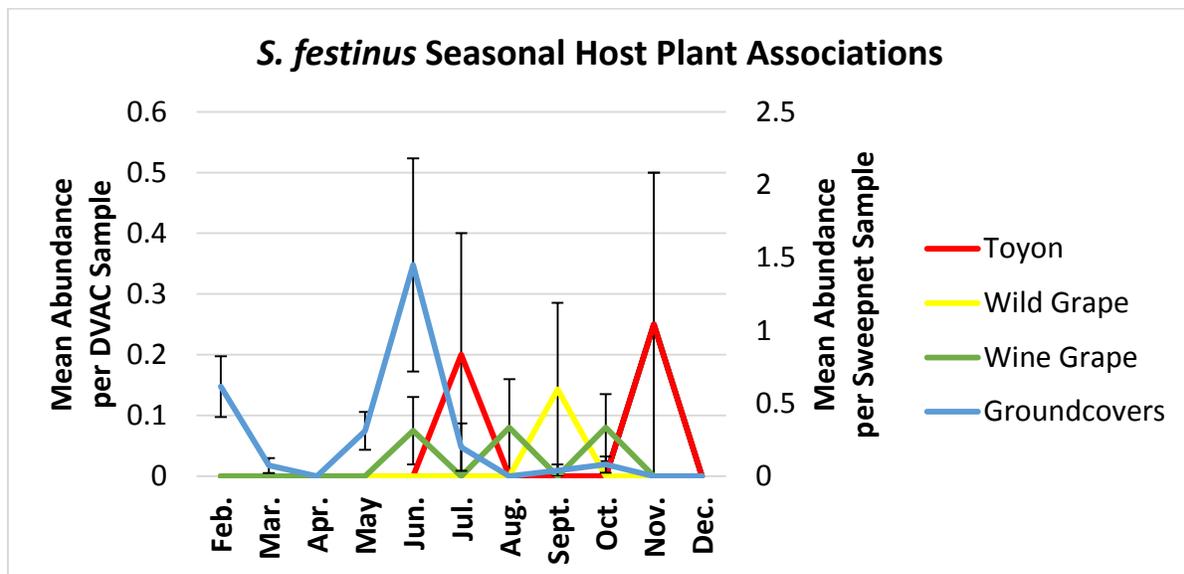
summary of the insect community found on wild grapes in our survey (**Table 2**). Diptera (flies) and western grape leafhopper make up >50% of the insects found on wild grape and >90% of organisms are represented when we include the parasitic Apocrita (parasitoid wasps), spiders, Formicidae (ants), *Empoasca* spp., Coleoptera (beetles), *Chrysoperla* sp. (green lacewings), *E. variabilis* (variegated leafhopper), *Osbornellus* sp., Psocoptera (book lice), Trichoptera (caddisflies), aphids, and Miridae. From this group, only *E. elegantula*, *Empoasca* spp., *E. variabilis*, *Osbornellus* sp., aphids, and the Miridae are likely to feed directly on wild grape tissue and only *E. elegantula* and *E. variabilis* are known to successfully reproduce on it.

**Table 2.** Arthropod community on wild grapes and cultivated wine grapes. Data shows mean annual abundance per sample  $\pm$  SEM and percentage of total arthropods found on the plant.

Order	Family	Genus/Species	Wild Grape		Wine Grape		
			Abundance	%	Abundance	%	
Araneae			0.39 $\pm$ 0.12	6%	0.02 $\pm$ 0.02	2%	
Coleoptera	Galerucinae		0.02 $\pm$ 0.02	<1%	0.01 $\pm$ 0.01	<1%	
	Cantharidae		-	-	<0.01	<1%	
	Other		0.18 $\pm$ 0.09	3%	0.08 $\pm$ 0.02	2%	
Dermaptera			0.04 $\pm$ 0.03	1%	-	-	
Diptera	Syrphidae		-	-	<0.01	<1%	
	Other		2.80 $\pm$ 0.68	41%	1.24 $\pm$ 0.14	28%	
Hemiptera	Acanaloniidae		0.02 $\pm$ 0.02	<1%	-	-	
	Alydidae		-	-	<0.01	<1%	
	Anthocoridae	<i>Orius</i> sp.	0.04 $\pm$ 0.04	1%	0.03 $\pm$ 0.01	<1%	
	Aphididae		0.08 $\pm$ 0.05	1%	0.09 $\pm$ 0.02	2%	
	Berytidae		0.04 $\pm$ 0.03	1%	<0.01	<1%	
	Cicadellidae	<i>Acinopterus angulatus</i>		-	-	0.01 $\pm$ 0.01	<1%
		<i>Deltocephalus fuscinervosus</i>		0.02 $\pm$ 0.02	<1%	0.02 $\pm$ 0.01	<1%
		<i>Dikraneura rufula</i>		-	-	<0.01	<1%
		<i>Dikrella</i> sp.		0.02 $\pm$ 0.02	<1%	-	-
		<i>Empoasca</i> spp.		0.22 $\pm$ 0.13	3%	<0.01	<1%
		<i>Erythroneura elegantula</i>		0.80 $\pm$ 0.43	12%	1.51 $\pm$ 0.44	35%
		<i>Erythroneura variabilis</i>		0.14 $\pm$ 0.07	2%	0.47 $\pm$ 0.19	11%
		<i>Graphocephala atropunctata</i>		-	-	<0.01	<1%
		<i>Macrosteles quadrilineatus</i>		-	-	<0.01	<1%
		<i>Osbornellus</i> sp.		0.12 $\pm$ 0.10	2%	-	-
		<i>Scaphytopius</i> spp.		0.02 $\pm$ 0.02	<1%	0.02 $\pm$ 0.01	<1%
		<i>Sophonia</i> sp.		-	-	<0.01	<1%
		Unknown		0.04 $\pm$ 0.03	1%	0.01 $\pm$ 0.01	<1%
	Geocoridae	<i>Geocoris</i> sp.		-	-	<0.01	<1%
	Lygaeidae		0.06 $\pm$ 0.05	1%	0.06 $\pm$ 0.04	1%	
Membracidae	<i>Spissistilus festinus</i>		0.02 $\pm$ 0.02	<1%	0.02 $\pm$ 0.01	<1%	
Miridae		0.08 $\pm$ 0.05	1%	<0.01	<1%		
Psyllidae		0.02 $\pm$ 0.02	<1%	0.02 $\pm$ 0.01	<1%		
Rhopalidae		0.02 $\pm$ 0.02	<1%	-	-		
Tingidae		-	-	0.01 $\pm$ 0.01	<1%		
Hymenoptera	Apoidea (non- <i>Apis</i> )		-	-	0.02 $\pm$ 0.01	<1%	
	Apocrita (parasitic)		0.57 $\pm$ 0.17	9%	0.17 $\pm$ 0.03	4%	
	Formicidae		0.37 $\pm$ 0.12	6%	0.01 $\pm$ 0.01	<1%	
	Vespidae		0.02 $\pm$ 0.02	<1%	-	-	
Ixodida	Ixodidae		0.04 $\pm$ 0.04	1%	-	-	
Lepidoptera			0.04 $\pm$ 0.04	1%	<0.01	<1%	
Neuroptera	Chrysopidae	<i>Chrysoperla</i> sp.	0.14 $\pm$ 0.12	2%	0.01 $\pm$ 0.01	<1%	
Orthoptera			0.02 $\pm$ 0.02	<1%	-	-	
Psocoptera			0.08 $\pm$ 0.05	1%	0.07 $\pm$ 0.02	2%	
Thysanoptera			0.04 $\pm$ 0.03	1%	0.22 $\pm$ 0.08	5%	
Trichoptera			0.08 $\pm$ 0.05	1%	<0.01	<1%	

Evaluating insect community overlap between wild grapes and wine grapes could help identify novel insect vectors of GRBaV. Organisms that were found on both wild and wine grape include aphids, Berytidae, *Chrysoperla* sp., Coleoptera, *Deltocephalus fuscineruosus*, Diptera, *Empoasca* spp., *E. elegantula*, *E. variabilis*, Formicidae, Galerucinae, parasitic Apocrita, Lepidoptera, Lygaeidae, *Spissistilus festinus* (three-cornered alfalfa hopper), Miridae, *Orius* sp., Psocoptera, Psyllidae, *Scaphytopius* spp., spiders, Thysanoptera, Trichoptera and a small number of unknown Ciccadellids. Of these organisms that co-occur on both wild and wine grape, *Deltocephalus fuscineruosus*, *Empoasca* spp., *E. elegantula*, *E. variabilis*, Lygaeidae, Miridae, Psyllidae, *Scaphytopius* spp., *Spissistilus festinus*, Thysanoptera, and the unknown Ciccadellids will likely feed directly on grape plant tissue and only *E. elegantula* and *E. variabilis* are known to reproduce on these species. The most commonly encountered organism on cultivated wine grape was *E. elegantula* (35%), followed by *E. variabilis* (11%), Thysanoptera (5%), aphids (2%) and Lygaeidae (1%). All other organisms represented <1% of the community found on wine grapes. From this group of likely feeders that occur on both wild and wine grape, we have conducted GRBaV transmission experiments with *E. elegantula* and *E. variabilis*, which represent some of the commonly encountered organisms on both wild and wine grape. Results from these trials have not indicated any ability of these insects to transmit the virus.

While it is notable that *S. festinus*, a known vector of GRBaV (Bahder et al. 2016), was found on both wild and wine grapes, on both plant species they represented <1% of total organisms. Regardless of the overall low populations encountered in vineyards, data on host plant associations of *S. festinus* (Figure 3) provides new information on population dynamics in vineyards. This species was primarily found in the late spring on groundcovers in and around the vineyard, which included various weedy grasses as well as overwintering grass / legume cover crops. As groundcovers died down, *S. festinus* was intermittently found in low abundance on wild grape, wine grape, toyon (*Heteromeles arbutifolia*), and coast oak (*Quercus agrifolia*) throughout the growing season. These are not necessarily reproductive hosts for this species and further work is needed to better understand the life cycle of *S. festinus* on the non-crop habitats in and around vineyards.



**Figure 3.** Seasonal host plant associations of *S. festinus* in North Coast vineyards. High densities of *S. festinus* were found on groundcovers in the late spring and then intermittently on wild grape, wine grape, coast oak and toyon. Plant species shown are not necessarily reproductive hosts. Right Y-axis denotes abundance on groundcovers, left Y-axis denotes abundance on all other plants.

### Establishing Colonies of Novel Vectors (2015-Present)

Due to the low abundance of novel candidate vectors (e.g. *Empoasca* spp., *S. festinus*, *D. fuscineruosus*), we have been working to establish colonies of these insects at the UC Berkeley greenhouse facilities in order to rear a large enough population suitable for GRBaV transmission experiments, which typically require >200 individuals per trial. Data is scant for many of these species and information on reproductive hosts is limited. As such, this spring

we collected candidate species from vineyards and introduced them into cages containing various potential host plants. So far, we have seen successful reproduction of *Aceratagallia* sp. and *Euscelidius schenki* on select host plants. We also collected large populations of *S. festinus* from alfalfa fields and are now seeing reproduction in our colonies.

### **Transmission Experiment with *S. festinus* (2016)**

A GRBaV transmission experiment was conducted with field collected *S. festinus* in July 2016. Individuals were collected from an organic alfalfa field and introduced into cages with GRBaV positive or negative vines. Each cage contained a single potted vine (11 cages each with a single GRBaV-positive vine and nine cages each with a single GRBaV-negative vine) and received 20 *S. festinus* adults. Adults could feed for 48 hours (AAP), after which the GRBaV-positive/negative vine was removed and a GRBaV-negative vine was introduced into each cage. The adults could feed on the negative vine for 48 hours (IAP) and were then removed from the vine. As with previous transmission experiments, the vines are now being held for a two-year period and will be tested for GRBaV every four months. While it has been demonstrated that *S. festinus* can vector GRBaV (Bahder et al. 2016), our goal is to first confirm these findings and then begin evaluating transmission efficiency of this species under laboratory and field conditions.

### **Evaluating *S. festinus* Overwintering Habitat and Seasonal Activity in Vineyards (March-October 2017)**

With the confirmation of *S. festinus* as a known vector of GRBaV, new information is needed on the seasonal ecology of this organism in vineyards.

#### **Overwintering Habitat**

Groundcovers and other non-crop plants in natural habitats adjacent to vineyards will be sampled in March to identify *S. festinus* overwintering habitat use. Sampling will take place in the natural habitats adjacent to Napa and Sonoma county vineyards. There will be at least four sites sampled each month. Natural habitat will consist of patches of riparian and/or oak woodland habitat > 400 m<sup>2</sup>. Sweep-nets will be used to sample groundcovers and perennial plant species in the natural habitats and at the periphery of adjacent vineyards. At each site, 10 sets of 30-sweeps will be collected from groundcovers using a 30.5 cm diameter sweep-net (BioQuip Products, Rancho Dominguez, CA). Groundcover species composition will be recorded. Sweep-nets will also be used to sample the canopy of at least 10 non-crop plant species at each site. For each sample, the sweep-net is held beneath the canopy while vigorously shaking the plant for 30 seconds to dislodge insects into the net.

#### **Seasonal Activity**

In February 2017 we established a study in five Napa and Sonoma county vineyards to evaluate the activity of *S. festinus* populations along transects that extend out from large patches of natural habitat into vineyards. At each site insects will be sampled along five parallel transects (positioned 20 meters apart) that extended out from the riparian or oak woodland habitat (i.e. “natural habitat”) into the vineyard. Each transect will be 160 meters long, 10 meters outside of the vineyard at the edge of the natural habitat and 150 meters into the vineyard. Along each transect samples will be taken at the edge of the natural habitat (0 meters) as well as at the vineyard edge (10 meters) and interior (150 meters).

Densities of *S. festinus* and other membracids, as well as *Erythroneura* leafhoppers and other hemipterans, will be monitored along the transects approximately every two weeks using a combination of yellow sticky-traps, sweep-nets and beat-sheet sampling. Two yellow sticky-traps (16 x 10 cm, Seabright Laboratories, Emeryville, CA) will be placed at each transect point. In the vineyard one trap will be placed in the vine canopy (approximately 3.5 feet above the ground surface) and another trap will be hung from irrigation lines (approximately 1.5 feet above the ground surface). In the natural habitat two sticky-traps will be hung from a pole at each transect point at a height equal to those in the vineyard (i.e. one trap 3.5 feet and the other 1.5 feet above the ground surface). Traps will be replaced approximately every two weeks from March to October. Sweep-nets will be used to sample groundcovers. At each transect point a set of 30-sweeps will be collected from the groundcovers using a 30.5 cm diameter sweep-net (BioQuip Products, Rancho Dominguez, CA). Groundcover species composition and percentage cover will be recorded. A modified beat-sheet will be used at each transect point to sample the canopy of grapevines (in the vineyard) and non-crop species (in the natural habitat). The beat-sheet consists of a one m<sup>2</sup> nylon funnel that feeds into a detachable one gallon plastic bag. For each sample the funnel is held beneath the canopy while vigorously shaking the plant (or vine) for 30 seconds to dislodge insects into the funnel and plastic

collection bag. Each month, vines along each vineyard transect point will be evaluated for signs of *S. festinus* feeding damage (i.e. girdling of leaf petioles). Each month at each vineyard transect point one shoot on 10 randomly selected vines will be visually inspected for leaf girdling. The total number of leaf nodes and girdles per shoot will be recorded.

### **Objective 3. Follow Disease Progression in Established Vineyard Plots to Collect Preliminary Data on Field Epidemiology**

#### **Large Block Mapping (One Site, 2009-2015)**

We have been studying grapevine leafroll disease (GLD) movement at one particular site in Napa Valley, beginning in 2009. The block is a 20 hectare newly planted (in 2008) block of Cabernet Sauvignon. Each year in September the incidence of GLD and more general “red leaf” symptoms were mapped at this site and location recorded with GPS. As early as 2009 many of the vines displayed “red leaf” symptoms but tested negative for grapevine leafroll-associated virus (GLRaV). In our subsequent surveys these symptoms appeared to spread through the vineyard, although most of these “red leaf” symptom vines continued to test negative for GLRaV over this period. We began testing vines for both GLRaV and grapevine red blotch-associated virus (GRBaV) in 2014 and found that 136 vines tested positive for red blotch, nine tested positive for leafroll, and 11 tested positive for both red blotch and leafroll. Plant material from the 2015 survey is still in the process of being tested, but we recorded about 250 “red leaf” symptomatic vines, all of which had tested negative for GLRaV in 2014. With the development of new and more complete primers for both leafroll and red blotch we are now in the process of re-testing plant material from the 2009-2013 survey to verify whether or not GRBaV is present in the “red leaf” symptom vines that previously tested negative for GLRaV.

In 2016, the “large block mapping” program was replaced with a “small block mapping” program (see below). Monitoring spread of GRBaV in small plots at multiple sites will allow for the comparison of spread patterns across multiple locations, each with their own unique set of features (variety-rootstock combination, environmental factors, insect communities, relation to natural habitats etc.). This type of multi-site comparison could potentially provide novel insights into the spatial and temporal dimensions of GRBaV spread. Smaller blocks does not necessarily mean less data, as the overall number of vines being monitored for GRBaV under this new “small blocks” program is actually greater than in the “large blocks” program.

#### **Small Block Mapping (Eight Sites, 2015-Present)**

In September 2015 we began to map and test for GRBaV (using the protocols described previously) at the same five established vineyards mentioned in objective 2. At each site an area consisting of six rows by 20 vines per row (120 vines/site total) was visually evaluated for GRBaV and petiole samples collected from each vine (three petioles/vine) for diagnostic testing. At some sites canes were sampled instead of petioles because samples were collected after vines had dropped their leaves. Cane samples consisted of a composite sample of three canes per vine. Each piece of cane material was taken from between nodes 1-5.

The idea is to return to these same blocks in September 2016 and 2017 to repeat this detailed mapping in order to evaluate if the virus appears to be spreading from vine to vine. In October 2015 we learned that one of these established vineyard sites (Napa - Yountville) was going to be removed due to intolerable levels of GRBaV incidence. In December 2015 we located an alternate site (Napa - Oakville 2) to replace the lost site and conducted the same detailed mapping protocol. Unfortunately this site was also subsequently replanted at the end of 2016, as was the Napa - Oakville 1 site. A new site has been located to replace these lost sites (Napa - Mt. Veeder). In fall 2016 additional sites in the Sierra Foothills were added to the mapping effort. See **Table 3** for a summary of the sites sampled over the past two years. Sampling in 2016 was expanded to include separate samples of three and six petioles from each vine to evaluate the sensitivity of virus detection. Visual evaluations were eliminated in 2016 as well, since it is now well-known that symptom expression does not correlate with GRBaV infection. Sampling in 2017 is now underway. Mapping sites in the Sierra Foothills have been sampled but the North Coast sites are currently inaccessible due to the recent wildfires.

**Table 3.** Sites sampled in the small block mapping program.

Site (County – Area)	Year Mapped		
	2015	2016	2017
Napa – Carneros	3 petioles	3 + 6 petioles	
Napa – Mt. Veeder		3 + 6 petioles	
Napa – Oakville 1	3 petioles	3 + 6 petioles	replant; sampling terminated
Napa – Oakville 2	3 canes	replant; sampling terminated	
Napa – St. Helena	3 petioles	3 + 6 petioles	
Napa – St. Helena	3 petioles	3 + 6 petioles	
Napa – Yountville	3 petioles	replant; sampling terminated	
Amador – Sutter Creek		3 canes	6 petioles + 3 canes
El Dorado – Placerville		3 canes	6 petioles + 3 canes

### Red Blotch Titers Survey

Concerns about the possibility of low GRBaV titer levels in potted vines used in the transmission trials (see objective 1) led us to initiate a broader survey to quantify GRBaV titer levels throughout grapevines over the course of the year. Between April 2015 - May 2016 plant material was collected each month from various parts (roots, trunk, canes, etc.) of at least 10 GRBaV positive vines at each of three vineyard sites in Napa Valley. The goal is understanding whether the virus localizes in certain regions of the grapevine during the year. If this is the case it could improve the focus of our search for novel vectors (i.e. vectors that preferentially feed on parts of the vine with high GRBaV titer levels).

### CONCLUSIONS

Findings from this research help improve our understanding of GRBaV transmission and field epidemiology in order to develop better recommendations and control programs for commercial growers. Greenhouse trials to evaluate GRBaV transmission by both suspected and novel insects aim to clarify which, if any, insects can transmit this virus and, if so, how efficiently they do so. Similarly, screening insects from field sites with suspected spread of GRBaV allows us to identify additional novel vectors for subsequent evaluation in greenhouse trials. Testing plant material from non-crop species in the natural habitats surrounding vineyards provides new information on potential reservoirs of GRBaV outside of the vineyard. Closer evaluation of the insects associated with non-crop reservoirs of GRBaV will further reinforce efforts to identify novel vectors. Detailed mapping of GRBaV at multiple sites where spread of this virus has been suspected will allow us to confirm if this is actually the case as well as evaluate spatial trends of infected vines relative to pertinent landscape features, such as riparian habitats or adjacent vineyard blocks with high levels of GRBaV infection. Finally, quantifying GRBaV titer levels throughout the vine will aid in the search for novel vectors that may feed on specific areas of the vine where the virus is concentrated.

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# QUANTIFYING VINE MEALYBUG SPATIOTEMPORAL DYNAMICS: ASSESSING INVASION RISK TO REFINE MANAGEMENT STRATEGIES

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**ABSTRACT**

The vine mealybug (*Planococcus ficus*) is a severe vineyard pest that contaminates fruit, debilitates vines, and transmits plant pathogens such as grapevine leafroll-associated virus-3. First reported in California from vines in the Coachella Valley, vine mealybug soon spread throughout much of the state, likely on infested nursery stock. It is currently found in most California grape-growing regions and its range continues to expand, making this pest a serious threat to other grape-growing regions of the United States. The ongoing expansion of vine mealybug in California and continued risk of its introduction into new areas necessitate better understanding of the factors driving its invasion. Here we use survey data on 2012-16 vine mealybug occurrence to characterize the factors associated with vine mealybug establishment and spread in Napa County, California. This work also identifies factors underlying hot spots in vine mealybug activity, quantifies spatiotemporal patterns in vine mealybug occurrence, and clarifies pathways that contribute to vine mealybug spread. All analyses are ongoing or pending. Ultimately, results of this investigation can improve understanding of the educational and regulatory steps needed to mitigate vine mealybug impact in Napa vineyards.

**LAYPERSON SUMMARY**

The invasive vine mealybug (*Planococcus ficus*) is an aggressive pest in California vineyards, where it reduces vine health and contaminates fruit. Vine mealybug management is challenging and costly (\$300 to \$500 per acre per year). Since vine mealybug has proven difficult to eradicate once established, these costs are often incurred yearly for the life of the vineyard. Vine mealybug distribution is still expanding within California, and there is continued risk of introduction to other grape-growing regions of the United States. Although vine mealybug biology and management have been intensively studied, the factors governing its invasion and spread are poorly characterized. Analyzing the patterns of vine mealybug occurrence in surveys conducted in Napa County from 2012 to 2016 will help explain why certain areas are heavily infested by this pest and what areas are most at risk of infestation in the future. An improved understanding of the pathways by which this insect disperses naturally or is moved by human activity also will inform regulatory steps and direct educational efforts toward mitigating spread by targeted risk reduction strategies. Ultimately, such information is critical for developing a statewide response to this important vineyard pest.

**INTRODUCTION**

Geospatial analyses and niche-based/species distribution modeling have previously been used to characterize plant, aquatic invertebrate, amphibian, and insect invasions. Results of these and similar investigations have been applied, with varying degrees of success, to develop early detection strategies, identify and prioritize management in high risk areas, and minimize monitoring expenditures (Thuiller et al. 2005, Bradley et al. 2010, Venette et al. 2010, Jiménez-Valverde et al. 2011, Vincente et al. 2016). An intriguing possibility is that information gained from geospatial analyses of invader spread and niche-based/species distribution modeling of suitable habitat for invaders may be used to simulate invader dispersal and predict invader distributions. Ensuing predictions of invader distributions could then guide detection and management efforts, as well as be evaluated and refined using field-collected data on invader occurrence. Here we use such tools to improve response to an important invasive insect in California vineyards, the vine mealybug (*Planococcus ficus*).

The vine mealybug is a severe vineyard pest that contaminates fruit, debilitates vines, and transmits plant pathogens such as grapevine leafroll-associated virus-3 (Daane et al. 2012, Almeida et al. 2013). Vine mealybug was first reported in California from vines in the Coachella Valley (Gill 1994) and soon spread throughout much of the state, likely on infested nursery stock (Haviland et al. 2005). It is currently found in most California grape-

growing regions (Godfrey et al. 2002, Daane et al. 2004a, 2004b). Despite the continued expansion of vine mealybug distributions in California, its current distribution in Napa County and areas at risk of vine mealybug introduction in this region are not well characterized.

Management of vine mealybug has proven challenging and often requires the use of multiple tactics, including biological control, mating disruption, and insecticides (Daane et al. 2008). Management can be particularly complicated in coastal winegrape growing regions where climatic conditions are favorable and Argentine ants (*Linepithema humile*) disrupt biological control (Daane et al. 2007, Gutierrez et al. 2008). Given that vine mealybug may complete three to ten generations per year under California climatic conditions, the insects have the capacity to develop large populations that contaminate fruit, causing yield losses and decreased fruit quality and presenting a serious risk of spread to new regions. Management costs may range from \$300 to \$500 per acre per year, and due to the aggressive nature of vine mealybug populations, these practices cannot be neglected.

## OBJECTIVES

Given the ongoing expansion of the vine mealybug in California and continued risk of its introduction into new areas, a better understanding is needed of what is driving its invasion. The overall goal of this research is to characterize the factors associated with vine mealybug establishment and spread in northern California vineyards, which will be addressed via the following objectives:

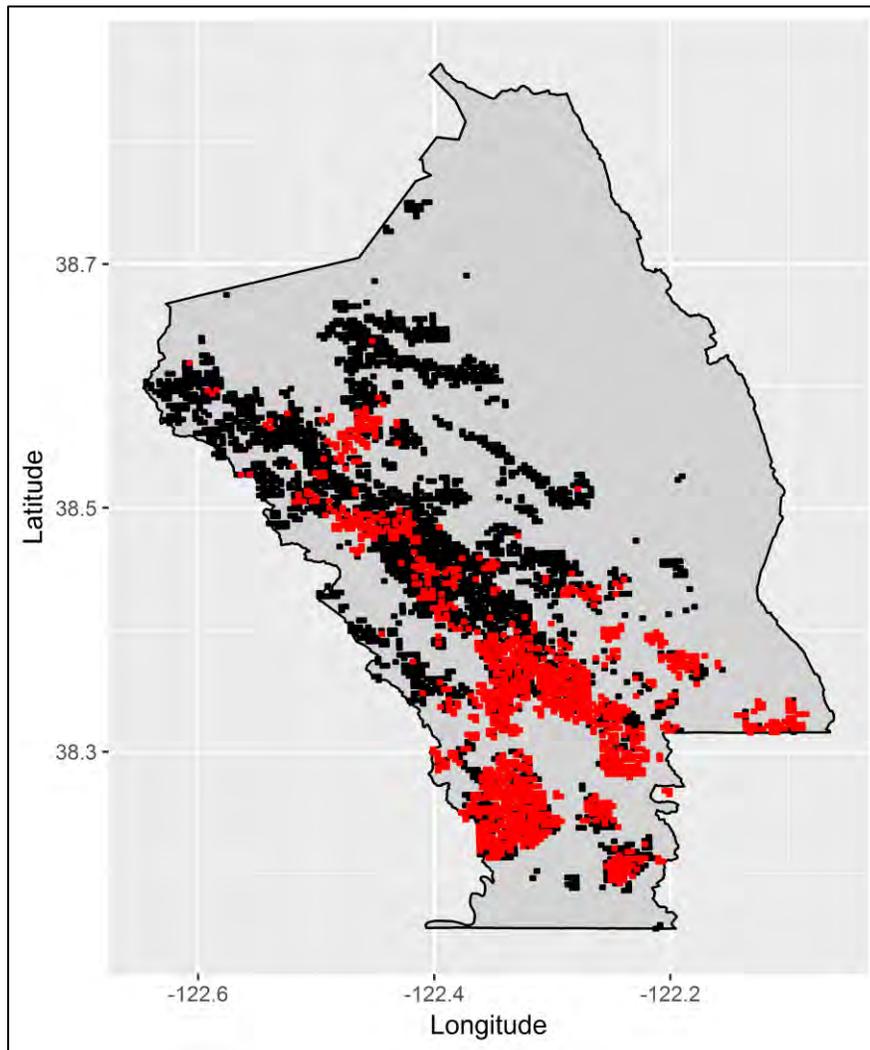
1. Quantify the spatiotemporal patterns in vine mealybug occurrence to identify invasion hot spots and patterns of spread.
2. Characterize the landscape, climatic, and anthropogenic factors associated with current vine mealybug occurrence to predict areas at risk of invasion.
3. Validate and update predictions of vine mealybug risk via in-field monitoring.

## RESULTS AND DISCUSSION

Survey data on 2012-16 vine mealybug occurrence have been received from the Napa County Agricultural Commissioner's Office and cleaned (i.e. removal of duplicate records, identifying missing information, rectifying data inconsistencies, etc.). Traps in each trapping year have been georeferenced relative to grid cells in the CDFA Statewide Grid System. Both the greatest number of traps recording captures and number of male vine mealybugs captured were recorded in 2016, but the number of male vine mealybugs caught varied considerably among trapping years (**Table 1**). A total of 4,004 traps were deployed in 2016; vine mealybug captures in this year appear to be highly clustered in grid cells located in the south-central region of Napa (**Figure 1**). Analyses on spatiotemporal trends in vine mealybug occurrence and abundance from 2012-16 are underway.

**Table 1.** Summary of 2012-16 cumulative trapping effort for vine mealybug in Napa County, California.

<b>Year</b>	<b># traps recording VMB captures</b>	<b># male VMB captured</b>
2012	577	49,327
2013	327	16,488
2014	296	43,444
2015	841	26,577
2016	1,415	49,785



**Figure 1.** 2016 trapping effort for vine mealybug in Napa County, with red cells denoting locations where vine mealybug was detected and black cells denoting where traps did not detect vine mealybug.

## CONCLUSIONS

Data cleaning of 2012-16 vine mealybug trapping effort has been completed and these data have been summarized. Our next step(s) will be to conduct spatial analyses to quantify spatiotemporal trends in vine mealybug occurrence and abundance, followed by analyses to identify environmental, climatic, or anthropogenic factors governing these spatiotemporal trends. Conclusions from these analyses are pending.

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## ECOLOGY OF GRAPEVINE RED BLOTCH VIRUS

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**ABSTRACT**

Grapevine red blotch virus (GRBV) is a new threat to the industry. Limited information is available on the ecology of GRBV although the three-cornered alfalfa hopper (*Spissistilus festinus*) is a recognized arthropod vector of epidemiological importance. Building on past studies on the spread of GRBV in a diseased vineyard, we characterized the transmission mode of GRBV by *S. festinus*. Gut clearing experiments on alfalfa, a nonhost of GRBV, following controlled feeding on GRBV-grapevines suggested a circulative transmission mode. This result was confirmed with localization experiments of GRBV in dissected organs of viruliferous *S. festinus*. Analyzing the seasonal abundance of vector candidate populations in a diseased vineyard for which spread is limited revealed an extremely low population of viruliferous *S. festinus* compared to a substantially higher population in a vineyard where spread is readily occurring. This result suggested that the rate of spread could be related to the dynamics of *S. festinus* populations. Surveys of legumes in cover crop species sown within middle rows of 10 diseased vineyards for GRBV and *S. festinus* did not reveal the occurrence of infected plants or vector specimens in spring 2017. These preliminary results suggested that legumes are unlikely to contribute to spread of GRBV in vineyard ecosystems. Nonetheless, this work needs to be replicated, particularly knowing that efforts to determine the experimental host range of GRBV via agroinoculation with infectious clones showed that bean can become infected with GRBV and *S. festinus* can transmit GRBV from infected to healthy bean plants. Research progress on the ecology of GRBV was disseminated to growers, farm advisors, and service providers at various winter grower conventions.

**LAYPERSON SUMMARY**

Grapevine red blotch virus (GRBV) is a new threat to the grape industry. This virus causes red blotch disease. Limited information is available on the ecology of red blotch disease. By investigating the localization of GRBV within isolated organs of viruliferous three-cornered alfalfa hoppers (*Spissistilus festinus*), experimental evidence in favor of a circulative transmission mode was obtained. These preliminary results will need to be confirmed. By contrasting the population and diversity of previously identified insect vector candidates in two vineyards with a differential spread of GRBV (one with limited spread and the other with efficient spread), a 25-fold reduction in the number of viruliferous *S. festinus* was observed on sticky traps in the vineyard with limited spread, while no *Melanoliarius* planthoppers were caught. These results suggest that the rate of spread could be related to *S. festinus* population dynamic attributes. In addition, surveying legume species used as middle row cover crops in diseased vineyards indicated that none of them hosted *S. festinus* or GRBV. However, agroinoculation experiments showed that bean and eventually vetch could be alternate hosts of GRBV. Thus, their role in the epidemiology of GRBV remains to be elucidated.

**INTRODUCTION**

Red blotch was described for the first time on Cabernet Sauvignon at the University of California Oakville Research Field Station in 2008 (Calvi 2011, Cieniewicz et al. 2017a, Sudarshana et al. 2015). Diagnosis based on symptoms can be challenging because of several confounding factors, including striking similarities between foliar symptoms elicited by red blotch and leafroll diseases, as well as several other biotic and even abiotic factors. Because symptom variation makes visual diagnosis of diseased vines difficult, only DNA-based assays are reliable for accurate diagnosis (Cieniewicz et al. 2017a, Sudarshana et al. 2015).

Fruit ripening issues have been documented with diseased winegrapes. Reductions of one to six degrees Brix have been consistently reported, as well as lower berry anthocyanin and skin tannins, particularly in red winegrapes such as Cabernet Franc and Cabernet Sauvignon (Calvi 2011, Cieniewicz et al. 2017, Reynard et al. 2017,

Sudarshana et al. 2015). Based on the effect of the virus on fruit quality and ripening, numerous vineyard managers are culling infected vines and replacing them with clean, virus-tested ones. The economic cost of grapevine red blotch virus (GRBV) is estimated to range from \$21,833 (for a 5% initial infection in year three and a 25% price penalty for infected grapes) to \$169,384 (for a 60% initial infection in year three and a 100% price penalty for the proportion infected grapes) per acre in Napa Valley; from \$12,023 to \$93,067 per acre in Sonoma; and from \$5,468 to \$39,140 per acre on Long Island in New York (Ricketts et al. 2017). These estimates highlight the economic impact of red blotch disease in different grape-growing regions in the United States.

GRBV is a member of the genus *Grablovirus* in the family *Geminiviridae* (Varsani et al. 2017). It has a circular, single-stranded DNA genome that codes for six open reading frames (Al Rwahnih et al. 2013, Cieniewicz et al. 2017a, Krenz et al. 2012, Sudarshana et al. 2015). We recently showed the causative role of GRBV in the etiology of red blotch disease using agroinoculation of tissue culture-grown grapevines with partial dimer or bitmer constructs of the GRBV genome (Fuchs et al. 2015).

GRBV was documented in all major grape-growing states in the USA. (Krenz et al. 2014). GRBV was also isolated from numerous table grape accessions at the USDA germplasm repository in Davis, California (Al Rwahnih et al. 2015) and in Canada (Poojari et al. 2017, Xiao et al. 2015). The widespread occurrence of GRBV in North America suggests that propagation material has played a significant role in its dissemination. The virus was also described in Switzerland (Reynard et al. 2017), South Korea (Lim et al. 2016) and India (GenBank accession number KU522121). The Virginia creeper leafhopper (*Erythroneura ziczac*) (Poojari et al. 2013) and the three-cornered alfalfa hopper (*Spissistilus festinus*) (Bahder et al. 2016a) have been shown to transmit GRBV from infected to healthy vines under greenhouse conditions. The epidemiological significance of these findings is unknown, stressing the need to carry out studies in diseased vineyards for vector identification. Interestingly, the transmission ability of *E. ziczac* was refuted (Bahder et al. 2016b), highlighting the need for additional studies, particularly to determine the role of *S. festinus* in GRBV transmission in vineyards and assess whether any other insects can vector GRBV. Recently, attributes of GRBV spread (Cieniewicz et al. 2017b) and the epidemiological role of *S. festinus* (Cieniewicz et al. 2017c) were documented.

The overarching goal of our research was to advance our understanding of the ecology of red blotch disease and its causal agent GRBV, with a major emphasis on transmission attributes and the epidemiological role of vineyard cover crops.

## OBJECTIVES

1. Characterize the spread of grapevine GRBV.
  - a. Describe the transmission mode of GRBV by *S. festinus*.
  - b. Test sentinel vines established in a diseased vineyard where spread is documented for the presence of GRBV.
  - c. Investigate the seasonal diversity and distribution of vector candidate populations in a diseased vineyard for which there is no evidence of spread.
2. Determine if vineyard cover crops can host GRBV and/or *S. festinus*.
  - a. Survey cover crops in Napa Valley vineyards for *S. festinus*
  - b. Survey cover crops in Napa Valley vineyards for GRBaV.
3. Determine the experimental host range of GRBV and *S. festinus*.
  - a. Agroinoculate commonly used vineyard cover crop species with infectious GRBV clones and assess virus infection.
  - b. Examine the reproductive potential of *S. festinus* on commonly used vineyard cover crop species.
4. Disseminate research results to farm advisors and to the grape and wine industry.

## RESULTS AND DISCUSSION

### Objective 1. Characterize the Spread of Grapevine Red Blotch Virus (GRBV)

Limited information is available on the attributes of GRBV spread in vineyards although substantial progress was recently made (Cieniewicz et al. 2017b). Nonetheless, limited information is available on the transmission mode and dynamics of dissemination. To investigate the transmission mode of GRBV by *S. festinus*, a colony of *S. festinus* was established on alfalfa in a growth chamber with controlled temperature, humidity, and photoperiod (Figure 1). Alfalfa is a host of *S. festinus* but not of GRBV (Cieniewicz et al. unpublished). Conditions to rear

*S. festinus* colonies were optimized so that a full development cycle, including oviposition, and the production of nymphs and adults, could be completed within two months.

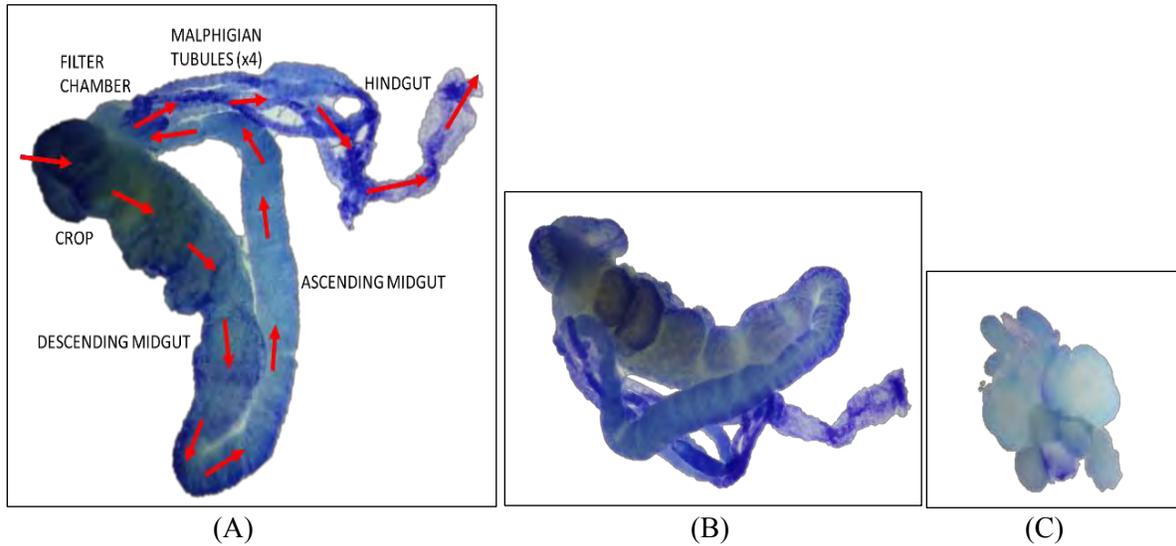


**Figure 1.** Colony of *Spissistilus festinus* in a growth chamber with nymphs on stems of alfalfa.

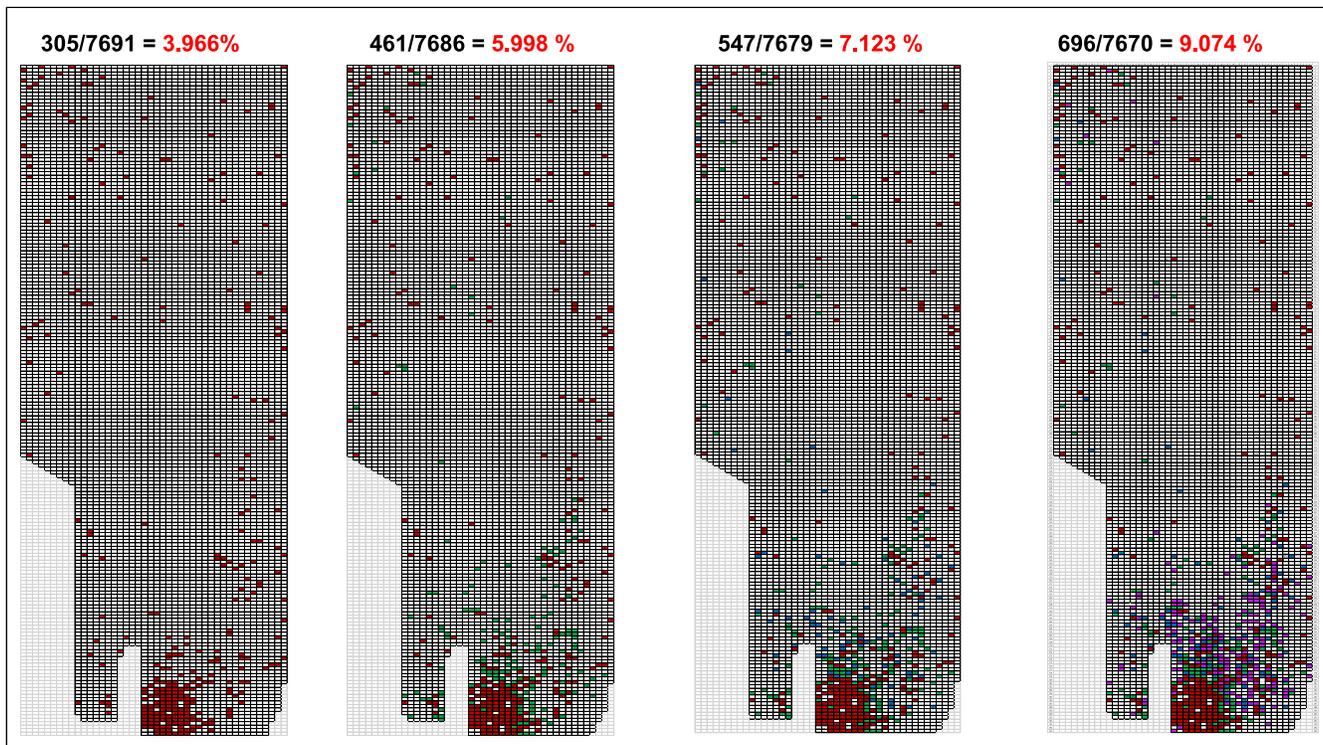
The transmission mode of GRBV by *S. festinus* is hypothesized to be circulative. To validate this hypothesis, specimens from the *S. festinus* colony were allowed to feed on GRBV-infected grapevines for 48 to 72 hours. Then, groups of two to four individuals were transferred to alfalfa and allowed to feed for two weeks. These assays were duplicated. Subsets of *S. festinus* were tested for the presence of GRBV after the virus acquisition step on infected grapevines and subsequent alfalfa feeding steps. After the acquisition period, six out of eight *S. festinus* in the first experiment, and three of five *S. festinus* in the second experiment were positive for GRBaV in multiplex polymerase chain reaction (PCR), confirming that *S. festinus* can ingest GRBaV. After feeding on alfalfa, most specimens tested (12 of 20) in the first experiment and 6 of 11 in the second experiment were positive for GRBaV. These results revealed that *S. festinus* is capable of keeping the virus even after a gut-clearing episode on a nonhost plant of GRBV. These findings revealed a circulative transmission of GRBaV. Additional experiments are underway to validate this observation.

To further characterize the transmission mode of GRBaV, the gut and salivary glands (**Figure 2**) were dissected and the hemolymph was collected from *S. festinus* individuals that were allowed to feed on GRBV-infected grapevines. Organs and the hemolymph were tested for GRBV by multiplex PCR (Krenz et al. 2014). Preliminary results indicated that 14 out of 14 gut organs tested positive for GRBV and 13 out of 14 hemolymph tested positive for GRBV. In addition, eight out of 14 salivary glands tested positive for GRBV in multiplex PCR. These results revealed that GRBV localizes in organ tissue of viruliferous *S. festinus*, including in salivary glands, demonstrating a circulative transmission mode. Additional experiments are ongoing to confirm these preliminary data.

To advance our understanding of the dynamics of GRBV spread in vineyards, sentinel vines, i.e. healthy Cabernet Franc on healthy 3309C, were established in spring 2015 in a diseased Cabernet Franc vineyard where spread of GRBV was extensively documented (Cieniewicz et al. 2017b,c) from 2014 to 2017 (**Figure 3**).



**Figure 2.** Description of *Spissistilus festinus* alimentary canal morphology. (A) Gut dissected in phosphate-buffered saline buffer and stained with toluidine blue dye with (B) alternative view, and (C) salivary glands. Organs are not shown to scale.



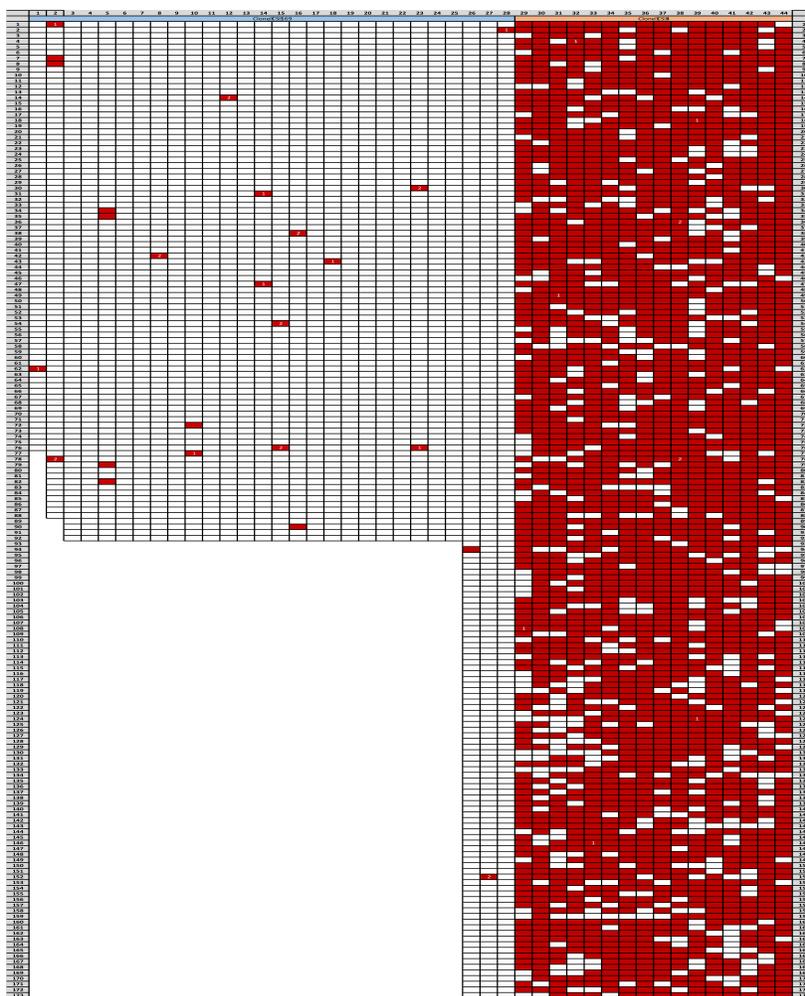
**Figure 3.** Spatiotemporal incidence of GRBV in a five-acre Cabernet Franc vineyard. Each cell represents the location of a vine. Colored cells represent diseased vines in 2014 (red, left panel), 2015 (green, left central panel), 2016 (blue, right central panel), and 2017 (purple, right panel).

Disease incidence increased from 3.9% in 2014 to 9% in 2017 in the study vineyard (**Figure 3**). Diseased vines were primarily aggregated throughout the vineyard and some were isolated (Cieniewicz et al. 2017). An increased aggregation of diseased vines was prominent at the bottom right corner of the vineyard (Cieniewicz et al. 2017b). In this corner, disease incidence increased from 30% to more than 80% in 2014-2017. In this area of the vineyard, approximately 100 sentinel vines were established in spring 2015. Visual observations and testing of GRBV using leaf samples collected in October 2017 indicated no red blotch infection. Identical data were obtained in 2016.

The fact that none of the sentinel vines became infected with GRBV in 2016 and 2017 while established in a vineyard area with a high rate of spread suggests that *S. festinus* might not be attracted to young vines or that the latency period for disease symptom expression in young vines and for GRBV to reach detectable levels following exposure to viruliferous *S. festinus* is at least two years. It will be interesting to continue monitoring the sentinel vines for GRBV infection in 2018.

To investigate the seasonal diversity and distribution of vector candidate populations in a diseased vineyard for which there is limited evidence of spread, a four-acre Cabernet Sauvignon vineyard was selected. This vineyard was established in 2008 with Cabernet Sauvignon clones 4 and 169. In 2011-2012, disease symptoms were apparent in the vineyard section with clone 4 vines, while the section with clone 169 vines was asymptomatic. This Cabernet Sauvignon vineyard is adjacent to the Cabernet Franc vineyard (to the southwest), 60 feet apart. Our hypothesis for a differential rate of GRBV spread in the Cabernet Franc and Cabernet Sauvignon vineyards are distinct *S. festinus* population dynamic attributes.

Analysis of the spatial distribution of GRBV-infected vines in October 2017 revealed that the majority of clone 4 vines were symptomatic (right) whereas only a few randomly dispersed clone 169 vines were diseased (left) (**Figure 4**). Previously, visual monitoring of vines in 2015 and 2016 followed by PCR testing did not document the existence of infected clone 169 vines. However, in October 2017, a few diseased clone 169 vines were identified in the Cabernet Sauvignon vineyard (**Figure 4**). Infected vines of clone 4 and 169 were primarily infected with GRBV clade 1 variants but also by GRBV clade 2 variants.



**Figure 4.** Spatial distribution of red blotch-diseased vines in a four-acre Cabernet Sauvignon vineyard. The majority of clone 4 vines are symptomatic (right) while only a few randomly dispersed clone 169 vines are diseased (left). Diseased vines are in red.

Insect yellow sticky traps were placed at the top of the selected Cabernet Sauvignon vineyard, spanning six rows of clone 4 vines and six rows of clone 169 vines (**Figure 5**). Traps were positioned on the middle trellis wire throughout the sampling area that spanned 12 rows, and six four-4-vine panels per row. In each row, a sticky card was placed in every other panel in alternating rows, such that each of the twelve rows contained three sticky cards (**Figure 5**). Sticky cards were removed weekly, placed in plastic bags, and shipped overnight from the vineyard to the laboratory in Geneva, New York for evaluation. Removed cards were replaced with new sticky cards. The survey was conducted from March to November 2017 to span the entire growing season. The same approach was used in a previous study in the Cabernet Franc vineyard to identify GRBV vector candidates (Cieniewicz et al. 2017c). This study led to the identification of *S. festinus* (Membracidae), *Melanoliarus* sp. (Cixiidae), *Osbornellus borealis* (Cicadelliade), and *Colladonus reductus* (Cicadellidae) as vector candidates (Cieniewicz et al., 2017c).



**Figure 5.** Sticky card traps in the Cabernet Sauvignon study vineyard in May 2017.

Insects caught on sticky card traps in the Cabernet Sauvignon were identified to genus and species where possible based on morphological characteristics. Specimens were identified and counted while still impacted on sticky cards. The number and identity of specimens was recorded for each sticky card to evaluate the abundance and diversity of *S. festinus*, *Melanoliarus* sp., *Osbornellus borealis*, and *Colladonus reductus*, the four previously identified vector candidates (Cieniewicz et al. 2017c).

Preliminary results revealed only three *S. festinus*, no *Melanoliarus* sp., only six *Osbornellus borealis*, and over 60 *Colladonus reductus* (**Table 1**). These specimens were individually removed from sticky cards using Goo Gone liquid degreaser to dissolve the adhesive and loosen the specimens for GRBV testing by multiplex PCR (Krenz et al. 2014). Preliminary results obtained so far indicate only one out of the three *S. festinus* being viruliferous (**Table 1**). This is in contrast to the data obtained in the Cabernet Franc vineyard for which 12 (48%) and 13 (52%) out of 25 *S. festinus* tested positive for GRBV in 2015 and 2016, respectively (Cieniewicz et al. 2017c). For *O. borealis* and *C. reductus*, no substantive differences were noticed between the Cabernet Sauvignon and Cabernet Franc vineyards, except that many more *C. reductus* were caught in the Cabernet Sauvignon vineyard. For example, for *O. borealis*, 13 (42%) and four (36%) specimens out of 31 and 11 in 2015 and 2016, respectively, tested positive for GRBV in the Cabernet vineyard (Cieniewicz et al. 2017c), and 67% (four of six) tested positive in 2017 (**Table 1**). For *C. reductus*, 14 (61%) and 12 (29%) specimens out of 23 and 41 in 2015 and 2016, respectively, tested positive for GRBV in the Cabernet vineyard (Cieniewicz et al. 2017c), and 30% (19 of 63) tested positive in 2017 (**Table 1**). It will be interesting to pursue this line of investigation to validate our hypothesis on the association between the rate of GRBV spread and *S. festinus* population dynamic attributes.

**Table 1.** Diversity and abundance of insects caught on sticky card traps placed from March to November in a Cabernet Sauvignon vineyard in which spread of GRBV is limited.

Family/Order	Species	Common Name	# Tested	# Positive	% Positive
Membracidae	<i>Spissistilus festinus</i>	three-cornered alfalfa hopper	3	1	33
Cicadellidae	<i>Colladonus reductus</i>		63	19	30
	<i>Osbornellus borealis</i>		6	4	67
	<i>Scaphytopius</i> sp.	sharp-nosed leafhopper	50	10	20
	<i>Euscelis</i> sp.		7	1	14
	<i>Empoasca</i> sp.	potato leafhopper	18	0	0
	<i>Erythroneura variabilis</i>	variegated leafhopper	25	0	0
	<i>Erythroneura elegantula</i>	western grape leafhopper	15	0	0
	Deltocephalinae		8	0	0
	<i>Xestocephalus</i> spp.		1	0	0
	<i>Japananus hyalinus</i>	Japanese maple leafhopper	2	0	0
	<i>Erythroneura ziczac</i>	Virginia creeper leafhopper	2	0	0
Delphacidae	nd	delphacid planthopper	2	0	0
Psyllidae	nd	psyllids	6	0	0
Thysanoptera	nd	thrips	13	0	0
Aphididae	nd	aphids	28	1	4
Miridae	nd	plant bugs	12	0	0
Lygaeidae	nd	seed bugs	2	0	0
Hymenoptera	nd	wasps, bees, ants	6	0	0
Diptera	nd	true flies	10	0	0
Coleoptera	nd	beetles	6	0	0
Psocoptera	nd	barklice	8	0	0
Aleyrodidae	nd	whiteflies	8	0	0
Phylloxeridae	nd	phylloxera (foliar-form)	8	0	0

### Objective 2. Determine if Vineyard Cover Crops Can Host GRBV and/or *S. festinus*

The preferred host range of *S. festinus*, a recognized vector of GRBV, includes species in the family Fabaceae. *S. festinus* is not generally considered a vineyard pest, but can cause yield loss in fabaceous crops, which are commonly sown as cover crops between vineyard rows. Therefore, it is important to determine whether some of the commonly used vineyard cover crop species can act as reservoir of the virus and host of *S. festinus*, and contribute to disease epidemics.

Preliminary 2016 survey results of vineyard cover crop species within and adjacent to the Cabernet Franc vineyard where spread of GRBV was documented in 2014–2016 (Cieniewicz et al. 2017b,c) did not yield any positive findings of GRBV. This work was repeated in 2017 with a special emphasis on legumes (Fabaceae) including vetch, peas, bean, and clover. Cover crop species in 10 vineyards of Cabernet Franc, Merlot, Cabernet Sauvignon, and Sauvignon Blanc were surveyed in March 2017 for *S. festinus* by sweep netting. The occurrence of GRBV was previously confirmed in these study vineyards by visual disease symptom observations and by PCR testing. In spite of extensive sweep netting (more than 15 hours) over three weeks, no *S. festinus* were caught on any cover crops within vineyard middle rows. Similarly, none of the more than 400 samples of vetch, pea, bean, and clover collected in March 2017 in 10 red blotch-infected vineyards tested positive for GRBV in multiplex PCR. This preliminary work seems to indicate that legumes, which are part of conventionally used cover crop seed mixes, are (i) not natural hosts of *S. festinus*, and (ii) unlikely to become infected with GRBV in vineyard ecosystems.

### Objective 3. Determine the Experimental Host Range of GRBV and *S. festinus*

It is important to complement surveys of vineyard cover crop species with inoculation experiments in the greenhouse to accurately determine if GRBV can infect legume cover crop species. Our GRBV infectious clones (Yepes et al. 2017) were used to agroinoculate clover, vetch, bean, and peas by needle pricking. Individual plants were agroinoculated and tested for the accumulation of GRBV at local and systemic sites. Virus replication was

verified by reverse transcription (RT) PCR at seven days post-inoculation in locally infected leaves. Systemic movement of the virus was detected by multiplex PCR at 14 days post-inoculation in apical tissue. An *Agrobacterium tumefaciens* strain containing the reporter gene  $\beta$ -glucuronidase (GUS) with an intron (Vancanneyt et al. 1990) was used to test the efficacy of DNA delivery needle pricking.

Preliminary results revealed bean and possibly vetch as alternate hosts of GRBV with more than half of the 20 agroinoculated plants of each species becoming infected, as shown by PCR and RT-PCR. In contrast, none of the 20 agroinoculated clover and pea plants became infected with GRBV, in spite of GUS histochemical staining of agroinoculated tissue (Cieniewicz et al. unpublished).

Agroinoculated bean plants infected by GRBV were used in transmission assays with *S. festinus*. Two to four specimens were deposited on GRBV-infected bean in insect-proof cages and allowed to feed for 48 to 72 hours. Then, individual *S. festinus* were transferred to healthy bean plants and maintained for 72 hours in insect-proof cages in the greenhouse. Bean tissue was tested by PCR and RT-PCR for GRBV two weeks post-transmission. Data indicated that eight of 20 bean plants became infected by GRBV following *S. festinus*-mediated transmission, revealing that GRBV can be transmitted from bean to bean by *S. festinus*. These preliminary results will need to be confirmed in replicated experiments to ascertain the experimental host range of GRBaV.

#### **Objective 4. Disseminate Research Results to Farm Advisors and the Industry**

Research findings were communicated to the industry via regular communications with extension educators. Presentations at winter grower conventions were also used to disseminate information, as follows:

- Cieniewicz E, Fuchs M. 2017. Grapevine red blotch virus in free-living *Vitis* sp. Cornell Recent Advances in Viticulture and Enology (CRAVE) conference, Nov. 14, Ithaca, NY (participants = 60).
- Fuchs M. 2017. Update on the ecology of red blotch virus. Sustainable Ag Expo, Nov. 14, San Luis Obispo, CA (participants = 500).
- Fuchs M. 2017. Viruses: Biology, ecology and management. Sustainable Ag Expo, Nov. 13, San Luis Obispo, CA (participants = 550).

Together, we reached out to over 1,200 growers.

#### **CONCLUSIONS**

*S. festinus* is an arthropod vector of GRBV of epidemiological importance in a diseased vineyard (Cieniewicz et al. 2017c). Limited information is available on the ecology of GRBV. *S. festinus* gut clearing experiments in combination with localization experiments of GRBV in dissected organs of viruliferous *S. festinus* indicated a circulative transmission mode. These results need to be validated. The abundance of viruliferous *S. festinus* was found substantially lower in a diseased vineyard for which spread is low, suggesting an association between the rate of spread and vector population dynamics. Legume cover crops in diseased vineyards are not likely to contribute to the spread of GRBV; however, additional studies are needed to confirm this observation. Research on the ecology of GRBV is anticipated to improve disease management in vineyards.

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## RESISTANCE TO GRAPEVINE FANLEAF VIRUS IN ROOTSTOCKS

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**Reporting Period:** The results reported here are from work conducted July 7, 2016 to December 20, 2017.

### ABSTRACT

Grapevine fanleaf virus (GFLV) causes fanleaf degeneration and is responsible for severe losses. Fanleaf management primarily relies on prophylactic measures and the use of rootstocks with resistance to the dagger nematode (*Xiphinema index*), the vector of GFLV. No source of resistance to GFLV has been identified in wild or cultivated *Vitis* species. Therefore, we are exploring RNAi to confer resistance to GFLV in rootstocks. We developed several RNAi constructs from conserved genomic regions of GFLV, including from the recently recognized viral suppressor of silencing, and tested their anti-GFLV potential in transient assays with *Nicotiana benthamiana*, a systemic herbaceous host. A few promising RNAi constructs were transferred into embryogenic calli of grapevine rootstock genotypes 101-14 MGT, 110R, 3309C, and 5C via *Agrobacterium tumefaciens*-mediated transformation. Some putative transgenic plants of 101-14 MGT were obtained and transferred to soil in the greenhouse. Once putative transgenic rootstocks are well established, they will be characterized for transgene insertion and expression, and subsequently for resistance to GFLV. Disseminate information to stakeholders through presentations at conventions and workshops.

### LAYPERSON SUMMARY

Grapevine fanleaf virus (GFLV) is one of the most devastating viruses of grapevines worldwide. The virus is transmitted by the dagger nematode (*Xiphinema index*) and is primarily managed in diseased vineyards through the use of rootstocks that are resistant to *X. index*. Such rootstocks delay the debilitating effect of GFLV on vine health and production but do not prevent GFLV infection. Since no source of resistance to GFLV is known in wild or cultivated *Vitis* species (Oliver and Fuchs 2011), we explored the anti-viral pathways of RNA interference (RNAi), an innate plant defense system, to confer resistance to GFLV infection in grapevine rootstocks. Several RNAi constructs derived from different conserved regions of the GFLV genome, including a recently recognized viral suppressor of RNAi, were identified and engineered for expression *in planta*. These constructs were concatenated to substantially reduce the probability that genetically diverse GFLV variants from vineyard populations would defeat the resistance (Fuchs 2017). These constructs were used in transformation experiments of rootstocks and some putative transgenic 101-14 plants were established in the greenhouse. These plants will be characterized for transgenic insertion and expression as well as for resistance to GFLV. Research progress on the development of fanleaf-resistant rootstocks was disseminated to grape growers, farm advisors, and service providers at various venues.

### INTRODUCTION

Fanleaf is one of the most devastating viral diseases of grapevines (Andret-Link et al. 2004). It causes serious economic losses by reducing vigor and yield, altering fruit juice chemistries, shortening the productive life of vineyards, or causing vine death. The causal agent, grapevine fanleaf virus (GFLV), is specifically transmitted from vine to vine by the soil-borne, ectoparasitic dagger nematode (*Xiphinema index*) (Andret-Link et al. 2004, Fuchs et al. 2017).

GFLV belongs to the genus *Nepovirus* in the family *Secoviridae*. It has a bipartite, positive-sense single-stranded RNA genome. The two genomic RNAs are expressed as a polyprotein that is cleaved into individual proteins at specific proteolytic cleavage sites. RNA1 (7,342 nucleotides) codes for five proteins: 1A (unknown function), 1B<sup>Hel</sup> (putative helicase), 1C<sup>VPg</sup> (viral protein genome-linked), 1D<sup>Pro</sup> (proteinase), and 1E<sup>Pol</sup> (putative RNA-dependent RNA polymerase). These proteins are involved in proteolytic processing and replication (Andret-Link et al. 2004, Fuchs et al. 2017). RNA2 (3,774 nucleotides) codes for three proteins: 2A<sup>HP</sup> (homing protein), 2B<sup>MP</sup> (movement protein), and 2C<sup>CP</sup> (coat protein) that are involved in RNA2 replication, movement, and virion

formation, respectively. Both GFLV RNA1 and RNA2 are required for systemic plant infection (Andret-Link et al. 2004, Fuchs et al. 2017).

Fanleaf management primarily relies on prophylactic measures through sanitation and certification that facilitate the production of planting material derived from clean, virus-tested stocks. Control of the nematode vector *X. index* is another component of the GFLV management portfolio, however, this approach can be challenging due to the relative lack of effective nematicides and to harsh environmental consequences related to their use. Prolonged fallow periods (up to 10 years) can reduce nematode populations in infested soils, but lengthy fallow periods are not practical in high-value grape-growing areas (Andret-Link et al. 2004). Grapevines with resistance to *X. index* have been identified and rootstocks resistant to this dagger nematode have been developed (Andret-Link et al. 2004, Oliver and Fuchs 2011).

Fanleaf is primarily managed in diseased vineyards by the use of rootstocks that are resistant to *X. index*. These rootstocks are extensively used in grape-growing regions where GFLV is a major threat to productivity, including the Central Coast, North Coast, Sacramento Valley, and San Joaquin Valley in California. They substantially delay the debilitating effect of GFLV on vine health and production but do not prevent GFLV infection (Andret-Link et al. 2004, Oliver and Fuchs 2011). As a result, vines become infected through translocation of the virus from rootstocks to scions and the productive lifespan of vineyards is substantially reduced. In addition to conferring a limited long-term protection of grapevines from GFLV, some of the *X. index*-resistant rootstocks have undesirable viticultural characteristics such as high vigor and poor rooting ability or susceptibility to lime-induced chlorosis (Oliver and Fuchs 2011). Resistance to GFLV in rootstocks would be desirable for fanleaf control; however, no source of resistance to this virus has been identified in wild or cultivated *Vitis* species (Oliver and Fuchs 2011).

Exploiting the anti-viral pathways of RNA interference (RNAi), an innate plant defense system, and using RNAi constructs derived from conserved regions of the GFLV genome to transform some of the most popular grapevine rootstocks is an elegant approach to engineer resistance. RNAi is an innate immune defense mechanism against plant viruses. It is a post-transcriptional process that is triggered by double-stranded (ds) RNA for the silencing of gene expression in a nucleotide sequence-specific manner through the production of small dsRNAs called small interfering (si) RNAs, for which the guide strand is incorporated into the RNA-induced silencing complex to find mRNAs that have a complementary nucleotide sequence, resulting in their endonucleolytic cleavage. Silencing is associated with the production of 21 to 24 nucleotide dsRNA duplexes (siRNAs) and are generated from dsRNA precursors by ribonuclease III-type Dicer-like enzymes. The siRNAs are then incorporated and converted to single stranded RNAs (ssRNAs) in an Argonaute-containing RNA induced silencing complex. This complex targets RNA for cleavage, in particular mRNAs that are complementary to siRNAs, i.e. viral RNAs of an invading virus, by inducing their post-transcriptional gene silencing processing through endonucleolytic cleavage. As a result, viral RNAs are chopped and nonfunctional, hence resistance to virus infection. The formation of dsRNAs by hairpin (hp) RNAs facilitates the silencing of target viral mRNAs via RNAi, resulting in the accumulation of virus-specific siRNAs that guide the destruction of complementary viral RNA.

Viruses encode proteins that act as suppressors of RNA silencing. Their role is to counteract the innate defense system of the plant by interfering with critical steps of the antiviral pathways of RNA silencing. Thus, an RNAi strategy designed against viral RNA silencing suppressors (VRS) should be optimal to confer resistance to virus infection in plants. In the case of GFLV, a VRS remains elusive. Thus, research is needed to identify and characterize a VRS for GFLV and translate the corresponding information to engineer resistance against GFLV in rootstock.

Single or multiple virus gene sequences can be used to develop resistant plants (Fuchs 2017). However, pyramiding sources of resistance is essential for achieving broad-spectrum and durable resistance. Stacking resistance-conferring gene sequences into single crop genotypes is paramount for protection against commonly occurring infections by genetically diverse virus strains across diverse ecosystems. In addition, pyramiding sequences from different viral coding regions, particularly highly conserved segments that are involved in various steps of the virus infectious cycle, i.e. replication, cell-to-cell movement, virion assembly, and/or acquisition by a vector, will favor broad-spectrum and durable resistance (Fuchs 2017). Indeed, by stacking polygenic resistance sources into a single crop genotype, the probability of genetically diverse virus variants overcoming multiple

resistance-conferring gene sequences is substantially reduced compared to monogenic resistance sources (Fuchs 2017). This is because many mutations with a low probability of occurrence and a high fitness penalty would be required for virus adaptation to pyramided resistance genes. As a result, populations of viruses are less likely to defeat the resistance (Fuchs 2017).

## OBJECTIVES

The major objective of our research was to explore RNAi to confer resistance to GFLV in rootstocks. Our hypothesis is that silencing several GFLV-encoded genes, including a VRS, in rootstocks will confer practical resistance to GFLV. The specific objectives of our research were to:

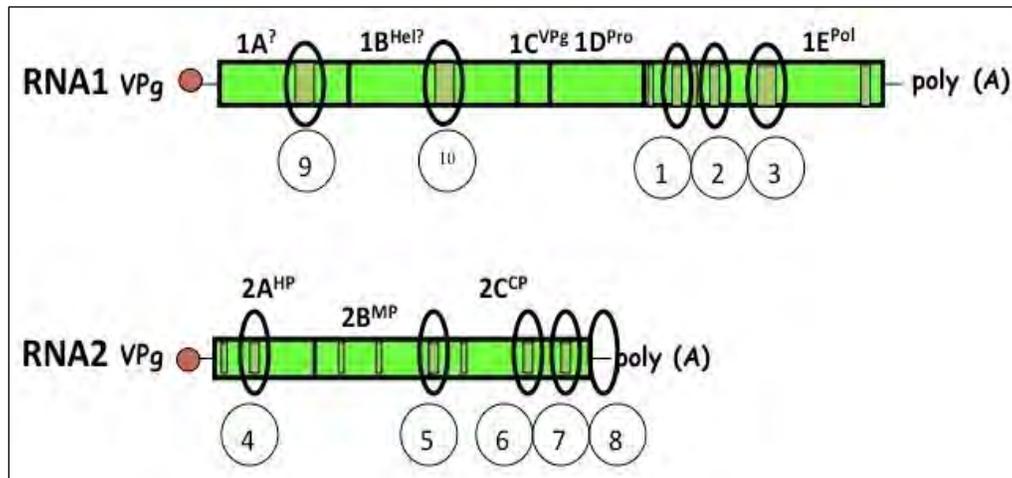
1. Develop RNAi constructs from conserved genomic regions of GFLV.
2. Test RNAi constructs for reduction of GFLV accumulation in transient assays.
3. Transfer promising RNAi constructs into grapevine rootstock embryogenic calli and develop transgenic clones.
4. Initiate phenotyping of transgenic RNAi grapevine rootstock clones by agroinfiltration with infectious GFLV constructs.
5. Disseminate information to stakeholders through presentations at conventions and workshops.

## RESULTS AND DISCUSSION

### Objective 1. Develop RNAi Constructs from Conserved Genomic Regions of GFLV

The goal was to mine the GFLV genome sequence and identify highly conserved genomic nucleotide sequence regions for the engineering of RNAi constructs.

The complete GFLV nucleotide sequences available in GenBank were downloaded and mined for short conserved nucleotide regions. Search parameters were 25 nucleotide stretches in length for which 85% of the positions were conserved amongst at least 95% of the sequences. Search outputs revealed 10 conserved regions throughout the GFLV genome (**Figure 1**).



**Figure 1.** Mapping of conserved nucleotide sequences on the GFLV genome. Conserved sequences are represented with light brown stripes. Fragments used for the production of concatenate RNAi constructs are circled and labeled 1-10. RNA1 coding regions are: 1A<sup>?</sup> (unknown function), 1B<sup>Hel?</sup> (putative helicase), 1C<sup>VPg</sup> (viral genomic-linked protein), 1D<sup>Pro</sup> (protease), and 1E<sup>Pol</sup> (RNA dependent-RNA polymerase). RNA2 coding regions are: 2A<sup>HP</sup> (homing protein), 2B<sup>MP</sup> (movement protein), and 2C<sup>CP</sup> (coat protein).

These conserved nucleotide stretches of 100-300 nucleotides in size are located on RNA1 (five conserved regions) and RNA2 (five conserved regions) (**Figure 1**). The conserved RNA1 regions are located in the 1A, 1B<sup>Hel</sup>, and 1E<sup>Pol</sup> coding regions. The conserved RNA2 regions are located in the 2A<sup>HP</sup>, 2B<sup>MP</sup>, and 2C<sup>CP</sup> coding regions, as well as in the 3' untranslated region (**Figure 1**).

Individual conserved regions were amplified by polymerase chain reaction (PCR) using specific primers and full-length cDNAs of GFLV RNA1 and RNA2 as template. Then, concatenate constructs resulting from the ligation of PCR products from different coding regions were produced (**Table 1**). Most concatenates were generated with fragments from different GFLV coding regions rather than from within a single coding region. This was done in expectation of broad-spectrum and durable resistance (Fuchs 2017).

**Table 1.** Concatenate constructs (100-300 nucleotides in size) designed in conserved regions of the GFLV genome.

Concatenate	Gene	Letter
5+8+2	2B <sup>MP</sup> +2C <sup>CP</sup> +1E <sup>Pol</sup>	A
7+1+4	2C <sup>CP</sup> +1E <sup>Pol</sup> +2A <sup>HP</sup>	B
4+6+3	2A <sup>HP</sup> +2C <sup>CP</sup> +1E <sup>Pol</sup>	C
3+7+5+1+6+8	1E <sup>Pol</sup> +2C <sup>CP</sup> +2B <sup>MP</sup> +1E <sup>Pol</sup> +2C <sup>CP</sup> +2C <sup>CP</sup>	D
2+4+5	1E <sup>Pol</sup> +2A <sup>HP</sup> +2C <sup>CP</sup>	E
1+6+8	1E <sup>Pol</sup> +2C <sup>CP</sup> +2C <sup>CP</sup>	F
6+7+8	2C <sup>CP</sup> +2C <sup>CP</sup> +2C <sup>CP</sup>	G
3+7+5	1E <sup>Pol</sup> +2C <sup>CP</sup> +2B <sup>MP</sup>	H
1+2+3	1E <sup>Pol</sup> +1E <sup>Pol</sup> +1E <sup>Pol</sup>	I

For example, fragment 245 encompasses conserved fragments of 1E<sup>Pol</sup> (conserved region #2 on **Figure 1**), 2A<sup>HP</sup> (conserved region #4 on **Figure 1**), and 2B<sup>MP</sup>/2C<sup>CP</sup> (conserved region #5 on **Figure 1**). Similarly, fragment 375 encompasses conserved fragments of 1E<sup>Pol</sup> (conserved region #3 on **Figure 1**), 2C<sup>CP</sup> (conserved region #7 on **Figure 1**) and 2B<sup>MP</sup>/2C<sup>CP</sup> (conserved region #5 on **Figure 1**). These fragments were cloned into the plasmid pEPT8 - a plasmid derived from pUC19 that contains the cauliflower mosaic virus 35 promoter sequence and nopaline synthase terminator sequence - and subsequently in binary plasmid pGA482G for mobilization into *Agrobacterium tumefaciens* strain C58 for plant transformation. The integrity of all cloned concatenate constructs was verified by restriction digestions and by sequencing at the Cornell Biotechnology Resource Center.

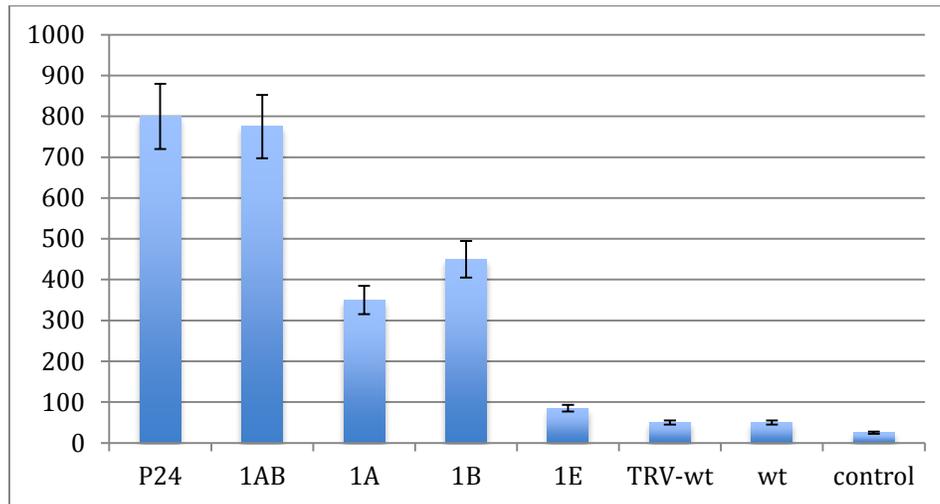
Advancing our understanding of GFLV-host interactions will undoubtedly provide new insights into how the virus hijacks the plant machinery and which viral protein domains are key to the plant-virus interactome. Along this vein, three additional RNAi constructs were engineered. These RNAi constructs were designed in the RNA1-encoded 1A and 1B<sup>Hel</sup> coding regions. The impetus for the RNAi constructs is that parallel research revealed that each of these coding regions has a weak VRS function while the fusion product 1A-1B<sup>Hel</sup> acts as a strong VRS (**Figure 2**).

The VRS activity of GFLV 1A-1B<sup>Hel</sup> is also illustrated by fluorescence measurement of detached leaves of transgenic *Nicotiana benthamiana* expressing green fluorescent protein (GFP) under ultraviolet illumination (**Figure 3**).

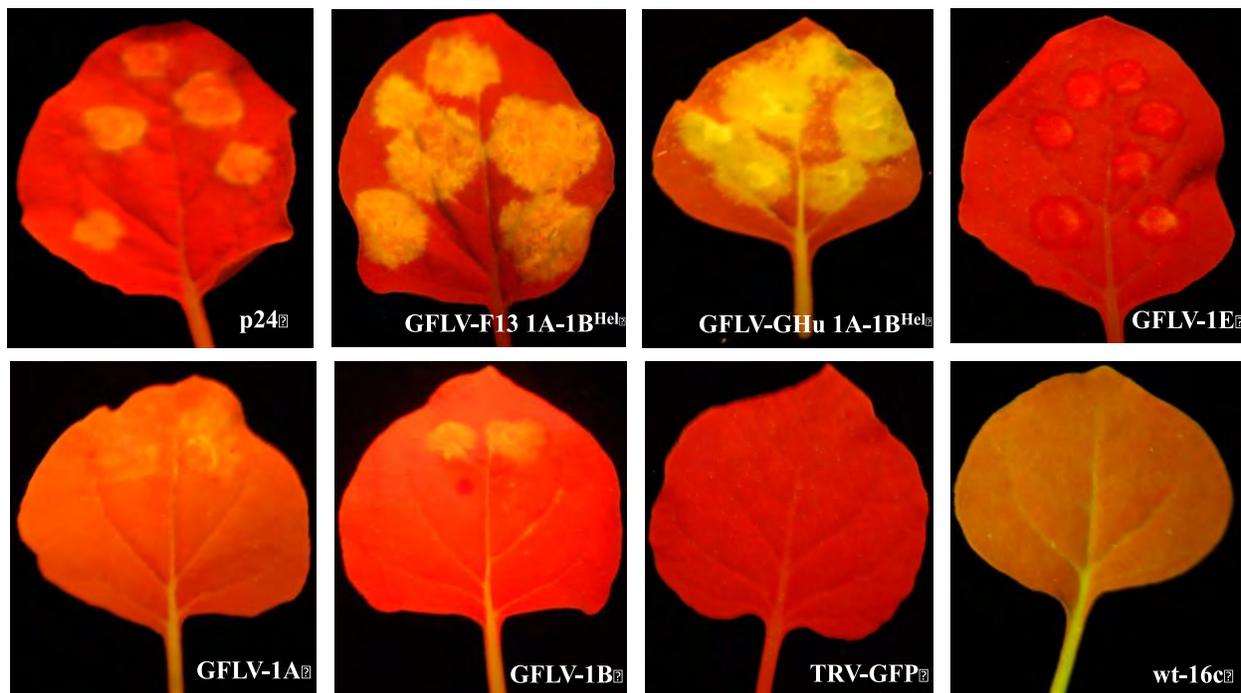
The VRS activity of GFLV 1A-1B<sup>Hel</sup> was as strong as p24, the VRS of GLRaV-2 (**Figures 2 and 3**). It is anticipated that RNAi 1A-1B<sup>Hel</sup> will have a strong anti-GFLV effect by interfering with RNAi silencing. Similar VRS features were assigned to the 1A, 1B<sup>Hel</sup>, and 1A-1B<sup>Hel</sup> fusion product of GFLV strains F13 and GHu (**Figure 3**).

## **Objective 2. Test RNAi Constructs for Reduction of GFLV Accumulation in Transient Assays**

The goal of this objective is to use a transient assay to screen the potential of RNAi constructs at interfering with GFLV multiplication. The development of grapevine rootstocks and the screening for resistance to GFLV is time consuming. Therefore, resistance to GFLV was evaluated first in the systemic herbaceous host *N. benthamiana* prior to its application to grapevines. Herbaceous hosts such as *N. benthamiana* offer the benefits of mechanical inoculation for resistance evaluation, short time to achieve systemic infection, and more expedient and high-throughput options to streamline the screening for resistance.



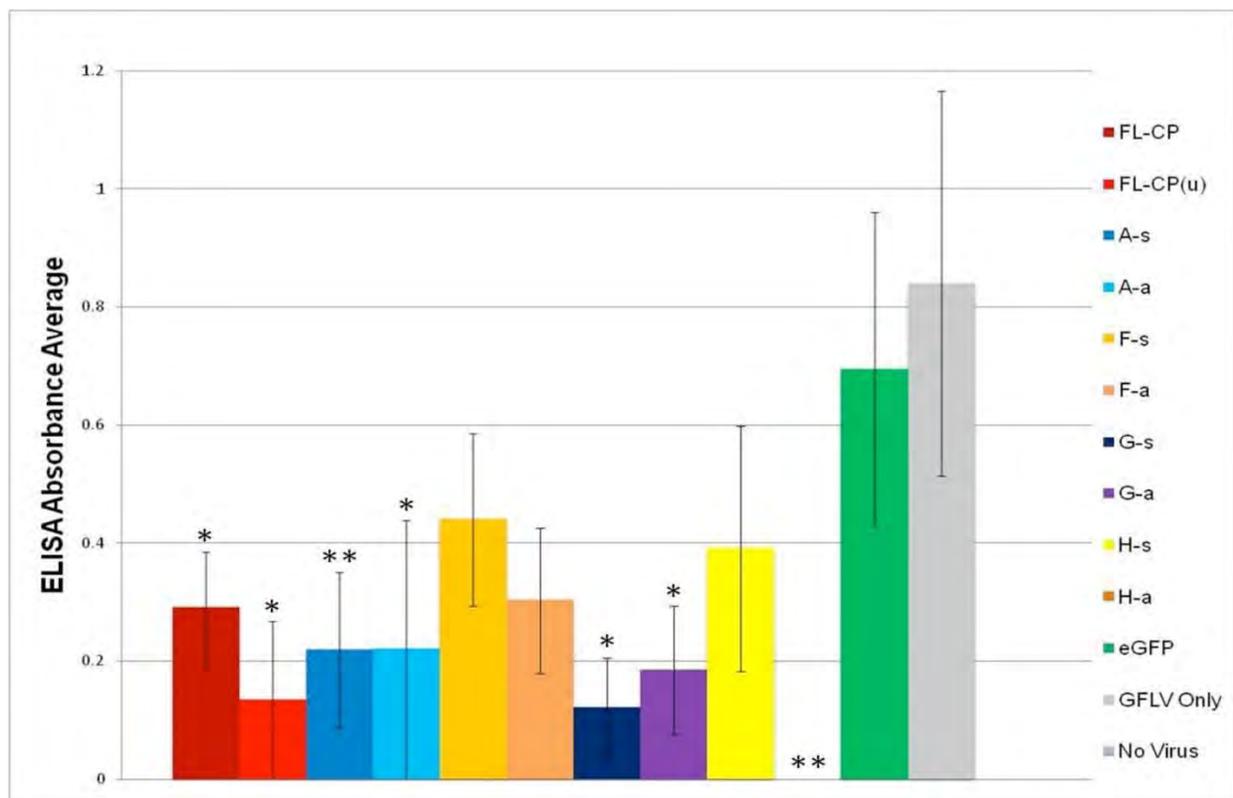
**Figure 2.** Expression of GFP from the jellyfish *Aequorea victoria* in transgenic *N. benthamiana* expressing GFP that were agroinoculated first with a chimeric tobacco rattle virus (TRV) containing GFP and then with different GFLV constructs. Measurements of GFP expression were taken at six days post-agroinoculation with GFLV constructs. P24: silencing suppressor of grapevine leafroll-associated virus 2 (GLRaV-2); 1AB: a fusion construction of GFLV 1A-1B<sup>Hcl</sup>; 1A; GFLV RNA-encoded 1A; 1B; GFLV RNA1-encoded 1B<sup>Hcl</sup>; 1E: GFLV RNA1-encoded RNA-dependent RNA polymerase; TRV-wt: wild-type TRV; wt: wild-type *N. benthamiana* expressing GFP; and control: unagroinoculated transgenic *N. benthamiana* expressing GFP. Measurements were taken with a fluorescence spectrophotometer (BioTeK, Winooski, VT, USA) using an excitation wavelength of 485 nm and an emission wavelength of 510 nm. Average values represent measurements from 5-10 plants per treatment. Error bars are shown.



**Figure 3.** Expression of GFP from the jellyfish *Aequorea victoria* in transgenic *N. benthamiana* expressing GFP that were agroinoculated first with a chimeric tobacco rattle virus (TRV) containing GFP and then with different GFLV constructs, including GFLV-F13 1A-1B<sup>Hcl</sup> fusion product, the GFLV-GHu 1A-1B<sup>Hcl</sup> fusion product, the GFLV-F13 1E<sup>Pol</sup>, the GFLV-F13 1A, and the GFLV-F13 1B<sup>Hcl</sup>. Controls were p24 of GLRaV-2, 16c agroinoculated with TRV-GFP, and wild-type 16c. Detached leaves of transgenic *N. benthamiana* expressing GFP were photographed under ultraviolet illumination.

Agroinfiltration was explored as a high-throughput and fast system for testing the capacity of RNAi constructs to interfere with GFLV multiplication following their transient expression. Infiltration was carried out using a needleless syringe in two lower true leaves per *N. benthamiana* plant, one of which received a control treatment [enhanced GFP (eGFP)] and the other of which received a GFLV RNAi construct. Other plants receiving eGFP treatments to both lower leaves were used for control comparisons. Experiments were repeated at least three times. Five days after lower leaves were agroinfiltrated, upper leaves of *N. benthamiana* plants were mechanically inoculated with GFLV using 1:50 dilutions of crude extracts of infected *N. benthamiana* leaves. Six days after mechanical inoculations with GFLV, leaf samples were collected and tested for GFLV accumulation by double antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) using specific antibodies. Thirteen days post-GFLV infection an additional leaf sample consisting of a single apical leaf was tested by DAS-ELISA to verify systemic infection.

Results suggested relatively reduced levels of GFLV accumulation in agroinfiltrated leaves receiving the RNAi construct, particularly RNAi constructs A, F, G, and H, versus those agroinfiltrated with *A. tumefaciens* containing an eGFP construct at six days post-inoculation (**Figure 4**). Plants that were not infiltrated with *A. tumefaciens*, but infected with GFLV, indicated the highest virus titers in all experiments. The next highest relative virus titers were observed in leaves receiving the eGFP control treatment, as expected. In contrast, several GFLV RNAi constructs, including A, F, G, and H, showed relatively lower virus titers versus control treatments. Particularly, construct H had potent anti-GFLV activity in repeated experiments (**Figure 4**).

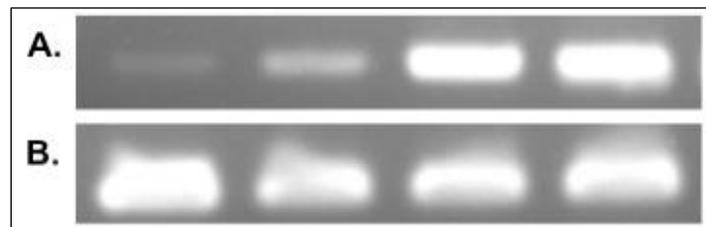


**Figure 4.** Relative GFLV titer measured by ELISA at six days post-inoculation in leaves agroinfiltrated with varied GFLV RNAi constructs. Absorbance value averages obtained across four experiments with five plants each are shown. Significant differences compared to control treatments are indicated \* ( $P < 0.05$ ) and \*\* ( $P < 0.01$ ).

Among the RNAi constructs tested so far, those with a consistent high anti-GFLV effect were H and G followed by A. Interestingly, RNAi construct H showed no detectable virus in any of the plants in all four experiments (**Figure 4**). The effect of RNAi construct F on GFLV accumulation was not significant. These results were consistent with the fact that some GFLV RNAi constructs suppressed virus accumulation in agroinfiltrated leaf patches. Nonetheless, GFLV was detected in apical leaves at 13 days post-inoculation, regardless of the level of

interference with GFLV accumulation in agroinfiltrated leaves. This suggested that expression of GFLV RNAi constructs should be stable in order to confer resistance.

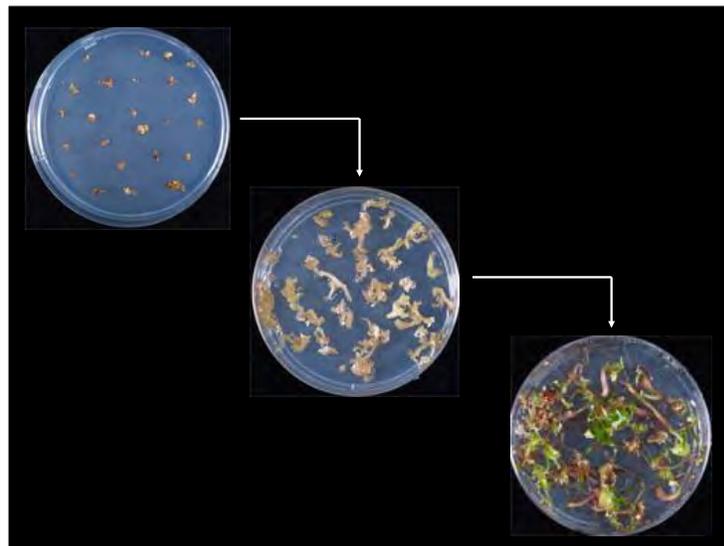
Semi-quantitative reverse transcription (RT) PCR was carried out on total RNA extracted from leaf disks of agroinfiltrated *N. benthamiana* leaves to further analyze the effect of RNAi constructs on GFLV accumulation. The ribulose 1,5-biphosphate carboxylase gene (*Rcb1*) was used as a housekeeping gene. A reduced GFLV RNA2 abundance was revealed in leaves that received RNAi constructs as compared to eGFP-infiltrated leaves from the same plant (**Figure 5**). These results confirmed the trend observed with the DAS-ELISA testing. It should be noted that primers used to detect GFLV were designed to bind within GFLV RNA2 in such a way that they did not yield a product in RT-PCR from the transgene constructs, allowing for specific detection of viral transcripts only. The transient assays will be further used to screen additional GFLV RNAi and hp RNAi constructs, particularly the RNAi 1A-1B<sup>Hel</sup> construct. In any event, agroinfiltration was validated as a high-throughput and fast system for testing the capacity of RNAi constructs to interfere with GFLV multiplication following their transient expression. These assays highlighted the potential of RNAi constructs H, G, and A at interfering with GFLV multiplication.



**Figure 5.** Semi-quantitative RT-PCR showing (A) lower relative GFLV RNA2 abundance in a *N. benthamiana* leaf agroinfiltrated with constructs A (two left lanes) versus a control infiltrated leaf at six days post-inoculation (two right lanes), (B) *Rcb1* internal RT-PCR control.

### Objective 3. Transfer Promising RNAi Constructs into Grapevine Rootstock Embryonic Calli and Develop Transgenic Clones

The goal of this objective is to transform embryogenic cultures with GFLV RNAi constructs and regenerate putative transgenic plants. Embryogenic cultures of rootstock genotypes 101-14 MGT, 3309C, 110R, and 5C were used for stable transformation experiments. GFLV RNAi constructs H, G, and 1A-1B<sup>Hel</sup> were transferred into rootstock embryogenic cultures (**Figure 6**).



**Figure 6.** Embryogenic calli of rootstock genotype 101-14 MGT following exposure to *A. tumefaciens* strain C58 containing GFLV RNAi construct H (left), elongating in the dark on a specific medium (middle), and regenerating into small plantlets (right).

Following transformation with *A. tumefaciens*, different degrees of elongation of embryogenic cultures were observed with the highest efficacy obtained with 101-14 MGT followed by 110R and 3309C. No elongation was observed yet for 5C. Additional transformation experiments of the four rootstocks are underway.

#### **Objective 4. Initiate Phenotyping of Transgenic RNAi Grapevine Rootstock Clones by Agroinfiltration with Infectious GFLV Constructs**

The goal of this objective is to characterize the insertion and expression of RNAi constructs in putative transgenic rootstocks, and agroinfiltrate transgenic plants with GFLV to identify resistant lines. This objective will be met once putative transgenic rootstocks are developed, established in soil in the greenhouse, and available for resistance screening. A few plants of the rootstock genotype 101-14 MGT were recently transferred to soil in the greenhouse (**Figure 7**).



**Figure 7.** Plant of a putative transgenic rootstock genotype 101-14 MGT established in soil in the greenhouse.

Once putative transgenic rootstocks are well established in the greenhouse, they will be characterized for transgene insertion and expression, and subsequently for resistance to GFLV.

#### **Objective 5. Disseminate Information to Stakeholders Through Presentations at Conventions and Workshops**

Research progress on the development of fanleaf-resistant rootstocks was disseminated to grape growers at various venues, as follows:

- Fuchs M. 2017. Viruses: Biology, ecology, and management. Sustainable Ag Expo, Nov. 13, San Luis Obispo, CA (participants = 250).
- Fuchs M. 2017. Innovations and insights in plant breeding. Cornell Center for Technology Licensing, Innovations in Food Systems: Feeding a Growing World. May 7, Ithaca, NY (participants = 100).
- Fuchs M. 2016. Genetically modified organisms. Finger Lakes Forum, Jan. 18, Geneva, NY (participants = 60).

Together, dissemination efforts on the research progress reached over 400 growers, extension educators, and service providers in California and New York.

#### **CONCLUSIONS**

Progress is made toward the development of grapevine rootstocks expressing RNAi constructs derived from conserved regions of the GFLV genome. Several RNAi constructs were engineered and stacked to facilitate durable and broad-spectrum resistance against vineyard GFLV populations. A few putative transgenic rootstocks were obtained and established in the greenhouse. Their evaluation for transgene insertion and expression as well

as resistance to GFLV will be the next important steps of our study. This research is anticipated to provide innovative solutions to manage grapevine fanleaf virus in diseased vineyards.

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## RESISTANCE TO GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3 AND THE GRAPE MEALYBUG

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### ABSTRACT

Grapevine leafroll-associated virus 3 (GLRaV-3) is the dominant virus causing leafroll disease, a devastating and widespread viral disease of grapevine. GLRaV-3 is primarily transmitted by the grape mealybug (*Pseudococcus maritimus*). Management of GLRaV-3 and grape mealybug remains challenging in diseased vineyards, essentially because there is no recognized host resistance. We are applying RNA interference (RNAi) to interfere with GLRaV-3 multiplication and down-regulate key genes involved in osmoregulation of the phloem sap diet of grape mealybug. The grape mealybug osmoregulatory genes aquaporin and sucrose were characterized by reverse transcription polymerase chain reaction (RT-PCR) using total RNA isolated from grape mealybugs and degenerate primers followed by sequencing. Corresponding sequences were used to engineer specific RNAi constructs. We also developed a specific RNAi construct against a grape mealybug gut double-stranded RNA (dsRNA) nuclease that was identified by RT-PCR using degenerate primers and total RNA. The stacking of these grape mealybug RNAi constructs is under way. In parallel, we engineered RNAi constructs against the coat protein, RNA-dependent RNA polymerase and silencing suppressor genes of GLRaV-3. Combinations of the GLRaV-3 RNAi will be stacked. The development of a transient assay based on detached leaves of *Vitis vinifera* Pixie to streamline the evaluation of the efficacy of varied RNAi constructs on grape mealybug mortality and GLRaV-3 multiplication is under way. In addition, stable transformation experiments with GLRaV-3 RNAi constructs were initiated. Research progress was communicated to grape growers at a winter convention.

### LAYPERSON SUMMARY

Six distinct viruses are associated with leafroll, a disease that is widespread in vineyards. Grapevine leafroll-associated virus 3 (GLRaV-3) is the dominant leafroll virus in diseased vineyards. This virus is primarily transmitted by mealybugs, which are sap-sucking insects and pests of grapes. The grape mealybug (*Pseudococcus maritimus*) is the most common vector of GLRaV-3. Since no source of resistance to GLRaV-3 or grape mealybug is known in cultivated or wild grape species, we are exploring RNA interference (RNAi) to achieve resistance in grapevine genotypes by activating an innate immune system against GLRaV-3 and by down-regulating key genes involved in osmoregulation of the sugar rich phloem sap diet of grape mealybug. We identified the grape mealybug osmoregulatory genes aquaporin and sucrose and engineered specific RNAi constructs. We also developed a specific RNAi construct against a grape mealybug gut double-stranded RNA (dsRNA) nuclease. In parallel, we engineered RNAi constructs against the coat protein, RNA-dependent RNA polymerase and silencing suppressor genes of GLRaV-3. Combinations of these RNAi will be stacked and their effect on grape mealybug mortality and GLRaV-3 multiplication will be evaluated first in transient assays, and subsequently in stable transformants. Research progress was communicated to grape growers at a winter convention.

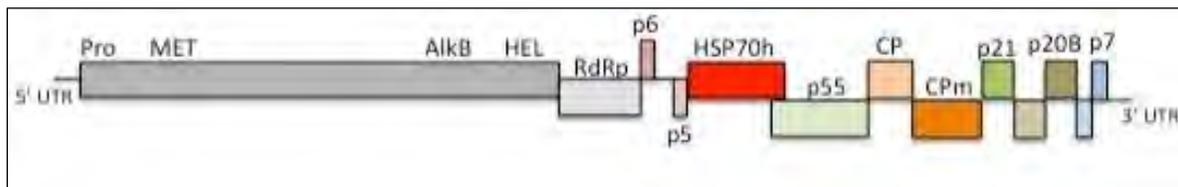
### INTRODUCTION

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. Leafroll can affect *Vitis vinifera*, *V. labrusca*, interspecific hybrids,

and rootstocks (Naidu et al. 2014). The economic cost of leafroll is estimated to range from \$12,000 to \$92,000 per acre in California (Ricketts et al. 2015) and from \$10,000 to \$16,000 in New York (Atallah et al. 2012).

Six viruses named grapevine leafroll-associated viruses (GLRaVs), e.g. GLRaV-1, -2, -3, -4, -7, and -13 have been identified in diseased vines (Fuchs et al. 2017, Naidu et al. 2014). These viruses belong to the genera *Ampelovirus* (GLRaV-1, -3, -4 and -13), *Closterovirus* (GLRaV-2), and *Velarivirus* (GLRaV-7) in the family *Closteroviridae*. GLRaV-1, -3 and -4 are transmitted by mealybugs while no vector is known for GLRaV-2 and -7. GLRaVs are phloem-limited and GLRaV-3 is the dominant leafroll virus in vineyards, including in California (Naidu et al. 2014).

The genome of GLRaV-3 consists of 12 open reading frames (ORFs) (**Figure 1**). It encodes a characteristic core of replication-associated genes, referred to as the replication gene block (RGB), at the 5' terminal portion of the genome and a more variable array of genes encoding structural and other proteins downstream of the RGB toward the 3' terminus (Naidu et al. 2015). The RGB proteins are expressed directly from the virion RNA and other proteins are expressed from a nested set of the 3' co-terminal subgenomic RNAs. The last set of ORFs includes proteins involved in suppression of host RNA silencing, in particular p19.7 or p20B (Gouveia et al. 2012). Distinct genetic variants of GLRaV-3 have been identified in diseased grapevines. They are referred to as genetic variant groups I to VI; they often exist in mixed infections although the biological significance of their genetic variability is unknown (Maree et al. 2013, Naidu et al. 2015).



**Figure 1.** Schematic representation of the genome organization for GLRaV-3. Blocks represent predicted ORFs. The replicase protein is shown in grey with the papain-like protease (Pro), methyltransferase (Met), alkB domain (AlkB) helicase (HEL), and RNA-dependent RNA polymerase (RdRp). Small transmembrane proteins (p6 and p5) are shown in pink, the heat shock protein 70 homolog (HSP70h) in red, the coat protein (CP) in salmon, and the minor coat protein (CPm) in orange. The silencing suppressor p19.7 or p20B is shown in light brown.

The transmission of GLRaV-3 by mealybugs is semi-persistent, with acquisition and inoculation occurring within one-hour access period of feeding by immature stages (Almeida et al. 2013). A single mealybug is sufficient to transmit the virus and initiate infection (Naidu et al. 2014). 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 all mealybug species may disseminate all transmissible strains of GLRaV-1, -3 and -4, and likely GLRaV-13.

Mealybugs are sap-sucking insects in the family Pseudococcidae. They are pests of grapes and many other important crops. At high densities, mealybugs can cause complete crop loss, rejection of fruit loads at wineries, and death of spurs, although small infestations may not inflict significant direct damage. In the feeding process on plant sap, mealybugs excrete honeydew that often becomes covered with a black sooty mold, which additionally damages fruit clusters. Several mealybug species feed on vines but the grape mealybug (*Pseudococcus maritimus*) is the most abundant and widespread in U.S. vineyards (Almeida et al. 2013).

Unassisted, mealybugs have limited mobility, but first instar immature mealybugs (crawlers) can be dispersed over long distances by wind and other means (Almeida et al. 2013). The grape mealybug is the most common vector of GLRaV-3 in diseased vineyards.

Current leafroll disease management options are essentially preventive and based on the use of planting material derived from clean, virus-tested certified stocks. In vineyards where infected vines are present, management strategies rely on the elimination of virus-infected vines and the reduction of mealybug populations to limit

vector-mediated spread through the application of systemic insecticides, e.g. spirotetramat. The level of mealybug control needed to limit virus spread is not known, although encouraging results were recently reported (Wallingford et al. 2015).

Management of leafroll viruses and their mealybug vectors remains challenging due to a lack of recognized host resistance (Oliver and Fuchs 2011). Innovative technologies to breed resistant grapevine material are needed to complement current strategies and address their limitations. Resistance can be achieved by applying RNA interference (RNAi), a relatively new paradigm for crop protection from pathogens and arthropod pests. The approach relies on the development of RNAi constructs targeting specific pathogen or insect genes and their use to specifically down-regulate the expression of the target genes in plants. The RNAi approach is highly specific and anticipated to reduce hazards of chemical pesticides. Conserved regions in the viral genome, including p19.7, the viral silencing suppressor, would be ideal for targeted RNAi-based control of GLRaV-3. Silencing the expression of p19.7 would enable the antiviral pathways of RNA silencing to be highly active against the virus.

The fact that mealybugs transmit leafroll viruses offers an opportunity to explore a two-pronged approach to simultaneously target the virus and its vector. Our research is to develop grapevines resistant to GLRaV-3 and the grape mealybug using RNAi. Our strategy is to combine RNAi against targets of the virus and the insect vector, 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. The overarching goal of this research is to explore innovative approaches to develop GLRaV-3- and grape mealybug-resistant grapevine genotypes by activating an innate immune system against the virus and by down-regulating key genes involved in osmoregulation of the sugar rich phloem sap diet of grape mealybug.

## OBJECTIVES

There are four specific research objectives:

1. Optimize RNAi constructs against grape mealybug.
2. Develop a high throughput transient expression system to test the efficacy of RNAi constructs.
3. Characterize stably transformed RNAi grapevines.
4. Disseminate information to stakeholders through presentations at conventions and workshops.

## RESULTS AND DISCUSSION

### Objective 1. Optimize RNAi Constructs Against Grape Mealybug

Perturbing the expression of osmoregulatory genes required for water balance, specifically aquaporin and sucrose genes, in the gut of phloem-feeding insects causes the insects to lose water from the body fluids and dehydrate, dying within two to three days (Karley et al. 2005, Shakesby et al. 2009, Tzin et al. 2015). Genes coding for aquaporin and sucrose are key osmoregulatory genes in the gut of phloem-feeding insects. Amplicons of aquaporin (*AQPI*) and sucrose (*SUCI*) genes have been obtained by reverse transcription polymerase chain reaction (RT-PCR) using total RNAs isolated from grape mealybug crawlers and degenerate primers. Amplicons of the expected size were successfully obtained for *AQPI* and *SUCI* and their nature was validated by sequencing. The sequence of *AQPI* and *SUCI* was used to design RNAi constructs.

Non-specific nucleases are expressed in the gut of insects and are known to degrade ingested double-stranded RNA (dsRNA) (Arimatsu et al. 2007, Christiaens et al. 2014, Luo et al. 2013). To further improve RNAi efficacy against *AQPI* and *SUCI*, RNAi constructs targeting dsRNA nucleases (*NUC*) are considered to improve grape mealybug mortality. This is because we hypothesize that delivery of *NUC* RNAi in combination *AQPI* and *SUCI* RNAi will result in higher killing of grape mealybugs, as shown previously for psyllid and whitefly pests (Luo et al. 2017, Tzin et al. 2015). A non-specific grape mealybug dsRNA nuclease was identified by RT-PCR with degenerate primers designed from databases and total RNA isolated from grape mealybugs. Amplicons of the expected size were obtained and validated by sequencing. RNAi constructs against *NUC* were developed. The stacking of RNAi *AQPI*, *SUCI*, and *NUC* constructs is under way with the objective to co-target two molecular functions with linked physiological function. Subsequently, these constructs will be cloned in a binary plasmid for expression *in planta*. We will engineer two types of constructs: the first will use the general promoter from cauliflower mosaic virus (CaMV) 35S; and the second will use the phloem-specific *sucrose-H<sup>+</sup> symporter* (*SUC2*) promoter, to target RNAi expression in the preferred feeding sites of grape mealybug.

## **Objective 2. Develop a High Throughput Transient Expression System to Test the Efficacy of RNAi Constructs Against Grape Mealybug or GLRaV-3**

Several RNAi against GLRaV-3 and grape mealybug as well as stacked RNAi against grape mealybug and GLRaV-3 will be developed. Handling numerous RNAi constructs in stable transformation can be challenging. Therefore, we develop a transient expression system to test the efficacy of RNAi constructs. This high throughput approach should help streamline the identification of the most promising RNAi constructs for stable transformation of grapevine genotypes.

Agroinfiltration assisted by vacuum was initially considered to deliver RNAi constructs to tissue culture -grown grapevines for transient expression. Preliminary experiments with tissue culture-grown grapevine plants indicated that feeding and survival of grape mealybugs on such material that was kept in sterile containers in a growth chamber was unexpectedly very low. Therefore, tissue culture-grown grapevines are not adapted to transient assays for evaluating their efficacy of RNAi. Therefore, we decided to develop a transient assay based on detached leaves of Pixie grapes. Pixie is a dwarf grape derived from *Vitis vinifera* cv. Pinot Meunier. It is used to maintain a colony of grape mealybugs that was established from populations collected in New York vineyards. To test the idea of a bioassay based on detached Pixie tissue, leaves and petioles were dissected and placed in microfuge tubes containing a food dye (**Figure 2**).



**Figure 2.** Development of a bioassay to test the efficacy of RNAi using detached leaves of the Pixie grape. The picture was taken 18 hours after exposure.

Red pigment was visible in the veins of treated Pixie leaves within one hour and more pigment continued to disperse in subsequent hours. This suggested that delivering RNAi to grape tissue via simple absorption is technically doable. Validation experiments with RNAi are under way. In addition, grape mealybug crawlers were feeding on detached leaves and their survival was minimal when deposited on detached leaves. These results are encouraging for the optimizing of a transient bioassay based on detached Pixie leaves for determining the efficacy of RNAi constructs against grape mealybug and GLRaV-3. This work will be continued because it is critical to develop a highly needed high throughput transient assay.

## **Objective 3. Characterize Stably Transformed RNAi Grapevines**

RNAi constructs against GLRaV-3 *CP* and *VSR* were used in stable transformation experiments via *Agrobacterium tumefaciens*-mediated transfer to embryogenic cultures of rootstock 1014 MGT and *V. vinifera* cv. Cabernet franc. Transformed embryogenic cultures are maintained in a growth chamber at 28°C in the dark for elongation and subsequent regeneration into plantlets.

#### Objective 4. Disseminate Information to Stakeholders Through Online Resources and Presentations at Conventions

Research findings were communicated at a winter growers convention (Fuchs M. 2017. Viruses: Biology, ecology, and management. Sustainable Ag Expo, Nov. 13, San Luis Obispo, CA (participants = 550).

#### CONCLUSIONS

The osmoregulatory *AQP1* and *SUC1* were characterized by RT-PCR using total RNA isolated from whole grape mealybug specimens and by sequencing. Sequences were used to engineer specific RNAi constructs. A dsRNA nuclease *NUC* was also characterized by RT-PCR using total grape mealybug RNA and sequencing, and a specific RNAi construct was developed. The stacking of these grape mealybug RNAi constructs is under way. In parallel, we engineered RNAi constructs against *CP*, *POL*, and *VSR* of GLRaV-3. Combinations of the GLRaV-3 RNAi will be stacked. We are developing and optimizing a transient assays to test the efficacy of varied RNAi constructs on grape mealybug mortality and GLRaV-3 multiplication based on detached leaves of *V. vinifera* Pixie. This assay is critical to streamline the evaluation of RNAi constructs. Finally, stable transformation experiments with GLRaV-3 RNAi constructs are under way.

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## TIMING OF FIELD TRANSMISSION OF GRAPEVINE RED BLOTCH VIRUS

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**Reporting Period:** The results reported here are from work conducted July 1, 2016 through November 7, 2017.

### ABSTRACT

The goal of this project is to determine when grapevine red blotch virus (GRBV) is spreading in the vineyard. Knowing when the virus is spreading will provide important information on effective management of GRBaV and help focus the efforts to identify additional vectors. This information will also help target control measures to times of the season when the virus is being transmitted in the field. Three vineyards where GRBV has been spreading are being used in this study. One vineyard has an adjacent riparian zone, with most virus spread occurring near the edge of the vineyard nearest the riparian zone. In this case the trap plants are placed in a grassy area between the riparian zone and the vineyard. The second vineyard has an alfalfa field adjacent to it, and since the one vector reported to transmit the virus is the three-cornered alfalfa hopper (*Spissistilus festinus*), the plants were placed perpendicular to the alfalfa field and within vineyard rows. The third vineyard has most spread adjacent to a recently disturbed wooded area. In each vineyard, every plant has a unique number and the location of each plant is being mapped so that where virus spread occurs in each vineyard can be determined. Fifteen plants are placed in each vineyard each month starting April 15, 2016 through September 15, 2016. After one month in the field the plants are returned to Corvallis, treated with a systemic insecticide, and maintained in a screenhouse. All 300 plants will be tested for GRBV in late October 2016 and then the plants will be overwintered and retested in the spring of 2017 and 2018.

### LAYPERSON SUMMARY

The goal of this project is to determine when grapevine red blotch virus (GRBV) is spreading in the vineyard. Knowing when the virus is spreading will provide important information on effective management of GRBV and help focus the efforts to identify additional vectors. This information will also help target control measures to times of the season when the virus is being transmitted in the field. Three vineyards where GRBV has been spreading were used in 2016 and four vineyards are being used in 2017. One vineyard has an adjacent riparian zone, with most virus spread occurring near the edge of the vineyard nearest the riparian zone. In this case the trap plants are placed in a grassy area between the riparian zone and the vineyard. The second vineyard has an alfalfa field adjacent to it, and since the one vector reported to transmit the virus is the three-cornered alfalfa hopper (*Spissistilus festinus*), the plants were placed perpendicular to the alfalfa field and within vineyard rows. This vineyard was removed after the 2016 season, and another nearby vineyard with GRBV was substituted for the 2017 field trials. The third vineyard has most spread adjacent to a recently disturbed wooded area. In 2017 a fourth vineyard was added to the study, adjacent to a grassy-wooded area, where GRBV movement has been observed. In each vineyard every plant has a unique number and the location of each plant is being mapped so that where virus spread occurs in each vineyard can be determined. Fifteen plants are placed in each vineyard each month starting April 15, 2016 and continuing through September 15, 2016, and starting May 2, 2017 and continuing until October 2017. After one month in the field the plants are returned to Corvallis, treated with a systemic insecticide, and maintained in a screenhouse. All 300 plants were tested for GRBV in November 2016 and were negative for GRBV in polymerase chain reaction (PCR) testing. After overwintering a set of 90 plants that represented trap plants for the 2016 growing season were tested by PCR in May 2017. Again, all plants were negative for GRBV. The entire set of 300 plants will be tested in September 2017 and again in September 2018. The plants from the 2017 trial will be tested in 2018 and 2019.

### INTRODUCTION

In 2012 a new virus was identified in Cabernet Franc plants in New York's Finger Lakes region and also in Cabernet Sauvignon plants in the Napa Valley. These plants exhibited leafroll-like symptoms but tested negative for leafroll viruses. At a meeting of the International Committee on the Study of Viruses and Virus-like Diseases

of Grapevine in October 2012, the name grapevine red blotch-associated virus (GRBaV) was agreed upon for this new virus. The name was changed to grapevine red blotch virus (GRBV) in spring 2017.

This research aims to determine when GRBV is spreading in the field. So far the three-cornered alfalfa hopper (*Spissistilus festinus*) has been shown to transmit GRBV, but this vector is very minor in many vineyards where the virus is spreading. Movement of GRBV in vineyards after planting has been documented and can be quite rapid, which clearly indicates the presence of an efficient vector or a vector that is present in very high numbers. An increase in the incidence of GRBV over time in young, healthy vineyards that are adjacent to infected vineyards also suggests the existence of a vector. There has been much work done on trying to identify the vector(s) of GRBV. Efforts looking at suspected vectors in California have resulted in the identification in early 2016 of the three-cornered alfalfa hopper as a vector. Regardless of whether this is the only vector or one of multiple vectors, the timing of transmission will be important information in developing a vector management plan.

If we know when the virus moves, efforts at vector control can be targeted to a specific timeframe rather than throughout the growing season. Also, knowing when the virus is moving in the vineyards will help focus on transient insects which may be present in vineyards for only a short period of time, or insects that feed on grapevines but have other preferred hosts. In either case these vectors could escape detection and identification in standard insect surveys. If transmission is more efficient in riparian areas adjacent to vineyards it will provide clues as to where one should look to identify potential vectors.

This project was started in March 2016 using in-house (USDA Agricultural Research Service) funds to ensure we could get the first year of field work done in 2016. Funding from the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board became available July 1, 2016 and is being used for the remainder of the project. Three hundred grapevines (Merlot on 3309 rootstock) were obtained via donation from Duarte nursery, repotted into three-gallon pots, and held in a screenhouse until used in the field, or held in a canyard near Corvallis isolated from any vineyards. Plants were tested for GRBV prior to use in the field experiment and all plants tested negative for GRBV in polymerase chain reaction (PCR) assays using two sets of primers. Beginning April 15, 2016, plants were placed in each of three vineyards for a one-month period (45 plants each month total). Then in mid-May 2016 these plants were returned to Corvallis, treated with a systemic insecticide, and stored in a screenhouse. The second set of plants was taken to the vineyards in mid-May 2016, and the process was repeated each month through September 2016. The last set of plants was returned to the greenhouse in Corvallis in mid-October 2016. There are a total of six sets of plants in each vineyard for a total of 270 trap plants, with an additional 30 plants that have not been taken to a vineyard and remained in the screenhouse or canyard during the summer. After the last set of plants was collected all 300 plants were tested for GRBV in November 2016. A subset of the plants were tested in May of 2017 and all will be tested in September 2017 and September 2018. The trap plants for the 2017 study will be tested in the fall of 2018 and 2019. In 2017 four vineyards are being used in the study: two in southern Oregon and two in the Willamette Valley. Again, 15 plants are being used per vineyard per month.

## **OBJECTIVES**

1. Determine the timing of field transmission of GRBV.

## **RESULTS AND DISCUSSION**

Three hundred plants were provided by Duarte Nursery for this work in 2016, AND 450 plants were provided in 2017. All plants were tested for GRBV prior to the start of the experiment in 2016 and a subset of the plants was tested for the trial prior to potting in 2017. Plants were potted in three-gallon pots and maintained in a canyard prior to taking them to the field. When plants were brought back to Corvallis from the fields they were treated with a systemic insecticide and maintained in a screenhouse.

The three vineyards were selected because of documented spread of GRBV in these vineyards in previous years. Vineyard #1 was near Jacksonville in southern Oregon and has a small riparian area adjacent to the east edge of the vineyard. The trap plants were placed in a grassy area between the riparian zone and the vineyard. Vineyard #2 was near Medford in southern Oregon, with the trap plants placed within the vineyard between every third plant in three rows near the west edge of the vineyard. There was an alfalfa field along the west edge of the

vineyard. This vineyard was removed after the 2016 season, and the second vineyard used in southern Oregon in 2017 was also near Medford, Oregon, with documented spread of GRBV. The third vineyard is in the Willamette Valley near Yamhill, Oregon. In this vineyard the spread is occurring throughout the vineyard, with high rates of spread along the east edge of the vineyard where there has been recent removal of adjacent woodlands. In this case the trap plants were placed between plants in a single row of the vineyard near the edge of where symptoms were observed. A fourth vineyard was added in 2017, another vineyard in the Willamette Valley, with spread of GRBV based on discussions with the grower.

Each plant was numbered (1-300 in 2016, and 1-400 in 2017) and the location of each plant and the month it was in the vineyard has been recorded. Thus, if GRBV spread is happening from the alfalfa field, we will know which plants were nearest the source as well as which month the plants were in the field and exposed to potential GRBV transmission.

All plants were tested for GRBV in November 2016 by PCR and all were negative for GRBV. A subset of 90 plants representing one vineyard in southern Oregon was tested in May 2017 and all were negative for GRBV. All plants from 2016 were tested in October 2017 and all were negative for GRBV. The last set of plants from the 2017 field experiments were brought back from the fields in mid-October. A subset of the 2017 plants (25% of the plants from the field) were tested the first week of November 2017, and all were negative for GRBV. In all cases the nucleic acid extracts were tested for the amplification of a plant gene to ensure the quality of the nucleic acid was such that it did not inhibit the enzymatic reactions of the PCR testing. All samples tested positive for the plant gene. Based on recent work from Marc Fuchs' lab at Cornell University showing the unreliability of testing for GRBV until two years after infection, the plan is to keep these plants for two full years after coming back from the field. The plants from 2016 and 2017 will be tested in the spring and fall of 2018 and 2019.

The experimental setup went according to plan and plant rotation went smoothly. We had feeding damage similar to that observed with three-cornered alfalfa hopper in one vine during the course of exposure in the vineyards. We placed sticky cards in the vineyard in the Willamette Valley and did not catch any three-cornered alfalfa hoppers. Recent work by entomologists Frank Zalom (University of California, Davis) and Vaughn Walton (Oregon State University) suggests that sticky cards are not effective for monitoring membracid insects. The entomologists will be doing the insect monitoring in 2017. Based on recent information from Marc Fuchs (May 2017 GRBV workshop in Davis, CA) it appears that detection of GRBV is very unreliable for the first two years after a plant is infected. Thus, the plan now is to maintain the trap plants for two full years after the end of the field part of the study and test them after one and two years.

The entomologists working on membracids in Oregon (Vaughn Walton and Rick Hilton) did catch several species of membracids in Oregon vineyards in 2016 and 2017, and feeding damage has been observed in the fields where we had our trap plants in 2017. Work on transmission by the membracid species identified from Oregon vineyards is ongoing by Vaughn Walton's group at Oregon State University.

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## **EDUCATION AND OUTREACH FOR THE GRAPEVINE CERTIFICATION AND REGISTRATION PROGRAM, AND AN ASSESSMENT OF RECENTLY ESTABLISHED PRODUCTION VINES FROM INCREASE BLOCKS**

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

### **ABSTRACT / LAYPERSON SUMMARY**

Grapevine viruses and other internal pathogens have been related to vineyard problems long before we ever knew they were there. Many issues troubling growers in the 1930s were later attributed to Pierce's Disease, fanleaf, and leafroll (Bioletti 1931, Matthews 2012). Likely due to the immediate destructive nature of Pierce's Disease as well as extensive outreach programs, growers in citrus and grapes combined their efforts to facilitate regional control of the vectors spreading the disease and the pathogen responsible for the disease decades ago. This type of effort has only recently been supported by industry for virus-related issues like leafroll. For many years viruses were perceived by growers as non-problematic. This false perception is likely attributed to the fact that many vineyards were previously established on rootstocks like AXR#1 and St. George (Wolpert et al. 1994), both of which are associated to the reduction of virus symptom expression (Golino 1993). After the failure of AXR#1, alternative rootstocks with varying levels of disease tolerance were grafted onto infected budwood from existing fields which led to many virus-related issues. It has taken decades since this turn in material to help growers understand the problems associated to certain viruses in vineyards in part due to the fact that virus symptoms are variable depending upon the season and different viruses cause different symptoms. Additionally, leafroll, a virus which reduces yield and limits sugar accumulation in the berry, easily spreads from one vineyard block to the next via its primary vector, common mealybugs. Decades after the failure of AXR#1, a pilot workgroup began in Napa with the intentions of managing leafroll regionally due to the rigorous efforts of our team (those mentioned in the heading as well as Monica Cooper, the farm advisor of Napa County). After five years of monthly meetings where growers shared the challenges and successes of their endeavors, growers in Napa feel they have leafroll under control. With the consistent extension and outreach explaining these work groups, growers across California have grown interested in replicating these efforts in their region. The overall intention of this project is to provide this opportunity to all grape/wine grape growing regions in California so that in the future, our investment in certified, virus tested material does not end at establishment. Additionally virus survey work will be completed in order to update protocols performed by the program.

### **INTRODUCTION**

Certified grapevine nursery stock consumers (grape producers) are concerned that the quality of the product they are purchasing from the clean plant program does not meet the standard they believe it should. Much of this concern stems from the expectation that certification offers something greater, in terms of freedom from virus contamination, than it scientifically can. With the discovery that grapevine leafroll-associated virus 3 is spreading in California, in addition to the discovery of grapevine red blotch-associated virus (Al Rwahnih et al. 2013, Golino et al. 2008), grape producers question the quality of certified vines. There is good evidence that clean plant programs work and that they have large economic benefits that can be shared by all actors in the supply chain (Fuller et al. 2015), but, as with all supply chains, in order for clean plant programs to work well, they require mutual trust between the actors in the chain. By defining the term "certified" according to the scientific sampling procedure and educating growers of the meaning of this term, we can bridge the current gap in perceptions that exists between the clean plant system and the purchasers of its products. However, because some viruses can be spread, unless a complete census of all certified vines was carried out every year, it is impossible for any certification program to reduce virus incidence to zero. The meaning of the term "certified" must be defined in relation to the statistical performance of the actual sampling plan used. In order for grower trust in the system to build, that meaning must be clearly articulated and appropriate expectations established for disease incidence in planting material emerging from a program using the definition. Additionally, it is unclear at this time what level of background infection per year occurs in nursery increase blocks, as well as a lack of understanding of potential

reinfection of increase blocks between sampling rotations. The intentions of this project are to provide quantifiable outreach and extension involving the certification program while addressing the background infection in nursery increase blocks and the potential reinfection in increase blocks between sampling bouts.

## **OBJECTIVES**

1. Develop a grower information pack and slide presentation to summarize the Grape Certification and Registration Program.
2. Hold grower meetings in key grape-growing regions of California to explain the functioning and efficacy and limitations of the certification program.
3. Quantify the impact of education and outreach by issuing pre-test and post-test surveys at grower meetings.
4. Assess the level of potential contamination or reinfection in newly established vineyard blocks when material is sourced from increase blocks.
5. Assess the level of reinfection of leafroll-3 and red blotch viruses in increase blocks between certification sampling bouts.

## **RESULTS AND DISCUSSION**

Since the project's initiation in October of 2016, efforts have been made by the above cooperators and the principal investigator to collaborate with farm advisors and industry related personnel across California. Meetings and presentations have been provided in order to notify the public of the potential for grower work group meetings in various parts of the state including the foothills, Bakersfield, Fresno, Paso Robles, Tulare, Lodi, San Diego, and Davis, California. Work group meetings have been scheduled and accomplished in Bakersfield and Fresno and field days have been provided in Mendocino and Carneros. Additionally we participated in the Calaveras Wine Alliance Vineyard Tour in order to spread the word about education, outreach, and vineyard sampling provided by this program. Collaboration with Mark Battany in the Central Coast is underway. Additionally, requests for vineyard blocks which are to be sampled for red blotch and leafroll have been made and contact with Joshua Kress has been established in order to analyze data provided by the certification program. Because the project recently began in October, there are no results to discuss at this time.

### **Objective 1. Develop a Grower Information Pack and Slide Presentation to Summarize the Grape Certification and Registration Program**

Multiple slide presentations have been produced and presented in numerous parts of the state, including Bakersfield, Fresno, Paso Robles, Tulare, Lodi, San Diego, Davis, and Calaveras, California.

### **Objective 2. Hold Grower Meetings in Key Grape-Growing Regions of California to Explain the Functioning and Efficacy and Limitations of the Certification Program**

Work group meetings were held in Bakersfield, Fresno, Mendocino, Carneros, and Calaveras.

### **Objective 3. Quantify the Impact of Education and Outreach by Issuing Pre-Test and Post-Test Surveys at Grower Meetings**

While discussing collaborative projects with Lynn Wunderlich, the farm advisor for Central Sierra Cooperative Extension, Lynn mentioned previous education and outreach presentations provided by Katherine Webb-Martinez, the current Associate Director of Program Planning and Evaluation in the UC Division of Agriculture and Natural Resources. Lynn and I contacted Katherine for more information on quantifying the impact of education and outreach. Her advice provided us the opportunity to more appropriately plan to assess impacts by way of a combination of retrospective pre-tests and post-tests. We are currently guiding our questions for the survey in that direction.

### **Objective 4. Assess the Level of Potential Contamination or Reinfection in Newly Established Vineyard Blocks When Material Is Sourced from Increase Blocks**

Samples have been collected from multiple vineyard locations in Mendocino, Bakersfield, Fresno, Calaveras, and Carneros.

## Objective 5. Assess the Level of Reinfection of Leafroll-3 and Red Blotch Viruses in Increase Blocks Between Certification Sampling Bouts

Joshua Kress at the California Department of Food and Agriculture has been contacted in order to access the diagnostic information when it becomes available.

### Publications and Presentations

- Arnold KL, Golino D, McRoberts N. 2016. A synoptic analysis of the temporal and spatial aspects of grapevine leafroll disease in an historic Napa vineyard and experimental vine blocks. *Phytopathology*. <http://dx.doi.org/10.1094/PHYTO-06-16-0235-R>.
- Arnold, KL, McRoberts N, Golino DA. North coast virus survey reveals improving health of vineyards over decades. *California Agriculture* (in press).
- Virus Workshop -- Utilized as a backbone to the workshop discussions for both the Bakersfield and Fresno groups on May 16 and 17. Both meetings were well attended and discussion ensued ranging from basic information to in depth management decisions. Attendees expressed their appreciation for the direction and atmosphere provided.
- Working with Work Groups -- Presented at the Red Leaf Disease Research Review Board meeting for PD/GWSS funding in Davis, California.
- Grapevine Certification: Viruses in Grapevines -- Presented at the Calaveras Winegrape Alliance educational meeting in Murphys, California.
- Attended Vineyard Tour in Calaveras in order to answer questions involving certification.
- *Viruses in Grapevines* -- Handout provided at field days in Mendocino, Calaveras, and Carneros (**Figure 1**).

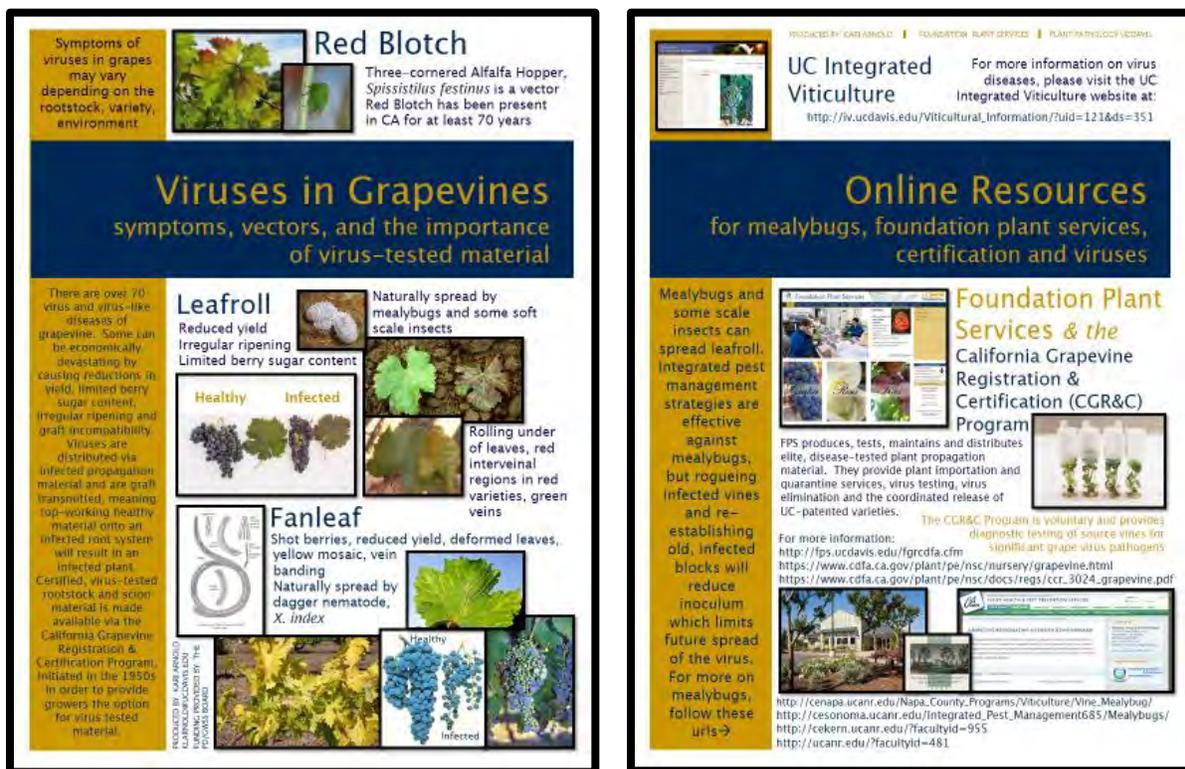


Figure 1. Handout provided at field days in Mendocino, Calaveras, and Carneros.

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#### **FUNDING AGENCIES**

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

## IDENTIFICATION OF GRAPE CULTIVARS AND ROOTSTOCKS WITH RESISTANCE TO VINE MEALYBUG

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### ABSTRACT

Vine mealybug (*Planococcus ficus*), an insect pest capable of causing direct and indirect damage to grape vineyards, costs California growers millions of dollars annually. Insecticide sprays used to manage the pest provide inconsistent results, and sustainable methods of control are needed. A previous study identified a single grape accession with resistance to other mealybug species, but did not evaluate vine mealybug. This work aims to evaluate the susceptibility of grape cultivars, rootstocks and species to vine mealybug.

### LAYPERSON SUMMARY

Vine mealybug (*Planococcus ficus*) is quickly becoming a major pest to the California grape industry. Growers spend an estimated \$123 to \$500 per acre each year to manage mealybugs, with losses still being observed. Insecticide sprays often provide inconsistent control due to problems associated with spray timing and poor contact with the insect. As concerns about the development of insecticide resistance increase, alternate systems for controlling mealybug are essential. Naturally resistant grape cultivars, though necessary, 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 insecticide applications. A single source of resistance to at least one species of mealybug has been identified in lab tests, but has not been tested against vine mealybug. Further work to identify new sources and test existing sources of resistance against vine mealybug in the lab and field is needed. This work will identify and evaluate grape material with natural resistance to vine mealybug in the lab and field for use as rootstocks and cultivar development. These materials will be made available to nurseries, researchers, and grape breeders.

### INTRODUCTION

Mealybugs are soft-bodied, sap-sucking insect pests of grapevines and other plants. Besides the direct losses attributed to damaged leaves and fruit in grape, mealybugs can transmit the economically important grapevine leafroll-associated virus (GLRaV). It is estimated that grapevine leafroll disease control costs growers \$12,106 to \$91,623 per acre annually in California (Ricketts et al. 2015). Of that expenditure mealybug control costs are estimated at \$50 per acre in vineyards with small mealybug populations and many natural predators, to \$500 per acre for vineyards with moderate populations and few parasitoids (Ricketts et al. 2015). Vine mealybug (*Planococcus ficus*) is one of six mealybug species that threaten the California grape industry. This introduced (ca. 1994) pest can rapidly reproduce and spread, outcompeting other mealybug species and making it the most important mealybug pest of grapes in California (Daane et al. 2012).

Vine mealybug development is temperature dependent, and the insect can complete its life-cycle during winter months if days are warm (**Figure 1**). This season-independent development leads to high population numbers, which has contributed to the difficulty of controlling this insect. For vine mealybug, up to seven generations per year have been observed in California vineyards compared to the two observed in grape mealybug (Geiger and Daane 2001, Gutierrez et al. 2008). Females reach maturity as soon as 30 days from egg, and once mature can produce 50 to 800 viable offspring, depending on nutrient availability (Waterworth et al. 2011, Berning et al. 2014). Even using a low estimate of 50 viable offspring, a single mealybug could produce millions of individuals over the course of a growing season.

Insecticides are the main form of control. Mating disruption and parasitoids have been implemented with success in vineyards, however, these forms of control are more expensive (Daane et al. 2007, Mansour et al. 2011, *UC IPM Pest Management Guidelines: Grape*). Optimization of insecticide control strategies (application timing and

efficacy) have garnered much attention. However, the vine mealybug spends much of its life and development on the roots and under the bark, protecting it from chemical sprays (Daane et al. 2012). This makes contact insecticides often ineffective, and systemic insecticides difficult to time. An effective complement to insecticides is the use of resistant grapes. Resistant grapes, and specifically resistant rootstocks, could directly reduce mealybug populations developing or overwintering under the bark and on roots in the vineyard.

Few sources of natural resistance to mealybug have been identified in grape. In Brazil, one study identified a single rootstock with lab-based resistance to mealybug (Filho et al. 2008, Figure 2). 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, were 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. While early work has shown that these two sources are likely to be resistant to mealybugs, further work is needed to confirm their use against vine mealybug outside of lab conditions, in addition to identifying new sources of resistance.

## OBJECTIVES

This project seeks to develop a novel control strategy for vine mealybug using host resistance as part of an integrated management program. This will be accomplished by identifying grape material with resistance to vine mealybug that can be used as rootstocks and a source of resistance for traditional cultivar breeding.

1. Develop a method to evaluate mealybug host resistance and identify grape material with leaf resistance to vine mealybug.
2. Evaluate grape materials with identified resistance to vine mealybug.
3. Determine multi-season sustainability of resistance to vine mealybug in identified grape rootstocks and cultivars.

## RESULTS AND DISCUSSION

### Objective 1

Potted grapevines of four accessions (**Table 1**) were planted into pots and are currently being grown in a growth chamber for detached and attached leaf assays for mealybug resistance.

**Table 1.** Grape accessions evaluated in objective 1.

Line	Type	Species	Special notes
Cabernet Sauvignon	Wine Grape	<i>V. vinifera</i>	Susceptible
Chardonnay	Wine Grape	<i>V. vinifera</i>	Susceptible
Flame Seedless	Table Grape	<i>V. vinifera</i>	Susceptible
17-01	Wild species	<i>V. champinii</i>	Potential resistant
IAC572	Rootstock	<i>V. caribbeae</i>	Potential resistant

### Objectives 2 and 3

In the summer of 2017, potted grapevines of seven accessions (**Table 2**) were placed into screen cages and evaluated for mealybug severity. Southern fire ants were detected among cultivars and were visibly maintaining mealybug colonies. Differences in mealybug severity and ant presence were detected among cultivars. The study is ongoing.

**Table 2.** Grape accessions evaluated for mealybug resistance in objectives 2 and 3.

Line	Type	Species	Special notes <sup>A</sup>
10-17A	Rootstock		Nematode resistance
IAC 572	Rootstock	<i>V. caribbeae</i>	Mealybug resistance
Cabernet Sauvignon	Wine grape		Known susceptible
17-01	Wild species	<i>V. champinii</i>	
17-02	Wild species	<i>V. candicans</i>	
PCO-349-11	Rootstock		Nematode resistance
17-03	Wild species	<i>V. australis</i>	

## CONCLUSIONS

The current study is ongoing, but early results suggest differences among grape cultivars in susceptibility to mealybugs and plant attractiveness to southern fire ants.

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## FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board, and by the Consolidated Central Valley Table Grape Pest and Disease Control District.

## INTEGRATIVE STUDIES OF VECTOR-RELATED FIELD EPIDEMIOLOGY FOR GRAPEVINE RED BLOTCH-ASSOCIATED VIRUS

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**Reporting Period:** The results reported here are from work conducted July 2017 to October 27, 2017.

**ABSTRACT / LAYPERSON SUMMARY**

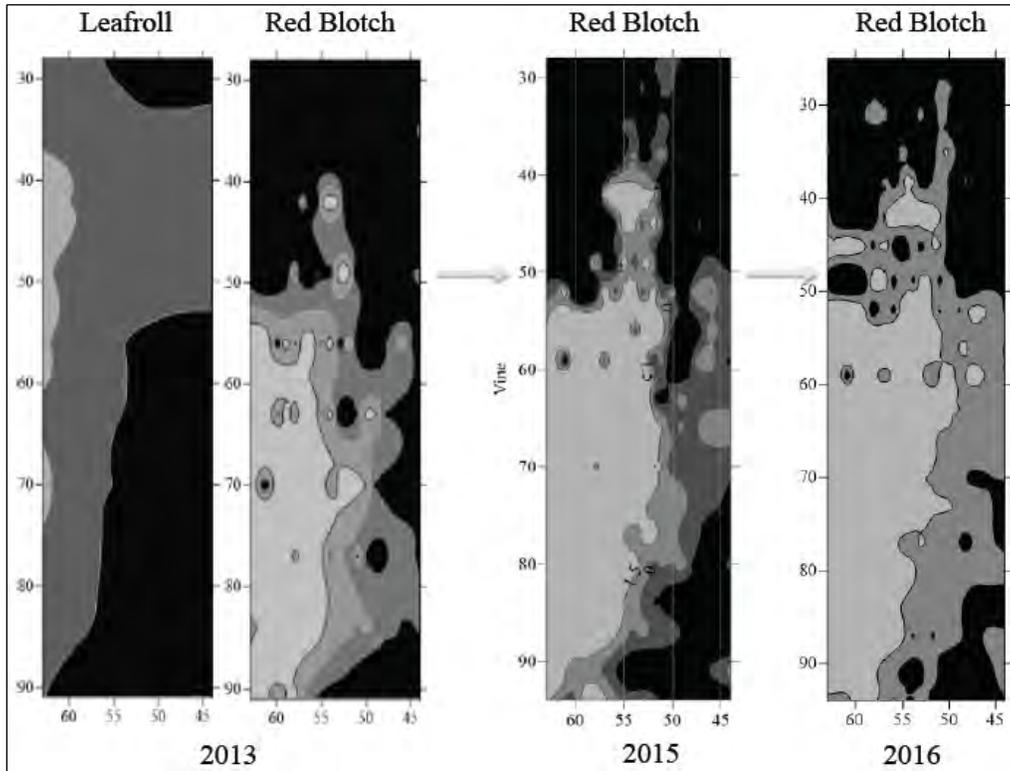
Red blotch is spreading. More information is needed on insect vectors. The current project aims to gain this information, in order to optimize future control strategies.

**INTRODUCTION**

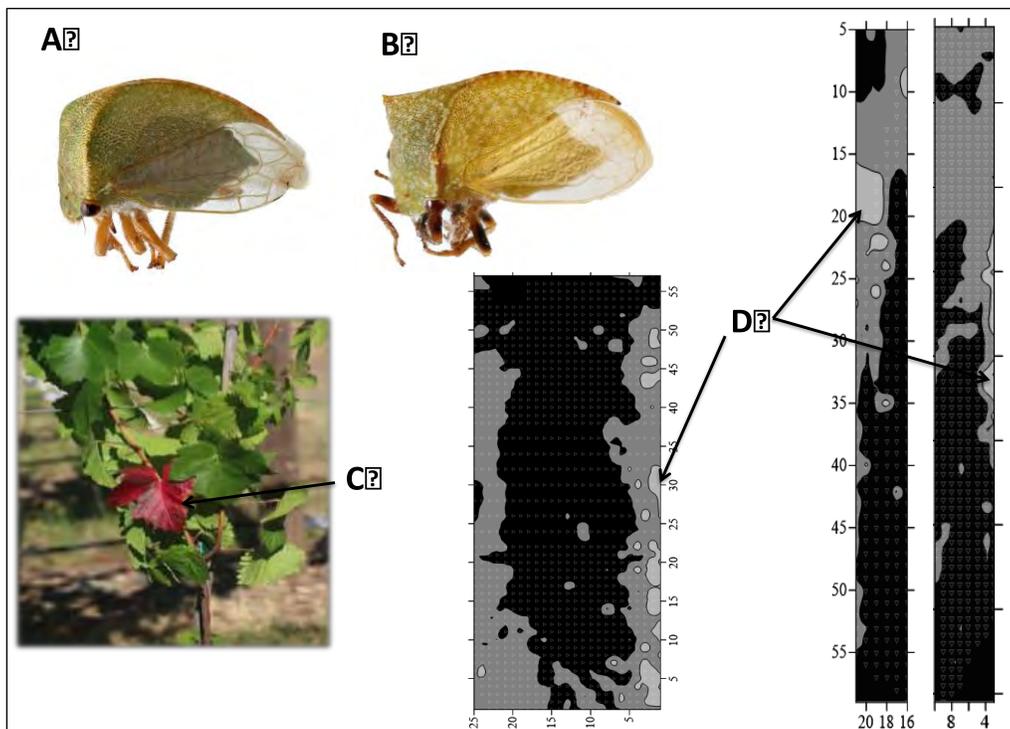
Grapevine virus diseases are of serious concern for vineyard managers and winemakers in all western production regions. Grapevine leafroll-associated virus (GLRaV) and grapevine red blotch-associated virus (GRBaV) impact grape berry quality. Growers and scientists alike have noticed a consistently lower °Brix at harvest of infected vines (Al Rwahnih et al. 2013, 2015), resulting in removal of symptomatic vines from vineyards. GRBaV is spreading; ecological mapping of GRBaV-positive vines, as verified by quantitative polymerase chain reaction (qPCR) during 2013-2016, showed a significant trend of virus increase in two of three areas studied in Oregon (**Table 1; Figure 1**). The spread of GRBaV is alarming, with doubling and 10x increases from 2014-2016 (**Table 1**). These viruses likely have separate insect vectors, and in-field distribution of the two viruses is independent of one another (**Figure 1**). Treehoppers (Membracidae) are the most likely vectors of GRBaV. Already one species, *Spissistilus festinus*, has been identified as a possible vector in California (Bahder et al. 2016a). During 2016 the treehopper species *Tortistilus wickhami* and *T. albidosparsus* were found feeding on grape shoots and leaves. Evidence of treehopper feeding (girdles) was predominantly found on vineyard edges (**Figure 2**) and mirrored in-field GRBaV distribution, strongly suggesting that these insects are vectors of GRBaV. Feeding observations were not conclusive, and we therefore initiated controlled transmission biology experiments adapted from Bahder et al. (2016a) during summer 2016.

**Table 1.** Red blotch virus infection in three Oregon grape-growing regions as determined by PCR from 2013 to 2016. Vines sampled in 2013-14 were re-tested for GRBaV through 2016.

Location	Year	Positive Vines	Assayed Vines	% Infection
Willamette Valley (Vineyard 1)	2013 & 2014	133	374	35.6%
	2015	172	374	46.0%
	2016	185	293	62%
S. Oregon (Vineyard 2)	2014	11	194	5.7%
	2015	58	194	29.9%
	2016	121	194	62.4%
S. Oregon (Vineyard 3)	2014	55	193	28.5%
	2015	33	193	17.1%
	2016	37	189	19.6%
E. Oregon (Vineyard 4)	2013 & 2014	4	396	1.0%
	2015	0	396	0.0%



**Figure 1.** Distribution of leafroll and distribution and spread of red blotch in a vineyard in Oregon (2013-2016). Data were generated using qPCR analysis and plotted using spatial distribution software. Light and dark-colored areas indicate presence and absence of virus.



**Figure 2.** Suspected insect vectors of red blotch: (A) *Tortistilus wickhami*, and (B) *T. albidosparsus*. These insects feed on canes and create girdles resulting in characteristic red leaves (C, red cultivars). (D) Feeding distribution symptoms are on vineyard edges.

The pathogen-vector-host complex is of significant economic importance, and available evidence indicates that only plants of the genus *Vitis* are hosts of GRBaV (Bahder et al. 2016b). In Oregon, eight vineyards were selected for virus-vector studies in 2010. Three blocks have since been fully removed due to perceived losses of fruit quality. We propose in-depth vector-related studies with particular focus on the two *Tortistilus* species as potential vectors of GRBaV in grapevines. The proposed work will build on currently available information on potential insect vectors, with focus on their seasonal presence and spatial distribution. Information from this project will direct and support future control programs for GRBaV both regionally and nationally.

A coordinated extension and outreach is an essential and integral part of industry involvement. Without industry involvement, we would not have made the progress during 2016. We also believe that the basis of vineyard health from an industry perspective is to help growers make informed decisions regarding practices to minimize risk of virus. To this end we used multiple channels to disseminate the newest and relevant information to growers regarding red blotch epidemiology. During 2016 we reached ~800 growers through the different channels (see 2016 report). We strongly believe that extension serves two purposes: To inform industry, and to work with growers to make progress on important crop production issues.

The proposed study is focused on filling the gaps in knowledge and extending information to the industry. The goal of this proposal strongly focuses on increasing knowledge of red blotch epidemiology by looking at virus spread and the role of potential vector insects. The planned methods have the goal of gathering needed knowledge on vector biology and potential non-crop hosts. One of the key objectives is to share information from this work with industry through various extension activities, and to work with growers to enhance the quality of industry-relevant information.

## OBJECTIVES

1. Follow insect vector distribution, and disease progression in relation to management.
2. Conduct controlled transmission biology experiments.
3. Obtain baseline information on current levels and extent of red blotch.
4. Extension of information on grapevine red blotch-associated virus, and insect vectors.

## RESULTS AND DISCUSSION

Our studies are coordinated with other research groups dealing with vector transmission biology (Kent Daane, Sudarshana Mysore, Frank Zalom), virology and non-crop hosts (Sudarshana Mysore, Frank Zalom), and viticulture (Anita Oberholster, Rhonda Smith). The proposed procedures will create clearer knowledge of the regional and national epidemiology of GRBaV. We will coordinate our viral transmission work with California collaborators. With assistance from our California collaborators (Frank Zalom and Sudarshana Mysore), the work will be complemented by PCR analysis of the gut contents of insect specimens collected in Oregon vineyards with high incidence of GRBaV.

### **Objective 1. Follow Insect Vector Distribution, and Disease Progression in Relation to Management**

This work is essential to better understand the role of vectors, surrounding vegetation, and spread of GRBaV in vineyards (Perry and Dixon 2002, Al Rwahnih et al. 2013).

#### **i. Follow Insect Vector Distribution and Incidence**

Six vineyards representing Southern Oregon (2), the Willamette Valley (2), and Eastern Oregon (2) will be visually surveyed for treehoppers, and beat sheet sampling will be conducted. At each site we will focus insect collections on Membracidae. We will sample a gridded pattern to include a minimum of 60 locations equally divided to include the riparian habitat, the interface between the riparian habitat and the vineyard, and the vineyard. Plants within the riparian habitat will include known perennial non-crop alternate hosts for *T. wickhami* and *T. albidosparsus* including coastal white oak (*Quercus garryana*), manzanita (*Arctostaphylos patula*), Pacific madrone (*Arbutus menziesii*), wild hazelnut (*Corylus cornuta*) seedling apple (*Malus domestica*), and seedling pear (*Pyrus spp.*) (Yothers 1934, Gut 1985, Valenti et al. 1997, Swieki et al. 2006). The interface between the riparian habitat and the vineyard will include *Cirsium arvense* (Canada thistle), *C. californicum* (California thistle), *C. occidentale* (cobwebby thistle) and *C. proteanum* (red thistle). Both *T. wickhami* and *T. albidosparsus* have commonly been found on these thistle species in past studies (Goeden and Ricken 1985, De Smet-Moens 1982).

The sampling methodology will allow spatial analysis and ecologically relevant association or dissociation of potential vectors with virus-infected vines and potential alternate hosts (Perry 1995, 1996, Perry and Dixon 2002). Sampled vineyards will contain at least 20 rows and 10 pole-to-pole “bays” (Charles et al. 2009) containing three to six vines per bay. Data collected during 2017-19 will be combined with earlier collected data of all potential insect vectors and analyzed using standard analysis of variance (ANOVA) in order to compare the seasonal presence of insects (Walton et al. 2013). Data will be compared using both ANOVA (insect species, alternate hosts, and location as variables) and correlation analyses to determine whether there is a pattern to GRBaV spread as compared to the distribution of potential vectors and alternate hosts (see **Figure 1** and **Figure 2**).

#### **ii. *Tortistilus* spp. Reproduction on Host Plants**

We will determine the reproduction and life cycle of *Tortistilus* spp. on *Vitis vinifera*. We will additionally place *T. wickhami* and *T. albidosparsus* adults on various cover crops, annual weed species, and perennial plants that commonly occur in West Coast vineyards (see previous section). These studies will be conducted in order to determine the importance of the alternate host plants in the lifecycle of the treehopper species.

#### **iii) Disease Incidence and Progression Coupled with Vector Feeding**

During 2016 we mapped all vines (similar protocol as described above) in a vineyard block showing symptoms of GRBaV (**Figure 2**), together with signs of *T. albidosparsus* feeding. We systematically analyzed vines for GRBaV using qPCR analysis. In 2017 and 2018 the rate of GRBaV increase for vines with treehopper feeding damage in 2016 will be compared to vines without treehopper feeding damage. Information from this experiment will provide a clearer understanding of field correlations of virus infection and feeding symptoms.

In addition, in 2016 we selected four samples on each of a subset of 36 vines with a total of 41 sample comparisons (i.e. five vines had two sets of four samples per vine) that showed treehopper feeding symptoms but were visually asymptomatic of GRBaV. Locations of samples were designated as: (1) above girdle, (2) below girdle, (3) opposite side of the vine at same height as the area above the girdle, and (4) opposite side of the vine at same height as the area below the girdle. Tissue samples from the girdled areas of the plants will be compared to tissues of the same vines far from girdles. This experiment will show if pruning of vine tissues soon after feeding damage will result in lower rates of virus acquisition, and will continue during 2017-2018. This work is coupled with the planned controlled greenhouse experiments to show the period of virus latency before appearance of symptoms.

#### **iv. Key Plant Material and Insect Management Techniques**

This proposed activity focuses on setting a low tolerance for virus-infected plants and insects that may vector GRBaV. All known virus-infected vines, as well as vines growing in non-crop regions, will be removed in order to minimize possible sources of virus infection. Insect populations will be minimized using integrated control techniques. We will work closely with managers in two affected vineyards to minimize the presence of treehoppers and other possible vectors.

These experiments will (a) investigate the impact of insecticide controls on pest and beneficial insects, and (b) assess the disease progression of the virus between seasons as described under (ii) above. We will determine the effect of the systemic insecticide spirotetramat (Movento, Bayer CropScience) on scale insects, aphids, and treehopper populations. These applications may be combined with additional applications of Applaud or Venom if insect populations are found to increase. Movento will be applied early in the season to minimize treehopper numbers and prevent insect vector buildup during the first part of the growing season.

#### **Objective 2. Conduct Controlled Transmission Biology Experiments**

Standard transmission biology tests have consistently resulted in the most reliable results to determine the vectors of viruses. Previous tests conducted on adults of *Erythroneura* leafhopper species involved low numbers of insect individuals (30-50) and acquisition access periods up to five days (Tsai et al. 2008, 2010, Blaisdell et al. 2015). During 2016 we initiated controlled experiments to determine successful transmission of GRBaV virus by both *T. wickhami* and *T. albidosparsus*. The initial virus status of all plant material was verified using qPCR. Field-collected live insects were placed on known GRBaV virus-infected plant material for 48 hours and were then transferred to plants confirmed to be without GRBaV for another 48 hours. All surviving insects were

subsequently transferred at one-week intervals to a new set of plants with no virus infection. This process was repeated weekly until all insects had died. Plants from the initial transmission biology experiments are currently being kept under greenhouse conditions and are tested at regular intervals using qPCR to determine the epidemiology of GRBaV. These experiments will be repeated during 2017 in order to create a robust dataset.

### **Objective 3. Obtain Baseline Information on Current Levels and Extent of GRBaV**

In order to characterize the prevalence of GRBaV in Oregon vineyards we will complete the planned survey work (see progress report data) among the three major growing areas: Southern Oregon, Willamette Valley, and Eastern Oregon. An additional ~120 GRBaV samples will be collected in Southern Oregon during 2017. Northern Willamette Valley surveys will include an additional 50 samples, and in Eastern Oregon we will collect 200 samples in the Milton-Freewater region. Sampling will occur in blocks that have not been sampled for GRBaV to date but where infection is suspected. Three sites in Southern Oregon, two in the Willamette Valley, and three sites in Eastern Oregon will be sampled. The sampling for each site will total a minimum of 25 plants, consisting of 20 symptomatic plants and an additional five asymptomatic plants. If fewer than 20 symptomatic plants are found at a given site, then all symptomatic vines will be sampled and the balance of the sample will be taken from asymptomatic vines. From each sampled vine, five leaves will be removed and placed into a cooler and transported to the laboratory for processing and analysis.

### **Objective 4. Extension of Information on the Importance of Vectors, GLRaV, and GRBaV in Oregon Vineyards**

Results will be provided to growers, grape industry representatives, and Oregon State University Cooperative Extension personnel through webinars, grower reports, seminars, and national webinars. We plan to organize a regional vineyard workshop on vectors and vineyard disease transmission for growers and industry in 2017. Vaughn Walton, Frank Zalom, Clive Kaiser, and Rick Hilton are the statewide and regional extension agents in the affected regions. They have given numerous presentations on grape insect pests at grower and research symposia. Results will also be published in popular and scientific journals (see report for the list of applicable publications from 2008-2016). Walton, Kaiser, and Hilton are strongly committed to the grape industry and have a good relationship with growers, consultants, and industry personnel that will aid in research and extension.

In Oregon, we presented results of earlier and work for this grant to growers in two locations, Salem, Oregon (25 attendees), and Milton Freewater, Oregon (30 attendees).

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## BIOLOGY AND ROLE OF TREEHOPPERS IN GRAPEVINE RED BLOTCH DISEASE

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### ABSTRACT

This project is in its first year, beginning July 1, 2017. It builds upon studies initiated earlier by the Zalom and Sudarshana labs at UC Davis, and the Daane lab at UC Berkeley. Results presented to date include monitoring the population dynamics of three-cornered alfalfa hopper (*Spissistilus festinus*; 3CAH) in vineyards and surrounding landscapes over the 2017 season in vineyards and along transects from vineyards to natural areas, preliminary field transmission studies, and greenhouse studies of the feeding and reproductive status of various weeds and cover crops found in vineyards as they relate to 3CAH feeding and reproduction. Methods and present status for additional studies proposed as objectives of this project are described.

### LAYPERSON SUMMARY

The results of this project are expected to better define the role of the three-cornered alfalfa hopper (*Spissistilus festinus*; 3CAH) in the epidemiology of grapevine red blotch virus (GRBV), and to examine the role of grapevines, cover crops, and non-crop vegetation in and around vineyards in sustaining 3CAH populations. Possible transmission by other treehoppers found in vineyards where GRBV is spreading will also be confirmed. 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 where nearby GRBV source are known to occur.

### INTRODUCTION

In 2007, a grapevine disease with symptoms that resembled those of leafroll in Napa County vineyards was found to be a distinct malady displaying red veins and blotches (Calvi 2011). The disease was named red blotch disease and further investigations revealed a new DNA virus initially named grapevine red blotch-associated virus (GRBaV), tentatively grouped in the family Geminiviridae (Al Rwahnih et al. 2013, Sudarshana et al. 2015). A similar virus was also found in grapevines in New York, Oregon, and Washington state (Krenz et al. 2012, Poojari et al. 2013, Seguin et al. 2014). The virus is now known to be widely distributed in the United States and has been found in Canada, China, India, and South Korea. California vineyards with the disease, especially those planted to red varieties, are known to impact quality of the grapes and the value of these grapes are substantially reduced.

Details of red blotch disease epidemiology are not well known. Although some researchers initially believed that the virus did not spread to or within established vineyards, observations by growers, consultants, and other researchers strongly suggested spread was occurring in some vineyards that was consistent with that of an insect vector. The virus was discovered in wild grapevines, mainly open-pollinated *Vitis californica*, (Bahder et al. 2016, Perry et al. 2016), even at a considerable distance from commercial vineyards. Among the many insect species found in commercial vineyards with red blotch disease, the three-cornered alfalfa hopper (*Spissistilus festinus*; 3CAH) was found capable of transmitting grapevine red blotch virus (GRBV) under laboratory conditions by Bahder et al. (2016). Subsequently, other treehoppers of the genus *Tortistilus* were observed feeding on grapevines with red blotch disease in California, Southern Oregon, and the Willamette Valley (Zalom and

Sudarshana, unpublished; Walton, unpublished), but the status of these species as GRBV vectors is not known. Although some aspects of 3CAH biology is mentioned in the scientific literature, the majority of this information comes from annual cropping systems where it is considered to be a pest of leguminous crops such as soybeans, peanuts, and of course, alfalfa. The biology of 3CAH and more especially the other treehoppers in vineyards is little known. A better understanding of their seasonal biology in and around vineyards and their role in virus transmission is essential for developing management guidelines to prevent spread of red blotch disease within vineyards and to uninfected vines. This research began in 2016 with grant funding from the CDFA Specialty Crops Block Grant Program (Sudarshana and Zalom), USDA Agricultural Research Service National Program funds (Sudarshana), and American Vineyard Foundation (Daane), all of which ended in June 2016. We finally received funding for this grant from the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board on October 10 due to complications in contracting between UC and CDFA. However, we pursued many elements of the proposed research initiated prior to July 2017 in anticipation of receiving the funds so some of the results reported hereafter chronologically precede the initiation of this grant.

## **OBJECTIVES**

The long-term objectives of this proposed study address a better understanding of the ecology and epidemiology of GRBV in California vineyards so that appropriate measures for preventing infection and spread of red blotch disease can be developed. The primary goal is to document the prevalence of treehoppers, focusing on 3CAH and *Tortistilus* species, in California vineyards and the surrounding landscape, and to understand their role in the spread of GRBV between grapevines and regionally.

The specific objectives of this project are:

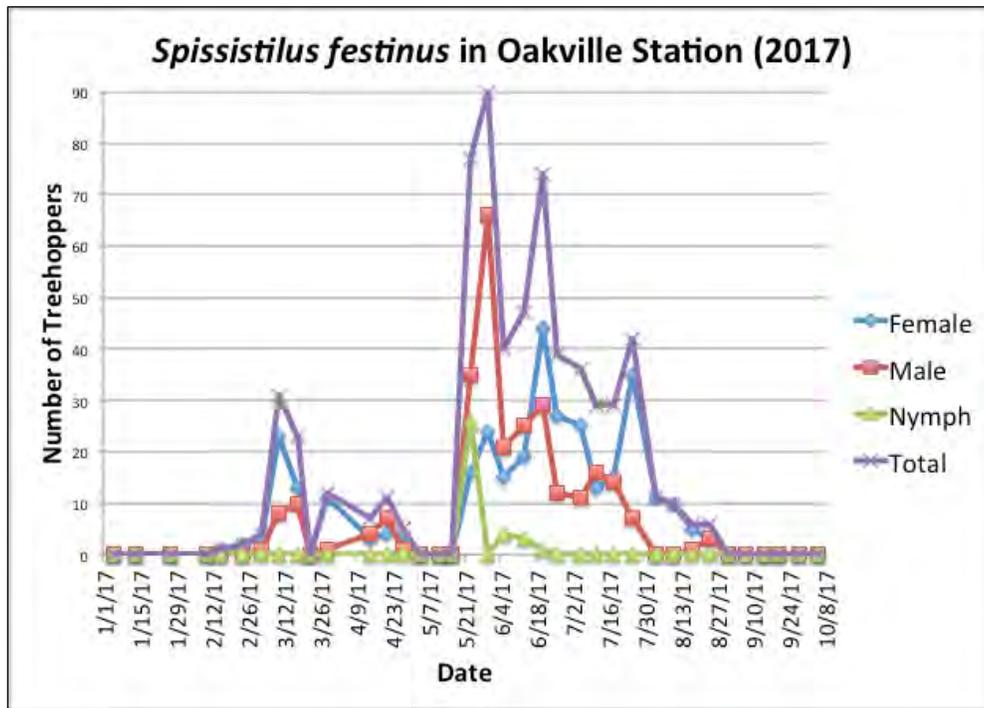
1. Monitor the population dynamics of 3CAH in vineyards and surrounding landscapes over the season.
2. Conduct GRBV transmission studies using treehoppers collected from vineyards with red blotch disease, and detect GRBV in the salivary glands of insects collected. Monitor field transmission by 3CAH.
3. Determine the transmission efficiency of 3CAH to identify virus acquisition periods and persistence in the insect.
4. Evaluate the role of cover crops on the 3CAH in vineyards.
5. Determine the status of common weed and cover crops as feeding and reproductive hosts for 3CAH.

## **RESULTS AND DISCUSSION**

### **Objective 1. Monitor the Population Dynamics of 3CAH in Vineyards and Surrounding Landscapes Over the Season**

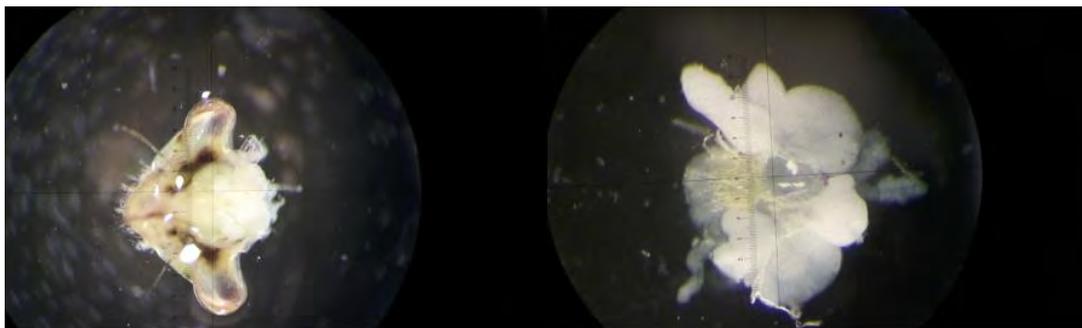
This objective was addressed by both the Zalom and Sudarshana labs at UC Davis, and by the Daane Lab at UC Berkeley.

In the study by the Zalom and Sudarshana labs and primarily conducted by Ph.D. student Cindy Preto, ground cover located in and around a 53-row Cabernet Sauvignon block at the UC Davis Oakville Research Station and the perimeter of the reservoir pond at that site was sampled weekly by sweep net since March 2016. The vineyard block consists of 53 rows. All odd-numbered rows were tilled late March and were therefore not sampled. Each even-numbered row was subdivided corresponding to the six proximal vines on each row border and the middle 18 vines, and ground cover within these areas was sampled separately for treehopper adults and nymphs, and adults were sexed. Sampling will continue through March 2018 when the vineyard is scheduled for removal due to increasing red blotch disease incidence. The first 3CAH adult collected for the current season was on February 15, 2017. We now believe that this marks the return of the overwintering generation to the vineyard. Bud break occurred on April 6, seven weeks after the first 3CAH adult was found in the vineyard. The first nymphs were collected on May 23, coinciding with an increase in adult 3CAH captures (**Figure 1**) and the phenological marker of bloom. Increase in captures of later-instar nymphs increased in concert with adult captures, and we posit that this indicates the first in-field generation of 3CAH. Subsequent 3CAH generations overlap one another. Veraison was noted on August 3 and vineyard weeds, which constitute the ground cover sampled at Oakville, started to noticeably dry by August 10, corresponding with a drop in adult 3CAH. The weeds in the vineyard and surrounding the irrigation pond were mowed by August 31 and no 3CAH adults were collected from ground cover thereafter. Vineyard floor weeds have not regrown since mowing, probably due to lack of rain.



**Figure 1.** Weekly sweep net sampling of vineyard ground cover for 3CAH at Oakville in 2017.

Salivary glands were extracted from the 3CAH collected at the Oakville vineyard to test for presence of GRBV biweekly beginning March 3, 2017, just prior to bud break. A total of 96 usable samples were collected. Salivary glands from 3CAH reared from eggs were dissected on each collection date, and served as negative controls. The salivary glands were removed, placed in 180 uL ATL and 20 uL proteinase K incubated four hours at 56°C, and are currently stored in a -80°C freezer at UC Davis awaiting GRBV detection (**Figure 2**).



**Figure 2.** 3CAH salivary gland dissections showing salivary glands within head capsule (left) and removed from head capsule (right).

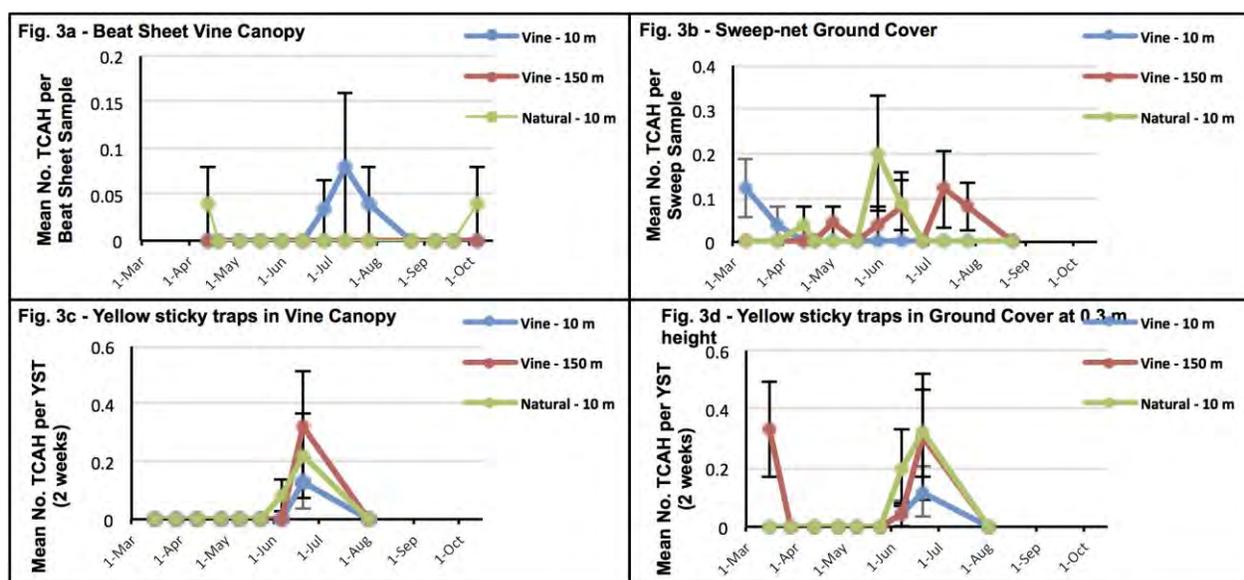
In a related study conducted by Houston Wilson of the Daane lab, changes in 3CAH populations and crop damage along transects that extend out from natural habitats into vineyards were evaluated at approximately two-week intervals between March and October 2017 using a combination of yellow sticky traps, sweep-nets, and beat-sheet sampling. Field sites consisted of vineyard blocks >2 acres in size adjacent to riparian and/or oak woodland habitat located in Napa and Sonoma counties. At each site, insects were sampled along five parallel transects (positioned 20 meters apart) that extended out from the riparian or oak woodland habitat (i.e. “natural habitat”) into the vineyard. Each transect was 160 meters long – going 10 meters into the natural habitat and 150 meters into the vineyard. Along each transect samples were taken at the interior of the natural habitat (10 meters into the habitat) as well as at the edge and interior of the vineyard (10 and 150 meters into the vineyard, respectively). There were five total study sites in all, and all vineyard blocks were red varietals that were at least five years old and located on level ground with similar trellis and irrigation systems. All plots were maintained insecticide free

throughout the course of the study. Two yellow sticky traps (16 x 10 cm, Seabright Laboratories, Emeryville, CA) were placed at each transect point in the vine canopy and on the drip irrigation line at ~ 0.3 meters above the soil surface. In the natural habitat, two sticky traps were hung from a pole at each transect point at a height above the ground surface equivalent to those in the vineyard. On each sampling date, proportion of ground cover to bare soil was recorded along with species composition and ground cover status. At each transect point, a set of 30 sweep net samples were used to sample the ground cover. A modified beat-sheet was used at each transect point to sample the canopy of grapevines (in the vineyard) and non-crop species (in the natural habitat). The beat-sheet consisted of a one meter<sup>2</sup> nylon funnel that fed into a detachable one gallon plastic bag. For each sample, the funnel was held beneath the canopy while vigorously shaking the plant (or vine) for 30 seconds in order to dislodge insects into the funnel and plastic collection bag.

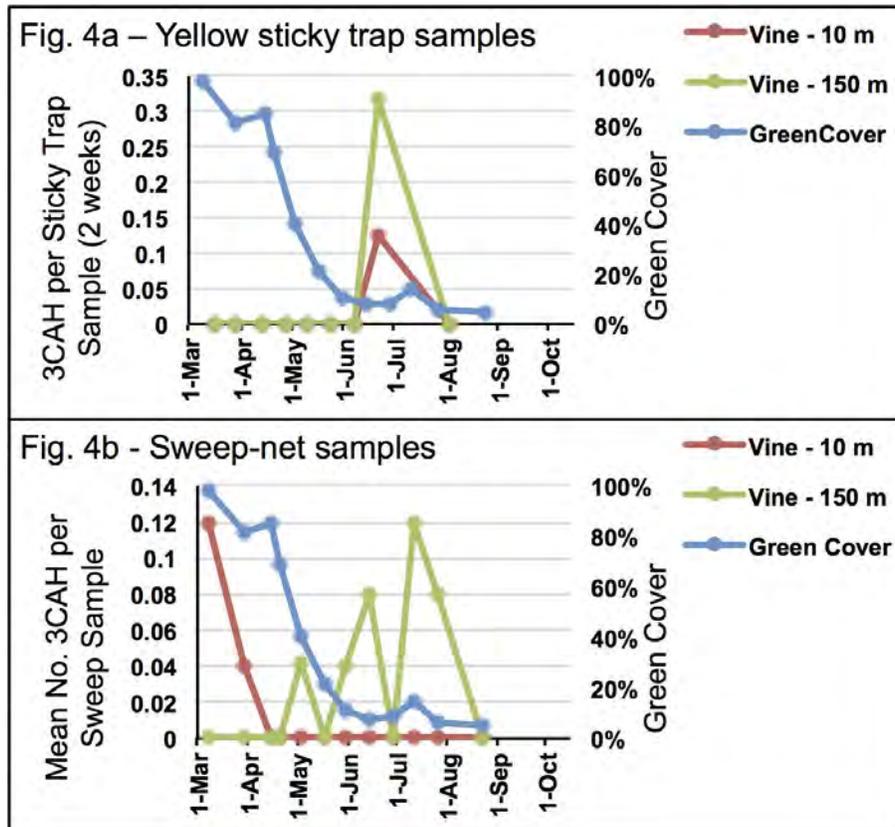
Each month, vines along each vineyard transect point were evaluated for signs of 3CAH feeding damage (i.e. girdling of leaf petioles). At each vineyard transect point, one cane from each of 10 randomly selected vines was visually inspected for leaf girdling. Total leaf nodes and leaf girdles per cane were recorded for each vine. Petiole girdling became apparent in August 2017 with a higher proportion of girdles located at the vineyard interior. This increase in girdling in August follows increased 3CAH densities observed in the vine canopy between June and August.

Preliminary findings indicate that 3CAH activity showed a strong temporal trend, with densities generally increased between June and August along with some activity observed in March (Figure 3). While there was no clear gradient of 3CAH activity across the transect points, densities on the yellow sticky traps were slightly elevated in natural habitats in early June just prior to increases observed in the vine canopy at both the vineyard edge and interior in the following round of sampling (Figures 3c and 3d). Comparing the different sampling techniques for 3CAH from the vine canopy and natural habitat, the highest 3CAH densities were recorded on yellow sticky traps, followed by sweep-nets and beat sheets. Changes in 3CAH densities between the ground covers and vine canopy were not always clearly reflected in the data. While densities in the vine canopy did increase as the proportion of healthy/green ground covers diminished (Figure 4a), some 3CAH could still be found on the little bit of ground cover that remained later in the season (Figure 4b). Surprisingly, these late season 3CAH adults were most frequently encountered on ground covers in the vineyard interior (Figure 3b).

Changes in 3CAH densities along these transects may provide evidence of seasonal movement of the insect between natural habitats and vineyards, while differences in 3CAH abundance on ground covers and in the crop canopy, along with petiole girdling, may indicate the timing of vine colonization and feeding.



**Figure 3.** 3CAH densities sampled along the transect using (3a) beat sheet in the vine canopy or perennial vegetation canopy; (3b) sweep-net on ground covers; (3c) yellow sticky traps in the vine canopy or at vine canopy height; and (3d) yellow sticky traps at ground cover height (~ 0.3 meters).



**Figure 4.** 3CAH densities in the vine canopy increased as the proportion of healthy/green ground covers diminished (4a), although some 3CAH persisted on ground covers late into the season (4b).

**Objective 2. Conduct GRBV Transmission Studies Using Treehoppers Collected from Vineyards with Red Blotch Disease, and Detect GRBV in the Salivary Glands of Insects Collected. Monitor Field Transmission by 3CAH**

Michael Bollinger of the Zalom lab at UC Davis has been collecting *Tortistilus* treehoppers in Napa and Sonoma County vineyards where GRBV has been occurring since May 2016, when we became aware of a large population of adults present and actively feeding on grapevines, but we have been unable to establish a reproducing colony in the laboratory. We attempted GRBV greenhouse transmission studies with field-collected ‘horned’ and ‘unhorned’ *Tortistilus* during 2016 that have yet to confirm transmission by quantitative polymerase chain reaction (qPCR). A larger study was initiated on May 24, 2017, when a very large population of wild *Tortistilus* was found feeding on vines in a Pope Valley vineyard. *Tortistilus* collected on that date and for several weeks thereafter were separated into ‘horned’ and ‘unhorned’ morphs, and individuals of each were placed onto qPCR GRBV confirmed positive Ghv-24-392 (clade II) and onto Ghv-32-377 (clade I) Cabernet Sauvignon source vines. qPCR confirmed test healthy Ghv-37 Cabernet Sauvignon source vines served as a negative control. Transmission was attempted both by placing individuals of both morphs that had fed on GRBV infected source vines into clip cages on the uninfected vines or in large cages containing eight uninfected vines and 20 male and 20 female *Tortistilus* of each morph. qPCR analysis of these plants will begin shortly. Also, in order to test acquisition in a more natural environment, field captured *Tortistilus* collected on May 30, 2017 were placed inside cages wrapped around separate qPCR confirmed positive and negative Cabernet Sauvignon field vines located at the Pope Valley vineyard and similarly on qPCR GRBV confirmed wild grapevine located in the vicinity for an acquisition access period of six days, then transferred to qPCR confirmed healthy Cabernet Sauvignon recipient vines and allowed an inoculation access period of six days. The GRBV testing of these plants will begin at about five months post-inoculation. All qPCR testing for these studies is being done by the Sudarshana lab at UC Davis.

All *Tortistilus* removed from the grapevines post-inoculation were placed inside 1.5 ml tubes filled with 95% ethanol for salivary gland removal and GRBV testing. Salivary glands from *Tortistilus* collected from the test positive Cabernet Sauvignon in the field have not yet been tested for presence of the virus, but 15 salivary glands removed and ran from the test positive wild grapevine have been tested with only one of the 15 testing positive.

At three intervals during summer and fall 2016, ten adult 3CAH that were allowed to feed on clade 1 or clade 2 GRBV infected vines for at least three days were caged on each of five virus free Cabernet Sauvignon grapevines that had been planted on the UC Davis Plant Pathology Field Station in 2015 by the Sudarshana lab (**Figure 5**, left photo). Testing of these vines for GRBV presence during 2017 had not documented transmission to date, but testing will continue through 2018. We have also been monitoring the vines for 3CAH girdles during 2016 and 2017, and many girdles were found in the planting. If transmission was successful from the caged inoculation attempts during 2016, we anticipate that this site will provide a controlled model for studying details of GRBV spread.



**Figure 5.** Cabernet Sauvignon grapevines on Freedom planted in 2015 at the UC Davis Plant Pathology Field Station. Above left: Caged grapevines for 3CAH release. Above right: Grapevines showing treehopper feeding damage with girdled shoots that turned red.

**Objective 3. Determine the Transmission Efficiency of 3CAH to Identify Virus Acquisition Periods and Persistence in the Insect**

Studies related to this objective have not yet been initiated.

**Objective 4. Evaluate the Role of Cover Crops on the 3CAHs in Vineyards**

In 2016-17, common cover crops were planted in replicated plots at three vineyard locations by Ph.D. student Cindy Preto of the Zalom lab at UC Davis, and sampled by sweep net for presence of treehoppers. **Figure 6** shows an example of a grass (left) and legume (right) cover crop replicate at one of the sites. In 2017-18, we will compare overwintering success of 3CAH on five cover crops, bell beans, Magnus peas, blando brome, California red oats, mustard, and unplanted resident. Each type of ground cover vegetation will be replicated four times in a randomized block design at the Armstrong Tract D2 block located south of Davis, and were planted on October 24, 2017. In January 2018, three plants in each replicate (12 plants per variety) will be caged with three male and three female 3CAH, and this will be repeated in February and March. Destructive sampling of all caged plants will take place in April, documenting girdling, oviposition, nymphs, and adults (alive and dead).

**Objective 5. Determine the Status of Common Weed and Cover Crops as Feeding and Reproductive Hosts for 3CAH**

Feeding and reproductive weed and cover crop hosts of 3CAH were determined in the greenhouse in a series of no-choice experiments that began in late 2016 and are still in progress. This study is part of the dissertation research of Cindy Preto in the Zalom lab at UC Davis. Three female and three male 3CAH were caged onto individual pots of weeds or cover crops (**Figure 7**). The cages were opened weekly for four weeks to determine adult survival, girdling, oviposition, and nymph emergence. Purple vetch was used as a positive standard in each run of the no choice experiment because of our previous laboratory and field observations of successful feeding and oviposition. The common vineyard weeds evaluated as feeding and reproductive hosts of 3CAH to date are presented in **Table 1**. The common cover crops that have been evaluated to date or are currently being evaluated for status as feeding or reproductive hosts are presented in **Table 2**. The relative reproductive success of 3CAH on each host will be compared to its success on purple vetch at the conclusion of all no choice tests.



**Figure 6.** A grass (left) and legume (right) cover crop plot from our winter 2016-17 study.



**Figure 7.** Weeds and cover crops caged with 3CAH in a greenhouse study at UC Davis.

**Table 1.** Status of common vineyard weeds as feeding and reproductive hosts for 3CAH in a laboratory study.

<b>Weeds as Feeding and Reproductive Hosts</b>	
<b><u>Host</u></b>	<b><u>Non-host</u></b>
Spanish Clover	*Wild Carrot
Birdsfoot Trefoil	Bermuda Grass
Field Bindweed	Sharppoint Fluvellin
Dandelion	Buckhorn Plantain
Common Groundsel	*Kentucky Bluegrass

\*Feeding host only

**Table 2.** Status of common vineyard weeds as feeding and reproductive hosts for 3CAH in a laboratory study, and cover crops being evaluated at present..

<b>Cover Crop as Feeding and Reproductive Hosts</b>		
<b><u>Host</u></b>	<b><u>Non-host</u></b>	<b><u>Current study</u></b>
Crimson clover	Mustard	Zorro fescue
Purple vetch	CA red oats	Red fescue
Bell beans		Annual ryegrass
Magnus peas		Merced rye
Blando brome		Barley
Subterranean clover		Daikon radish
Woollypod vetch		
Black medick		

In an effort to evaluate preference of 3CAH to confirmed reproductive cover crop and weed reproductive hosts when presented a choice, three groups of five plants containing four known reproductive hosts from the completed no-choice experiment will be randomly arranged in a large dome-shaped cage in the greenhouse and replicated three times (**Table 3**). Purple vetch will be included in each evaluation as a standard. Ten male and ten female 3CAH will be released into each cage and allowed to freely feed and oviposit. All adults will be removed from the cages after one week. Nymphs will be counted and collected from individual plants on weeks two and three. Destructive sampling of all plants and collection of nymphs will be conducted at week four. The four plant varieties with the most 3CAH nymphs collected from the first run of the experiment, plus the purple vetch standard, will be evaluated again in a subsequent repetition of the experiment.

**Table 3.** Cover crops and weeds identified as reproductive hosts of 3CAH that will be compared in an anticipated preference study.

<b>Group One Cover Crops</b>	<b>Group Two Cover Crops</b>	<b>Group Three Weeds</b>
Purple vetch	Purple vetch	Purple vetch
Black medick	Blando brome	Field bindweed
Dutch white clover	Crimson clover	Spanish clover
Subterranean clover	Bell beans	Birdsfoot trefoil
Woollypod vetch	Magnus peas	Dandelion

## CONCLUSIONS

This newly-funded project is intended to address important gaps in the knowledge of the transmission and spread of GRBV in California vineyards that were identified in our earlier studies. Members of our team confirmed transmission of the virus by 3CAH, and this current project hopes to confirm transmission in the field as well as details of the transmission process. Observations by our team and researchers at Oregon State University suggest that other treehopper species may also transmit the virus, but transmission has not been confirmed to date. This is being addressed by our project as well. Studies on alternate feeding and reproductive hosts of 3CAH in the greenhouse and field that were initiated in the last year will be completed as a result of this project. This information will be directly applicable to management of red blotch disease in California vineyards.

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