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The reports in this document have not been peer reviewed.

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REPORTS

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Xylella fastidiosa

and

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THE EPIDEMIOLOGY OF NOVEL *PDR1* RESISTANT GRAPEVINES: EPIDEMIC AND VECTOR MOVEMENT MODELS TO SUPPORT INTEGRATED DISEASE MANAGEMENT

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ABSTRACT

Resistant cultivars of agricultural crops are integral to sustainable integrated disease management strategies. Our previous work indicated that grapevines expressing the *PdR1* 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 *PdR1* resistant and susceptible genotypes in our vector transmission experiments and integrating greater biological detail into our epidemic modeling work. Our preliminary experimental results suggest that vector transmission from *PdR1* grapevines follows our theoretical predictions and exhibits non-linear dynamics. Specifically, while *PdR1* resistant grapevines provide promising resistance, under some conditions, we see greater transmission rates from *PdR1* resistant vines than from susceptible vines. This may be caused by an interaction between the resistance trait and vector feeding preference. These results, while preliminary, complicate integration of *PdR1* grapevines into Pierce's disease management strategies for growers. Moreover, growers may be able to benefit from *PdR1* resistant cultivars without planting all of their acreage to them. We are exploring tradeoffs between disease resistance and economic profit of *PdR1* plants through bio-economic modeling, with the ultimate goal of developing management recommendations for the optimal planting of *PdR1* grapevines. 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 *PdR1* 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*). Our research confirms previous findings that *PdR1* grapevines are partially resistant to *Xf* colonization. While deployment of *PdR1* traits represent a promising management strategy, they will have to be deployed as part of an integrated management strategy, involving additional actions to slow the spread of *Xf* within and among vineyards. Moreover, growers may be able to benefit from *PdR1* resistant cultivars without planting all of their acreage to them. Our work is integrating biological information about how *Xf* spreads within a vineyard with economic scenarios on how grower profits may be impacted by growing *PdR1* grapevines. Our work will provide growers with recommendations on the optimal mixtures of *PdR1* resistant and susceptible grapevines for effective disease management, based on particular environmental conditions.

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 vs. tolerance traits on pathogen spread and disease prevalence can differ dramatically (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 *Xf* in California – blue-green sharpshooter (*Graphocephala atropunctata*; BGSS) and glassy-winged sharpshooter (*Homalodisca vitripennis*) - 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 *PdR1* locus (Walker and Tenscher, 2016). These hybrid vines do not suffer from Pierce's disease symptoms to the same extent of susceptible lines (Krivanek and Walker, 2005; Krivanek et al., 2006). Furthermore, from our previous results, *PdR1* 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 *PdR1* locus (Walker and Tenscher, 2016). Specifically, we ask, under what ecological conditions and spatial arrangements will the use of *PdR1* vines reduce *Xf* spread and maximize economic benefits to growers? The research consists of three objectives:

1. Test the effects of *PdR1* resistant plants on vector feeding preference and transmission of *Xf*.
2. Model the optimal mixture of *PdR1* and susceptible grapevines to reduce *Xf* spread and maximize economic return.
3. Estimate dispersal of insect vectors from field population data.

RESULTS AND DISCUSSION

Objective 1. Test the Effects of *PdR1* Resistant Plants on Vector Feeding Preference and Transmission of *Xf*

In 2017, we investigated the interplay between vector feeding preference and transmission of *Xf* from *PdR1* resistant and susceptible grapevine genotypes. We inoculated two *PdR1* resistant genotypes (labeled 094 and 102) and two susceptible genotypes (007 and 092) with *Xf* STL strain. At 2, 5, 8, and 14 weeks post-inoculation, we introduced eight BGSS into a cage with one inoculated plant (from one of the four genotypes) and one *Xylella*-free test plant, of either susceptible genotype. We included eight replicates of each combination of week since inoculation and genotype, and each replicate was independent - using different plants and vectors in each trial. We recorded which plant the vectors were feeding on at regular intervals over a four-day period, estimated *Xylella* populations in the source plants using culturing, assessed Pierce's disease symptoms in the source plants, and assessed transmission by culturing from *Xylella*-free test plants three months after the trials. We are in the process of estimating *Xylella* populations in vectors using quantitative polymerase chain reaction (qPCR.)

We estimated attraction rates and leaving rates of the BGSS by fitting data collected on the number of insects on each plant to the Consumer Movement Model described in Zeilinger et al. (2014). We used generalized linear models with quasi-Poisson or Poisson link functions to test for differences in genotypes and time since inoculation (2, 5, 8, and 14 weeks) in *Xf* populations in source plants, *Xf* populations in vectors, and in Pierce's disease symptom severity. For Pierce's disease symptom severity, we used the index described in Guilhabert and Kirkpatrick (2005). To test for differences in the percent of test plants infected with *Xf*, we combined data for the resistant genotypes and the susceptible genotypes then fit these data to multiple linear and non-linear ecological models.

Linear model: $y = ax + b$

Ricker: $y = axe^{-bx}$

Holling Type IV: $y = \frac{ax^2}{b+cx+x^2}$

Logistic growth: $y = \frac{1}{1+e^{-(a+bx)}}$

Michaelis-Menten: $y = \frac{ax}{b+x}$

In these equations, y is the proportion of test plants infected, x is the weeks post-inoculation, and *a*, *b*, and *c* are model-specific parameters. The non-linear models were selected based on *a priori* hypotheses on the dynamics of infection in our experiment. The Ricker and Holling Type IV models exhibit a unimodal or "humped" functional response, whereas the Logistic Growth and Michaelis-Menten models exhibit an asymptotic or saturating functional response (Bolker, 2008). The model that fit the data best was selected for the *PdR1* resistant and susceptible genotypes separately using Aikake's Information Criterion corrected for small sample size (AIC_c).

BGSS vectors showed significant preference for *Xylella*-free test plants compared to inoculated susceptible plants (007 and 092 genotypes) at 14 weeks post-inoculation. Likewise, BGSS vectors showed a preference for *Xylella*-free test plants at eight weeks post-inoculation (**Figure 1**). Both of the susceptible genotypes exhibited deteriorating Pierce's disease symptoms over time and were significantly worse than the resistant genotypes (**Figure 2A**; week x genotype interaction: $F_{3, 102} = 9.83$, $P < 0.0001$). For population sizes of *Xf* in the inoculated source plants, the two susceptible genotypes had significantly greater populations than the resistant genotypes (**Figure 2B**, $F_{3, 115} = 23.70$, $P < 0.0001$) and populations increased over time across genotypes ($F_{1, 115} = 4.92$, $P < 0.03$). To date, we have assayed, using qPCR, 610 out of 917 total BGSS recovered from our experiment for *Xf* infection. Preliminarily, the proportion of vectors in each trial that acquired *Xf* increased over time; vector acquisition also appears to be greater from susceptible genotypes than resistant genotypes (**Figure 2C**).

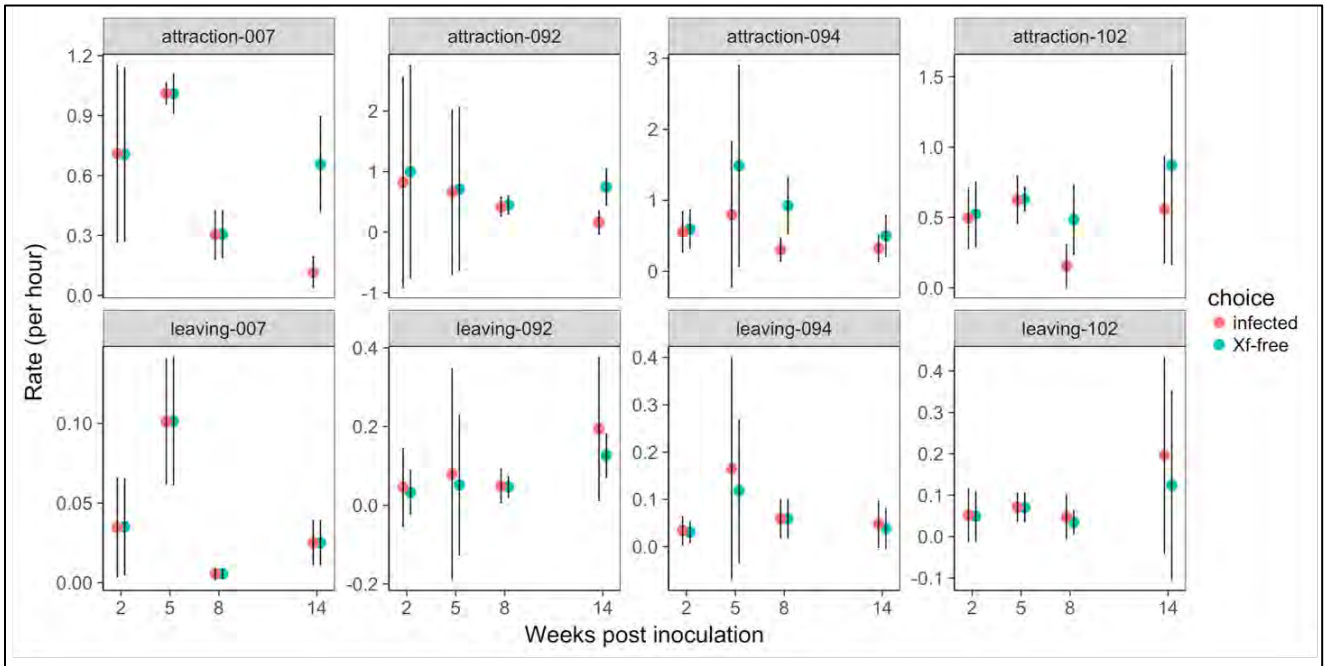


Figure 1. BGSS attraction rates (top four panels) varied significantly between infected and *Xylella*-free plant choices, particularly for susceptible genotypes (007 and 092) at 14 weeks post-inoculation. Leaving rates (bottom four panels) did not differ significantly. Error bars represent 95% confidence intervals.

The proportion of *Xylella*-free test plants that became infected exhibit clear non-linear dynamics over time post-inoculation (**Figure 2D**). The best model for the resistant genotypes was the Holling Type IV whereas the best model for the susceptible genotypes was the Ricker model, suggesting significant differences in the transmission dynamics between the resistant and susceptible genotypes (**Table 1**). These models suggest distinct biological processes underlying these dynamics, which we are exploring using additional modeling.

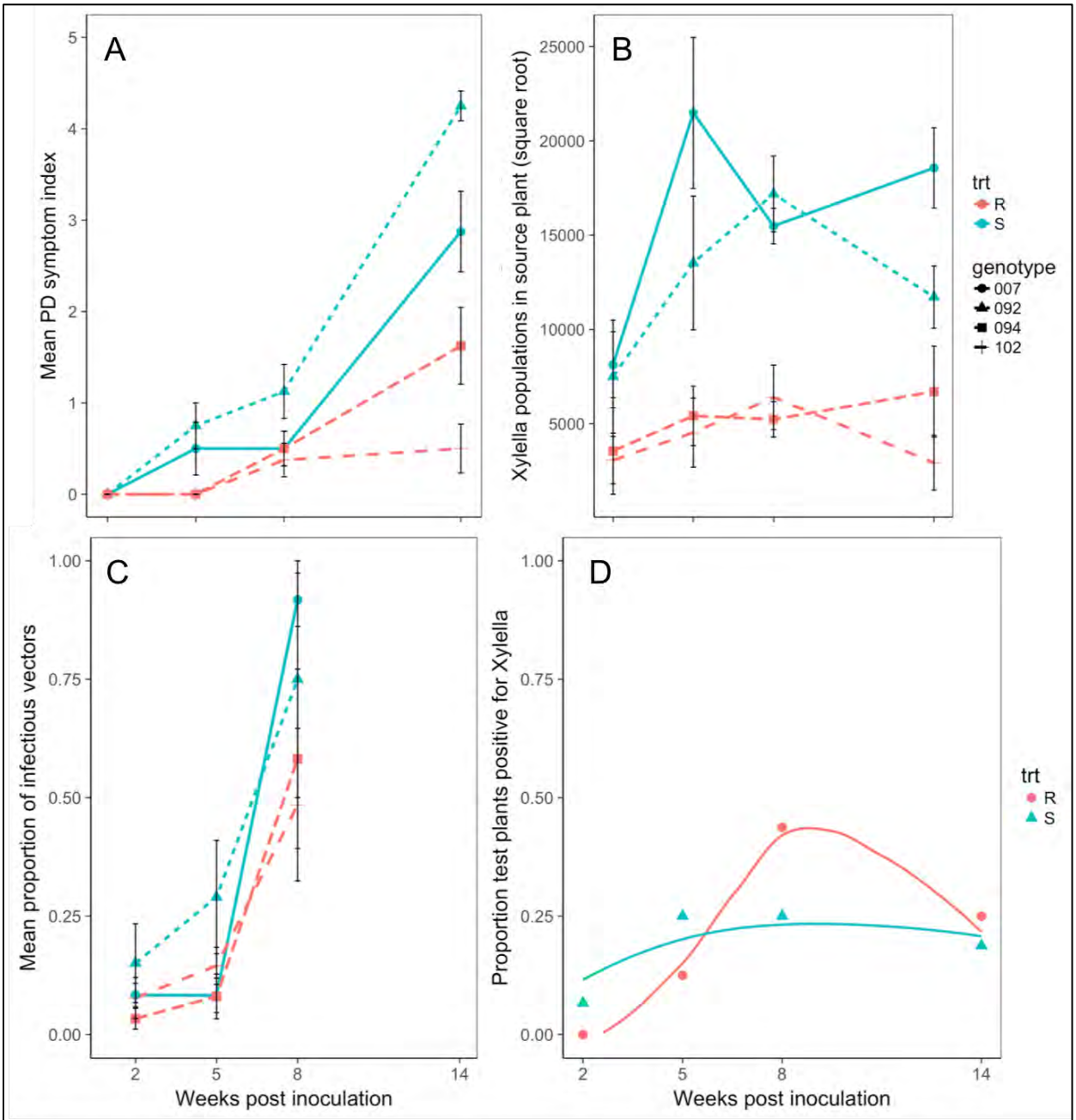


Figure 2. Mean PD symptom severity (A) and *X. fastidiosa* density in inoculated plants (B) were greater for susceptible genotypes (S, blue lines) than resistant genotypes (R, red lines). Proportion of vectors infectious with *X. fastidiosa* tended to be greater in susceptible genotypes than resistant genotypes (C). Proportion of test plants infected with *X. fastidiosa* were significantly different between resistant and susceptible genotypes, based on our model selection process (D). Resistant and susceptible genotypes were combined for analysis of transmission (D). Error bars represent ± SE.

Table 1. Results from model selection for transmission dynamics. The model that bests fits the data has the lowest ΔAIC_c value. df = degrees of freedom (i.e., number of parameters).

Model	AIC_c	ΔAIC_c	df
Resistant genotypes			
Holling Type IV	17.0	0	3
Linear	19.9	2.9	2
Ricker	19.9	2.9	2
Michaelis-Menten	20.1	3.1	2
Logistic Growth	22.5	5.6	2
Susceptible genotypes			
Ricker	16.4	0	2
Michaelis-Menten	17.0	0.6	2
Linear	17.9	1.6	2
Logistic Growth	18.0	1.7	2
Holling Type IV	18.7	2.3	3

Objective 2. Model the Optimal Mixture of *PdR1* and Susceptible Grapevines to Reduce *Xf* Spread and Maximize Economic Return

We have built a preliminary economic extension to our vector-susceptible-infected (SI) epidemic model, described in our proposal. We have included **Box 1** from our proposal, which describes the epidemic model that we previously developed.

We consider a scenario where two vineyards are grown adjacent to each other - one composed of a grape cultivar susceptible to Pierce's disease, Patch 1, and another composed of *PdR1* resistant grapevines, Patch 2. Then we can define the state variables in **Box 1** for each patch, such that S_j, E_j, $H_{C,j}$, and $H_{I,j}$, where $j = 1, 2$, to represent hosts in either Patch 1 or Patch 2, respectively.

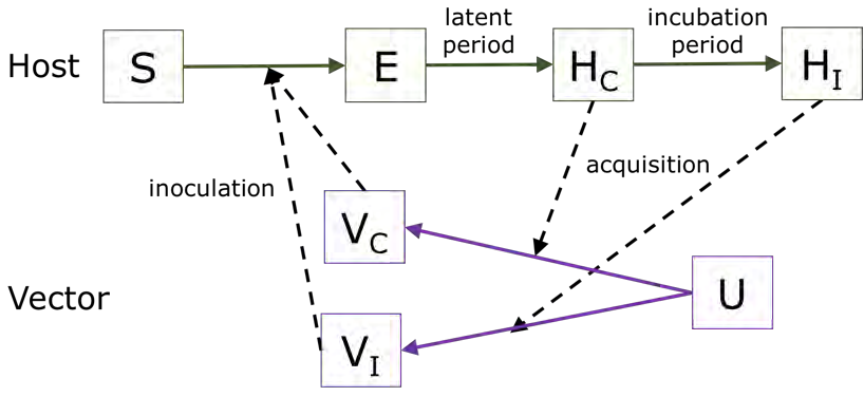
For the preliminary economic model, we followed the framework of Macpherson et al. (2017) and assumed that yield is proportional to the density of healthy or asymptomatic hosts at harvest time ($t = \tau$). In our epidemic model (**Box 1**), hosts in the compartments S_j, E_j, and $H_{C,j}$ are healthy, whereas hosts in $H_{I,j}$ are diseased. Then total yield, Y, is defined as:

$$Y = c_1 M_1(t = \tau) + + c_2 M_2(t = \tau)$$

where $M_j = S_j + E_j + H_{C,j}$ and represents the total density of healthy hosts. The parameters c_j modulate the relative value of the two cultivars. For instance, if the resistant cultivar has a lower value per unit of harvested grapes, then we set $c_2 < c_1$. We set $\tau = 500$ to ensure that the epidemic model dynamics reach equilibrium. In addition, as a first approximation, we assume that all healthy hosts produce the same yield and all diseased hosts produce no yield.

We first explored the sensitivity of our bioeconomic model to variation in economic value of resistant grapevines and the area planted to resistant grapevines. We varied the value of the c_2 parameter between 0.01 and 10, while setting $c_1 = 1$ constant. For the epidemic model parameters, we used the mean values from our 2016 experimental results, as described in **Box 1**. Given our epidemic model parameters, the value of grapes from the resistant cultivar has a strong effect on total yield, indicating that yield from the susceptible patch is relatively poor and unimportant (**Figure 3A**). Unsurprisingly then, there is much higher yield when the resistant patch is larger.

In the initial simulation, we used epidemic parameter estimates from our experimental results. We also sought to explore the effects of uncertainty in the parameter estimates. Again, increasing the area planted to *PdR1* resistant grapevines increases the total expected yield (**Figure 3B**). At the same time, we see a large amount of uncertainty in the results as well, with a slight increasing in the 95% confidence intervals with increasing area planted to resistant grapevines.



$$\frac{dS}{dt} = \eta(E + H_C + H_I) - \beta_c - \beta_I$$

$$\frac{dE}{dt} = \beta_C + \beta_I - \delta E - \eta E$$

$$\frac{dH_C}{dt} = \delta E - \gamma H_C - \eta H_C$$

$$\frac{dH_I}{dt} = \gamma H_C - \eta H_I$$

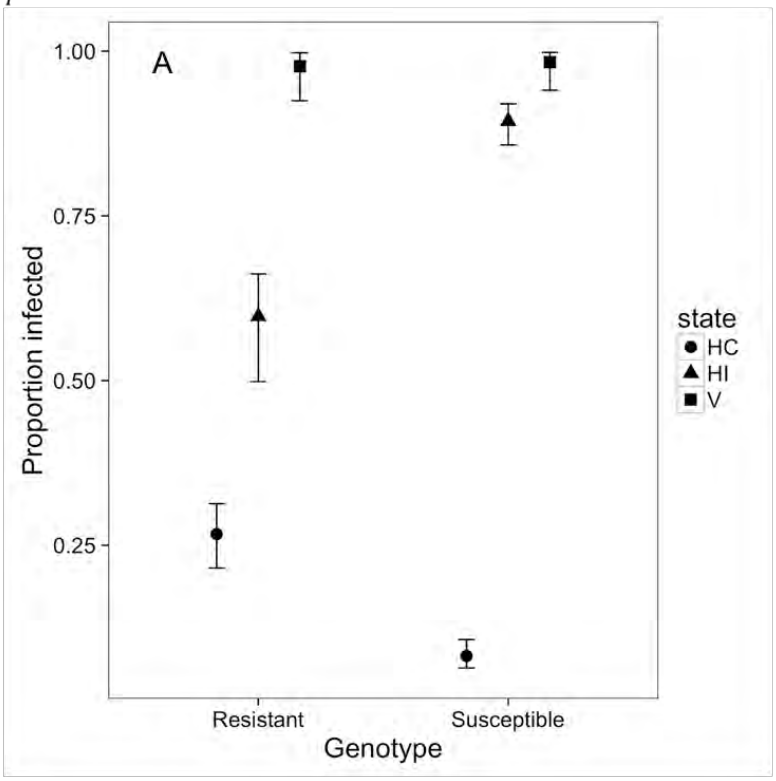
$$\frac{dU}{dt} = \lambda V_C + \lambda V_I - \alpha_C - \alpha_I$$

$$\frac{dV_C}{dt} = \alpha_C - \lambda V_C$$

$$\frac{dV_I}{dt} = \alpha_I - \lambda V_I$$

$$\beta_i = \frac{p_i(1 - e^{-b_i T})V_i S}{N}$$

$$\alpha_i = \frac{\mu_i(1 - e^{-a_i T})H_i U}{N}$$



Box 1. We modeled the spread of *X. fastidiosa* through simulated *PdR1* Resistant and Susceptible vineyards using a continuous-time SI-vector compartmental model. The model included compartments for non-infected hosts and vectors (S and U), exposed hosts (E), asymptomatic infected "Carrier" hosts (H_C), diseased infected hosts (H_I), and infectious vectors that acquired infection from either the H_C or H_I compartments (V_C and V_I). Inoculation and acquisition rates, β_i and α_i where i = C, I, were adapted from Madden et al. (2000). We used experimental data to estimate values for vector attraction rate (p_i), vector leaving rate (μ_i), inoculation probability (b_i), infectious period (δ^{-1}), incubation period (γ^{-1}), and host recover (η). Vector acquisition probability (a_i) was set as proportional to inoculation probability, pending data collection of *X. fastidiosa* populations in vectors. Vector recovery (λ) was set at 0.083. Time spent feeding (T) was calculated from Almeida and Backus (2004). $N = S + E + H_C + H_I$. Based on experimental results, estimates for i = C parameters were taken from 3-week trials while estimates for i = I parameters were taken from 12-week trials. We calculated standard errors for each experimentally-derived parameter and used Monte Carlo simulations (n = 5,000) to estimate mean and 95% confidence intervals for densities of infected hosts, H_C and H_I (filled circles and triangles, Panel A), and vectors, $V_C+V_I = V$ (filled squares), for *PdR1* Resistant and Susceptible vineyard scenarios. More detail and R code can be found at https://github.com/arzeilinger/pdr1_preference.

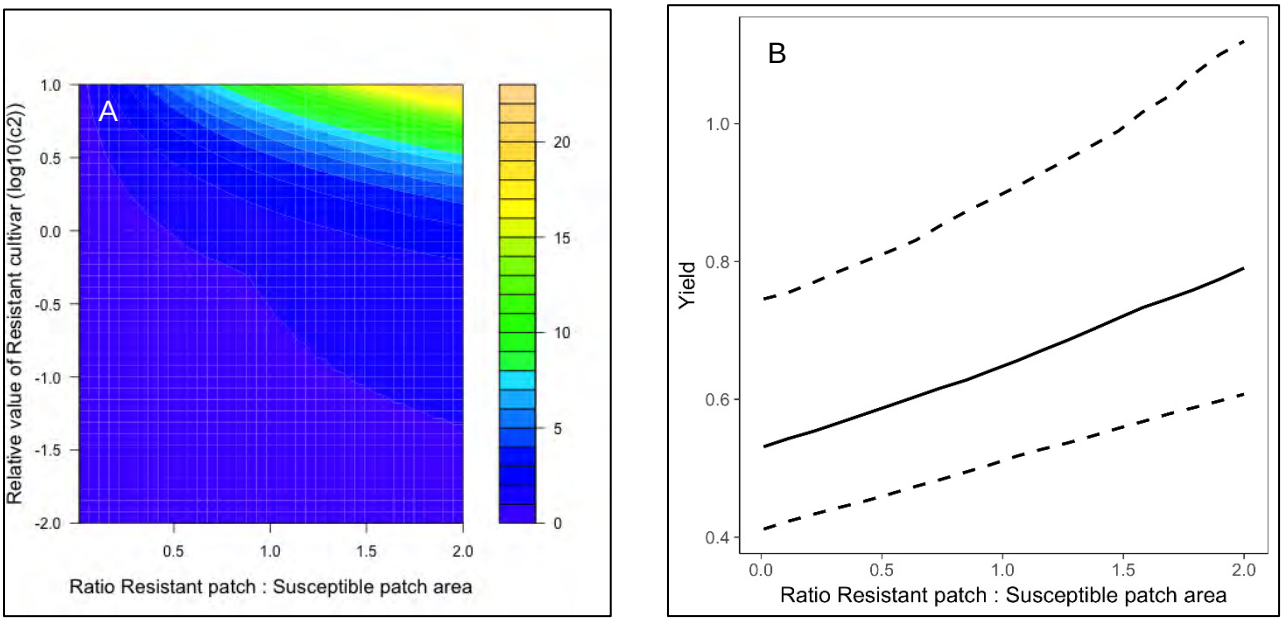


Figure 3. (A) Contour plot showing the expected total yield from varying the area of the Resistant patch (relative to the Susceptible patch area), and the relative value of grapes from the Resistant cultivar (c_2). Note that we varied c_2 from 0.01 to 10 and log10 transformed the y-axis. The colors indicate total yield, *Y*. (B) Median expected total yield (solid line) increases with increasing area planted to Resistant grapevines, but so too does uncertainty increase (95% confidence intervals, dashed lines). Confidence intervals were calculated from 600 Monte Carlo simulations of epidemic parameter values derived from our *PdR1* transmission experimental results. For these simulations, $c_1 = 1$, $c_2 = 0.1$.

Overall, our preliminary economic analysis suggests that planting *PdR1* resistant grapevines at high densities would be the most economically efficient strategy under epidemiological conditions measured in our 2016 experiments (*data not shown*). Our next steps will be to simulate economic outcomes from our 2017 experimental results (above), which appear more robust than our 2016 experiment.

We are also exploring different mathematical forms of transmission of *Xf* within the model. Our model in **Box 1** assumes a frequency-dependent form of transmission. Preliminary explorations of alternative forms of transmission suggest that the economic outcome may be quite different. Specifically, if we use a density-dependent form of transmission instead, a mixture of *PdR1* resistant and susceptible grapevines appears to produce the greatest economic return (*results not shown*). We are working to assess the robustness of these results and explore the effects of additional epidemiological and economic aspects of the model.

Objective 3. Estimate Dispersal of Insect Vectors from Field Population Data
Work on Objective 3 is ongoing and will be informed by results in Objectives 1 and 2. There are no results to report at this time.

CONCLUSIONS

Overall, our results confirm previous work in that *PdR1* resistant plants exhibit partial resistance to *Xf*, resulting in reduced bacterial populations and reduced Pierce's disease symptom severity. However, because *Xf* is able to reach moderate population sizes in resistant plants, there is still significant vector transmission from these plants. Importantly, because of reduced symptom severity and vector feeding preference for healthy grapevines, transmission dynamics are complex - transmission from resistant plants can be worse under some conditions (e.g., eight weeks post-inoculation within our experiments). These results suggest that there may be a window of time - during disease progression - where *PdR1* grapevines could act as reservoir hosts, amplifying vector transmission.

A critical question remains, under what ecological conditions, and for how long, could *PdR1* vines amplify transmission? We are working to address this question through epidemiological modeling (**Box 1**). We also are working to describe conditions under which different mixtures of *PdR1* resistant and susceptible grapevines would maximize economic return for growers. While there is some concern that *PdR1* vines could enhance *Xf*

spread in the field, our results suggest that these partially resistant vines hold promise to greatly improve Pierce's disease management. The key question remains to develop strategies to optimize their use in vineyards.

REFERENCES CITED

Bolker BM. 2008. *Ecological Models and Data in R*. Princeton University Press, Princeton, NJ.

Cronin JP, Rúa Megan A, Mitchell CE. 2014. Why is living fast dangerous? Disentangling the roles of resistance and tolerance of disease. *Am Nat* 184:172-187. doi: 10.1086/676854.

Erlanger TE, Keiser J, Utzinger J. 2008. Effect of dengue vector control interventions on entomological parameters in developing countries: A systematic review and meta-analysis. *Med Vet Entomol* 22:203-221. doi: 10.1111/j.1365-2915.2008.00740.x.

Guilhabert MR, Kirkpatrick BC. 2005. Identification of *Xylella fastidiosa* antivirulence genes: Hemagglutinin adhesins contribute to *X. fastidiosa* biofilm maturation and colonization and attenuate virulence. *Mol Plant Microbe Interact* 18:856-868.

Krivanek AF, Riaz S, Walker MA. 2006. Identification and molecular mapping of *PdR1*, a primary resistance gene to Pierce's disease in *Vitis*. *Theor Appl Genet* 112:1125-1131. doi: 10.1007/s00122-006-0214-5.

Krivanek AF, Walker MA. 2005. *Vitis* resistance to Pierce's disease is characterized by differential *Xylella fastidiosa* populations in stems and leaves. *Phytopathology* 95:44-52. doi: 10.1094/PHYTO-95-0044.

Macpherson MF, Kleczkowski A, Healey JR, et al. 2017. The effects of invasive pests and pathogens on strategies for forest diversification. *Ecol Model* 350:87-99.

Mundt CC. 2002. Use of multiline cultivars and cultivar mixtures for disease management. *Annu Rev Phytopathol* 40:381-410.

Perring T, Farrar C, Blua M, et al. 2001. Proximity to citrus influences Pierce's disease in Temecula Valley vineyards. *Calif Agric* 55:13-18.

Roy BA, Kirchner JW. 2000. Evolutionary dynamics of pathogen resistance and tolerance. *Evolution* 54:51-63.

Walker MA, Tenscher AC. 2016. Breeding Pierce's disease resistant winegrapes. *Proceedings of the 2016 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA, pp. 167-177.

Zeilinger AR, Daugherty MP. 2014. Vector preference and host defense against infection interact to determine disease dynamics. *Oikos* 123:613-622. doi: 10.1111/j.1600-0706.2013.01074.x.

Zeilinger AR, Olson DM, Andow DA. 2014. A likelihood-based biostatistical model for analyzing consumer movement in simultaneous choice experiments. *Environ Entomol* 43:977-988. doi: 10.1603/EN13287.

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EVALUATING POTENTIAL SHIFTS IN PIERCE'S DISEASE EPIDEMIOLOGY

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Reporting Period: The results reported here are from work conducted July 2017 to June 2018.

ABSTRACT
In this report we summarize recent activities in this project. We show that the *Xylella fastidiosa* (*Xf*) infection rate of the blue-green sharpshooter (*Graphocephala atropunctata*) in Napa/Sonoma populations fluctuates during the year, being higher in late fall/winter. A population genomics study indicated that California grape-growing regions have genetically distinct populations of *Xf*, although the biological meaning of these findings remains to be determined. A data mining effort led to a publication demonstrating that severe pruning of Pierce's disease-infected plants does not lead to healthy plants. Finally, data on the biology of the meadow spittlebug (*Philaenus spumarius*), a vector of *Xf* commonly found in northern California vineyards, is presented.

LAYPERSON SUMMARY
A Pierce's disease (PD) epidemic emerged in Napa and Sonoma Counties. Very high PD prevalence was reported throughout the region, with a large number of stakeholders reaching out to University of California Cooperative Extension Farm Advisors. In the summer of 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 PD in the North Coast is usually below 1-2% per vineyard, but several vineyards surveyed had over 25% of vines symptomatic. Second, historically, PD is closely associated with riparian zones in the North Coast, but we have visited several vineyards where PD 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 project is to determine what factors are driving this epidemic, so that ecology-based disease management strategies can be devised and immediately implemented, as was successfully done in the past when disease drivers appear to have been different.

INTRODUCTION
Pierce's disease (PD) 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 PD epidemic is emerging. The goal of this project is to determine what factors are driving this epidemic, so that ecology-based disease management strategies can be devised and immediately implemented, as was successfully done in the past when disease drivers appear to have been different. In this report we summarize progress made recently, covering some but not all objectives of this project.

OBJECTIVES

1. Vector, pathogen, and host community surveys to inform the development of a quantitative model to assess future PD risk and develop integrated management strategies.
2. *Xylella fastidiosa* (*Xf*) colonization of grapevines and the role of overwinter recovery in PD 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 PD information.

RESULTS AND DISCUSSION

We provide a summary of work that has been performed, as well as novel preliminary data when available. This section is not a complete summary of all activities; please refer to previous reports or contact the Principal Investigator or Cooperators for additional information. For example, in Objective 1 we report on the most recent data we have, which has not been included in previous reports, and provide the first estimates of the *Xf* infection rate of blue-green sharpshooter (*Graphocephala atropunctata*; BGSS) for Napa and Sonoma Counties. On the other hand, we do not report on BGSS populations.

Objective 1. PD Patterns

As part of Objective 1, we have now conducted two years of PD surveys in 32 vineyards throughout Napa and Sonoma Counties, in the fall of 2016 and again in the fall of 2017. As a first step toward understanding the condition changes that may have triggered the recent PD 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% PD (**Table 1**). Two other sites located in Sonoma County ("NEWS" and "V7") are adjacent to riparian corridors, with PD prevalence ranging between approximately 8 and 20% (**Table 1**).

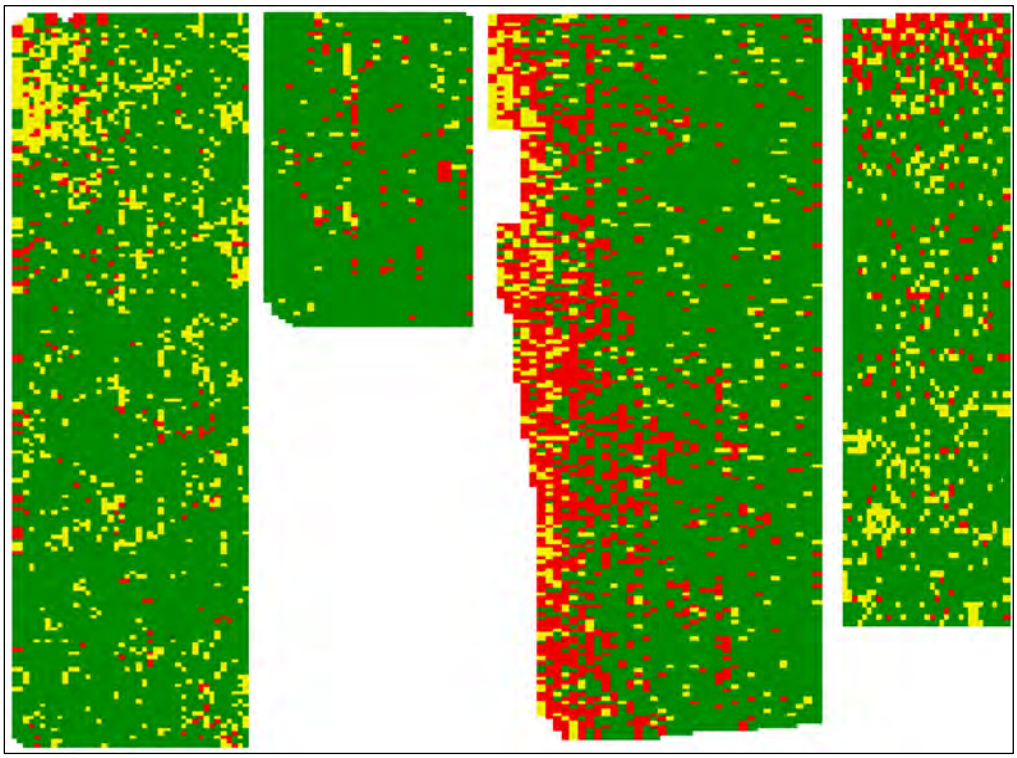

Figure 1. Mapping results for PD at four representative sites in the fall of 2016. Red pixels denote vines with PD, yellow are dead, missing, or replant vines, and green denotes apparently healthy vines. Sites (L to R) are: CDV, TREF, NEWS, V7. Maps are on the same approximate scale, but each is oriented 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, PD, dead, replant, or missing, and collected tissue samples from up to 20 PD vines to confirm infection by *Xf*. The mapped distributions of initial disease prevalence were then subjected to a suite of analyses to look for (1) non-random distribution (i.e., clustering) of PD cases, (2) spatial association between PD cases and other non-healthy disease categories (i.e., dead, missing, or replant vines), and (3) non-uniform distribution of PD 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 PD (Brunson and Comber, 2015). The tests were significant for all four of the sites (**Table 1**). This suggests significant clustering of PD cases at all sites, though the scale of clustering varied from below five vine spaces for site TREF to over 15 vine spaces at site NEWS. Next, a similar L means test was used for PD 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), PD vines are more likely to be found near dead, missing, or replant vines than expected by chance.

Table 1. Summary statistics for PD 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 PD symptoms, L means test for clustering of PD cases, L means test for co-clustering between PD cases and missing, dead, or replant vines, and test for uniformity in the distribution of PD cases across the vineyard block (i.e., no disease gradient).

				PD clustering		**Co-clustering**		**Uniformity**		
Site	**Riparian**	**# vines**	***% PD***	***u***	***P***	***u***	***P***	χ^2	**df**	***P***
CDV	N	7,406	2.85	144.17	0.01	6.670	0.01	1.0172	2	0.6013
TREF	N	2,220	4.68	37.158	0.01	5.050	0.12	1.7144	2	0.4243
NEWS	Y	6,608	20.11	256.4	0.01	17.832	0.01	9.6049	2	0.0082
V7	Y	3,355	8.29	107.45	0.01	0.5741	0.01	21.663	2	<0.0001

In the third analysis of PD 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 PD 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 PD declined significantly at greater distances, with most cases within approximately 60 meters of the riparian corridor but with still a handful of cases at much greater distances (**Figure 2**).

Vector Natural Infectivity in Napa and Sonoma. As part of our monitoring activities, we regularly collected BGSS on nearly 400 sticky traps and tested them via quantitative polymerase chain reaction (qPCR) to determine the fraction that were positive for *Xf*. Thus far more than 1,800 unique BGSS have been assayed from collections made at more than 30 vineyard sites between December 2016 and April 2018.

Of the insects tested, overall, approximately 14% (256 of 1,812) were positive for *Xf*, with a range between sites of approximately 2% to over 25% (**Figure 3**). We compared the fraction of samples testing positive in two related analyses. First, we analyzed the overall differences in infectivity in a GLMM, which included fixed effects of site type (i.e., sites with riparian habitat nearby vs. non-riparian sites) and trap location (i.e., traps within the vineyard vs. bordering the vineyard), a random effect of site identity to account for autocorrelation stemming from repeated measurements made at each site over time, and binomial error. A second GLMM was conducted on just those 11 sites at which there were sufficient samples over the season to estimate an effect of time (month irrespective of year; as a fixed effect), a fixed effect of trap location, a random effect of site identification to account for

autocorrelation, and binomial error. In both analyses, model selection (via Akaike Information Criterion [AIC] rankings) was used to identify the minimum adequate model.

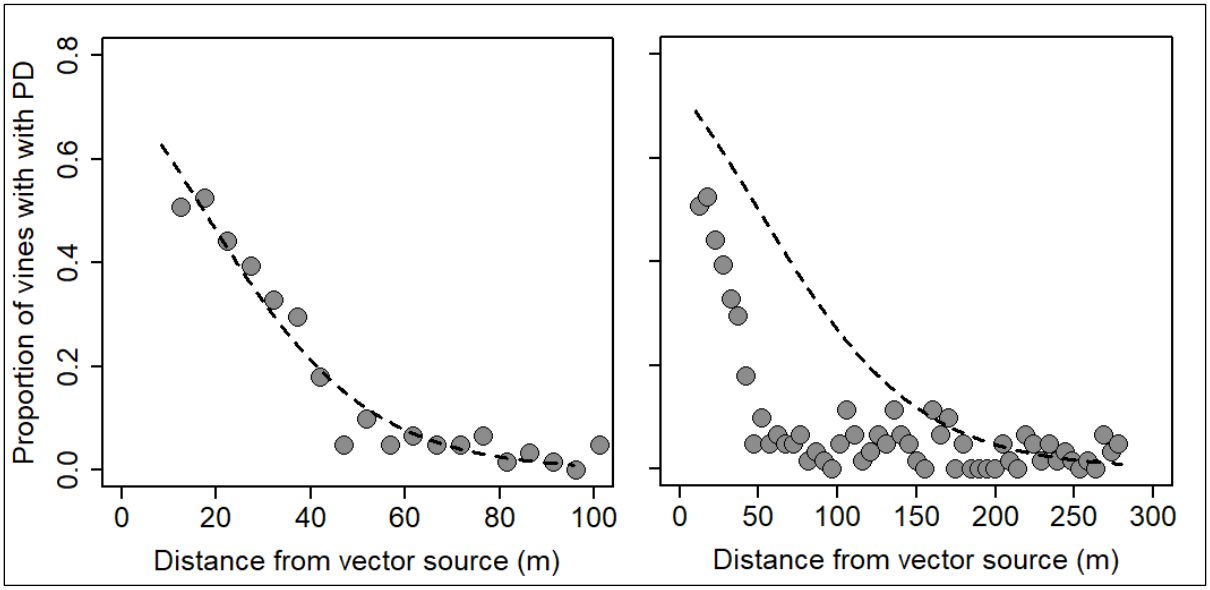

Figure 2. Gradients in PD prevalence as a function of distance from riparian habitat. Sites: left = NEWS, right = V7. Points reflect proportions of vines with PD of 50-100 vines at different binned distances. Dashed lines denote model fit.

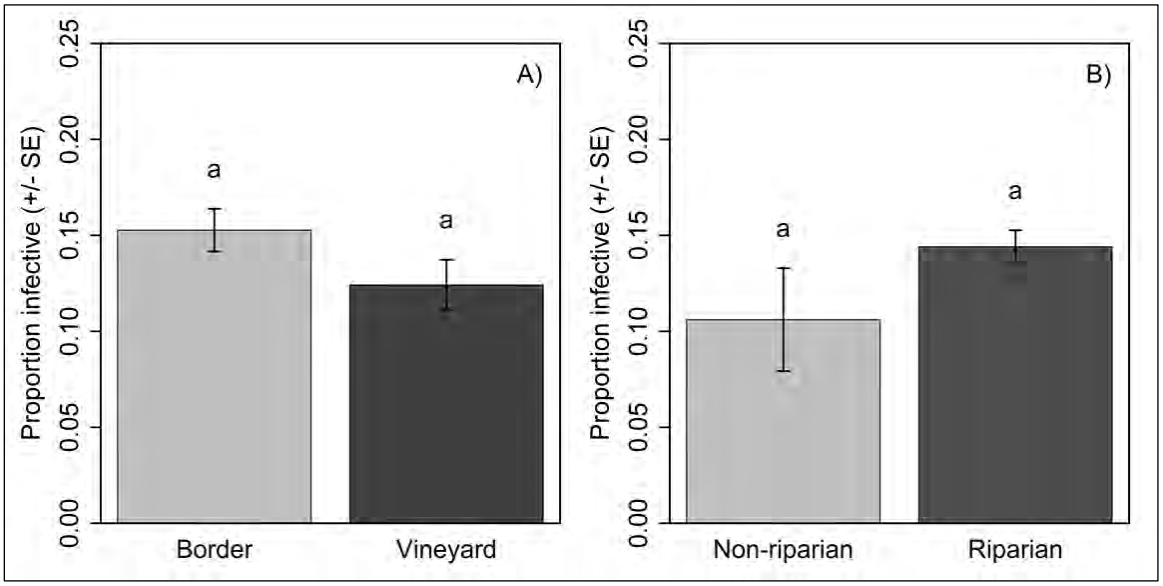

Figure 3. Overall proportion of BGSS testing positive for *Xf* between (A) trap locations, and (B) site types.

For the first analysis, the preferred model included only a non-significant effect of trap location ($\chi^2 = 0.782$, $df = 1$, $P = 0.3765$). Although, overall, the fraction of BGSS collected from traps bordering vineyards (i.e., nearby riparian habitat or other source habitat) testing positive for *Xf* was higher than those BGSS collected within vineyards, the difference was not significant. Similarly, although the overall fraction of BGSS testing positive at riparian sites was slightly higher than at non-riparian sites, the difference was not significant. These results suggest that *Xf* infectivity within BGSS populations is pretty well mixed, at least with respect to these relatively crude categories of site type and trap location at a site.

For the second analysis, the preferred model included only a significant effect of time (χ^2 = 173.67, *df* = 11, *P*<0.0001). The fraction of BGSS testing positive varied from a low of approximately 1% in May to a high of 50% in November (**Figure 4**). These results suggest there is substantial variability in BGSS infectivity, with low infectivity over much of the growing season and far higher infectivity during the late and dormant seasons.

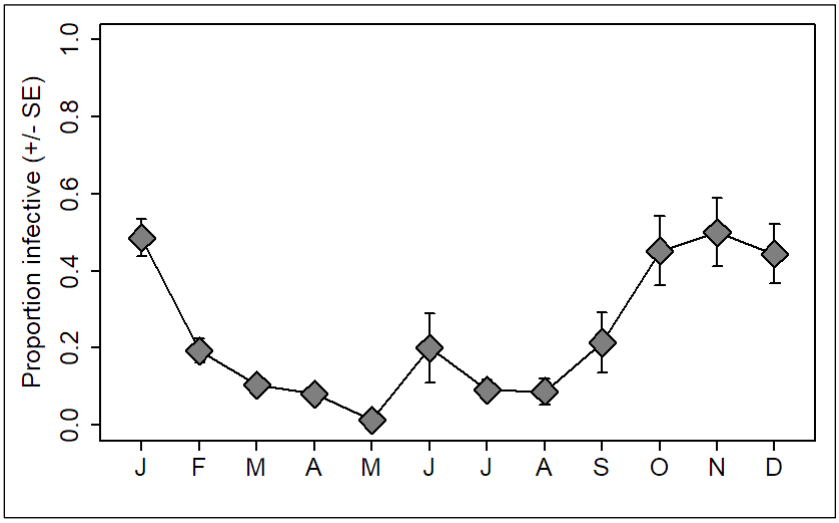

Figure 4. Seasonal variability in the fraction of BGSS testing positive for *Xf*.

Xf Population Genomics. We also surveyed *Xf* populations in California to determine if the recent epidemic was the consequence of a newly virulent strain. Populations from five regions were sampled. Below (**Figure 5**) is a phylogenetic tree based on genomic sequences of 122 *Xf* isolates collected from grapevines expressing PD symptoms in California. Each point represents one sample; colors represent different regions in the state. *Xf* isolates were region specific, with a few exceptions.

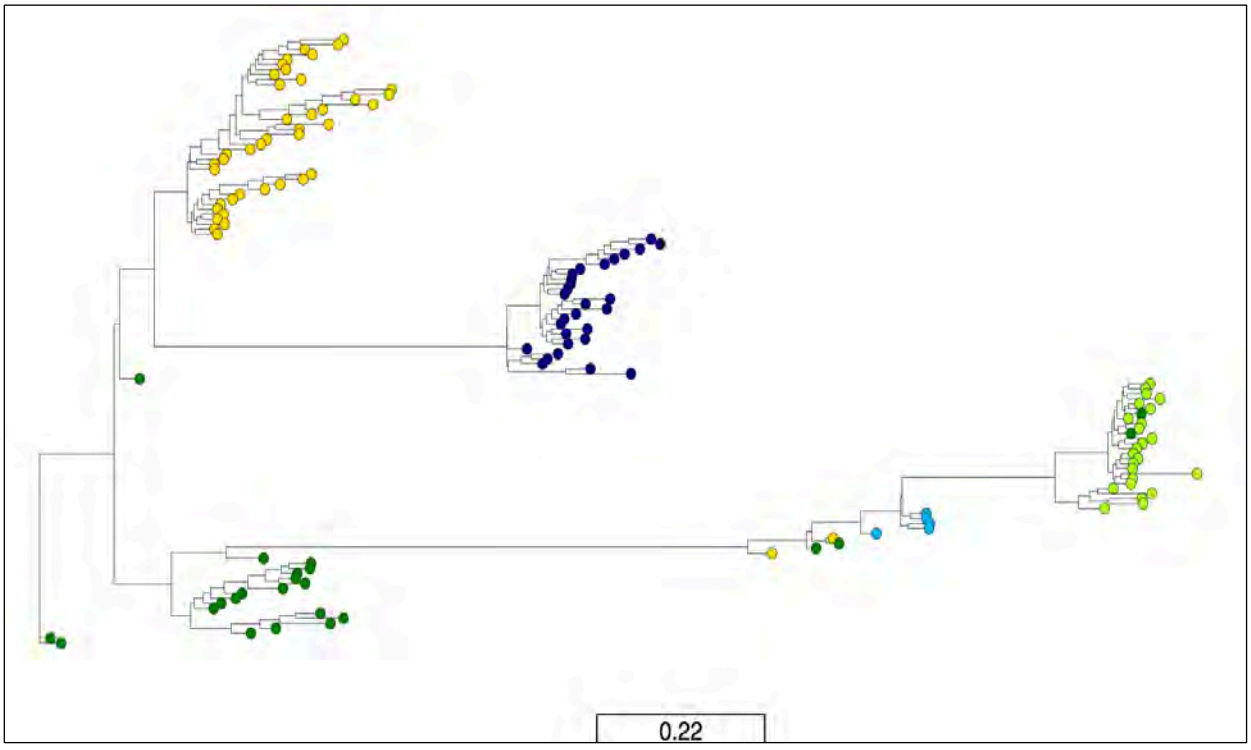

Figure 5. Phylogenetic tree based on genomic sequences of 122 *Xf* isolates collected from grapevines expressing PD symptoms in California

Objective 2. Estimating the Importance of Climatic Conditions for Driving PD Incidence

As a first step toward understanding whether climatic conditions in recent years have contributed to the ongoing PD resurgence in the North Coast, we compared historic data from 11 weather stations from throughout the grape-growing regions of Napa and Sonoma Counties, which include up to 70 years of data. All else being equal, a lack of cold conditions over the winter and early spring should contribute to PD incidence, by reducing the fraction of vines recovering from infection (Feil and Purcell, 2001) and potentially contributing to greater vector population densities (Gruber and Daugherty, 2012). To address this prediction, we compared historic averages with more contemporary observed dormant season (i.e., November through April) temperatures between 2011 and 2016, focusing on two metrics: (1) mean daily minimum temperature and (2) number of days with winter temperatures below 4.4°C (Lieth et al., 2011).

For each of the 11 sites, we calculated historic averages for both of the temperature metrics, and the corresponding values for each of the seasons between 2011 and 2016, a span of time that conservatively captures the onset of the most recent PD epidemic in the North Coast. Next, to facilitate comparisons among sites, the contemporary year estimates were standardized by dividing by the historic average for that site. Thus, values of relative daily minimum temperature greater than 1 correspond to a dormant season that is warmer than the historic average, whereas values of the relative frequency of days with minimum temperatures below 4.4°C of greater than 1 indicate conditions colder than the historic average (**Figure 6**).

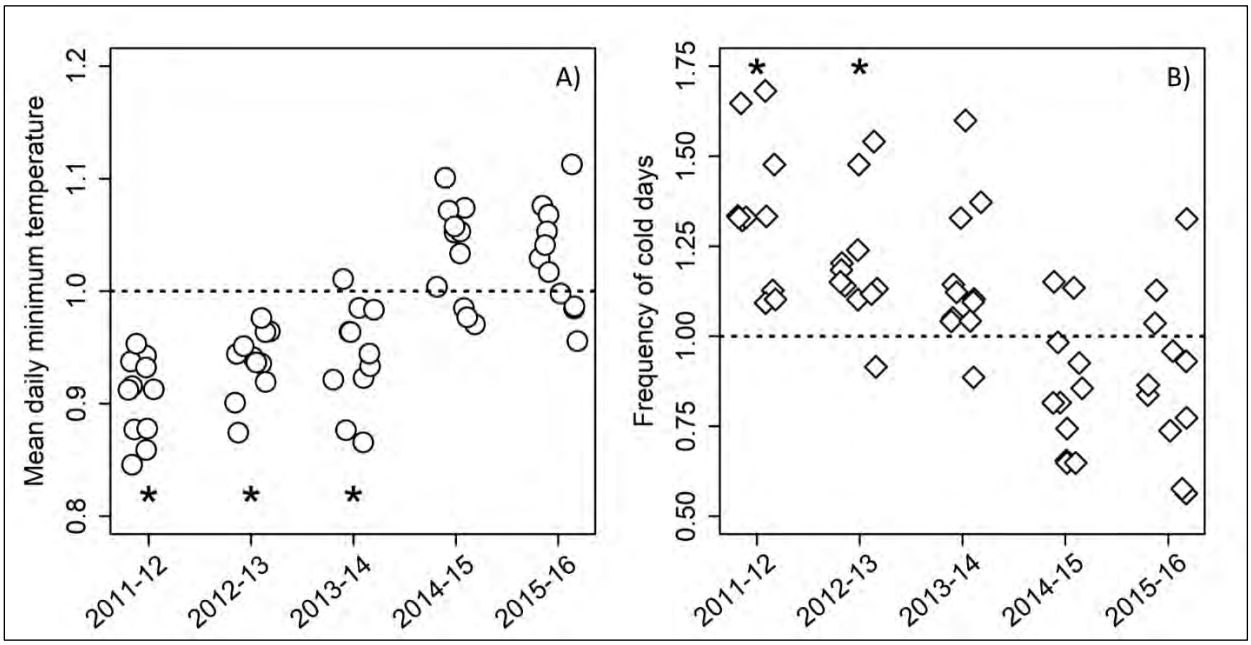

Figure 6. Relative (A) daily minimum temperature and (B) number of days with minimum temperatures below 4.4°C over the dormant season (November - April) compared to historic averages at 11 sites in Napa and Sonoma Counties. *Denotes yearly means that are significantly different than the historic average.

Objective 3. Ecology of Spittlebug Vectors

In 2016, 2017, and 2018 we surveyed sites in Napa and Sonoma Counties for nymphs of the meadow spittlebug (*Philaenus spumarius*). At the site in Sonoma two vineyards were surveyed, while at the Napa site only one vineyard was surveyed. Except in cases of extreme weather, the vineyard sites were surveyed biweekly. These surveys consisted of randomly selecting 10 plots in each vineyard during each sampling period. Each plot consisted of two vine-rows and one inter-row and had an approximate area of 7 x 15 ft^2.

Nymph sampling consisted of randomly tossing six 2 x 2 ft^2 quadrats in each plot and collecting all nymphal spittle masses within each quadrat. Nymphs were removed from spittle masses and individually counted back in UC Berkeley. Associated nymphal host plants were identified in the field and any unknown host plants were collected and preserved for identification back at UC Berkeley. For 2017 and 2018, we summarized the most

common nymphal host plants across all sites by counting the number of survey plots where meadow spittlebug nymphs were found on a given host plant (**Figure 7**).

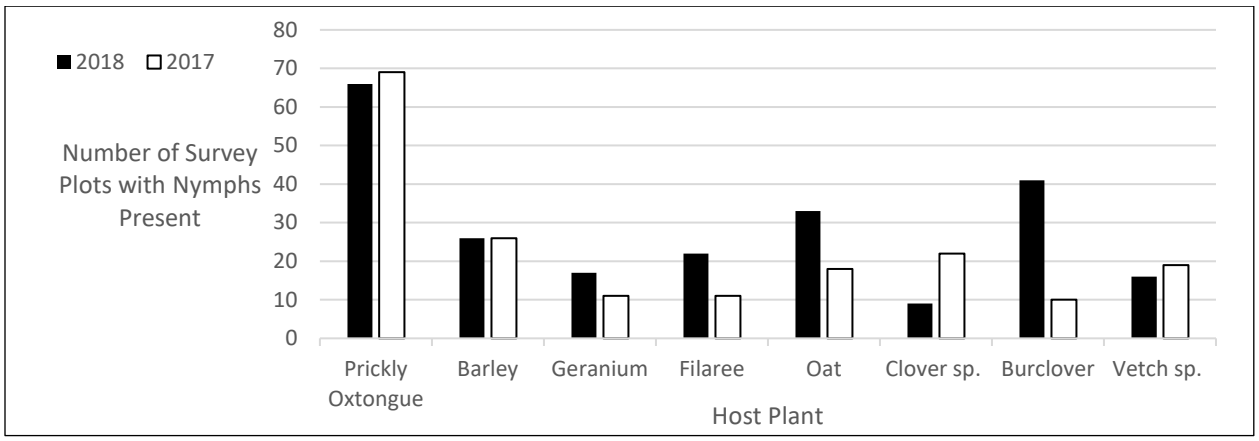

Figure 7. Number of survey plots with nymphs of the meadow spittlebug present, by most common hosts.

In late March to early April of each field season, biweekly surveys for the adult meadow spittlebugs began at our two sites in conjunction with observations of this species' phenological development. In each of the 10 plots, the two vine-rows and the inter-row were each subjected to 25 sweeps with a sweep net. Additionally, a yellow sticky trap (Seabright Labs) was hung on the middle trellis wire of each plot's two vine-rows and checked biweekly (weather permitting) for captured adults of meadow spittlebug. Below (**Figure 8**) we present preliminary data that has not been analyzed.

Objective 4. Data Mining of Unpublished Data

Our efforts for this objective focused on one particular project, which asked if severe pruning of *Xf*-infected grapevines would cure plants from infection (**Figures 9 and 10**). This has remained a major question for vineyard managers, where the practice is still attempted, primarily because there were no studies available on the topic demonstrating the fact the concept is likely not viable. A large effort to mine data associated with 20-year old experimental data led to a publication: Daugherty MP, Almeida RPP, Smith RJ, Weber EA, Purcell AH. 2018. Severe pruning of infected grapevines has limited efficacy for managing Pierce's disease. *American Journal of Enology and Viticulture* 69:289-294. The conclusion of that work is: "These results suggest that severe pruning does not clear *Xf* infection from grapevines to an extent that would justify its adoption for disease management."

CONCLUSIONS

Information generated in this project is shedding light onto old questions. First, natural infectivity of BGSS appears to fluctuate during the year, being higher during the winter. *Xf* infecting grapevines in California is diverse and geographically structured. Details of the biology of the meadow spittlebug vector are reported, although the role of this species on PD epidemics remains to be determined. Severe pruning of plants with PD does not cure plants from *Xf* infection.

FUNDING AGENCIES

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

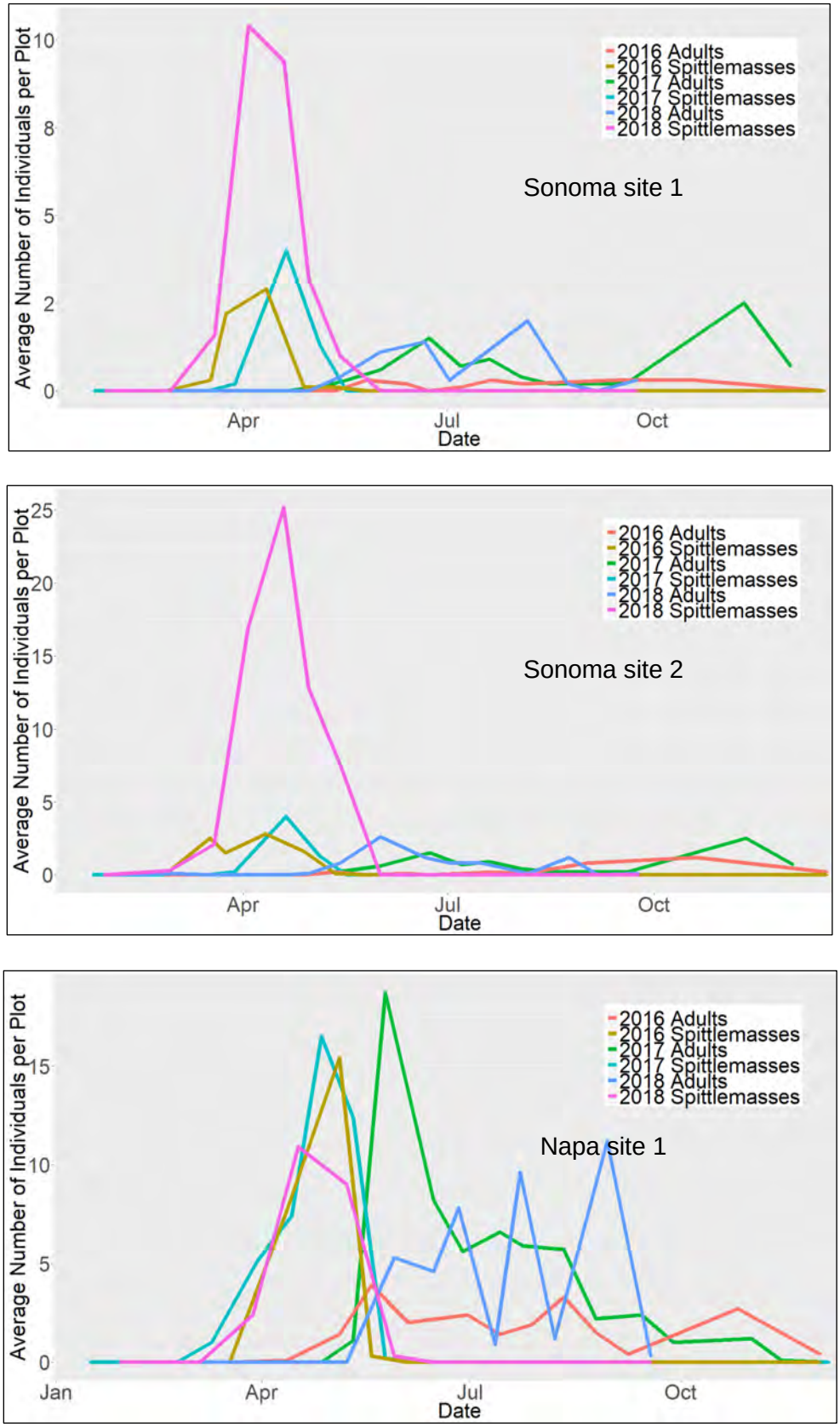

Figure 8. Average number of meadow spittlebug adults and spittle masses per plot at three sites in 2016 - 2018.

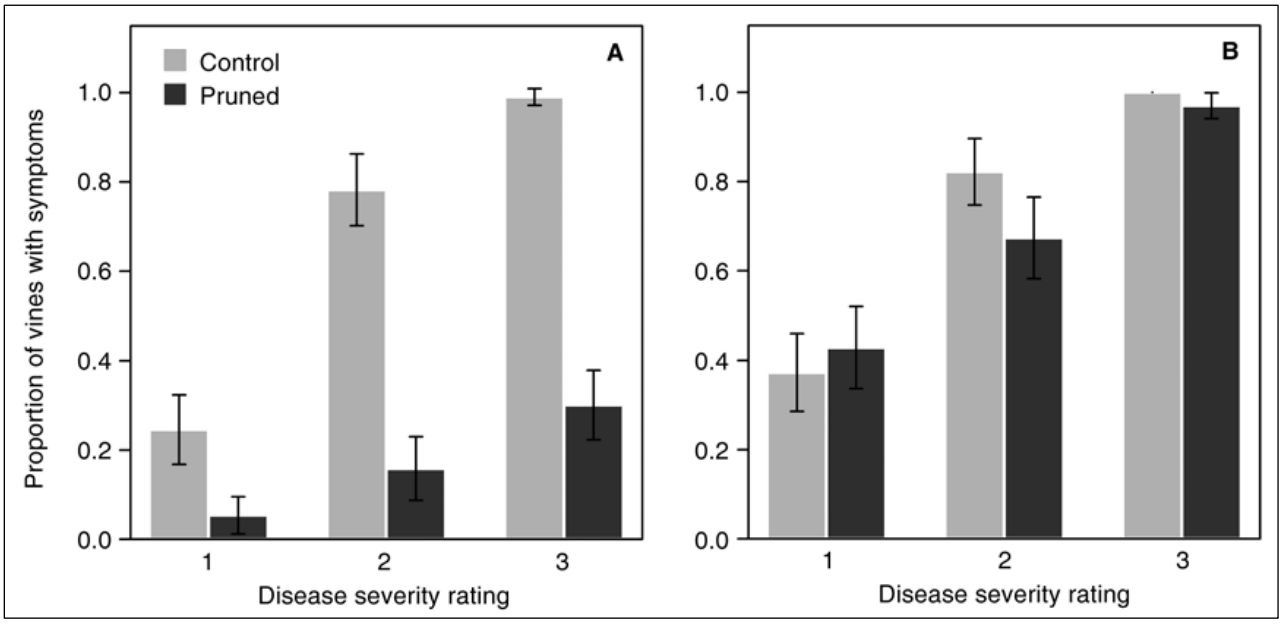

Figure 9. Return of PD symptoms in severely pruned or control (conventionally pruned) vines from three disease-severity categories after (A) one year or (B) two years. Each column represents the average proportion of vines with symptoms, for groups of 101 to 133 vines spread among six vineyard blocks. Error bars denote 95% confidence intervals.

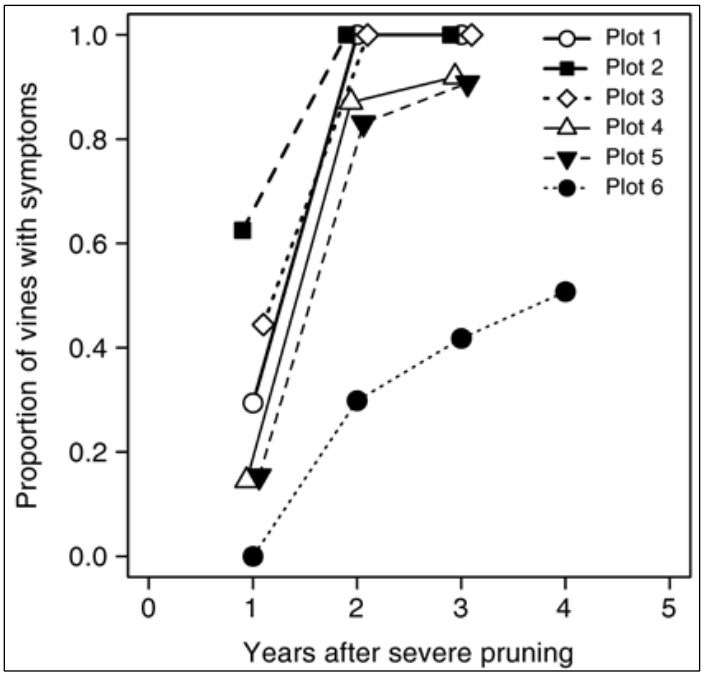

Figure 10. Return of PD symptoms after severe pruning of vines in the most severe disease category, for the six vineyard plots. Some plot symbols offset slightly for clarity. Points represent the overall proportion of vines that showed symptoms for up to 67 replicate severely pruned vines per block.

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

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Reporting Period: The results reported here are from work conducted July 1, 2018 to October 4, 2018.

ABSTRACT

This project was only recently approved, as such there are no results to present or discuss. Below we summarize the rationale for this project, as well as its goals.

LAYPERSON SUMMARY

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

OBJECTIVES

This research project has three objectives, which were identified as pressing issues that need to be addressed to improve our understanding of Pierce's disease epidemiology.

1. Role of spittlebugs in Pierce's disease epidemiology.
2. Mathematical modeling of Pierce's disease spread.
3. *Xylella fastidiosa* (*Xf*) population genomics.

Objective 1. Role of Spittlebugs in Pierce's Disease Epidemiology

Our previous work on spittlebugs focused on the meadow spittlebug (*Philaenus spumarius*), which was found in vineyards with unique Pierce's disease spatial distribution patterns. This insect has previously been studied in California as a vector of *Xf*. However, in addition to *P. spumarius*, it has become evident that species of *Aphrophora* (spittlebugs that lack a common name) and *Pagaronia* (a leafhopper) are associated with vineyards. These species have not been studied in vineyards, or in the context of Pierce's disease, but their ubiquity in vineyards in Napa and Sonoma where neither *P. spumarius* nor the blue-green sharpshooter (*Graphocephala atropunctata*) have been observed raised their profile as insects to be studied as vectors *of Xf*.

Objective 2. Mathematical Modeling of Pierce's Disease Spread

The current hypothesis explaining Pierce's disease spread in the North Coast is that blue-green sharpshooters overwinter as adults in riparian zones, where these insects acquire *Xf* from host plants and then migrate to the vineyard to infect vines within close proximity to the riparian vegetation. This conceptual model implies that disease in vineyards does not increase exponentially and is, in several ways, largely independent of Pierce's disease prevalence during the prior year. However, disease patterns and incidence rates observed in field plots (monitored in Napa and Sonoma counties since 2016) suggest there may be gaps in this model. Specifically, it is plausible that Pierce's disease prevalence during the prior year may be linked to rates of Pierce's disease spread in

the following year. If so, this could elevate the importance of vine removal as a component of Pierce's disease management, a strategy that is not currently recommended. It could also lead to alternative timing of vector management programs. Because this alternative hypothesis on Pierce's disease spread would, if correct, lead to fundamental changes to Pierce's disease management, we propose to first approach the problem from a mathematical perspective, prior to executing future field trials.

Objective 3. *Xf* Population Genomics

It has been assumed that *Xf* populations causing Pierce's disease in California, and elsewhere, were genetically and phenotypically homogenous. This assumption has both theoretical and practical implications, as well as potential impacts on the transportation of plant material through the state. We used a population genomic approach to demonstrate that *Xf* populations infecting grapevines in California are region specific. In other words, *Xf* populations causing Pierce's disease in Napa County are, for example, genetically distinct from *Xf* causing Pierce's disease in Sonoma County. Our goal in this objective is to identify and study these genetic differences in more detail. Another goal is to continue following *Xf* infections of mature commercial vines over time, to determine how the pathogen evolves as well as how it moves within plants. Evaluation of symptom development is a primary goal of this study, as it has implications for disease management.

FUNDING AGENCIES

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

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

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Reporting Period: The results reported here are from work conducted July 2017 to September 2018.

ABSTRACT
This project is a continuation of previously funded projects to evaluate the field efficacy of transgenic grapevine rootstocks expressing a chimeric antimicrobial protein (CAP) or a polygalacturonase inhibitory protein (PGIP) to provide protection to the grafted scion variety from developing and succumbing to Pierce's disease (PD). A total of 120 independent lines corresponding to seven versions of CAP and five versions of PGIP exploiting components optimized and tested in previously funded projects have been introduced successfully and expressed in Thompson Seedless (TS) as well as in commercially relevant rootstocks 101-14 and 1103. One-half or 60 lines each with six plant replicates for a total of 379 plants were planted in the field in August 2018, as transgenic rootstocks grafted to Chardonnay as scion. Next spring (2019) the remaining 60 lines will be planted to conclude the field planting stage of this project. Once expressed in the rootstock these proteins will move into the grafted PD sensitive scion variety Chardonnay and this study aims at evaluating the ability of these proteins to control the development of the disease. These two proteins CAP and/or PGIP control the spread and severity of the disease by controlling the bacteria-plant interaction but do so by disrupting different interacting surfaces. The CAP proteins disrupt the bacterial surface that includes the lipopolysaccharide layer while, the action of PGIP is indirect by preventing/ interfering with the disruption of the plant pectin layers found in the plant middle lamella and exposed in pit membranes in xylem tissues. In this project, we will evaluate in the field the effectiveness of rootstocks expressing either these two proteins in limiting the disease development in the scion while maintaining vine health and productivity. Elite rootstock lines identified in this project will be good candidates for commercialization.

LAYPERSON SUMMARY
This project continues the field efficacy evaluation of standard grapevine rootstocks expressing individually, seven chimeric antimicrobial proteins and five polygalacturonase inhibitory proteins to provide protection to the grafted scion variety from developing Pierce's disease. A total of 120 rootstock lines expressing these proteins individually have been created with six plant replicates each to give a total of 720 plants to be tested in the field grafted to Chardonnay scion that is sensitive to Pierce's disease. We have planted this summer (2018) half of these, 60 lines represented in 379 plants in the field; the remaining 348 plants corresponding to ~60 lines will be planted in the spring of 2019. Once planting and training have been completed the vines will be challenged by infection with *Xylella fastidiosa* to identify rootstock lines that can protect the scion from developing Pierce's disease while maintaining their productivity. Elite rootstock lines identified in this project will be good candidates for commercialization.

INTRODUCTION
The focus of this study is to evaluate the rootstock-based expression of chimeric antimicrobial proteins (CAP; Dandekar et al., 2012a) and polygalacturonase inhibitory protein (PGIP; Agüero et al., 2005, 2006) to provide transgraft protection of the scion grapevine variety against Pierce's disease (PD). A field trial testing four lines of CAP-1 and four lines of PGIP corresponding to PGIP-1, PGIP-2, PGIP-3 and PGIP-4 was recently concluded (Dandekar et al., 2018). Twelve plants corresponding to each of the eight lines (independent transgenic events) were planted in 2011 as transgenic rootstocks grafted to wild-type scion with both rootstock and scion being Thompson Seedless variety (TS). We had previously demonstrated that both PGIP and CAP-1 are secreted into the xylem where they were able to protect the vines from developing PD (Agüero et al., 2005; Dandekar et al., 2012a). The purpose of the field trial was to evaluate the ability of the transgenic rootstock to transgraft protect the wild-type scion from developing and/or succumbing to PD. The inoculations were performed yearly starting in 2012 and from 2013 till 2015 all 12 replicates of each of the transgenic lines were inoculated only in the grafted

scion portion at a point at least 100 cm above the graft union. Disease symptoms, vine death, and other parameters were evaluated each year and the field trial was concluded in 2017. The data generated over the four seasons of evaluation clearly indicated that both rootstocks (CAP and PGIP) were able to transgraft protect the scion at a point that was at least 100 cm above the graft union. A significant decrease in vine mortality was observed for vines grafted to transgenic CAP or PGIP expressing rootstock as compared to wild-type rootstocks. Vines grafted to transgenic rootstocks harbored lower pathogen titers compared to those grafted to wild-type rootstocks. Spring bud break, a parameter of vine health, was much higher for vines grafted to either transgenic rootstock or much lower for the wild-type rootstock. This present study builds on earlier work and incorporates advances in transformation of commercially relevant grapevine rootstocks as well as incorporates improvements in individual components present in CAP and PGIP constructs. A method to successfully transform two commercially relevant rootstocks, 101-14 and 1103 (Christensen, 2003), was successfully developed (Dandekar et al., 2011, 2012b) and the method was further improved by David Tricoli in the Plant Transformation Facility at UC Davis. The original CAP-1 construct (Dandekar, 2012a) was improved upon by identifying grapevine-derived components (Chakraborty et al., 2013, 2014b). The surface interacting component (neutrophil elastase) was replaced with P14a protein from *Vitis shuttleworthii* that also displays serine protease and antimicrobial activity (Chakraborty et al., 2013; Dandekar et al., 2012c, 2013) and more recently with PrtA that displays serine protease and antimicrobial activity (Gouran et al., 2016). The antimicrobial peptide component (cecropin B; CB) was replaced with HAT52 and/or PPC20 that were identified using novel bioinformatics tools developed by us (Chakraborty et al., 2013, 2014a) and the efficacy of their antimicrobial activity of the selected peptides were verified by their ability to kill *Xylella fastidiosa* (*Xf*) cells (Chakraborty et al., 2014b). Improvements in the secretion of PGIP were also made based on an earlier study on the characterization of xylem sap proteins, whose signal peptides could be identified and have been used instead of the natural one expressed in the peel tissue of pear fruit (Agüero et al., 2005, 2008). The field introduction of these transgenic rootstocks is aimed at evaluating different lines to identify those with good efficacy in protecting grafted, sensitive scion cultivar Chardonnay from developing PD.

OBJECTIVES

The goal of this project is to field-test transgenic rootstocks expressing CAP and/or PGIP proteins to determine their ability to transgraft protect a sensitive scion grapevine from developing and succumbing to PD.

1. Develop commercially relevant transgenic rootstock lines expressing CAP and/or PGIP.
2. Field test the efficacy of commercially relevant transgenic rootstock lines expressing CAP and/or PGIP proteins to transgraft protect a sensitive grapevine cultivar from developing and spreading PD.

RESULTS AND DISCUSSION

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

This objective translates the results of two previously funded projects (11-02040-SA and 12-0130-SA). Project 12-0130-SA, titled “Building a Next Generation Chimeric Antimicrobial Protein to Provide Rootstock-Mediated Resistance to Pierce's Disease in Grapevines,” led to the development of additional CAP proteins with components derived from grapevine and other proteins (Dandekar et al., 2013). Project 11-0240-SA, titled “Engineering Multi-Component Resistance to Pierce's Disease in California Grapevine Rootstocks,” led to the development of a method to transform commercially relevant rootstocks 101-14 and 1103 (Dandekar et al., 2011, 2013). David Tricoli at the Plant Transformation Facility at UC Davis has further improved upon the grapevine rootstock transformation protocol and carried out all of our transformations. Shown in **Figure 1** are all of the CAP vectors being field tested in this project. CAP-1 is the original vector that was field tested in TS rootstocks and several lines showed efficacy (Dandekar et al., 2016, 2018). CAP-2 has the original components as described earlier (Dandekar et al., 2012a), however, the expression of the CAP has been improved by including a translational enhancer (omega), an efficient secretion sequence (Ramy3D), and the CAP-2 protein has an epitope tag (FLAG) to enable detection of the protein in transgenic tissues. CAP-3 to 6 are four vector constructs to test the *Vitis* derived components (**Figure 1**). The CAP-3 and CAP-4 are designed to test the *Vitis* component replacing protease from CAP-1. The CAP-3 vector, pDP13.35107, tests the VsP14a protein by itself. The VsP14a component is present in *Vitis shuttleworthii* (*Vs*) and has a similar function to the CAP-1 protease (Dandekar et al., 2014; Chakraborty et al., 2013). Expression of VsP14a by itself confirmed its protease and antimicrobial activity against *Xf* (Dandekar et al., 2014). The fourth vector, pDP13.36122 (CAP-4), expresses VsP14a linked to CB, the antimicrobial peptide domain used successfully in CAP-1 (Dandekar et al., 2012a). The fifth, CAP-5, pDM14.0708.13 (**Figure 1**), links the VsP14a to a 52-amino acid segment of the HAT protein from *Vitis vinefera* that displays a moderate clearance activity against *Xf* (Chakraborty et al. 2014b; Dandekar et al. 2013). The sixth,

CAP-6, pDM14.0436.03 (**Figure 1**), links the VsP14a to a 20-amino acid segment of the PPC protein from *Vitis vinifera* that has very good antimicrobial activity against *Xf* (Chakraborty et al. 2014a; Dandekar et al. 2013). The seventh and final, CAP-7, pDG14.01 (**Figure 1**), expresses PrtA, a protease that displayed antimicrobial action against *Xylella* in a tobacco system (Gouran et al., 2016). All of these seven vectors, CAP-1 to CAP-7, were transformed in the Plant Transformation Facility and transgenic grapevine rootstocks have been obtained.

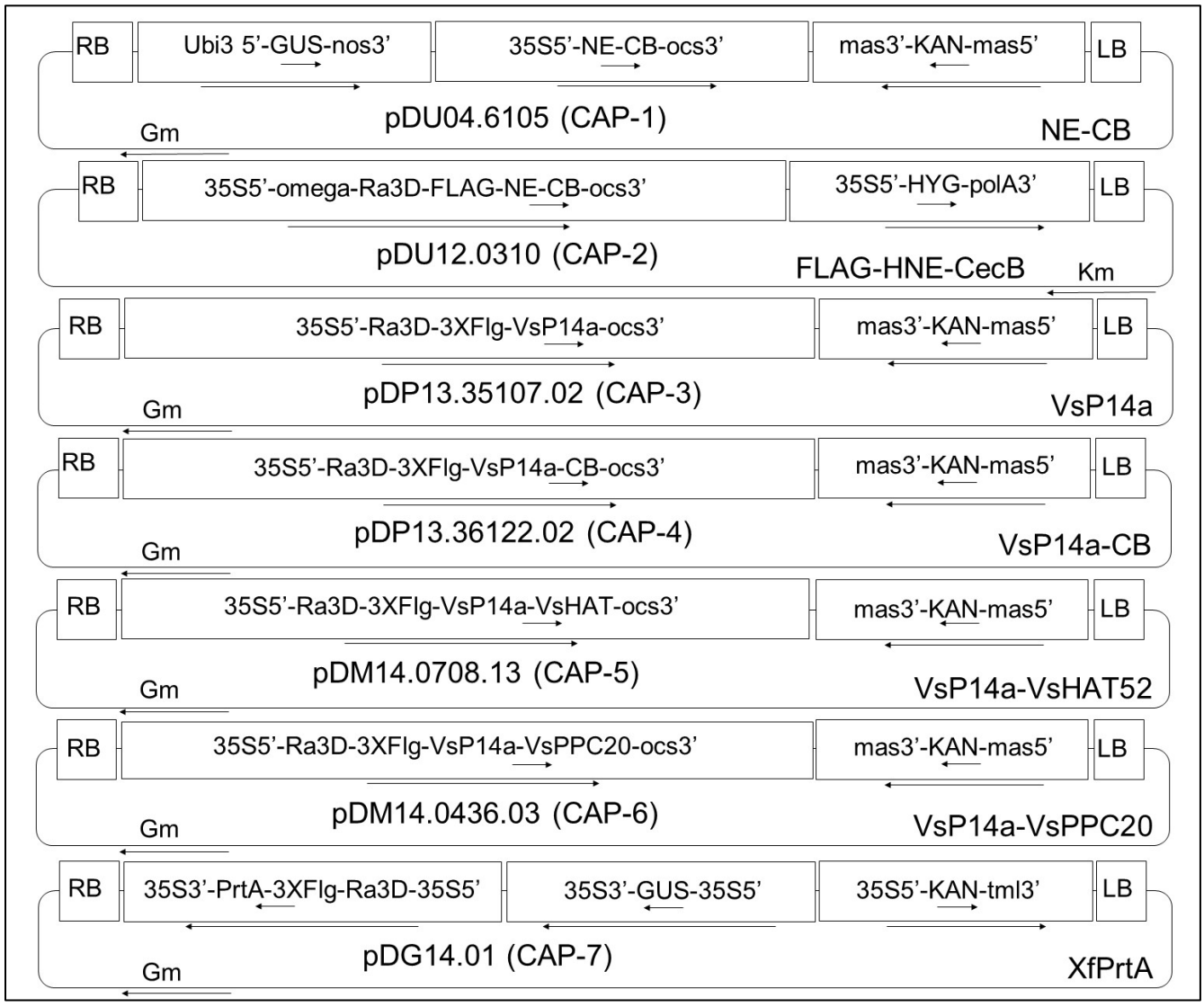

Figure 1. CAP vectors used in this study to develop transgenic rootstocks that will be evaluated in the field.

In addition to the seven CAP constructs we will also be evaluating the five PGIP constructs shown in **Figure 2**. The PGIP-1, pDU94.0928 (**Figure 2**) construct is the original pear PGIP expressed in grapevine and shown to provide resistance/tolerance to PD (Aguero et al., 2005). PGIP-2, pDU05.1002, encodes a pear PGIP sequence with its native signal peptide deleted and is referred to as mPGIP as it is similar in sequence to the mature form of PGIP found in plant tissues. PGIP-3, pDU05.1910 (**Figure 2**), contains a pear PGIP coding sequence fused to the signal peptide from the nt-protein of grapevine whose sequence was reported by Aguero et al., (2008). PGIP-4, pDU06.0201 (**Figure 2**), contains the mPGIP coding sequence fused to the signal peptide from the chi protein from grapevine whose sequence was reported by Aguero et al., (2008). PGIP-5, pDA05.XSP (**Figure 2**), contains the mPGIP coding sequence fused to the signal peptide from a xylem abundant protein from cucumber, and PGIP-6, pDU05.0401 (**Figure 2**), links the mPGIP sequence to the Ramy3D signal peptide from the rice alpha-amylase protein.

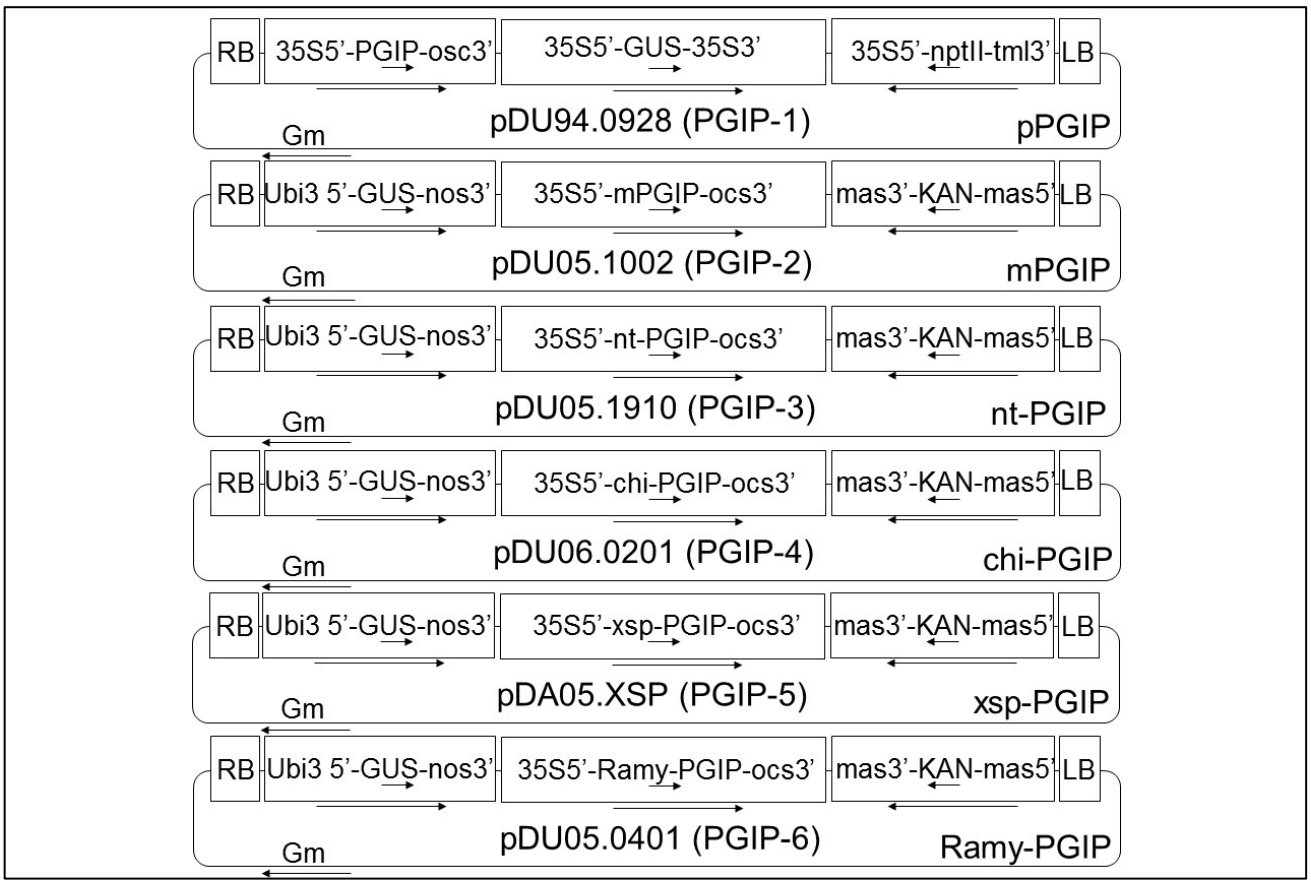

Figure 2. PGIP vectors used in this study to develop transgenic rootstocks that will be evaluated in the field.

Table 1. List of transgenic lines in the greenhouse generated from the vectors shown in Figures 1 and 2.

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

All seven of the CAP and five PGIP constructs have been successfully transformed into grapevine by the Plant Transformation Facility and transgenic plants have been steadily appearing. It takes 18 months to get back transformed plantlets. This task has been completed and **Table 1** (above) lists all of the lines that we have obtained so far. It is quite a challenge to transform 101-14 lines and more so for 1103 (**Table 1**). Two types of analysis are carried out with each of the transformed lines as they emerge from the Plant Transformation Facility

to confirm that they are transgenic and that they express the protein. DNA and proteins are extracted from leaves obtained from each plantlet. The extracted DNA is used to confirm integration of transfer DNA (T-DNA) through positive polymerase chain reaction (PCR) amplification of the Kan/Hyg gene present in all of our CAP and PGIP vector constructs as previously described (Dandekar et al., 2012a). The extracted proteins are separated on a gel and then a western blot analysis is carried out using antibodies raised against FLAG, an epitope that is present in CAP proteins 2-7 but not CAP-1. In some cases we also isolate RNA to confirm expression of the CAP transgene, first as a positive PCR product using complementary DNA (cDNA) copied from the RNA and then to quantitate the amount of expression using quantitative real-time (qRT) PCR as described (Dandekar et al., 2012a). Lines with no PCR product for the Kan/Hyg genes are not propagated and are discarded.

Table 2. List of constructs and lines that were planted in the field in August 2018.

No	Construct	Binary Vector	Transgene	Number of novel lines			Plants in Field
				101-14	1103	TS	
1	CAP-1	pDU04.6105	NE-CB	6			47
2	CAP-2	pDU12.031	NNE-CB		9		53
3	CAP-3	pDP13.35107	VsP14a	7			42
4	CAP-4	pDP13.36122	VsP14a-CB	3			12
5	CAP-5	pDM14.0708	VsP14a-VvHAT52	1	3		24
6	CAP-6	pDM14.0436	VsP14a-VvPPC20	7	4		63
7	PGIP-2	pDU05.1002	mPGIP			4	24
8	PGIP-3	pDU05.1910	nt-PGIP			4	24
9	PGIP-4	pDU06.0201	chi-PGIP			4	24
10	PGIP-5	pDA05.XSP	xsp-PGIP			4	24
11	PGIP-6	pDU05.0401	Ramy-PGIP			4	24
12	WT			1	1	1	18
	Total number of lines and plants			60			379

Objective 2. Field Test the Efficacy of Commercially Relevant Transgenic Rootstock Lines Expressing CAP and/or PGIP Proteins to Transgraft Protect a Sensitive Grapevine Cultivar From Developing and Spreading PD

This objective focuses on the field-testing of all seven CAP and five PGIP lines shown in the last column of **Table 1**. **Table 2** shows the lines that were propagated last fall (2017) to create mother plants that were transferred to the lath house. Foundation Plant Services (FPS) at UC Davis helped with creating the grafted plants for the field planting. First cuttings were harvested from mother plants in the lath house after the plants went dormant. In the spring of 2018 these cuttings were rooted to make plants that were later budded with the scion variety Chardonnay, creating the vines that were planted in the field. FPS was able to successfully propagate and graft 70% of our lines (**Table 2**); the remaining 30% were successfully propagated and bud grafted by us. Shown in **Table 3** below is the map of the field planting of 379 plants. There are currently 11 gaps that remain, and these plants are being generated and will be planted in the spring of 2019. On August 1, 2018, we planted the first batch of plants that were grafted by FPS and which constituted 70% of the planting, and on August 19, 2018, we planted the remaining 30% that were grafted by us. We are currently maintaining stock or back-up plants of all of the plants indicated in **Table 2**, so we can replace any plants that are lost in the field. We are currently evaluating 66 remaining lines that have emerged from the Plant Transformation Facility and that are transitioning from the lab to the greenhouse. These vines are being tested for protein expression and for the presence of the selectable marker gene. The remaining lines will be propagated to create grafted plants for field introduction next year.

Table 3. Field-planting map of the 60 lines shown in **Table 2**. The row number appears on the top and the vine number on the side

R/V	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**
30	P4.78	C2.6	C1.182	P6.33	P2.29	C5.6	P6.32	C3.3	C1.183	P3.08	P5.23	P6.33	C6.2
29	P4.78	C2.6	C1.182	P6.33	P2.29	C5.6	P6.32	C3.3	C1.183	P3.08	P5.23	P6.33	C6.2
28	P4.78	C2.6	C1.182	P6.33	P2.29	C5.6	P6.32	C3.3	C1.183	P3.08	P5.23	P6.33	C6.2
27	C3.3	C1.186	C5.3	P5.21	C6.9	C3.1	C2.5	C2.2	P2.22	C5.5	C3.5	C2.4	P3.27
26	C3.3	C1.186	C5.3	P5.21	C6.9	C3.1	C2.5	C2.2	P2.22	C5.5	C3.5	C2.4	P3.27
25	C3.3	C1.186	C5.3	P5.21	C6.9	C3.1	C2.5	C2.2	P2.22	C5.5	C3.5	C2.4	P3.27
24	C6.12	P4.7	P3.16	P2.27	M-WT	C5.6	C6.4	C6.1	P4.7	P6.4	C5.1	****	C6.11
23	C6.12	P4.7	P3.16	P2.27	M-WT	C5.6	C6.4	C6.1	P4.7	P6.4	C5.1	****	C6.11
22	C6.12	P4.7	P3.16	P2.27	M-WT	C5.6	C6.4	C6.1	P4.7	P6.4	C5.1	C3.4	C6.11
21	C1.187	C6.8	C1.184	P3.07	C2.7	C6.1	****	P4.56	****	C1.185	C3.6	P4.77	P5.21
20	C1.187	C6.8	C1.184	P3.07	C2.7	C6.1	C2.9	P4.56	C6.9	C1.185	C3.6	P4.77	P5.21
19	C1.187	C6.8	C1.184	P3.07	C2.7	C6.1	C2.6	P4.56	C6.9	C1.185	C3.6	P4.77	P5.21
18	C6.3	P5.27	P2.35	C6.3	P3.08	P6.32	****	C1.182	C6.7	P4.78	C6.12	C6.8	C2.6
17	C6.3	P5.27	P2.35	C6.3	P3.08	P6.32	C1.187	C1.182	C6.7	P4.78	C6.12	C6.8	C2.6
16	C6.3	P5.27	P2.35	C6.3	P3.08	P6.32	C1.187	C1.182	C6.7	P4.78	C6.12	C6.8	C2.6
15	C6.6	C6.4	C2.8	C1.186	C2.2	P2.22	P3.27	P2.35	T-WT	C6.3	M-WT	C6.1	P5.02
14	C6.6	C6.4	C2.8	C1.186	C2.2	P2.22	P3.27	P2.35	T-WT	C6.3	M-WT	C6.1	P5.02
13	C6.6	C6.4	C2.8	C1.186	C2.2	P2.22	P3.27	P2.35	T-WT	C6.3	M-WT	C6.1	P5.02
12	C6.1	C2.1	C1.185	C4.3	C3.3	C2.3	P-WT	P2.27	C1.184	P-WT	C1.186	C3.1	C2.3
11	C6.1	C2,1	C1.185	C4.3	C3.3	C2.3	P-WT	P2.27	C1.184	P-WT	C1.186	C3.1	C2.3
10	C6.1	C2.1	C1.185	C4.3	C3.3	C2.3	P-WT	P2.27	C1.184	P-WT	C1.186	C3.1	C2.3
09	P4.77	T-WT	P6.4	P5.23	C3.7	C4.2	P6.3	P6.3	P2.29	****	C5.6	****	****
08	P4.77	T-WT	P6.4	P5.23	C3.7	C4.2	P6.3	P6.3	P2.29	****	C5.6	****	****
07	P4.77	T-WT	P6.4	P5.23	C3.7	C4.2	P6.3	P6.3	P2.29	C3.3	C5.6	C6.6	P3.7
06	C2.9	C2.5	C3.6	C1.182	C3.5	C3.4	C3.2	C4.3	C2.8	P3.16	P3.07	C5.3	C3.2
05	C2.9	C2.5	C3.6	C1.182	C3.5	C3.4	C3.2	C4.3	C2.8	P3.16	P3.07	C5.3	C3.2
04	C2.9	C2.5	C3.6	C1.182	C3.5	C3.4	C3.2	C4.3	C2.8	P3.16	P3.07	C5.3	C3.2
03	C6.2	C5.1	C1.183	C2.4	P4.56	C6.11	P5.02	P5.27	C1.182	C2.7	C4.2	C2.1	C1.186
02	C6.2	C5.1	C1.183	C2.4	P4.56	C6.11	P5.02	P5.27	C1.182	C2.7	C4.2	C2.1	C1.186
01	C6.2	C5.1	C1.183	C2.4	P4.56	C6.11	P5.02	P5.27	C1.182	C2.7	C4.2	C2.1	C1.186

Figure 3. View of field planting (August 2018).

Table 4. Lines currently in the lab and greenhouse that are being evaluated and propagated for field introduction in 2019.

No	Construct	Binary Vector	Transgene	Number of novel lines			Plants
				101-14	1103	TS	
1	CAP-3	pDP13.35107	VsP14a	17	1		108
2	CAP-4	pDP13.36122	VsP14a-CB	21	1		132
3	CAP-5	pDM14.0708	VsP14a-VvHAT52	2	1		18
4	CAP-7	pDG14.02	XfPrtA	9		14	138
				66			396

CONCLUSIONS

The goal of this project is to field-test transgenic rootstocks expressing CAP and/or PGIP proteins to determine their ability to transgraft protect a sensitive scion grapevine from developing and succumbing to PD. We have successfully introduced 60 independent events corresponding to 11 constructs of CAP and PGIP yielding 379 plants. These plants are composed of transgenic rootstock grafted to a wild-type Chardonnay scion planted in the field in August 2018 in 13 rows of 30 plants in each row. We have 11 gaps that remain where the plants need to be planted. We are currently evaluating and propagating the remaining 66 lines corresponding to four constructs that will be used to create the remaining plants to complete the field introduction in the summer of 2019.

REFERENCES CITED

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

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

Agüero CB, Thorne ET, Ibáñez AM, Gubler WD, Dandekar AM. 2008. Xylem sap proteins from *Vitis vinifera* L. Chardonnay. *Am. J. Enol. Vitic*. 59(3):306-311.

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

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

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

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

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

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

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

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

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

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

Dandekar AM, Ibáñez AM, Jacobson A. 2016. Field testing transgenic grapevine rootstocks expressing chimeric antimicrobial protein and polygalacturonase-inhibiting protein. *Proceedings of the 2016 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, San Diego, CA, pp. 35-42.

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

Gouran H, Gillespie H, Nascimento R, Chakraborty S, Zaini PA, Jacobson A, Phinney BS, Dolan D, Durbin-Johnson BP, Antonova ES, Lindow SE, Mellema MS, Gulart LR, Dandekar AM. 2016. A secreted protease PrtA controls cell growth, biofilm formation, and pathogenicity in *Xylella fastidiosa*. *Nat Sci. Rep*. Aug 5;6: 31098. doi: 10.1038/srep31098.

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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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ABSTRACT

Genetic strategies for disease suppression and information characterizing the bacterial-plant interaction are high priority areas in the Pierce's disease research program. Plants bearing transgenes from the laboratories of Dandekar, Powell, Lindow, and Gilchrist were tested extensively under greenhouse and field conditions in USDA APHIS approved field environments. Two types of genetically modified plants bearing single constructs of test genes have been evaluated under *Xylella fastidiosa* inoculated disease conditions: whole plant transgenics, and transgenic rootstocks that were grafted to non-transformed Pierce's disease (PD)-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 paired combinations of the transgenes will be introduced into individual rootstocks adapted to California grape growing regions to which are grafted PD-susceptible Chardonnay scions.

LAYPERSON SUMMARY

The first phase of field testing to evaluate grapevines expressing potential Pierce's disease (PD) suppressive transgenes under field conditions began in 2010 and was terminated in 2017. A second phase field experiment will continue evaluation of resistance to PD 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 inoculated with *Xylella fastidiosa*. PD symptoms including classical foliar symptoms and cane death occur within 24 months. The initial field tests have shown positive protection against PD by five different DNA constructs. A new planting is in progress that will consist of untransformed PD-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 PD. This research also will address the ability of the pathogenic bacteria to colonize and move within the xylem of the grape plant downward from the inoculated scion to the transgenic rootstock. The latter analysis will determine if the transgenic rootstock is differentially protected against *Xylella*-induced death of the rootstock. The grafting, planting, and training of the vines will be guided by Josh Puckett and Deborah Golino (Foundation Plant Services, University of California, Davis) for trellising and plant management to reflect commercial production standards.

INTRODUCTION

The individual laboratories of the principal investigator (PI) and co-princial investigators established transgenic plants and field tested the genes listed in **Table 1** 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 PD in cultivated grapes. The new rootstock combination with paired transgenes each were evaluated first in the laboratory and then the greenhouse before moving to the field. The highest expressing rootstocks will be grafted to susceptible non-transgenic

Chardonnay scions to assess potential cross-graft protection of the scion. The primary objective for expressing genes in combination is to create durable resistance, resistance to *Xylella fastidiosa* (*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 (1-10).

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

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

OBJECTIVES

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

RESULTS AND DISCUSSION

Objective 1. Complete the Current Field Evaluation of Transgenic Grape and Grape Rootstocks

Final field evaluations of this planting were completed in June 2016.

Objective 2. Destruction of Existing Planting

The field experiment that began in 2010 was terminated under Objective 2 of this proposal according to the regulations specified in the USDA APHIS permit (**Figure 1**). This has now been followed by establishment of the second phase of field testing approved by the Product Development Committee to develop transgenic rootstocks incorporating stacked genes (dual constructs) to be grafted to non-transformed PD-susceptible Chardonnay scions to test for potential cross-graft protection against PD (Objective 3).

Figure 1. Final destruction of the plants at the Solano County field site by burning on June 7, 2017. Following removal of poles and wires, undercutting, and piling of plants, the material was burned and the ashes incorporated by disking.

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 incorporation of the ashes was completed as soon as the field dried in the following spring. The entire field was then cross-disked multiple times and leveled in preparation for future planting.

Objective 3. Establishment and Management of New Planting with Stacked Gene Transgenic Rootstocks
Figure 2 shows the physical location of the new planting in relation to the 2010 planting. 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 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 *Xf* 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 PD. Test plants included transgenic plants expressing genes from Dandekar, Powell, Lindow, and Gilchrist projects compared with non-transgenic PD-susceptible Thompson Seedless and Freedom rootstock plants as controls. The results through 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 PD 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 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.

Figure 2. 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 x 470 feet for planting, which equals 1.8 acres accommodating up to 32 new rows (excluding the 50-foot buffer areas surrounding the plots). The new area will accommodate ~900 new plants in 2018-19. Current area (red box) equaling 1.6 acres including the 50-foot buffer areas surrounding the plots is the area that is now cleared of plants and all plant material burned as shown in **Figure 1**.

The grafting, planting, and training of the vines will be guided by Josh Puckett and Deborah Golino (Foundation Plant Services, University of California, Davis) working with PI Gilchrist for grafting the scions and field planting. They also will provide guidance for trellising and plant management to reflect commercial production standards. The field plot design will enable experimental *Xf* inoculations, pathogen and disease assessments, as well as grape yield. Land preparation and planting of the experimental area is 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. All plants will be maintained under a newly installed drip irrigation system. An image of the completed phase 1 of the field planting is shown in **Figure 3**.

Figure 3. Planting configuration for the dual constructs. This image illustrates the new planting of the dual construct transformed rootstocks grafted with an untransformed scion of Chardonnay. This first phase of the planting (left image) was completed August 1, 2018. Image on the right shows growth on October 10, 1018.

The Following Protocols Are Being Followed as the Planting and Management Proceed:

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

b. After the first year, the canes will be tied down during the dormant season and trimmed to the appropriate length or shorter if the cane girth is not over 3/8-inch in diameter. The shoots that push will be suckered to remove double shoots and to achieve a shoot (and hence spur position) spacing of about four to five inches between them.

c. Grape fruit yield will be measured after the second or third year depending on the fruit set.

d. Evaluation of the experimental plants for plant morphology, symptoms of PD infection, and the presence of the bacteria will follow past protocol. Each parameter will be determined over time by visual monitoring of symptom development and detection of the amount and movement of the bacteria in plant tissues (mainly leaves and stems) by quantitative polymerase chain reaction (qPCR) assays. The analysis will be done in the Gilchrist lab by the same methods and laboratory personnel as has been done with the current planting. A comparative quantitative determination by qPCR of the presence of *Xylella* in non-transgenic scions and grape rootstocks will be compared with conventional grape and grape rootstocks.

e. Both symptom expression and behavior of the inoculated bacteria will provide an indication on the level of resistance to PD infection and the effect of the transgenes on the amount and movement of the bacteria in the non-transgenic scion area.

f. The area is adjacent to experimental grape plantings that have been infected with PD for the past two decades with no evidence of spread of the bacteria to uninfected susceptible grape plantings within the same

experiment. Hence, there is a documented historical precedent for the lack of spread of the bacteria from inoculated to non-inoculated plants, an important consideration for the experiments carried out for this project and for the granting of the 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 PI Gilchrist and conducted by Bryan Pellissier, the Field Superintendent employed by the Department of Plant Pathology. The field crew work closely with PI Gilchrist to determine timing and need of each of the management practices, including pruning and thinning of vegetative overgrowth as necessary.

h. Regular tilling and hand weeding will maintain a weed-free planting area. Plants will be pruned carefully in March of each year, leaving all inoculated/tagged branches and numerous additional branches for inoculation and sampling purposes in the coming year. All pruning material will be left between the rows to dry, then flail chopped and later rototilled to incorporate the residue per requirements of the USDA APHIS permit.

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.

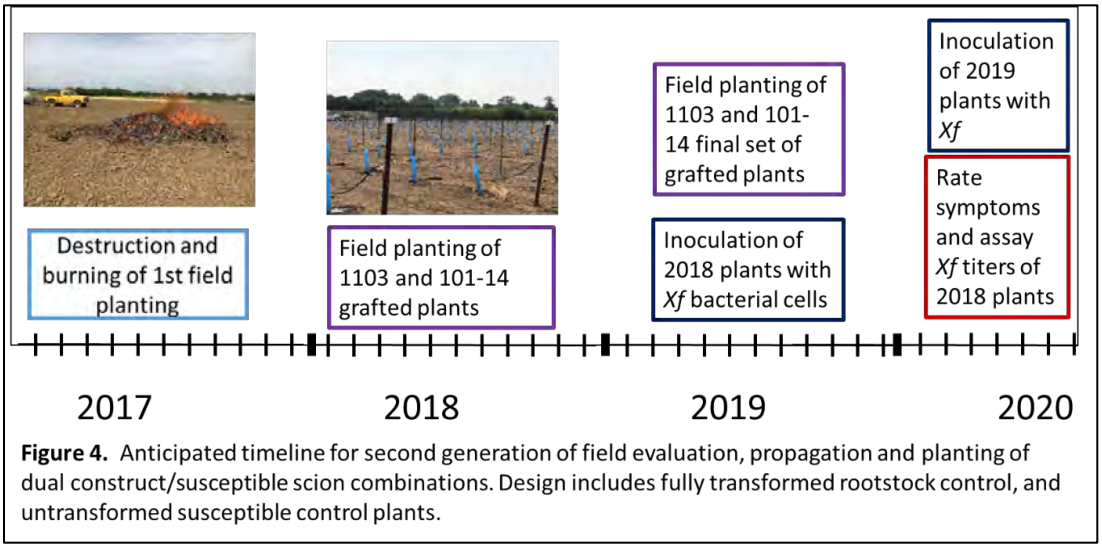

Figure 4. Anticipated timeline for second generation of field evaluation, propagation and planting of dual construct/susceptible scion combinations. Design includes fully transformed rootstock control, and untransformed susceptible control plants.

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

Four years beginning with 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 2018-2019 funding cycle and will depend on the results of the field evaluation up to that point. The field area has been designated legally available for planting the specified transgenic grapes by USDA APHIS under permit number 7CFRE340 that is held by Co-PI Dandekar. The protocols for managing the existing and the new plantings with the dual constructs have been used successfully over the past five years (1-9). These protocols include plant management, inoculation with *Xf*, development of classical symptoms of PD exhibiting the range from foliar symptoms to plant death, and the assessment of protection by a set of transgenes selected by molecular techniques to suppress the symptoms of PD and/or reduce the ability of the pathogenic bacteria to colonize and move within the xylem of the grape plant downward from the inoculated scion to the transgenic rootstock.

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 PD 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 were evaluated first in the laboratory and then the greenhouse before moving to the field. The dual gene expressing rootstocks were grafted to susceptible non-transgenic PD-susceptible Chardonnay scions to assess potential cross-graft protection against PD. The field area has been permitted by the USDA APHIS for this experiment. The protocol for constructing the rootstocks and grafted scions, planting, and commercial style management of the vines is in place and will be coordinated by Josh Puckett and Deborah Golino. Beginning with initial planting in 2018 and followed by additional plantings as experimental plants become available in the second year. Inoculation and evaluation will begin when the plants have been in the ground for one year and will continue annually until the field planting is terminated. Funding for completion of the fourth and any following years will be proposed in the 2018-2019 funding cycle and will depend on progress of the field evaluation up to that point.

REFERENCES CITED

1. Gilchrist DG, Lincoln JE. 2014. Field evaluation of grape plants expressing PR1 and UT456 transgenic DNA sequences for protection against Pierce's disease. *Proceedings of the 2014 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA, pp. 126-132.
2. Dandekar AM. 2015. Chimeric antimicrobial protein and polygalacturonase-inhibiting protein transgenic grapevine field trial. *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. 18-26.
3. Gilchrist DG, Lincoln JE. 2015a. Field evaluation of grape plants expressing potential protective DNA sequences effective against Pierce's disease. *Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes.* December 2015. California Department of Food and Agriculture, Sacramento, CA, pp. 49-53.
4. Gilchrist DG, Lincoln JE. 2015b. Field evaluation of grape plants expressing PR1 and UT456 transgenic DNA sequences for protection against Pierce's disease. *Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes.* December 2015. California Department of Food and Agriculture, Sacramento, CA, pp. 54-58.
5. Gilchrist DG, Lincoln JE, et al. 2016a. Transgenic rootstock-mediated protection of grapevine scions by introduced single and dual stacked DNA constructs. *Proceedings of the 2016 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA, pp. 59-66.
6. Gilchrist DG, Dandekar AM. 2016b. 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.
7. Gilchrist DG, Lincoln JE, et al. 2017a. Transgenic rootstock-mediated protection of grapevine scions by introduced single and dual stacked DNA constructs. *Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes.* December 2017. California Department of Food and Agriculture, Sacramento, CA, pp. 35-42.
8. Gilchrist DG, Lincoln JE, et al. 2017b. Field evaluation of cross-graft protection effective against Pierce's disease by dual and single DNA constructs. *Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes.* December 2017. California Department of Food and Agriculture, Sacramento, CA, pp. 29-34.
9. Lindow S. 2015. Continued field evaluation of diffusible signal factor producing grape for control of Pierce's disease. *Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes.* December 2015. California Department of Food and Agriculture, Sacramento, CA, pp. 127-141.
10. Lincoln JE, Sanchez JP, Zumstein K, Gilchrist DG. 2018. Plant and animal PR1 family members inhibit programmed cell death and suppress bacterial pathogens in plant tissues. *Molecular Plant Pathology* 19(9): 2111-2123.

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

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ABSTRACT

Collectively, a team of researchers (Lindow, Dandekar, Labavitch/Powell, and Gilchrist) identified, constructed, and advanced to field evaluation five novel DNA constructs (**Table 1**) that, when engineered into grapevines, suppress symptoms of Pierce's disease (PD) 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 PD symptoms. Each of the five transgenes, when expressed as single genes, reduced the disease levels under field conditions both as full plant transgenics and as transgenic rootstocks grafted to a non-transformed PD-susceptible scion. This initial field trial consisting of single gene constructs was begun in 2010 and evaluated until discontinued at the end of the 2016 growing season. The field experiment is to be replaced with a second field trial designed to evaluate untransformed scion protection by rootstocks bearing paired combinations of the five constructs. This approach involves "stacking" a combination of distinct protective transgenes in a single rootstock line, which is intended to foster not only durability but also more robust protection of the non-transformed scion against PD.

LAYPERSON SUMMARY

Xylella fastidiosa (*Xf*) is the causative agent of Pierce's disease (PD). Collectively, a team of researchers (Lindow, Dandekar, Labavitch/Powell, and Gilchrist) has identified five novel genes (DNA constructs) (**Table 1**) which, when engineered into grapevines, suppress symptoms of PD by reducing the titer of *Xf* in the plant, reducing its systemic spread in the plant, or blocking *Xf*'s ability to trigger PD symptoms. These projects have moved from the proof-of-concept stage in the greenhouse to characterization of PD resistance under field conditions where current data 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. This approach involves "stacking" a combination of distinct protective transgenes in a single rootstock line, which is intended to foster not only durability but also more robust protection of the non-transformed scion against PD.

INTRODUCTION

Briefly, this report will describe information on the history, likely function, and impact of each of the genes deployed as single transgenes in fully-transformed plants in the initial field study in USDA APHIS approved field trials wherein test plants were mechanically inoculated with *Xf* to induce Pierce's disease (PD). The experimental materials of this project are five specific DNA constructs (Table 1) that were shown to be effective in PD suppression under field conditions as single gene constructs, as described by Lindow, Dandekar, and Gilchrist in previous reports. They will now be evaluated for potential cross-graft-union protection. 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. If

successful, the obvious benefit would be that any unmodified (non-transgenic) varietal wine grape scion could be grafted to and be protected by transformed rootstock lines.

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

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

PGIP and CAP (Abhaya Dandekar)
The Dandekar lab has genetic strategies to control the movement and to improve clearance of *Xf*, the xylem-limited bacteria. 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, DSF (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). Accumulation of DSF in *Xf* cells causes a change in many genes in the pathogen with the overall effect of suppressing 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 the inappropriate activation of a genetically conserved process of programmed cell death (PCD) 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 Two of these grape sequences (PR1 and UT456), when expressed as transgenes in grape, suppressed PD symptoms and dramatically reduced bacterial titer in inoculated plants under greenhouse and field conditions (Lincoln, Sanchez, and Gilchrist, 2018).

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, Tricoli et al., 1995 describes the stacking of several genes for virus resistance in squash (note: D. Tricoli 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). This project will evaluate potential synergism in suppression of PD 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.
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 messenger RNAs (mRNAs) expected to be produced by the inserted genes, before they are subjected to grafting and greenhouse inoculation assays for transgene movement and resistance to PD.
3. The second major step in the process after verification of the genotypic integrity of the transgenic plants is production of the clonal ramets of each plant line to enable two cane growth development of the rootstocks and grafting of the Chardonnay scions.
4. Evaluate the resulting lines for efficacy by inoculation with *Xf* in a preliminary greenhouse experiment to identify the most protective lines from each combination of genes. A total of three independent transgenic lines of each dual construct in each rootstock with be selected to be bulked up to five copies of each for field planting at the USDA APHIS approved site in Solano County. (note: the greenhouse inoculation step was eliminated once it was clear that greenhouse-based foliar symptoms did not provide a reliable indicator of disease response. There were discernable differences among the individual plants based on bacterial counts within each of the10 dual combinations but spurious leaf burn symptoms were confounding and not characteristic of PD. However, the PCR confirmation of dual transformation was successful and was carried forward as the selection criteria.)

RESULTS AND DISCUSSION

Construction of Dual Gene Expression Binaries

The strategy is to prepare dual plasmid constructs bearing a combination of two of the protective genes on a single plasmid with a single selectable marker 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 transfer DNA (T-DNA) were constructed by J. Lincoln (Gilchrist et al., 2016).

All plasmids were transformed into *Agrobacterium* strain EHA105, the preferred transformation strain for grape plants. As a check on 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 successful 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 enables 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 1**). The transformation progress, following verification of insert integrity, for each line is shown in **Table 3**.

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

Transgene Transcripts	Number of Plants	Percent of Plants
two	230	67
one	99	29
none	12	4

Analysis of the Transgenic Rootstocks to Confirm Dual Insertions Transcripts

This analysis is performed by isolating the RNA from transgenic grape leaves and purified by a modification of a cetyl trimethylammonium bromide (CTAB) protocol and includes LiCl precipitation. The RNA is converted to complementary DNA (cDNA) by oligo dT priming and reverse transcriptase. PCR reactions are set up using the synthesized cDNA as template and specific pairs of primers are 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 rate of two genes into a given transgenic plant was 67 percent of the total plants provided by the transformation facility (**Table 2**). This underscores the need for dual transcript verification prior to moving plants forward to grafting, 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.

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 (**Figures 2 and 3**).

Evaluation of the Lines by Inoculation with *Xf* in the Greenhouse

Preliminary inoculations were initiated in the greenhouse and selections made based on qPCR analysis of *Xf* titre in the tissue above the inoculation site under original objective 4. The qPCR tests will be repeated after the scions are inoculated in the field, which our experience deems more reliable. 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 PD symptom scoring. The time frame from receipt of plants, analysis and selection of the individuals for field planting has been 9-13 months. Total number of plants to screen if all plants are verified transgenics will be at least 1,070 including 70 untransformed control plants.

Table 3. Transcript profiling of the dual construct transformed transgenic rootstocks. The totals do not include whole transformed and untransformed PD susceptible controls (200 plants).

Genotype	Construct code	Construct	# lines grafted 2018	# plants to field 2018
1103	AB	pCA-5oP14HT-5oUT456	6	36
101-14	AB	pCK-5oP14HT-5oUT456	4	24
1103	AC	pCA-5fCAP-5oP14HT	6	36
101-14	AC	pCK-5fCAP-5oP14LD	0	0
1103	AD	pCA-5PGIP-5oP14HT	6	36
101-14	AD	pCK-5PGIP-5oP14LD	6	36
1103	AF	pCA-5oP14HT-5orpfF	0	0
101-14	AF	pCK-5oP14LD-5orpfF	1	6
1103	BC	pCA-5fCAP-5oUT456	6	36
101-14	BC	pCA-5fCAP-5oUT456	0	0
1103	BD	pCA-5PGIP-5oUT456	0	0
101-14	BD	pCK-5PGIP-5oUT456	6	36
1103	BF	pCA-5oUT456-5orpfF	4	24
101-14	BF	pCK-5oUT456-5orpfF	0	0
1103	CD	pCA-5PGIP-5FCAP	0	0
101-14	CD	pCK-5PGIP-5FCAP	0	0
1103	CF	pCA-5fCAP-5orpfF	6	36
101-14	CF	pCK-5ofCAP-5orpfF	0	0
1103	DF	pCA-5PGIP-5orpfF	6	36
101-14	DF	pCK-5PGIP-5orpfF	6	36
			63	378

Table 4. Dual-construct transformed 1103 and 101-14 rootstocks grafted to untransformed Chardonnay for planting in the USDA APHIS regulated field on August 1, 2018.

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

Initially each of the first transgenic lines of 1103 were inoculated with *Xf* in the greenhouse per the original Objective 4. Within the inoculation experiment, samples were 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 under these greenhouse conditions were not reliably diagnostic of the disease severity nor related to the relative bacterial titer in the inoculated canes. Hence, we have found the more reliable indicator of the integrity of the transformation was the insert-dependent transcript analysis. Hence, the greenhouse inoculations were discontinued and molecular analysis was used to select the transgenic rootstocks to be moved forward to grafting. After verification of dual inserts the selected lines were moved to a lath house for final stem development prior to rooting of the transformed rootstock prior to grafting (**Figure 1**).

Figure 1. Plants selected as rootstock source material. Image shows selected dual construct containing plants in the lath house as the final site to produce material for rootstock development, for grafting on non-transgenic scions and field evaluation.

Figure 2. J. Puckett harvesting transgenic rootstock canes for bud grafting to untransformed Chardonnay. Packet tag indicates rootstock and paired gene combinations expressed in this rootstock.

Figure 3. Bud grafting of wild-type PD-susceptible Chardonnay to the dual construct transformed rootstocks and planting of the grafted individuals in the USDA APHIS regulated field.

Figure 4. Planting of the dual constructs. This image illustrates the new planting of the dual construct transformed rootstocks grafted with an untransformed clone of Chardonnay. This first phase of the planting was completed August 1, 2018.

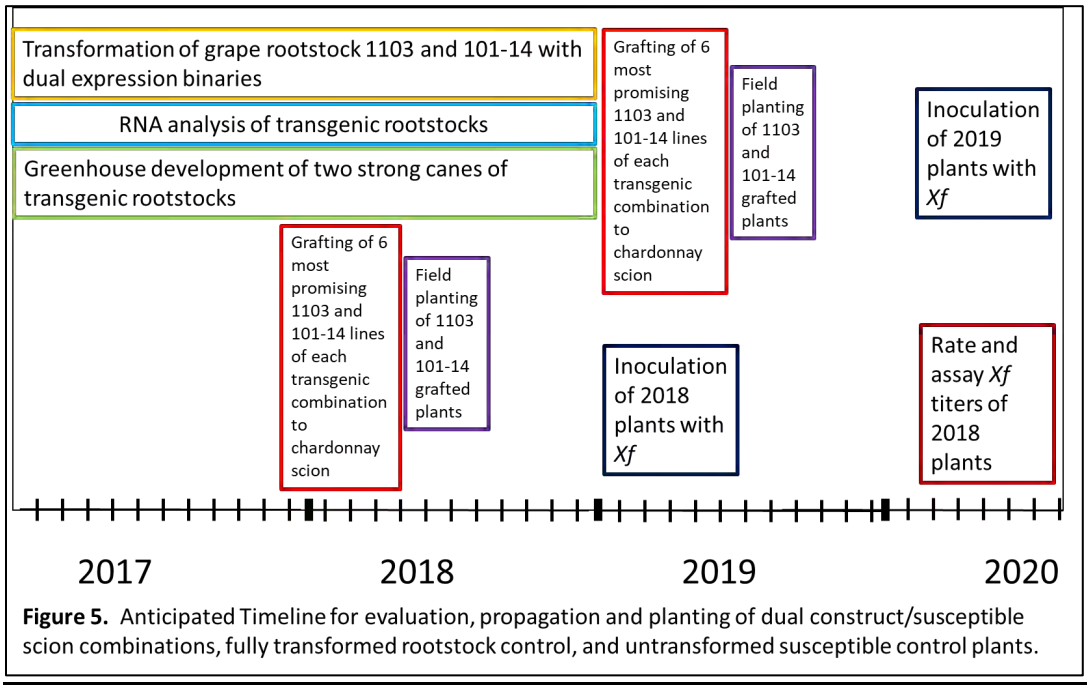

Figure 5. Anticipated Timeline for evaluation, propagation and planting of dual construct/susceptible scion combinations, fully transformed rootstock control, and untransformed susceptible control plants.

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

REFERENCES CITED

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

Dandekar AM, Gouran H, Ibáñez AM, Uratsu SL, Agüero CB, McFarland S, Borhani Y, Feldstein PA, Bruening G, Nascimento R, Goulart LR, Pardington PE, Chaudhary A, Norvell M, Civerolo E, Gupta G. 2012. An engineered innate immune defense protects grapevines from Pierce disease. *Proc Natl Acad Sci USA* 109(10): 3721-3725.

Escobar MA, Civerolo EL, Summerfelt KR, Dandekar AM. 2001. RNAi-mediated oncogene silencing confers resistance to crown gall tumorigenesis. *Proc Natl Acad Sci USA* 98(23):13437-13442.

Gilchrist DG, et al. 2016. Transgenic rootstock-mediated protection of grapevine scions by introduced single and dual stacked DNA constructs. *Proceedings of the 2016 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA, pp. 59-66.

Hajdukiewicz P, Svab Z, Maliga P. 1994, The small versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. *Plant Mol Biol* 25:989-994.

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

Lindow, SE. 2013. Continued field evaluation of diffusible signal factor producing grape for control of Pierce's disease. *Proceedings of the 2013 Pierce's Disease Research Symposium.* California Department of Food and Agriculture, Sacramento, CA, pp. 136-144.

Tricoli, DM, Carney KJ, Russell PF, McMaster JR, Groff DW, Hadden KC, Himmel PT, Hubbard JP, Boeshore ML, Quemada HD. 1995. *Nature Biotechnology* 13:1458-1465. doi:10.1038/nbt1295-1458.

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

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ABSTRACT

Burkholderia phytofirmans strain PsJN was found to be capable of extensive growth and movement within grape after both needle and spray inoculation. *B. 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. The population size of *Xylella fastidiosa* (*Xf*) is greatly reduced in plants in which *P. phytofirmans* is either co-inoculated at the same time and location, inoculated at the same time but at other nearby locations, and even inoculated at other locations either three weeks before or after that of the pathogen. The dramatic reductions in population size of *Xf* are observed in all grape varieties tested. Reductions in pathogen population are similarly large when *P. phytofirmans* is inoculated by spraying in a suspension containing 0.2% Break-Thru, an organosilicon surfactant with very low surface tension, as when directly inoculated into plants using a needle. While *P. phytofirmans* can achieve large population sizes in inoculated grapes within three to four weeks after inoculation, and spread up to one meter away from the point of inoculation, it's 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 of the pathogen alone. Substantial control of Pierce's disease in Cabernet Sauvignon was seen in field trials. The largest reductions in disease severity were observed in plants treated with *P. phytofirmans* applied by droplet puncture or by spray application in a penetrating surfactant either at the same time as, or up to three weeks after, that of the pathogen.

LAYPERSON SUMMARY

A naturally occurring *Paraburkholderia* strain that is capable of extensive 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* in both greenhouse studies as well as field studies. 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 since plants can be relatively easily inoculated by various methods.

INTRODUCTION

As the extensive movement of *Xylella fastidiosa* (*Xf*) through the plant is essential for both symptom development and successful transfer between plants by insect vectors, the pathogen has adapted a cell density-dependent transcriptional modulation system based on accumulation of fatty acid signal molecules known as diffusible

signal factor (DSF) to coordinate expression of opposing traits required for growth and movement in the plant and those enabling acquisition by insect vectors, since the phenotypes required for these two processes are somewhat incompatible. A novel disease control strategy aimed at achieving "pathogen confusion" based on production of DSF in transgenic plants harboring the pathogen DSF synthase yielded plants of much lower susceptibility to Pierce's disease (PD). Alternative strategies of producing DSF in plants that did not require the use of transgenic plant varieties and which could be employed in currently existing crops would be an attractive procedure. One such scheme could employ naturally-occurring endophytic microorganisms into which the genes from *Xf* encoding DSF synthesis could be added. A variety of endophytic bacteria recovered from surface-sterilized, field-grown grapes and other plant species have been described. Most of these reports are rather qualitative, with the simple presence of a given taxon noted. Moreover, the population sizes of such bacteria within the aboveground parts of various woody plants were typically rather low (< 103 cells/g). A relatively large and well distributed population of any such organism to be used in biological control would probably be needed. In contrast to the apparently low population sizes achieved by many bacteria that gain entrance into woody plants, *Paraburkholderia phytofirmans* PsJN (formerly known as *Burkholderia phytofirmans* and *Pseudomonas* sp. PsJN) has been reported to achieve large population sizes in plants, including grape. Nearly all studies of *P. phytofirmans*, however, have been qualitative studies of seedling plants, often inoculated via application to roots. We report here the extensive colonization of mature grapevines with strain PsJN, documenting its exceptional ability to grow and move within this woody plant. Furthermore, we report the unexpected results of the striking efficacy of *P. phytofirmans* PsJN itself, without addition of *Xf* DSF synthase, in blocking colonization of grape by *Xf* and dramatically reducing symptoms of PD. Studies of the population dynamics of this strain, as well as of *Xf* in co-inoculated plants suggests a likely, DSF-independent, mechanism by which *P. phytofirmans* inhibits *Xf* in plants and also reveals practical means by which plants can be inoculated with this biological control agent to achieve control of PD.

OBJECTIVES

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

RESULTS AND DISCUSSION

Objective 1. Biological Control with *P. phytofirmans* PsJN

While the biological control of PD 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 when re-inoculated. We have recently, however, found that *P. phytofirmans* strain PsJN, which had been suggested to be an endophyte of grape seedlings, multiplied and moved extensively in mature grape plants (**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 *B. phytofirmans* revealed that it has a homologue of *Xf rpfF*. While we have no evidence for its production of a DSF species to which *Xf* could respond, the promiscuous nature of RpfF in *Xf* and other species suggested that it might make DSF species to which *Xf* would respond under some circumstances, such as when growing within plants. Our studies have shown that co-inoculation of *Xf* and *B. phytofirmans* resulted in greatly reduced disease symptoms compared to plants inoculated with *Xf* alone; whereas the number of infected leaves of plants inoculated with *Xf* alone increased rapidly after week 12, very little disease was observed in plants inoculated with *Xf* and *B. phytofirmans* (**Figure 1**).

P. phytofirmans was able to inhibit PD 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 PD was greatly suppressed compared to that of plants inoculated with *Xf* alone (**Figure 2**). While the greatest reduction in disease severity was conferred in Cabernet Sauvignon, a variety somewhat more resistant to PD than either Thompson Seedless or Chardonnay, *P. phytofirmans* conferred a very high level of disease resistance (**Figure 2**). 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.

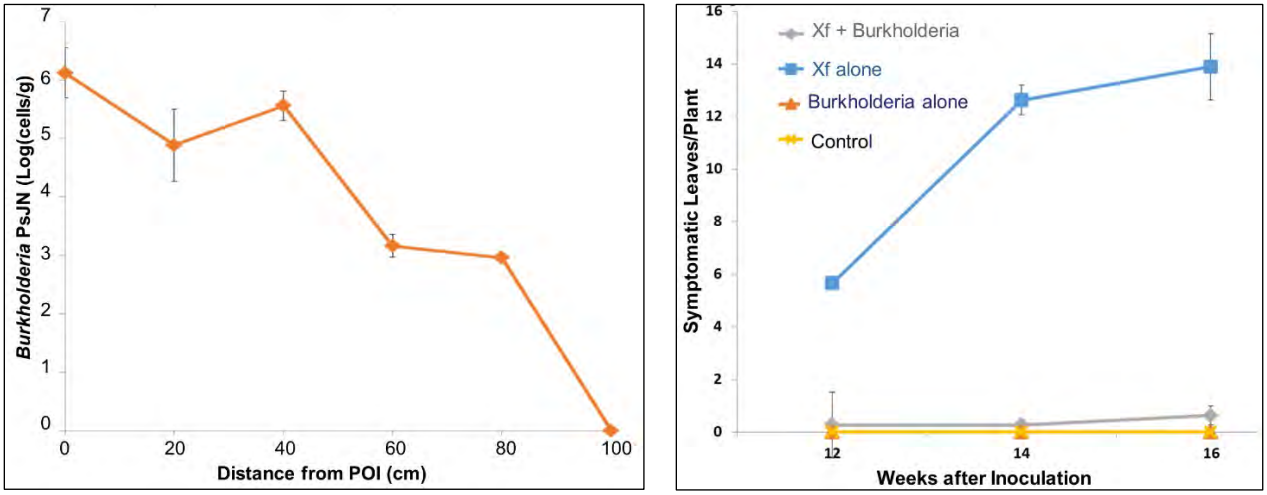

Figure 1. Left: Population size of *B. phytofirmans* in Cabernet Sauvignon grape at various distances from the point of inoculation after six weeks incubation. Right: Severity of PD of Cabernet Sauvignon at various times after inoculation with *Xf* alone (blue) or when co-inoculated with *B. phytofirmans* (gray) or when inoculated with *B. phytofirmans* alone (red).

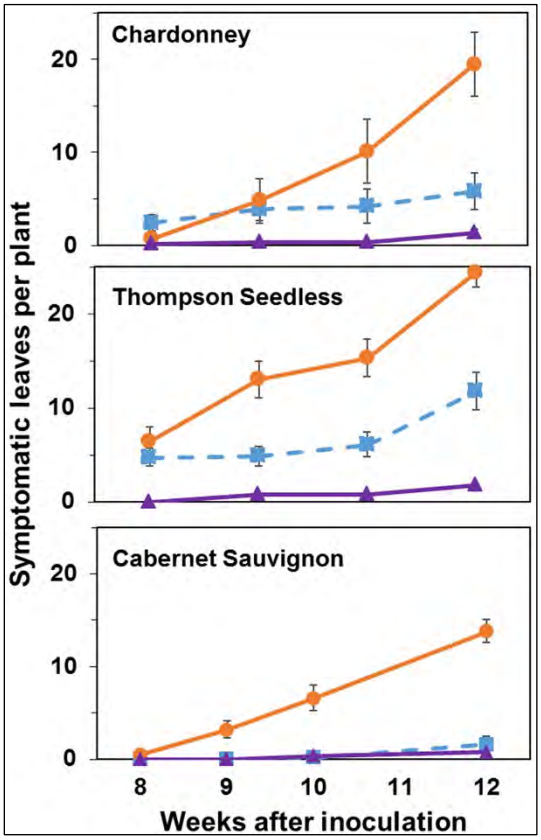

Figure 2. Severity of PD observed in different grape varieties needle inoculated at the same time but at different locations with *Xf* and *P. phytofirmans* (blue line) compared to that inoculated only with *Xf* (orange line), or with *P. phytofirmans* alone (purple line). The vertical bars represent the standard error of the determination mean disease severity.

The large reductions in the severity of disease when *Xf* was co-inoculated with *P. phytofirmans* PsJN was associated with the apparent elimination of viable cells of the pathogen both at the point of inoculation as well as at various distances distal to the point of inoculation either four or eight weeks after inoculation (**Figure 3**). In contrast, the population size of *Xf* increased progressively after its inoculation into grape in the absence of *P. phytofirmans*, reaching population sizes of approximately 10^6 cells at all sites within about 60 cm from the point of inoculation, and moved to at least 120 cm from the point of inoculation within eight weeks after inoculation (**Figure 3**). Such large populations throughout the plant were associated with a high severity of disease, which increased between 11 and 14 weeks after inoculation (**Figure 4**). In contrast, no viable cells of *Xf* were detected at any location in these plants either four or eight weeks after inoculation together with *P. phytofirmans* (**Figure 4**), and no evidence of PD was observed even by 14 weeks after inoculation (**Figure 4**). By four weeks after inoculation, population sizes of *P. phytofirmans* of about 10^4 cells/g were observed at all points up to 60 cm distal to the point of inoculation (**Figure 3**). Curiously, while readily detected up to 90 cm or more from the point of inoculation when assessed eight weeks after inoculation, *P. phytofirmans* population sizes were consistently about 10-fold lower at a given distance from the point of inoculation than at four weeks (**Figure 3**). *P. phytofirmans* population sizes were often slightly lower at a given sampling time and location when co-inoculated into plants with the pathogen compared to when it was inoculated alone (**Figure 3**). Large reductions in population sizes of *Xf*, often to undetectably low numbers, in plants inoculated with *P. phytofirmans* at various times, and in various ways, was always observed in the many experiments undertaken.

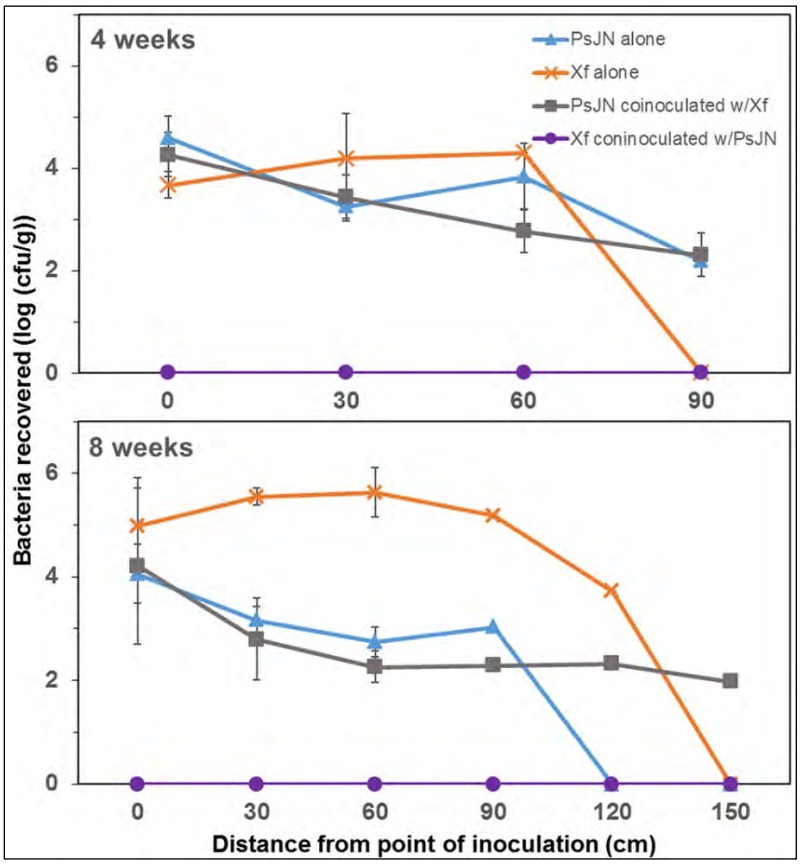

Figure 3. Population size of P. *phytofirmans* PsJN in Cabernet Sauvignon grape stems when needle inoculated alone (triangles, blue line) or together with *Xf* (squares, black line), and *Xf* when inoculated alone (X's, orange line), or together with PsJN (circles, purple line) at various distances from the point of inoculation after four weeks incubation (top panel) or after eight weeks incubation (bottom panel). The vertical bars represent the standard error of mean log-transformed bacterial population sizes.

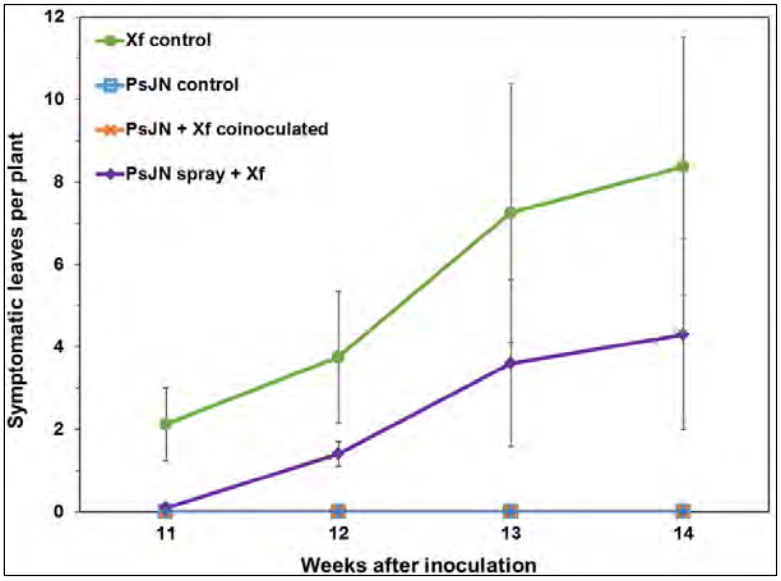

Figure 4. Severity of PD symptoms on Cabernet Sauvignon grape (experiment also described in **Figure 5**) inoculated only with *Xf* (circles, green line), needle inoculated with a mixture of *Xf* and *P. phytofirmans* PsJN (X's, orange line), inoculated with *Xf* immediately after spray inoculation of PsJN in 0.2% Break-Thru (diamonds, purple line), or needle inoculated only with PsJN (squares, blue line). The vertical bars represent the standard error of the mean number of symptomatic leaves at a given assessment time.

To determine whether the inhibitory effects of *P. phytofirmans* on the process of PD was dependent on any direct interactions between it and *Xf* that might have occurred because of their mixture together at the point of inoculation, we compared the dynamics of disease process in plants in which the pathogen and strain PsJN were applied as a mixed inoculation in the same site with that in plants in which they were inoculated separately up to 10 cm apart but at the same time. As previously observed, the severity of PD in plants in which the pathogen and strain PsJN were applied as mixed inoculum in the same site in the plant was greatly reduced at a given time after inoculation compared to plants inoculated only with the pathogen (**Figure 5**). Importantly, disease severity for plants inoculated at the same time but at different locations with these two strains was usually only nearly as low as that in plants receiving a mixed inoculum. Both treatment schemes resulted in very large reductions in disease severity compared to that of control plants inoculated only with the pathogen (**Figure 5**).

Given that close physical proximity of *Xf* and *P. phytofirmans* at the time of inoculation of the pathogen is apparently not required to achieve large reductions in disease, we explored methods of inoculation of plants with strain PsJN that might ultimately prove more practical for implementation under field conditions than injection into stems. Spray application of bacterial inoculum might readily be adoptable by growers because of similarities in methodology and equipment used in other pest management procedures. Topical application of suspensions of *P. phytofirmans* of 10^8 cells/ml in buffer alone resulted in only very low internalized population sizes of this strain within either petioles or leaf lamina when assessed at different times after spray application (**Figure 6**). In contrast, the population size of strain PsJN applied in buffer containing 0.2% Break-Thru, an organo-silicon surfactant conferring extremely low surface tension to aqueous solutions (similar to that of Silwet L77), were about 1,000-fold higher than that within leaves sprayed with bacterial suspensions in buffer alone (**Figure 6**). Furthermore, the population size of strain PsJN was about 100-fold higher within the lamina of the leaf compared to that within the petioles. Not only were large internalized populations of *P. phytofirmans* achieved immediately after inoculation ($> 10^3$ to 10^5 cells/g), but these endophytic bacterial population sizes increased with time for at least nine days after spray inoculation (**Figure 6**). In many other experiments in which strain PsJN was topically applied with 0.2% Break-Thru, the population size of strain PsJN within leaves immediately after inoculation was as high as 10^5 cells/g (data not shown). The leaves sprayed with bacterial suspensions containing this surfactant immediately acquired a water-soaked appearance, indicative of water infiltration into the leaf (**Figure 7**). The number of bacteria introduced into the plant by such sprays appeared to be influenced by the water status of the plant and whether stomata were fully open, both of which influenced the degree of water infiltration. It thus appears that *P. phytofirmans* can be readily inoculated into grape by simple spray application when appropriate surfactants are used.

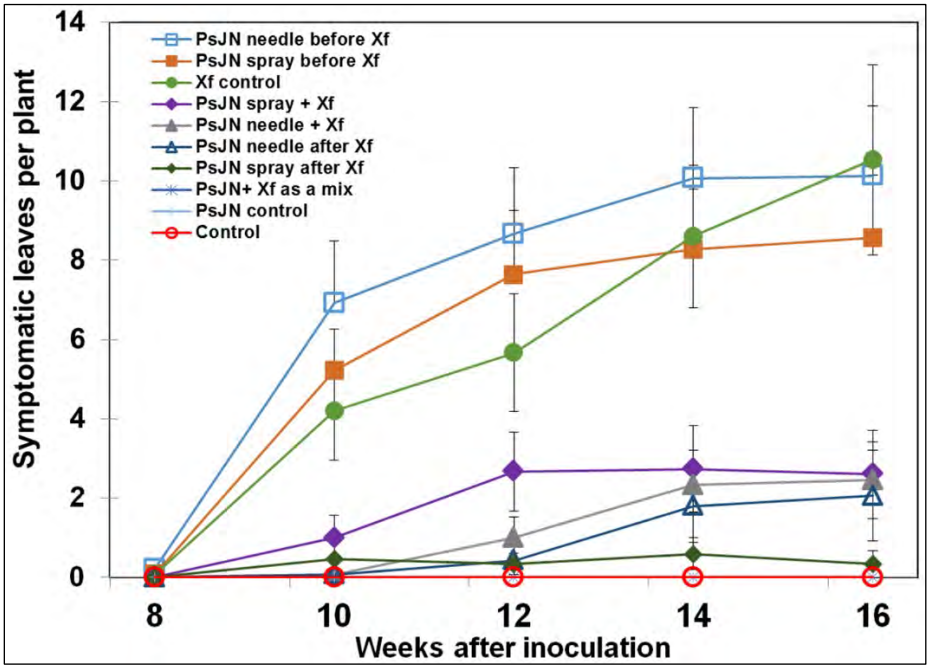

Figure 5. Severity of PD symptoms on Cabernet Sauvignon grape inoculated only with *Xf* (filled circles, green line), needle inoculated with a mixture of *Xf* and *P. phytofirmans* PsJN at the same site (X's, light blue line), needle inoculated at the same time with *Xf* and *P. phytofirmans* PsJN but at different sites on the base of the plant (filled triangles, gray line), inoculated with *Xf* immediately after spray inoculation of PsJN in 0.2% Break-Thru (filled diamonds, purple line), needle inoculated with PsJN 30 days before inoculation with *Xf* (open squares, light blue line), sprayed with PsJN in 0.2% Break-Thru 30 days before inoculation with *Xf* (filled squares, brown line), sprayed with PsJN in 0.2% Break-Thru 30 days after inoculation with *Xf* (filled diamonds, green line), needle inoculated with PsJN 30 days after inoculation with *Xf* (open triangles, blue line), needle inoculated only with PsJN (vertical slash, light blue line), or on uninoculated plants (open circles, red line). The vertical bars represent the standard error of the mean number of symptomatic leaves assessed on each of 15 replicate plants for each treatment at a given assessment time.

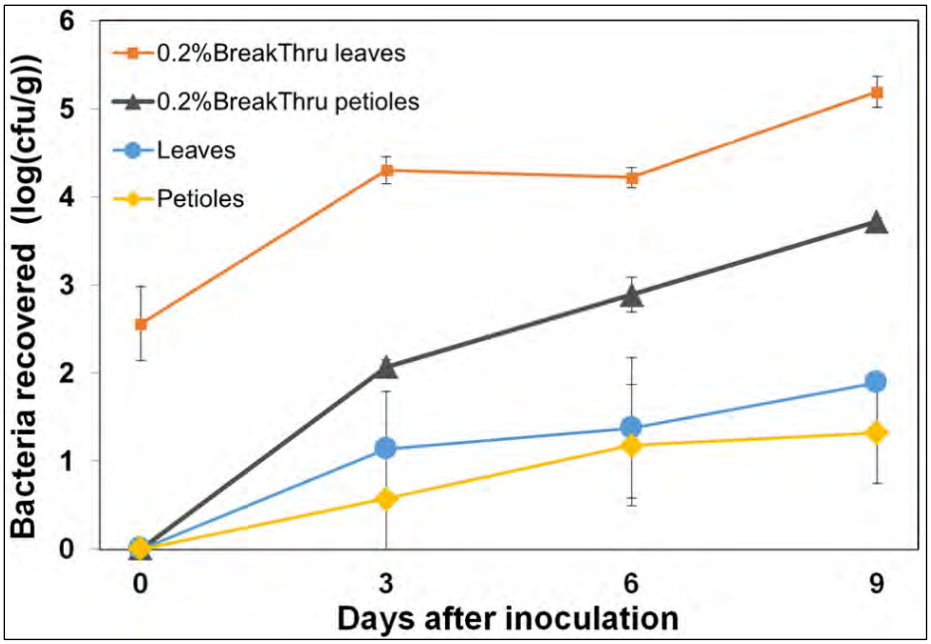

Figure 6. Population size of *P. phytofirmans* PsJN within leaves (squares, orange line) or petioles (triangles, black line) when sprayed onto Cabernet Sauvignon grape with 0.2% Break-Thru, or within leaves (circles, blue line) or petioles (diamonds, yellow line) when sprayed onto plants in buffer alone, when sampled at various times shown on the abscissa after inoculation. The vertical bars represent the standard error of the mean of long-transformed population sizes.

Figure 7. Water-soaked spots in leaves of Cabernet Sauvignon grape 10 minutes after topical applications of a suspension of *P. phytofirmans* PsJN suspended in 0.2% Break-Thru.

The severity of PD on plants sprayed with *P. phytofirmans* immediately before inoculation with *Xf* was significantly lower than on control plants inoculated with the pathogen alone (**Figures 4, 5, and 8**). While the disease severity of plants sprayed with *P. phytofirmans* at the same time as inoculation with the pathogen was often slightly higher than that on plants that were puncture-inoculated with this strain at the same time as the pathogen when assessed at a given time, the degree of disease severity in both treatments was always much less than that of control plants inoculated only with the pathogen, and often did not differ significantly. It appears that topical application of *P. phytofirmans* with a surfactant that allows spontaneous stomatal infiltration is nearly as effective in mediating control of PD as direct inoculation of this biological control agent into xylem tissue.

While *Xf* and *P. phytofirmans* apparently do not need to be entirely spatially coincident at the time of inoculation of the pathogen in order to achieve suppression of PD symptoms, and substantial disease control was inevitably obtained when the two strains were inoculated at the same time into plants by various ways, insights as to the possible mechanisms contributing to disease control were obtained by inoculating strain PsJN into plants at various times relative to that of the pathogen. Surprisingly, the extent to which the severity of PD was reduced when *P. phytofirmans* was inoculated into plants either by injection or spray application four weeks prior to inoculation with *Xf* was invariably less than when the two strains were applied at the same time when made by the same method of PsJN application. For example, in some experiments, PD severity in plants treated with *P. phytofirmans*, either by needle inoculation or spraying four weeks before that of the pathogen, did not differ from that of plants inoculated with the pathogen alone, while simultaneous inoculation with strain PsJN by either method conferred very large reductions in disease severity compared to control plants (**Figure 5**). In other experiments, pre-treatments of plants with *P. phytofirmans* either by needle inoculation or spraying conferred significant reductions in disease severity compared to that of control plants, yet the extent of disease control was substantially less than that conferred by corresponding needle or spray inoculation at the same time as the pathogen (**Figure 8**). Disease severity in plants sprayed with *P. phytofirmans* was, however, consistently less than that in plants to which strain PsJN had been inoculated by puncturing before the pathogen (**Figures 5 and 8**). Even more surprising, however, was the observation that disease control achieved by puncture or spray inoculation of *P. phytofirmans* into plants three to four weeks after inoculation of the pathogen was as great as, and often greater than, that achieved by simultaneous inoculation by a given method (**Figures 5 and 8**). Given that population sizes of *Xf* typically increase and spread extensively in inoculated plants within four weeks (**Figure 3**), it was remarkable to find, as in other experiments, very low or undetectable population sizes of *Xf* subsequent to such treatments with *P. phytofirmans*, even though it was applied four weeks after that of the pathogen (data not shown).

Insight as to the surprising observation that pre-treatment of plants with *P. phytofirmans* inevitably reduced its efficacy in biological control of PD compared to simultaneous or post inoculation treatments was provided by analysis of the temporal patterns of colonization of plants by strain PsJN. We frequently observed that while

relatively large population sizes of *P. phytofirmans* could be detected throughout grape within a few weeks after inoculation, this population size often subsequently decreased, often dramatically so (**Figure 3**; data not shown). A more systematic examination of *P. phytofirmans* populations when co-inoculated with *Xf* in grape as a function of time revealed that its population size and distribution distal to the point of inoculation both increased for at least three weeks after inoculation, but then started to decrease by five weeks (**Figure 9**). As in most other experiments, viable cells of *P. phytofirmans* often became undetectably low within 10 weeks after inoculation (data not shown). As in all experiments, when inoculated in the absence of *P. phytofirmans* both the population size and extent of distribution of *Xf* distal to the point of inoculation tended to increase with time (**Figure 9**) while viable cells of the pathogen were not detected at any time or distance from the point of inoculation when co-inoculated with strain PsJN (**Figure 9**).

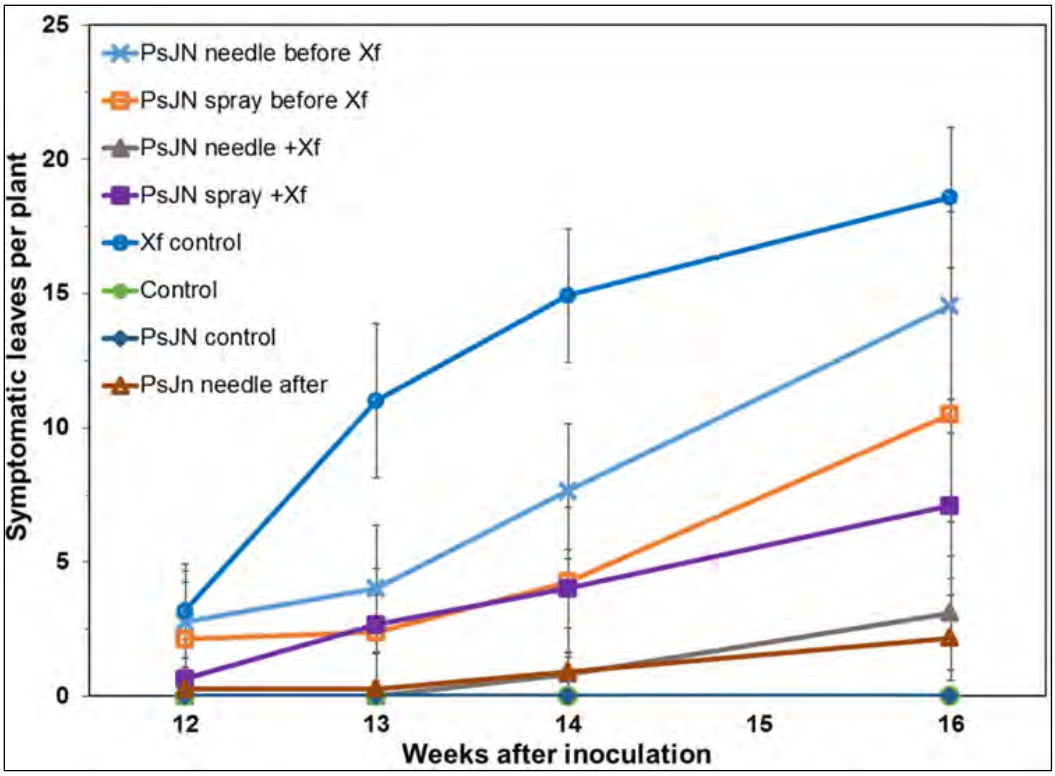

Figure 8. Severity of PD symptoms on Cabernet Sauvignon grape inoculated only with *Xf* (circles, dark blue line), needle inoculated with a mixture of *Xf* and *P. phytofirmans* PsJN (filled triangles, gray line), inoculated with *Xf* immediately after spray inoculation of PsJN in 0.2% Break-Thru (filled squares, purple line), needle inoculated with PsJN 30 days before inoculation with *Xf* (X's, light blue line), sprayed with PsJN in 0.2% Break-Thru 30 days before inoculation with *Xf* (open squares, orange line), inoculated with *Xf* 30 days after needle inoculation with PsJN (open triangles, red line), or needle inoculated only with PsJN (diamonds, dark blue line), or on uninoculated plants (filled circles, green line). The vertical bars represent the standard error of the mean number of symptomatic leaves at a given assessment time.

Objective 2. Mechanisms of Biological Control

As discussed in Objective 1, it seemed possible that *Paraburkholderia* may alter the behavior and survival of *Xf* by inducing changes in grape plants themselves, such as by stimulating innate plant immunity. Plant innate immunity serves as an important mechanism by providing the first line of defense to fight against pathogen attack. While grape apparently does not successfully recognize and therefore defend against infection by *Xf*, it might be possible that plants could be "primed" to mount a defense against *Xf* by another organism such as *Paraburkholderia*. Certain beneficial microorganisms such as *P. phytofirmans* PsJN have been shown to prime innate defenses against various pathogens in model plant system such as *Arabidopsis*, and a recent study suggest that it could also do so in grapes. Further, the bacterium induces plant resistance against abiotic stresses, apparently by changing patterns of gene expression in host plants. We thus explored whether the reduced disease symptoms and lower pathogen population seen in plants inoculated with *Paraburkholderia* either before or after

that of *Xf* is mediated by the activation of plant innate immunity. To test this hypothesis, we measured the expression of various genes in grape that are responsible for, or reflective of, responses to pathogens and mechanical and abiotic stresses in (1) control plants with no treatment, (2) plants injected with the *Paraburkholderia* strain alone, (3) plants injected with both *Paraburkholderia* and *Xf* strains simultaneously, and (4) plants inoculated only with *Xf*.

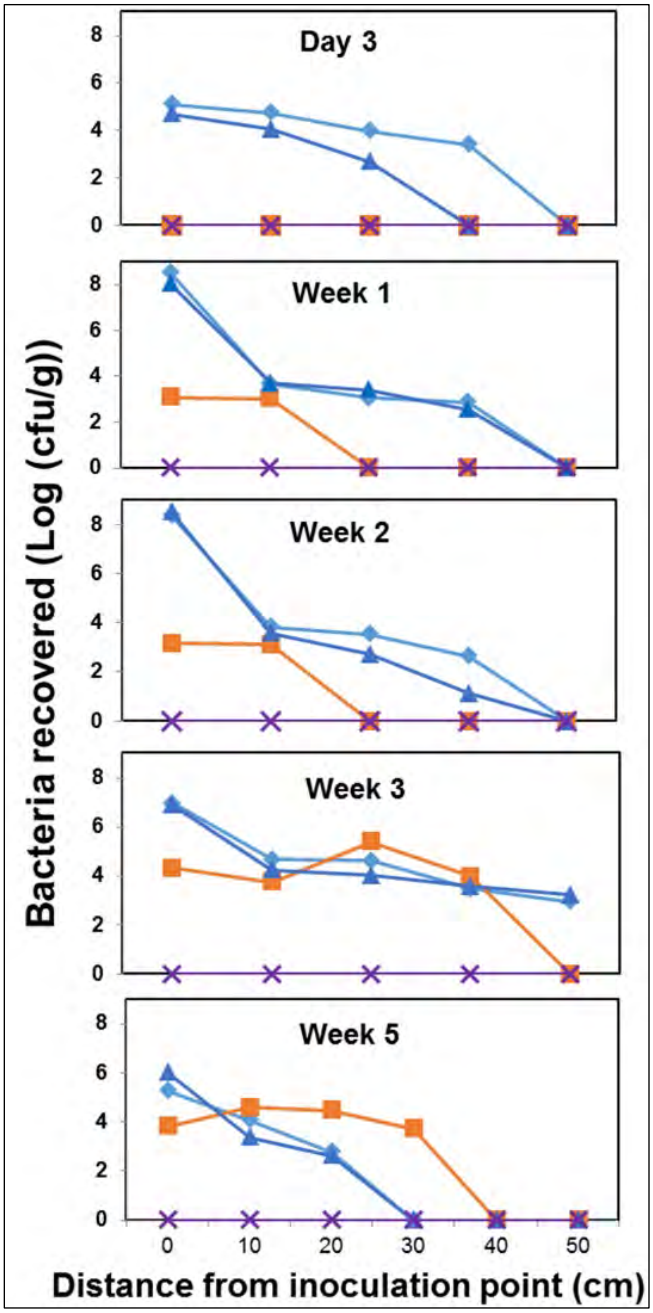

Figure 9. Population size of *P. phytofirmans* PsJN in Cabernet Sauvignon grape stems when needle inoculated alone (diamonds, light blue line) or together with *Xf* (triangles, dark blue line), and *Xf* when inoculated alone (squares, orange line), or together with PsJN (X's, purple line) at various distances from the point of inoculation. Each panel shows population sizes at a given time after inoculation.

The abundance of PR1 indicative of induction of salicylic acid-mediated host defenses, JAZ1 indicative of jasmonic acid-mediated host defenses, and ETR1 reflecting ethylene-dependent responses were determined in RNA isolated from petioles collected from near the point of inoculation of plants by semi-quantitative reverse

transcription polymerase chain reaction (RT-PCR). The abundance of EF1a, expected to be constitutively expressed, was used as an internal control to account for the efficiency of RNA isolation. The abundance of these indicator transcripts was compared in plants inoculated only with *P. phytofirmans*, *Xf*, or co-inoculated with the pathogen and strain PsJN weekly after inoculation as well as in mock-inoculated plants. Little expression of JAZ1 was detected in any of the plants, irrespective of the sampling time after inoculation (**Figure 10**). In contrast, some PR1 transcript was seen soon after inoculation of plants only with *P. phytofirmans*, with lesser amounts subsequently detected. Low levels of PR1 transcript were also observed within one week of inoculation of plants only with *Xf*, with reductions thereafter. Most notably, the highest levels of PR1 transcript were observed in plants co-inoculated with *P. phytofirmans* and *Xf*, with the apparent abundance of this transcript increasing with time up to three weeks (**Figure 10**). The abundance of PR1 transcript in these plants decreased rapidly thereafter (data not shown). Very low levels of ETR1 transcript were observed in all plants except those co-inoculated with *P. phytofirmans* and *Xf* (**Figure 10**). This suggests that an interaction between *P. phytofirmans* and *Xf* induces both the SA- and ethylene-dependent signal transduction pathways in grape to levels higher than that mediated by either strain alone.

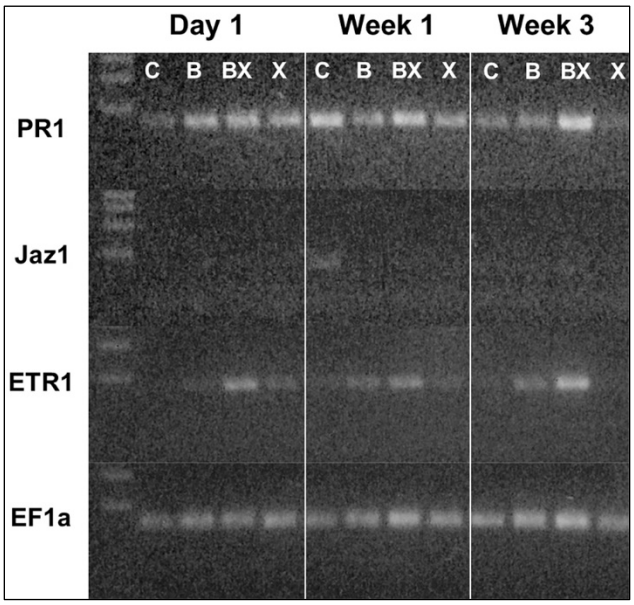

Figure 10. Products obtained after PCR amplification of complementary DNA (cDNA) obtained from RNA that had been subjected to reverse transcriptase that was isolated from petioles of Cabernet Sauvignon grape near the point of inoculation of plants that were inoculated only with buffer (C), inoculated with *P. phytofirmans* PsJN alone (B), inoculated with both PsJN and *Xf* (BX), or were inoculated with *Xf* alone (X). 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 lane.

We have observed in the many experiments in which grape has been inoculated with *Paraburkholderia* that population sizes of this biological control agent are maximal in plants within a few weeks after inoculation, but that populations in the plant seem to decrease thereafter. We are continuing work to test the hypothesis that *Paraburkholderia i*s a very efficient colonizer of grape, but one that may be self-limiting. Specifically, we hypothesize that the plant may locally recognize and respond to the colonization of *Paraburkholderia* in a way that leads to a reduction in its own population size. In fact, it may be this response of the plant to *Paraburkholderia* that is also responsible for the dramatic reductions in *Xf* populations in plants inoculated with *Paraburkholderia*. If, as we hypothesize, such a host response is relatively local to the plant region colonized by *Paraburkholderia*, the patterns of biological control that we have observed could be explained. Specifically, biological control of PD would be expected if *Paraburkholderia* was applied at the same time as or even after that of the pathogen if the rapid movement of *Paraburkholderia* throughout the plant mediated a defensive reaction either before the plant had been colonized by *Xf* or before the pathogen had achieved population sizes sufficient to incite disease symptoms. In this model, the spatial movement and persistence of *Paraburkholderia* in the plant would determine the efficacy of biological control (**Figure 11**). Our ongoing studies to investigate the spatial

movement and temporal persistence of *Paraburkholderia* in plants after inoculation relative to that of the pathogen when inoculated at different times and locations are central to our understanding of how to optimize biological control of PD.

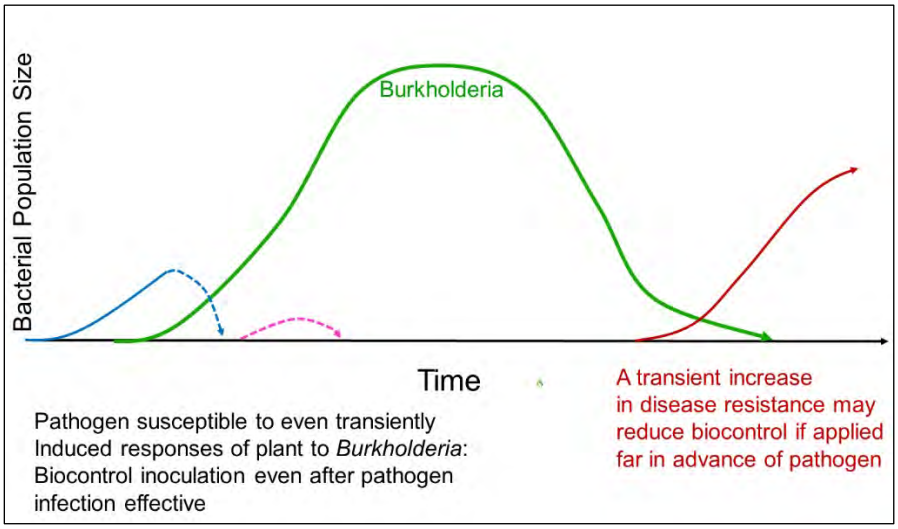

Figure 11. A model describing the expected temporal growth and persistence of *Paraburkholderia* in grape plants after inoculation (green line) and the expected effects on population sizes of *Xf* inoculated at various times relative to that of *Paraburkholderia* (blue, pink, and red lines) based on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

Objective 3. Field Efficacy of Biological Control of PD

Large-scale field studies in a replicated field site managed by the Department of Plant Pathology at the University of California, Davis were initiated in 2018 that evaluated the extent to which the factors which we found to control the efficacy of biological control under greenhouse conditions were directly applicable to the control of PD in a field setting. The study was also designed to enable us to evaluate the effectiveness of spray applications of *Paraburkholderia* relative to that of direct needle inoculation. A large planting of Chardonnay, Cabernet Sauvignon, and Pinot noir were established from so-called "Uber" plants generously provided by Duarte Nurseries. The grapevines were planted in late April 2017 and were sufficiently large by the spring of 2018 to inoculate with the pathogen and *Paraburkholderia*. In 2018 both Cabernet Sauvignon and Pinot noir were inoculated, while Chardonnay will be inoculated in 2019. The overall experimental design involves the following treatments: (1) challenge plants with *Xf* relatively soon after needle inoculation or topical treatment with *Paraburkholderia*; (2) challenge plants with *Xf* several weeks after inoculation of plants with *Paraburkholderia* in different ways; (3) inoculate *Paraburkholderia* into plants in different ways only after challenge inoculation with *Xf* to assess the potential for "curative effects" after infection has occurred; and (4) challenge inoculate plants treated with *Paraburkholderia* with *Xf* on multiple occasions, spanning more than one growing season, to reveal the persistence of the biological control phenomenon. Greenhouse studies in our current project have also indicated that topical applications of a DSF-like molecule, palmitoleic acid, with a penetrating surfactant can also confer disease resistance. This treatment was therefore compared with the various biological control treatments. Each treatment consisted of 10 plants for a given grape variety. For individual vines, one on each of the four cordon arms for a given plant were inoculated. The details of the experimental design are shown in **Figure 12**.

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

Figure 12. Experimental design and treatment listed for field trials conducted in 2018. Columns represent treatments made at a given time indicated in the headings. Note that on some occasions more than one treatment was applied at a given inoculation time. Unless otherwise noted, all inoculations were made at the base of the vines. Xy or *Xylella* = inoculation made with *Xf* strain STL via droplet puncture. B needle = inoculation made with *P. phytofirmans* PsJN via droplet puncture. B and Xy mix = inoculum of both *P. phytofirmans* and *Xf* were mixed and inoculated as a single droplet puncture. Bspray = inoculation made by spraying *P. phytofirmans* PsJN in 0.2% Break-Thru. B trunk = inoculation of the trunk of vines (ca. 30 cm from soil level) made with *P. phytofirmans* PsJN via droplet puncture. Soap = spray application of 2% palmitoleic acid. Year 2 = challenge inoculation with *Xf* to be made in spring 2019 in plants that were inoculated in spring 2018 with *P. phytofirmans* in different ways.

As was observed under greenhouse conditions, topical applications of *P. phytofirmans* with 0.2% Break-Thru to leaves was found to be an efficient way to introduce this bacterium into grape tissues under field conditions. Water-soaking was quite apparent within one minute after application to leaves (**Figure 13**). Despite the fact that the water suspensions dried relatively rapidly on the leaves under the relatively warm and often windy conditions in which they were applied, water-soaking was quite extensive and persisted for approximately 15 minutes after inoculation. Large population sizes of strain PsJN were immediately introduced into leaves in this process, and these populations remained high for many days after inoculation (**Figure 13**).

Substantial levels of disease control were conferred by application of *P. phytofirmans* PsJN in various ways to both Cabernet Sauvignon and Pinot noir grapevines, either before or after challenge inoculations with *Xf* (**Figure 14**). At the time of this report, statistical analyses of disease assessments of Pinot noir were still underway, and so disease control obtained in Cabernet Sauvignon will be discussed. Disease severity was measured as the proportion of the total leaves on a given inoculated shoot that exhibited symptoms of leaf scorching. Disease severity was measured approximately every three weeks beginning in mid-August; three separate assessments of disease severity were made. Differences in disease severity were determined after calculating the area under the disease progress curve (AUDPC) for disease measured over time. Very high levels of disease were observed in control vines in which *Xf* was inoculated a single time (treatment #6) or on two occasions (treatment #17), or when treated only with the surfactant Break-Thru after inoculation (treatment #12). As expected, no symptoms of PD were observed on control plants that were not inoculated with *Xf* (treatments #7, 8, and 9). Very high levels of disease control were observed in plants treated with *P. phytofirmans* applied in different ways. While the greatest degree of disease control was achieved when both *P. phytofirmans* and *Xf* were co-inoculated together at a single site into vines (treatment #2), a very high degree of disease control was also observed when *P. phytofirmans* was either injected or sprayed onto plants several weeks after inoculation with *Xf*

(treatments #10 and #11, respectively), or inoculated at the same time as, but at different locations within, a vine (treatments #1 and #3). Surprisingly, disease control conferred by a single inoculation of *P. phytofirmans* made after that of the pathogen provided higher levels of disease control than multiple such applications (compare treatments # 10 and #11 with treatments #13 and #14). In contrast to what had been observed in greenhouse studies, injection of *P. phytofirmans* into plants three weeks before they were inoculated with *Xf* also led to high levels of disease control (treatment #4). Given that field-grown plants have a large trunk on which cordons on the vines are borne, unlike the single stems resulting from rooted cuttings in greenhouse studies, we evaluated the direct injection of *P. phytofirmans* into the base of the trunk to determine if a systemic and distal effect on disease control could be conferred. Disease reductions from trunk injection were similarly large as those made directly into the vines in which *Xf* was inoculated (compare treatment #18 with treatments #1 and #10). Repeated topical application of palmitoleic acid also appeared efficacious for disease control (treatment #19).

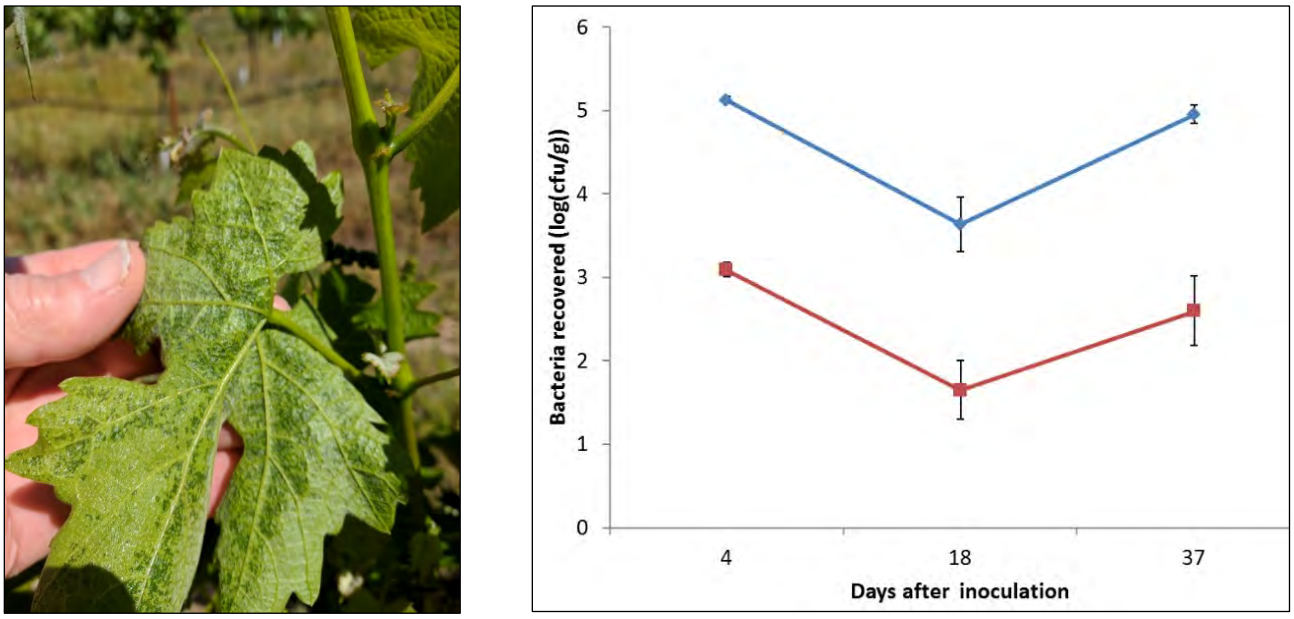

Figure 13. Left: water-soaking appearance of Cabernet Sauvignon leaves approximately two minutes after topical application of a suspension of *P. phytofirmans* PsJN in 0.2% Break-Thru in a field trial. Right: population size of *P. phytofirmans* PsJN recovered from surface sterilized lamina of spray-inoculated leaves (blue line) or surface sterilized petioles (red line) at various times after spray inoculation. The vertical bars represent the standard error of log-transformed viable bacteria recovered per gram of plant tissue.

In addition to measuring the severity of disease as the proportion of symptomatic leaves on a given inoculated shoot (as shown in **Figure 14**), we also assessed the extent to which the pathogen moved from each of the four inoculated shoots on a given plant to infect and cause symptoms on adjacent shoots. We thus counted the number of additional shoots on a given plant that exhibited symptoms of PD (**Figure 15**). Even within the short time since plants were inoculated with the pathogen alone (Treatments #6, 12, and 17), symptoms could be observed on a large number of adjacent vines on a given plant (**Figure 15**). In contrast, many fewer adjacent vines exhibited any symptoms of PD on plants treated with *P. phytofirmans* in various ways. Generally, those treatments such as treatment #2 that conferred the greatest reduction in disease severity on inoculated vines also conferred the greatest reduction in spread of disease symptoms to adjacent vines on a given plant (**Figure 15**). It was noteworthy that the direct inoculation of *P. phytofirmans* into the trunk of these mature plants also greatly reduced any spread of disease symptoms away from the inoculated vines (treatment #18), suggesting that it's basal inoculation site may have maximized any potential systemic induction of disease resistance that is postulated as a mechanism of action of *P. phytofirmans*. The high levels of disease control seen after inoculation with *P. phytofirmans* are exciting and suggest that even higher levels of disease control could be conferred after further exploration of practical questions of optimum timing and application methods.

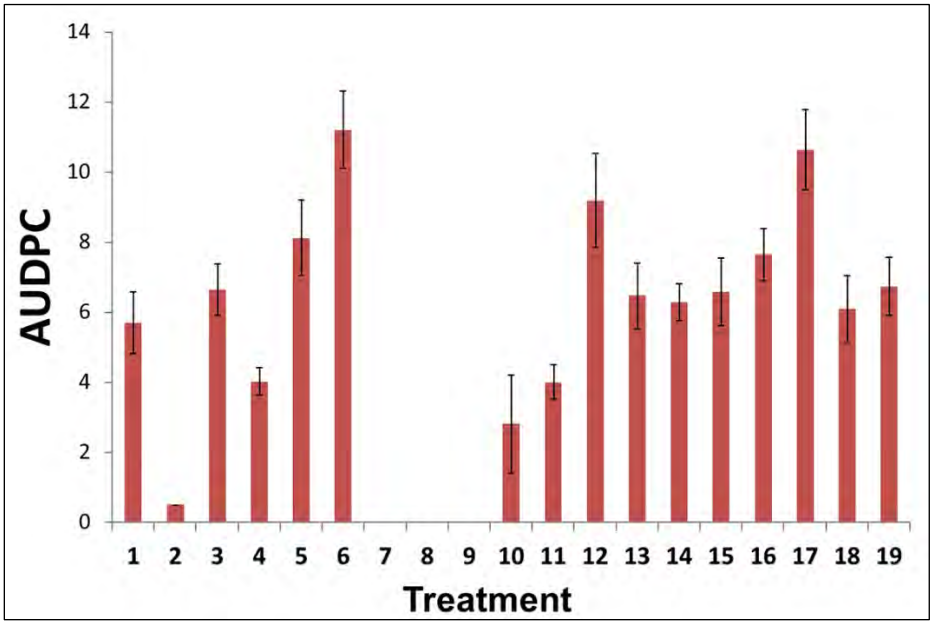

Figure 14. Disease severity of Cabernet Sauvignon grapevines shown as the area under the disease progress curve for disease assessments made on three occasions in the summer of 2018. The treatment numbers refer to the treatments described in **Figure 12**.

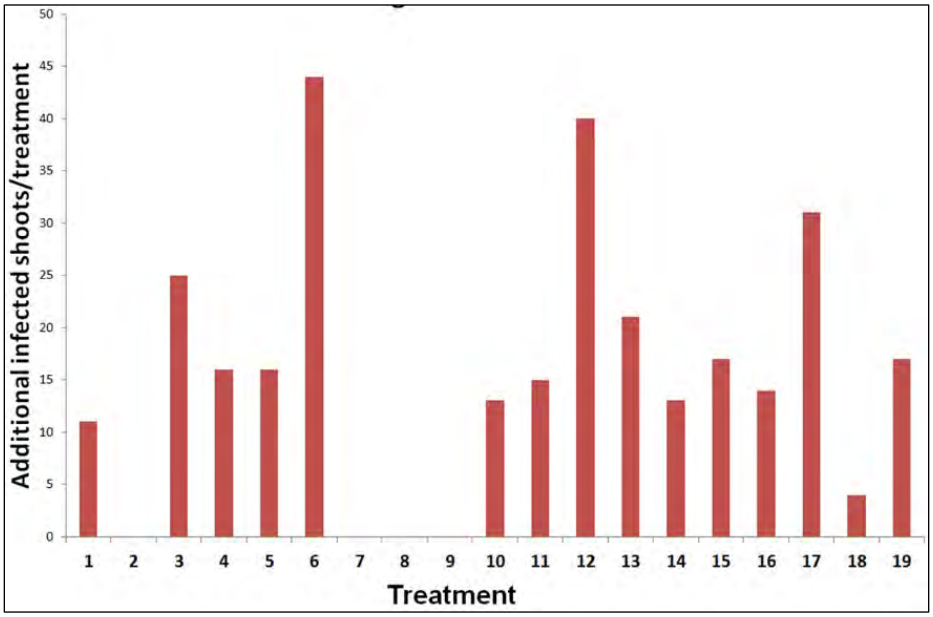

Figure 15. The number of additional shoots on a given plant that were not directly inoculated with *Xf* that exhibited symptoms of PD by late September 2018. Shown is the total number of shoots on plants inoculated with *Xf* on the 10 plants receiving a given treatment (described in **Figure 12**) that exhibited symptoms of PD.

CONCLUSIONS

The studies directly address practical strategies of control of PD. Our results reveal that *P. phytofirmans* continues to provide levels of biological control under greenhouse conditions that are even greater than what we would have anticipated, and encouraging results were obtained of practical means for introducing this strain into plants such as by spray applications. In addition, the fact that it seems to be active even when not co-inoculated with the pathogen is a very promising result that suggests this method of disease control might also be readily implemented. The high levels of disease control seen after inoculation with *P. phytofirmans* are exciting and suggest that even higher levels of disease control could be conferred after further exploration of practical

questions of optimum timing and application methods. 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
Finding 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 2018.

ABSTRACT
Transgenic plants of several different winegrape varieties, as well as rootstock varieties, have been made in an effort to produce significant levels of diffusible signal factor (DSF) in plants to achieve "pathogen confusion." In these plants, either the unmodified *rpfF* gene encoding the DSF synthase from *Xylella fastidiosa* is expressed under the control of a strong constitutive plant promoter, or a variant of *rpfF* encoding a protein with sequences that should direct the enzyme to the chloroplasts in plants is expressed. 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. Several greenhouse malfunctions have delayed the testing of the remaining plants, although all testing should be completed by the end of 2018 and clones of the plants prepared for field planting by early 2019.

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 UC 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. 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 2019. 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 grapes as own-rooted plants as well as rootstocks, for susceptible grape varieties to Pierce's disease.
2. Determine the 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 project number 14-0143-SA titled "Comparison and Optimization of Different Methods to Alter Diffusible Signal Factor Mediated Signaling in *Xylella fastidiosa* in Plants to Achieve Pierce's Disease Control," which was funded by the CDFA 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 *Xf* RpfF into several different grape cultivars. This and other projects in the previous nine years had described a cell density-dependent gene expression system in *Xylella fastidiosa* (*Xf*) mediated by a family of small signal molecules called diffusible signal factor (DSF) which we have now characterized as 2-Z-tetradecenoic acid (hereafter called C14-cis) and 2-Z-hexadecenoic acid (C16-cis). 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 endogluconase which are required for digestion of pits 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 the 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 number 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 close to completion. The work of this new continuing project is to establish field trials in Solano County in 2019 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 number 14-0143-SA grape variety Thompson Seedless as well as the advanced rootstock varieties 1103, 101-14, and Richter were transformed with the *rpfF* 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 bisphosphate 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 Plant Transformation Facility at UC Davis.

Our goal was to obtain between 5 and 10 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 *rpfF* 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.

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 for 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 of each combination that have been delivered to UC Berkeley.

Variety	Gene Introduced	
	Untargeted RpfF	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. As noted above, screening for disease resistance of the non-targeted RpfF plants already delivered is mostly complete. Unfortunately, there have been a series of mishaps in the greenhouses harboring these plants that have delayed our progress. A major greenhouse malfunction in August 2017 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. Recently, a series of plants for which clones had been laboriously produced were in the process of being evaluated for disease control when a pesticide application in the greenhouse caused severe damage to the leaves, making it impossible to assess disease severity. Because the process of testing plants for disease severity is such a long one, spanning 30 weeks or more from the time plants are initially propagated until disease assessment is complete, there have been numerous opportunities for pest damage to interfere with disease assessments, or more commonly, for unintentional damage from pesticides that are needed to maintain the health and vigor of these plants under greenhouse conditions for such a long period of time to occur. Overall, the process of evaluating the various lines for disease resistance has proved to be slower than expected. 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, and disease testing is now nearly complete. The most highly-resistant transformants for each of these grape varieties has now been identified, and we are in the process of producing sufficiently large numbers of rooted cuttings and grafted plants for establishment in the field in 2019. The grafting process will add an additional three months to the process of generating plants for use in field studies, but we expect 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	Gene Introduced		Untransformed Plants
	Untargeted RpfF	Chloroplast-targeted RpfF	
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. An 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 uninoculated 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, however. 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 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 observed 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 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 method of polymerase chain reaction, 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 sizes 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 and especially to plants infected naturally with sharpshooter vectors. The earlier work, therefore, has provided solid evidence that this strategy is a useful one for managing Pierce's 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 Pierce's Disease and Glassy-winged Sharpshooter Board.

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

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ABSTRACT

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

LAYPERSON SUMMARY

Pierce's disease (PD), caused by *Xylella fastidiosa* (*Xf*), has economically impacted the California grape industry since the 1990s. Growers lose an estimated $56 million annually in decreased production and vine replanting. Breeding efforts have resulted in new wine grape cultivars using a single source of PD resistance. This source has been effective against a few strains of *Xf*, but its durability in the field is unclear. The range in virulence (amount of disease a given isolate can cause) of *Xf* in California is not known, and regional differences appear likely. Research is needed to better understand the variability of *Xf* in California and how this might impact PD resistant grape breeding. This proposal will evaluate *Xf* virulence and the sustainability of PD resistant material.

INTRODUCTION

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

Virulence comparisons among *Xf* strains are also useful to understand the biology of this pathogen. In *Nicotiana tabacum* (tobacco), different subspecies of *Xf* are capable of colonizing and causing leaf scorch symptoms (1,10), and show differences in host colonization and symptomatology (9,18). Tobacco has been used as a model system to understand changes in host mineral and nutrient composition caused by *Xf* infection (6,11), bacterial gene function (2,12), and the impact of new DNA acquired from natural competence and recombination (10). Tobacco assays could be a useful tool to predict isolate virulence on grapevine. Using tobacco to test multiple strains saves considerable greenhouse space and time, as it can take half the time of a grape experiment.

Pierce's disease (PD) resistance has been identified in multiple *Vitis* species (**Figure 1**). How these sources differ in durability (sustainability of resistance when exposed to multiple strains) of resistance is unclear. A single source of resistance *PDR1* from a *V. arizonica*, a wild southwestern grape, accession has been used to develop high quality wine grapes with PD resistance (breeding efforts by Andy Walker, UC Davis). Table grape breeding efforts also use this same source. Plants with *PDR1* have no disease symptoms and low bacterial populations when inoculated with *Xf*. *PDR1* has maintained efficacy in field trials in Texas and northern California, but its durability to individual isolates is unclear. Other sources of resistance or tolerance have been identified, but their efficacy against multiple isolates of *Xylella* has not been evaluated.

Figure 1. Wild species and hybrids with potential PD resistance.

OBJECTIVES

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

1. Evaluate the virulence diversity of *Xf* strains from California.
2. Evaluate known sources of PD resistance against diverse strains of *Xf*.

RESULTS AND DISCUSSION

Funding was received by the PI in September 2018, and projects are currently in process. A brief description and expected outcomes for each objective are as follows.

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

For this objective, each year 40 *Xf* strains will be selected from the collection of cooperator R. Almeida and will be assessed for virulence in both tobacco (sub objective a) and a subset on grape (sub objective b). We anticipate that ~120 strains will be assessed for virulence in tobacco during the course of this project. Virulence assessments in tobacco ('Petite Havana') (6) will be conducted in the greenhouse facilities at Auburn University. During two time-points (at the beginning and full onset of leaf scorch symptoms) petiole samples will be collected at different positions in plants and *Xf* populations will be quantified by qPCR (4,6,7) to assess movement inside the xylem. Differences in virulence as evidenced by symptom development or bacterial spatial movement will determine phenotypic groups that will be used to select strains to be tested in grapevines (Subobjective b).

In grapevine, virulence assessments will be conducted in a greenhouse at the USDA ARS San Joaquin Valley Agricultural Sciences Center (SJVASC) in Parlier, CA. Plants will be evaluated weekly to monitor disease progression using a 0 to 5 based scale indicating disease severity for 25 weeks. An area under the disease progression curve (AUDPC) will be calculated for each line to determine the rate and severity of disease progression for each isolate. At the end of the experiment, petioles will be collected from each plant and bacterial populations will be estimated using qPCR. Differences in isolate virulence will be used to detect geographic variability among *Xf* strains in grape. Virulence data collected will be shared with the cooperator (Dr. Almeida) and evaluated for potential genetic/virulence associations using genetic pipelines currently employed in his lab. While the small sample size would not allow for conclusive association of genetic markers with virulence, it could serve as preliminary data for more in-depth studies.

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

Grape breeding for Pierce's Disease resistance uses on a single source of a resistance (*V. arizonica*) to *Xf*. The ability of this and other sources of resistance to maintain an acceptable level of resistance to multiple, diverse strains of *Xf* is unknown. A panel of ten *Xf* strains with virulence and genetic variability determined in Objective 1a will be used to determine the durability of resistance for each grape line. Plants will be evaluated weekly to monitor disease progression using a 0 to 5 based scale indicating disease severity for two growing seasons. Symptoms will be evaluated until plants become dormant in the fall, and will resume after bud break the

following season. An area under the disease progression curve (AUDPC) will be calculated for each line to determine the rate and severity of disease progression for each isolate. At the end the first and second growing seasons, petioles will be collected from each plant and bacterial populations will be estimated using quantitative PCR. Differences in durability of resistance will be determined for each source of resistance/tolerance and information will be provided to public and private breeding programs on which sources of resistance will be most durable or should be combined to improve resistance durability.

Table 2. Grape lines with resistance to *Xylella fastidiosa* subsp. *fastidiosa* used for testing durability against diverse strains of *Xylella*.

Line	Species	Type of Resistance
8909-08	*V. arizonica* x *V. rupestris*	*PDR1*
Norris	*Interspecific hybrid*	multigenic
IAC 572	*V. caribbeae*	Unknown
B43-17	*V. arizonica*	*PDR1*
BD5-117	*Interspecific hybrid*	multigenic
Tampa	*V. aestivalus*	multigenic

REFERENCES CITED

1. Alves E, Kitajima EW, Leite B. 2003. Interaction of *Xylella fastidiosa* with different cultivars of *Nicotiana tabacum*: A comparison of colonization patterns. *Journal of Phytopathology-Phytopathologische Zeitschrift* 151:500-506.
2. Chen H, Kandel PP, Cruz LF, Cobine PA, De La Fuente L. 2017. The major outer membrane protein MopB is required for twitching movement and affects biofilm formation and virulence in two *Xylella fastidiosa* strains. *Molecular Plant-Microbe Interactions* 30(11):896-905.
3. Coletta-Filho HD, Bittleston LS, Lopes JRS, Daugherty MP, Almeida RPP. 2015. Genetic distance may underlie virulence differences among isolates of a bacterial plant pathogen. *J Plant Path* 97:465-469.
4. Cruz LF, Cobine PA, De La Fuente L. 2012. Calcium increases *Xylella fastidiosa* surface attachment, biofilm formation, and twitching motility. *Applied and Environmental Microbiology* 78:1321-1331.
5. Daugherty MP, Lopes JRS, Almeida RPP. 2010. Strain-specific alfalfa water stress induced by *Xylella fastidiosa*. *European Journal of Plant Pathology* 127:333-340.
6. De La Fuente L, Parker JK, Oliver JE, Granger S, Brannen PM, van Santen E, Cobine PA. 2013. The bacterial pathogen *Xylella fastidiosa* affects the leaf ionome of plant hosts during infection. *PLoS One* 8.
7. Francis M, Civerolo EL, Bruening G. 2008. Improved bioassay of *Xylella fastidiosa* using *Nicotiana tabacum* cultivar SR1. *Plant Disease* 92:14-20.
8. Kandel PP, Lopez SM, Almeida RPP, De La Fuente L. 2016. Natural competence of *Xylella fastidiosa* occurs at a high frequency inside microfluidic chambers mimicking the bacterium's natural habitats. *Applied and Environmental Microbiology* 82:5269-5277.
9. Lopes JRS, Daugherty MP, Almeida RPP. 2010. Strain origin drives virulence and persistence of *Xylella fastidiosa* in alfalfa. *Plant Pathology* 59:963-971.
10. Lopes SA, Ribeiro DM, Roberto PG, Franca SC, Santos JM. 2000. *Nicotiana tabacum* as an experimental host for the study of plant-*Xylella fastidiosa* interactions. *Plant Disease* 84:827-830.
11. Navarrete F, De La Fuente L. 2015. Zinc detoxification is required for full virulence and modification of the host leaf ionome by *Xylella fastidiosa*. *Molecular Plant-Microbe Interactions* 28:497-507.
12. Oliver JE, Cobine PA, De La Fuente L. 2015. *Xylella fastidiosa* isolates from both subsp *multiplex* and *fastidiosa* cause disease on southern highbush blueberry (*Vaccinium sp.*) under greenhouse conditions. *Phytopathology* 105:855-862.
13. Oliver JE, Sefick SA, Parker JK, Arnold T, Cobine PA, De La Fuente L. 2014. Ionome changes in *Xylella fastidiosa*-infected *Nicotiana tabacum* correlate with virulence and discriminate between subspecies of bacterial isolates. *Molecular Plant-Microbe Interactions* 27:1048-1058.
14. Rogers EE. 2012. Evaluation of *Arabidopsis thaliana* as a model host for *Xylella fastidiosa*. *Molecular Plant-Microbe Interactions* 25:747-754.

15. Simpson AJG, Reinach FC, Arruda P, Abreu FA, Acencio M, Alvarenga R, et al. 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. *Nature* 406:151-157.
16. Van Sluys MA, de Oliveira MC, Monteiro-Vitorello CB, Miyaki CY, Furlan LR, Camargo LEA, et al. 2003. Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. *Journal of Bacteriology* 185:1018-1026.

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

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ABSTRACT / LAYPERSON SUMMARY

The bacterium *Xylella fastidiosa* (*Xf*) is the cause of Pierce's disease (PD) in grapes and is a major threat to fruit, nut, olive, and coffee groves. The most damaging effects of PD 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 PD symptoms such as 'matchstick petioles' and 'green cane islands' is not understood. Prior work 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 microRNA (miRNA) activities (both P_i and miRNAs are diffusible signals in plants). The data generated in three initial years of research support resulted in a new award in 2018. Results continue to strengthen support of 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 and petioles has been completed from three years of field collection and the datasets quality-assured. Further analysis of the sequence data and new samples collected in 2018 will allow a systematic and comprehensive view of gene activities and their roles in the etiology of PD. A clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 genome-editing approach has generated transgenic grapevine plants to directly test the model of anthocyanin regulation to determine the effector genes' 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 inorganic phosphate (P_i) (phosphite [Phi]; reduced P_i), which alters host and microbe phosphate homeostasis, can impact *Xf* growth and host PD etiology. This aspect could result in development of a novel management tool for PD complementary to the primary high-priority genome editing approach to engineer PD 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 experimental results offer a new paradigm for PD management with potential translational benefits for other crops.

INTRODUCTION

The overuse of phosphorous (P) fertilizer results in severe environmental pollution. As natural and anthropogenically-induced climatic changes occur, increased P limitation is expected to hinder biological productivity[1]. The P_i analogue phosphite (Phi) reduces populations of several insect species in the field[2] making it a potentially good fit for integrated pest management programs, although this aspect has not been developed, nor tested for Pierce's disease (PD), since its discovery[3]. There is evidence for host plant stress physiology (e.g., visual and/or olfactory cues related to host metabolites) associated with glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) deterrence[4]. A few studies have determined that some anthocyanin and

derivative tannic compounds can reduce insect feeding[5], including sap-sucking insects[6, 7], which provides a plausible basis for observed PD infection susceptibility differences between anthocyanless and red cultivars[8-11]. However, similarity in GWSS PD transmission rates among cultivars harboring different bacterial populations in petioles[12] suggests that variability in pathogen distribution within-plant[13] or phase of the life cycle (biofilm versus motile) may be important for vector transmission and/or disease etiology. Quality improvements depend on applying new genetic insights and new technologies to accelerate breeding through improved genotyping and phenotyping methods, and by increasing the available diversity in germplasm[14-16]. The genetic identity of traditional cultivars used for wine discourages breeding approaches because markets and statutes dictate cultivar choice, thus varieties lack recombination and the resultant opportunity to select/screen for adaptability, e.g., PD resistance and P metabolism.

MicroRNAs (miRNAs) and small interfering RNAs (siRNAs) are the specificity "guide" for nucleases of the ARGONAUTE (AGO) class which cleave or otherwise repress protein-coding transcripts in a nucleotide sequence-specific manner[17, 18]. Evidence shows that miRNAs and siRNAs operate systemically by moving through vasculature, raising prospects of genetic engineering of grapevine rootstocks for PD resistance in non-genetically modified organism (GMO) scions[19-21]. Microbes and viruses utilize plant miRNAs to facilitate pathogenesis, and plants have co-opted miRNAs for plant innate immunity[22-27]. Although the molecular mechanisms of RNA interference in plant-microbe interactions are poorly understood, there is mounting evidence that plant immunity to microbial pathogens requires post-transcriptional gene silencing (PTGS) pathways[28-35]. This suggests broader roles for plant and pathogen small RNAs (sRNAs) in environmental responses and evolutionary adaptations[36, 37], which may include microbe and/or vector feeding processes.

The general research objective of this project is to continue to test a coalescent model that specific siRNAs, namely *Trans-Acting small-interfering locus4 (TAS4*) and miR828 produced by the host, are key regulators of PD etiology subject to P modulation[38]. The long-term goal is to establish a new technology in grapes that will allow genetic manipulations that will not carry the negative connotation of "GMO." This is because the transgenes are removed by conventional backcrosses of the transgenics, resulting in only mutated endogenous effector genes, analogous to breeding approaches to introgress dwarfing or pathogen resistance genes that was the basis of the Green Revolution of the 1960s.

Understanding the molecular mechanisms of miR828/*TAS4* in biotic stress responses will provide cogent (e.g., miRNA-based) strategies for engineering stress-tolerance and productivity by increasing P uptake without increasing fertilizer application. We previously put forward a model and summarized the evidence for a role of deranged P_i, altered source-sink distributions of sucrose, and the stress hormone abscisic acid (ABA)[39] in regulating phytoalexin polyphenolic accumulations via miR828, *TAS4*, and their target MYB transcription factors (viz. MYBA6/7 and close homologues) important for PD. As an independent, partial test of the hypothesis, we initiated work on transgenic tobacco that overexpresses the *Arabidopsis* target of *TAS4* siRNA; *AtMYB90/ PRODUCTION OF ANTHOCYANIN PIGMENT2/PAP2*. Transgenic plants have a dominant phenotype of purple leaves[40] and functional endogenous genes for Nta-miR828[41] and *NtTAS4ab*[42] hypothesized to interact with the over-expressed MYB effector[43]. Results presented at the 2016 Pierce's Disease Research Symposium and in the 2017 *Research Progress Reports* and other project progress reports provided compelling confirmation, as previously shown in *Arabidopsis*[38, 39], for functional conservation of an autoregulatory loop where target AtMYB90/PAP2 overexpression induces expression of the endogenous negative siRNA regulator Nt*TAS4*-3'D4(-) and its upstream trigger Nt-miR828. The inverse correlations observed between both *Nt-TAS4*-3'D4(-), Nt-miR828, and *Xylella fastidiosa* (*Xf*) infection status in PAP2-overexpressing tobacco is strong evidence in support of our model. An unexpected result consistent with the *causative Xf* model is that *Xf*-infected transgenic genotypes show Nt*TAS4*-3'D4(-) and Nt-miR828 reductions correlate with disease symptom severity.

In addition to the phased, small interfering RNAs (phasiRNAs) generated from TAS4-3'D4(-) targeting of *VvMYBA6/A7*, we have shown an inverse correlation[44] of abundances of phasiRNAs significantly up-regulated by *Xf* infection and significant down-regulation of their cognate mRNA targets, namely disease resistance loci Pentatrico-Peptide Repeat (PPR) and Nucleotide-Binding Sequence/Leucine-rich Repeat Receptors (LRRs). Over 150 LRRs out of the 341 such genes annotated in grapevine[45] were differentially regulated by *Xf* infection in our datasets and produced phasiRNAs in inverse proportion to their target mRNA abundances. Such clustering of gene ontology in our RNA sequencing (RNA-Seq) and sRNA data ***very strongly support the working model*** that

Xf infection results in amplification of phasiRNAs for loci known to control pathogen resistance by silencing target genes. The diversity and conservation of phasiRNA loci across plant taxa[46-49] revealed by our results encompasses orthologues of *MYB*s triggered by miR828 in many species[50-58], including grape[59]; *TAS* effectors *Suppressor of Gene Silencing3 (SGS3), DCL2*[54, 60], and *AGO2* targeted by miR403[61], and the huge families of *LRR* and *PPR*s targeted by miR482[48, 50, 54] and *TAS1-3*/miR390/3627/4376/7122[58, 62], respectively. The collective loss of miRNAs targeting PTGS effectors, PPRs, and LRRs in bacteria-infected tissues that results in susceptibility[48, 63] demonstrates their functions as master regulators of defense and targets of pathogen virulence effectors.

In addition to the compelling evidence thus far generated that supports the working model, we generated novel results that Phi impacts *Xf* growth, which underscores the practical value of the project to develop a durable management tool while generating new knowledge about PD etiology and engineered resistance. In the first CDFA award #15-0214-SA (July 2015-Dec. 2017) we initiated production of clustered regularly interspaced short palindromic repeats (CRISPR)-edited grapevine genotypes targeting *VvMIR828, TAS4a, TAS4b, MYBA6*, and *MYBA7* and described independent evidences[64-71] directly supporting the P stress modulation model (Final Report, https://static.cdfa.ca.gov/PiercesDisease/reports/2018/rock_CDFA_final_report_15-0214SA_submit.pdf). We achieved our initial objectives within the time frame of two-and-a-half years' funding, and report here our ongoing progress in calendar year 2018 on characterization of the genome-editing effector transgenic grapevine materials for *VvMYBA6, MYBA7*, and *TAS4b*.

OBJECTIVES

1. Test the miR828, *TAS4*, and target *MYBA6/7* functions in PD etiology and *Xf* infection and spreading by genome editing using CRISPR/Cas9 transgenic technology.
2. Characterize tissue-specific expression patterns of *TAS4*, *MIR828* primary transcripts, sRNAs, and *MYB* and other miRNA target genes in response to *Xf* infections in the field and in edited genotypes.
3. Characterize the changes in control versus edited genotypes for (a) xylem sap [P_i], and (b) polyphenolic levels of *Xf*-infected canes and leaves. If results are conclusive based on greenhouse studies, in the future we will conduct field trials and collaborate to carry out insect diet preference/behavioral modification/fitness assays on defended transgenic materials. (c) Test the P_i analogue Phi as a durable, affordable, and environmentally sound protectant/safener for PD.

RESULTS AND DISCUSSION

Objective 1. Test the *miR828, TAS4,* and Target *MYBA6/7* Functions in PD Etiology and *Xf* Infection and Spreading by Genome Editing Using CRISPR/Cas9 Transgenic Technology

Successful regeneration of plantlets from somatic embryos produced from rootstock 101-14 grape transformations for five CRISPR binary transfer DNA vectors (plus empty vector control) in the lab of Cooperator David Tricoli was documented in the 15-0214-SA Final Report (https://static.cdfa.ca.gov/PiercesDisease/reports/2018/rock_CDFA_final_report_15-0214SA_submit.pdf). We have received said regenerant transgenic plantlets for six *MYBA6*, six *MYBA7*, two *TAS4b*, and two empty-vector (control) events from the Cooperator under duly issued USDA-APHIS-BRS permit # 17-342-101m, and transplanted them in the greenhouse. More regenerants, including for the remaining *MIR828* and *TAS4a* effectors, are outstanding, and two more *TAS4b* events are forthcoming in the next month from a third round of transformations initiated in late 2016.

CRISPR editing of grapevine *L-idonate dehydrogenase* gene (*VvIdnDH*), *phytoene desaturase (VvPDS),* and *VvWRKY52* transcription factor genes has been recently reported[72-74]. The VvWRKY52 knockout transgenics showed increased resistance to *Botrytis cinerea*, whereas the observed ratio of mutated cells for *PDS* was higher in older leaves compared to new upper leaves, suggesting that efficiency of double strand break (DSB) production in grapevine by Cas9 is time-dependent, or in cells of older leaves DSB repair may be decreased.

Figure 1 shows the results of molecular characterization for those events that have grown to sufficient size to harvest tissue samples. Genomic Southern blot evidences (**Figure 1bc**) support at least six independent events for MYBA6, five of six tested MYBA7 events, and both TAS4b tested events evidenced with unique restriction fragment lengths when probed with either the selectable marker *nptII* and the effector *cas9* genes (panels b,c, respectively). Interestingly, MYBA7.2 and 7.3 events are clones based on restriction fragment length patterns in the Southern blot. These clones are likely the result of secondary embryos that can develop off the primary transformed embryo, which break free from the primary transformant early in the process of regeneration and then

germinate into a plant that is a clone of the primary event. Further characterization of these and other lines is ongoing, including for immunoblot validation of Cas9 protein expression. Further characterization of genome editing events of target genes by polymerase chain reaction (PCR) amplification and sequencing is in process.

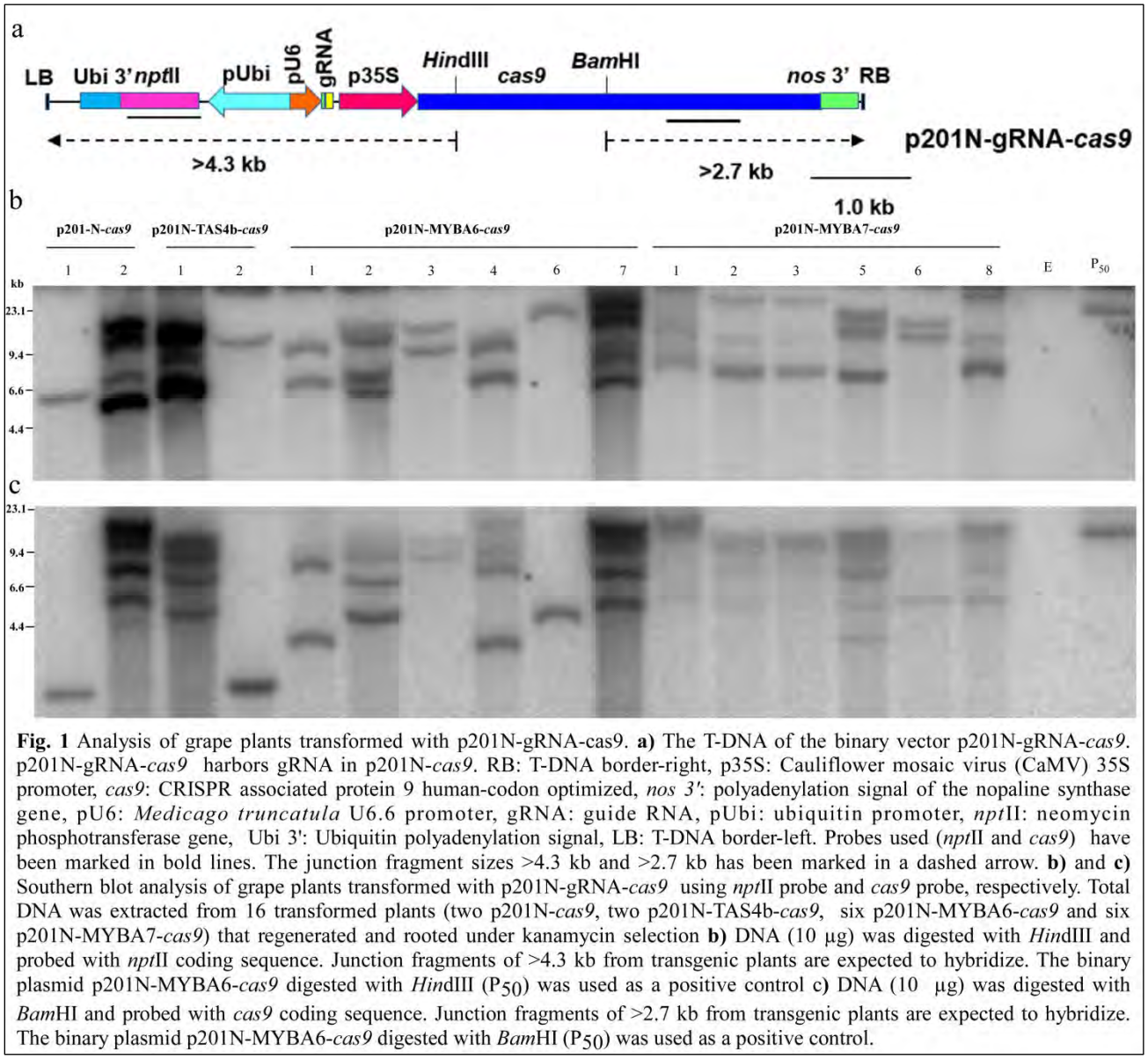

Fig. 1 Analysis of grape plants transformed with p201N-gRNA-cas9. **a)** The T-DNA of the binary vector p201N-gRNA-*cas9*. p201N-gRNA-*cas9* harbors gRNA in p201N-*cas9*. RB: T-DNA border-right, p35S: Cauliflower mosaic virus (CaMV) 35S promoter, *cas9*: CRISPR associated protein 9 human-codon optimized, *nos 3'*: polyadenylation signal of the nopaline synthase gene, pU6: *Medicago truncatula* U6.6 promoter, gRNA: guide RNA, pUbi: ubiquitin promoter, *npt*II: neomycin phosphotransferase gene, Ubi 3': Ubiquitin polyadenylation signal, LB: T-DNA border-left. Probes used (*npt*II and *cas9*) have been marked in bold lines. The junction fragment sizes >4.3 kb and >2.7 kb has been marked in a dashed arrow. **b)** and **c)** Southern blot analysis of grape plants transformed with p201N-gRNA-*cas9* using *npt*II probe and *cas9* probe, respectively. Total DNA was extracted from 16 transformed plants (two p201N-*cas9*, two p201N-TAS4b-*cas9*, six p201N-MYBA6-*cas9* and six p201N-MYBA7-*cas9*) that regenerated and rooted under kanamycin selection **b)** DNA (10 µg) was digested with *Hin*dIII and probed with *npt*II coding sequence. Junction fragments of >4.3 kb from transgenic plants are expected to hybridize. The binary plasmid p201N-MYBA6-*cas9* digested with *Hin*dIII (P_{50}) was used as a positive control c**)** DNA (10 µg) was digested with *Bam*HI and probed with *cas9* coding sequence. Junction fragments of >2.7 kb from transgenic plants are expected to hybridize. The binary plasmid p201N-MYBA6-*cas9* digested with *Bam*HI (P_{50}) was used as a positive control.

Figure 2 shows the results of polyacrylamide gel electrophoresis-based genotyping[75] for evidence of genome editing of target genes in the transgenic events. PAGE heteroduplex analysis is based on the rationale that DNA heteroduplexes migrate at a slower rate than homoduplexes in polyacrylamide gels. PCR amplification of target sequences results in mixture of amplicons including the edited allele harboring nucleotide deletions. Denaturation and renaturation of PCR products result in homo- and heteroduplexes with different migration rates. There is evidence of one candidate editing event for TAS4b, four editing events for MYBA6, and at least two editing events for MYBA7. The sequencing of PCR amplicons of target loci as an independent validation of editing events is in process.

Objective 2. Tissue-Specific Expression Patterns of *TAS4*, *MIR828*, sRNAs, and MYB and Other Targets
We received three NextSeq500 (~400 million reads per run) datasets in early 2018 for samples submitted in late 2017 to the Institute of Integrative Genome Biology, University of California, Riverside comprised of Illumina libraries with biological replicates for small RNAs, stranded mRNAs, and degradome samples from the 2017

'Calle Contento' Temecula field leaf samples, the 2016 replicated greenhouse *Xf* tobacco MYB90 overexpression experiment, and for 'matchstick petiole' samples from the 2017 Temecula field expedition. The latter experiment has scope for discovery of differential miRNA expressions associated with a diagnostic, yet pleiotropic and enigmatic PD symptom (abscission of the leaf blade but not at the typical petiole/cane junction) hypothesized to be due to deranged small RNA activities. There are six sRNA libraries and 18 degradome pooled samples, and 12 stranded mRNA-Seq transcriptome libraries sequenced separately. **Table 1** lists the cumulative grapevine sRNA and degradome library quality control parameters through data pre-processing to remove ribosomal RNAs, transfer RNAs, and small nucleolar RNAs (snoRNAs)[76] and genome[77] annotation stages of samples characterized to date. The statistical power from multiple replicates across years will drive defensible claims at the publication stage, which will be completed this year contingent upon sufficient statistical power manifesting from the multiple biological replicates for three years, 2015- 2017.

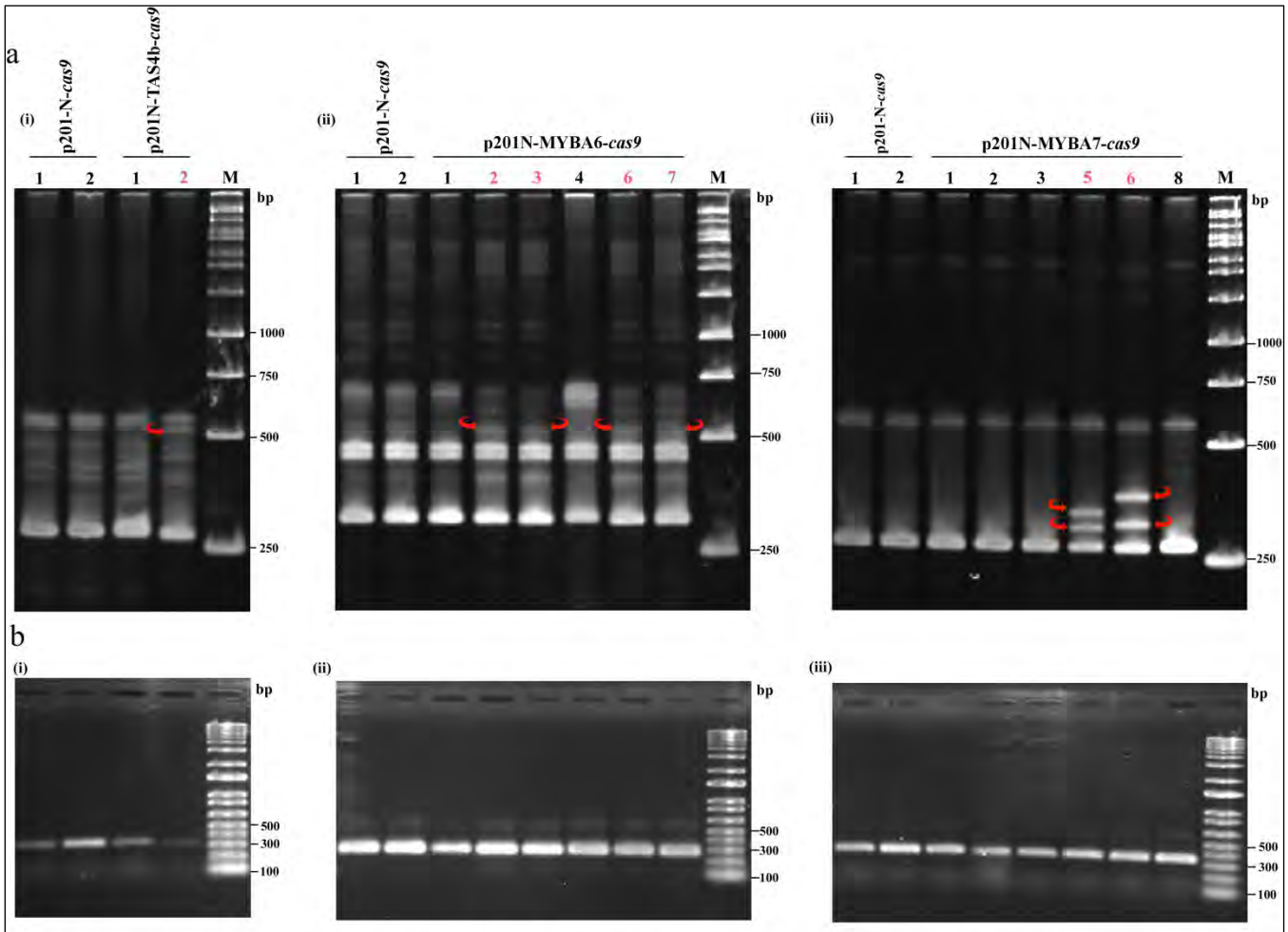

Fig. 2 Assay to evaluate genome edited events. **a)** PAGE heteroduplex assay for genome editing events. Target amplification of vector and gRNA transformed plant DNA (200 ng) was performed using (i)TAS4b-specific primers (ii) MYBA6-specific primers (iii) MYBA7-specific primers. PCR amplicon was denatured at 95°C and annealed at room temperature to form homoduplexes and heteroduplexes. Slow migration rate of heteroduplexes is an indication of editing event (red arrows). **b)** Targeted amplicon sequencing assay. The specific mutation in genome edited plants can be identified by targeted amplicon sequencing. Partial Illumina adapter sequences in the 5' end of gene specific primers ((i)TAS4b-specific primers (ii) MYBA6-specific primers (iii) MYBA7-specific primers) is used for target amplification. A ~300 bp amplicon for each transgenic event line was gel purified and submitted for fee-for-service sequencing (results pending).

Table 1. Quality control parameters* of sequenced sRNA libraries from 2015-2017 Temecula PD-infected samples.

sRNA libraries. Sample/Year	raw reads (million)	%rRNA, tRNA	%snoRNA	trimmed, clean reads (million)	%MIRNAs$
Leaf, PD2015	7.83	66.29	5.76	3.22	13.30
Leaf, Con2015	2.91	65.27	6.18	1.22	34.49
Leaf, PD2016.1	16.13	81.76	4.41	4.67	29.62
Leaf, PD2016.2	54.08	82.46	4.21	15.56	39.44
Leaf, Con2016.1	5.16	48.08	6.05	2.39	47.89
Leaf, Con2016.2	8.70	46.20	5.64	4.39	35.11
Leaf,PD2017.1	4.54	85.29	2.59	0.53	18.49
Leaf,PD2017.2	10.97	69.26	3.63	3.05	22.18
Leaf, Con2017.1	37.55	65.59	5.64	15.45	14.80
Leaf, Con2017.2	16.38	56.41	3.65	7.83	21.03
Petiole,PD2017	15.51	45.52	4.60	8.34	17.25
Petiole,Con2017	10.31	81.72	5.81	3.45	6.15
Degradome libraries. Sample/Year					
Leaf, PD2016	23.69	72.70	0.08	9.33	trace
Leaf, Con2016	27.49	46.85	0.03	16.04	trace
Leaf, PD2017.1	19.82	0.75	0.05	19.49	trace
Leaf, PD2017.2	21.82	54.09	1.12	11.27	trace
Leaf, Con2017.1	36.40	0.13	0.05	36.00	trace
Leaf, Con2017.2	24.16	5.80	0.38	22.48	trace
Petiole, PD2017	23.11	1.12	0.08	22.63	trace
Petiole, Con2017	25.58	1.21	0.04	25.03	trace
RNA-Seq transcriptome libraries. Sample/Year†					
Leaf, PD2016.1	38.67	1.10	0.33	38.24	trace
Leaf, PD2016.2	33.96	0.69	0.15	33.73	trace
Leaf, Con2016.1	39.52	0.79	0.06	39.21	trace
Leaf, Con2016.2	26.07	11.28	0.08	23.13	trace
Leaf, PD2017.1	17.68	3.14	0.01	17.46	trace
Leaf, PD2017.2	45.12	7.39	0.04	37.63	trace
Leaf, Con2017.1	29.42	1.23	0.01	28.49	trace
Leaf, Con2017.2	24.91	16.56	0.07	23.06	trace
Petiole, PD2017	36.30	0.07	0.03	36.26	trace
Petiole, Con2017	37.30	0.58	0.02	37.07	0.01

* Datasets mapped to *Vitis vinifera* 12X genome sequence, version NCBI RefSeq GCF_000003745.3 [77] with bowtie [89] after trimming adapter with fastx-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/).

$ Mapped to miRBase22 plant MIRNA hairpins (http://www.mirbase.org/).

† Mapped to ref transcriptome with kallisto-sleuth [84].

A fourth set of PD-infected and candidate control samples (> four biological replicates) was collected from the 'Calle Contento' vineyard in Temecula, CA on July 23-25, 2018. **Table 2** shows results of anthocyanin quantitations for these samples, which have significantly higher anthocyanin concentrations than healthy controls sampled from the same vineyard. These samples can be characterized by Illumina sequencing and included in a manuscript in preparation at the publication stage, if warranted based on results in process for three consecutive sample years.

Table 2. Anthocyanin quantitation of *Xf*-infected candidate Merlot leaves from 'Calle Contento' vineyard, Temecula, CA, July 2018.

Condition	µmoles cyanidin-O-glucoside equiv/mg fresh weight	s.e.m.	pval*
control healthy	8.5	0.9	
PD symptoms	22.7	3.3	0.02

* Significantly different than control, two-sided Student's t-test, unequal variance assumed (n = 4).

In a prior Progress Report (July 2017), we documented the down-regulation of miR398, miR399, miR828, and *TAS4ab* expressions from two independent *Xf* challenge experiments with transgenic tobacco over-expressing AtPAP2/MYB90 (target of TAS4 siRNA) and evidence for the importance of the miR828/TAS4/MYB autoregulatory module[39] in response to *Xf*. We also reported preliminary evidence from grapevine PD 2015 libraries for concordant down-regulation of miR156 *SBP* targets, miR162 target *DICER*, *SUPPRESSOR OF GENE SILENCING3*, miR168 target *ARGONAUTE*, miR399 target *VvPHTs*, and *PdR1* candidate Leucine-Rich Repeat receptor (2017 March Progress report) in strong support of the model. The tobacco-as-surrogate model system component of the study is mostly completed except degradome validation of miRNA activities on target mRNAs, which will be completed in parallel with ongoing grapevine miRNA/mRNA/degradome library analysis on multiple years of field samples. Here we report current analysis of differentially expressed (DE) *MIRNA*s and phasiRNA-producing loci from three years of PD and control sample Merlot field materials to date. **Figure 3** shows principal component analysis (PCA) of the differences in PD field samples versus controls across three years for 200,000 phasiRNA loci and 219 *MIRNA* loci called *de novo* by ShortStack and validated for all annotations in miRBase22[78]. The good clustering of PD versus controls for dimensions of treatment and replicates across years that encompass >60% of all variation demonstrates a robust experimental design for statistical inference.

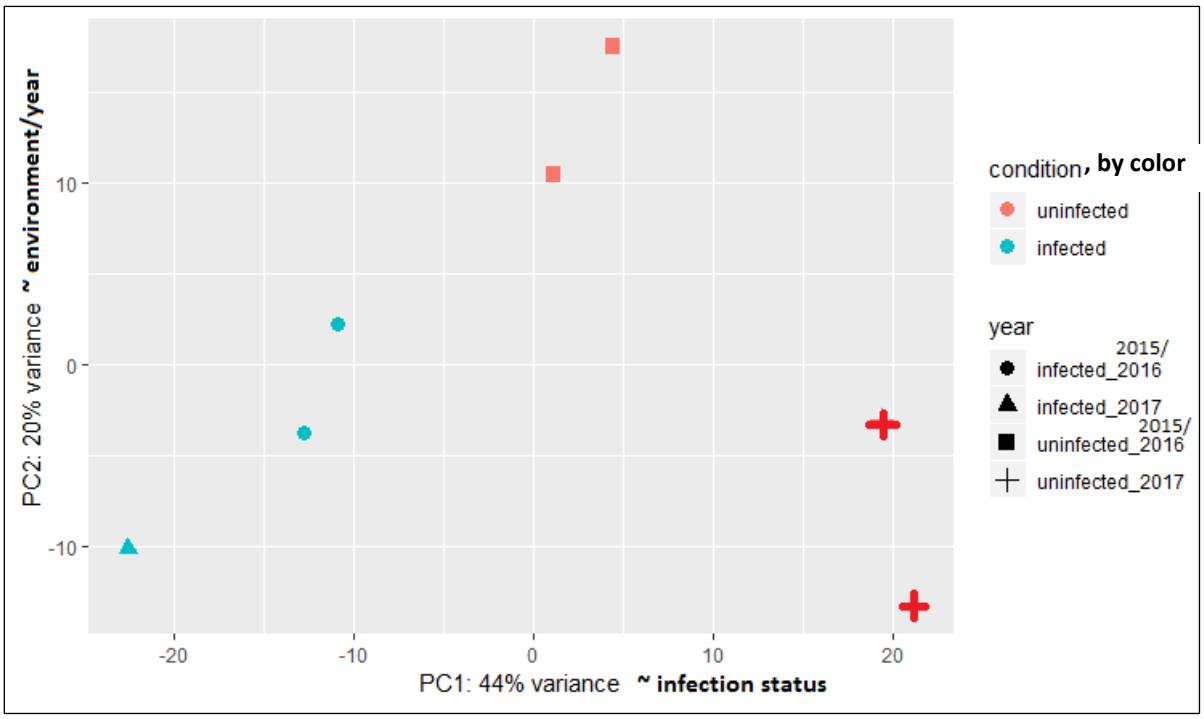

Figure 3. Principal component (PC) analysis of seven grape leaf libraries from Temecula, CA field samples representing sRNA-generating loci subjected to differential expression analysis. The percentage of variation is depicted in the PC1 and PC2 axes. Based on clustering of samples, PC1 represents the major dimension of PD symptoms that was the basis for sample collection and PC2 is inferred to capture the environmental variation across years.

Previous studies in soybean, tobacco, and *Arabidopsis* documented an association with Leucine-Repeat Receptor phasiRNA production by miR482/2118/*TAS5* and miR6019/6020 modules that correlate with virus susceptibility[48, 54, 63, 79, 80], but the broader functional significance of phasiRNA production in general, and in biotic stress and PD in particular, is unknown. We have obtained evidence that phasiRNA production from novel PHAS protein-coding genes and ncRNA loci is strongly correlated with PD infections: the percentage of PHAS loci (ShortStack Dicer phase scores > 30) for well-expressed grape mRNAs and ncRNAs is ~6.3% (n = 1,200 out of 99k moderately expressed clusters), but for multiple-test corrected differentially expressed loci in PD symptomatic leaves the percentage is ~10% ($p < 0.0008$; data not shown). The significance of this observation

will become clearer as we mine those PHAS loci for their biology and by discovering their miRNA effectors by running PhaseTank[81] on the degradome libraries. This observation establishes a key role of phasiRNAs in PD host response and supports our working model of PD etiology mediated by sRNAs.

Table 3 lists in descending order of statistical significance (false-discovery rate < 0.05) the top *MIRNA*s and select phasiRNA-producing loci differentially expressed in field samples manifesting PD symptoms. Their known biological functions and previous reports[64, 82, 83] of up-regulation in *Xf*-infected grapevine corroborates our preliminary results presented in previous progress reports and strongly establish the validity of our working model. The top DE miRNAs in our analysis are miR397 and miR408, which independently target laccases important for lignin biosynthesis, a novel finding that provides insight into the molecular mechanism underlying the enigmatic textbook symptom of 'green-island bark' on *Xf*-infected canes. Also relevant is the finding that miR858, which has been shown in Rosids and cotton to target other homologous MYBs than those MYBs targeted by miR828 involved in lignin and secondary metabolite biosynthesis[52, 53, 56], is also differentially expressed in response to *Xf*. This compelling result is consistent with observed down-regulation of miR408 with concordant increase in target *PLANTACYANIN*, and deranged expression of miR399 and miR827 that independently target phosphate transporters and phosphate homeostasis F-box effectors in *Arabidopsis*[66] and citrus infected with bacterial pathogens[25, 70, 71] including *Xf*[65]. An interesting observation that warrants further study is that *TAS4c* is up-regulated by *Xf* infection. We have observed in degradome analyses of ultraviolet light-mediated induction of miR828 and *TAS4* activities that TAS4c 3'-D4(-), which has a divergent nucleotide sequence from TAS4ab D4(-) species, shows slicing activity against *TAS4ab* primary transcript (data not shown). Thus, the up-regulation of *TAS4c* in response to *Xf* may be evidence of a homeostatic feedback loop by *TAS4c* to negatively control *TAS4ab* activities that antagonize anthocyanin effectors *MYBA5/6/7* (Sunitha et al., submitted). This would fit with the model that *Xf* pathogenicity towards its host is via sRNAs targeting anthocyanin and lignin metabolism.

Table 3. Differential expression of phasi sRNAs from select protein-coding genes and *MIRNA*s clusters in PD symptom field leaf samples, Temecula, CA, 2015-2017. Up-regulated loci in **bold**.

Locus Annotation	**baseMean expression**	**log_2Fold Change**	**P value**	**PhaseScore 21nt register**
Phe-Ammonia Lyase VIT_06s0004g02620	323	**6.11**	0§	NA†
Raffinose synthase VIT_11s0016g05770 [see 80]	861	**6.10**	0§	NA
Anthocyanidin synthase VIT_02s0025g04720	287	**6.89**	4.4E-11	NA
Chalcone synthase3 VIT_05s0136g00260	230	**5.43**	3.5E-08	NA
Xylella fastidiosa genome sRNAs	2731	**3.70**	1.7E-04	NA
TAS4b, targets MYBA5/6/7 → triggers phasiRNAs	5815	-2.96	6.7E-03	16018.6
vvi-miR397a-3p, targets laccases	29	-3.31	7.1E-03	828.7
vvi-miR408-3p, targets laccases	117	-2.32	7.2E-03	817.9
vvi-miR391-5p, targets *TAS3*, pentatricopeptide rpt	86	-2.65	8.1E-03	599.7
vvi-miR858, targets MYBs associated with lignin	9	-3.45	1.0E-02	14.6
MYB VIT_14s0066g01220, target of miR828 → triggers phasiRNAs	39	-3.08	1.1E-02	4416.8
vvi-miR399i, targets phosphate transporters	104	-3.13	1.3E-02	307.3
vvi-miR394c-5p, targets F-box	19	-3.36	1.4E-02	189.6
MYBA6 VIT_14s0006g01290, target of *TAS4* 3'-D4(-) tasiRNA → triggers phasiRNAs	6	-3.49	1.9E-02	69.0
vvi-miR827-3p, targets phosphate signaling F-box	807	-1.68	6.3E-02	547.6
TAS4c, targets MYBA5/6/7 → triggers phasiRNAs	230	**0.19**	n.d.§	4060.5
vvi-miR828-star (mature below detection limit)	1	-1.44	0.38	16.6
TAS4a, targets MYBA5/6/7 → triggers phasiRNAs	23428	-0.51	0.59	29108.5

† These clusters of sRNAs were not called by ShortStack as having a dominant DICER activity size class.
§ Not determined by DESeq2 due to independent filtering of assumed outliers defined by Cook's distance [84].

RNA-Seq data was mapped to the reference transcriptome with kallisto-sleuth[84]. We obtained 1,329 differentially expressed genes (793 up, 536 down; data not shown) with expression above a threshold (>30 reads mapped to a transcript per library on average), a log2-fold-change (LFC) of > |2|, and multiple-testing Bonferroni-adjusted

$p < 0.05$ for statistical significance. This is comparable to the 1,240 (977 up, 263 down) DE genes reported for a similar greenhouse *Xf* challenge experiment by the Dandekar group[82]. For the 13 genes claimed differentially regulated by *Xf* infection and quantification validated by real-time (RT)-PCR (in **Figures 3 and 6B** of Dandekar's *FPS* paper)[82], we observe a good concordance with our results (correlation coefficient = 0.75, $p < 0.05$), however, only eight of our results are statistically significant, suggesting differences exist between the published greenhouse results and our field and/or leaf samples, read depths of libraries (see **Table 2**), and/or methods (**Table 4**). When the Dandekar group Supplemental Datasets are made available by the publisher in due course (the paper is only just published, while the supplemental materials are not yet available), we will be able to conduct a genome-wide correlation of our results with the published claims using our independent methods[84] to ascertain whether those methods and results are comparable to ours or otherwise, which will shed light on questions about sample/experimental variability. **Table 5** compares our MapMan[44] RNA-Seq Gene Ontology classification results of 1,240 top *Xf* DE genes with the top 1,240 DE genes reported by the Dandekar group for greenhouse *Xf* challenge RNA-Seq[82]. Another recently published RNA-Seq transcriptomic analysis of early grapevine petiole responses to *Xf* at eight and 24 hours post-inoculation[85] will be useful for genome-wide analysis of concordance with our and others' results and integration into an epidemiological model of disease dynamics[86].

Table 4. Comparison between recently reported DE of 14 genes in *Xf*-challenged greenhouse leaves versus our RNA-Seq DE calculations on four Temecula, CA field sample biological replicates harvested in 2016/17. Significant LFC values in **bold.** Note the sole discordant result for Fold Change sign (line in *italics*) is for a very low-expressed gene and is therefore discounted.

Annotation	GeneID	Dandekar LFC	our LFC*	Mean expressed	our padj
PR-2/beta1,3-glucanase	VIT_06s0061g00100	6.64	6.43	4659	**2.75E-21**
PR-1	VIT_03s0088g00810	5.32	2.03	635	1.27E-01
PR-8; chitinase	VIT_05s0094g00200	1.58	2.30	21	**4.68E-02**
HSP18	VIT_08s0058g00210	4.32	4.21	6	**2.60E-04**
HSP17	VIT_04s0008g01520	3.58	4.97	50	**5.51E-14**
HSP4	VIT_07s0031g00670	2.32	1.90	303	**9.23E-04**
Nucleoredoxin-1	VIT_01s0127g00520	2.81	1.58	91	**3.23E-02**
Peroxidase	*VIT_00s1677g00010*	*1.14*	*-0.10*	*4*	*9.58E-01*
ferritin5	VIT_13s0067g01840	-1.00	-0.58	1066	4.17E-01
Sucrose synthase	VIT_07s0005g00750	3.46	1.62	7345	**4.40E-02**
Pectin lyase	VIT_14s0066g01060	1.72	0.23	24	8.25E-01
UDP-glycosyltransferase	VIT_17s0000g04750	1.07	0.37	857	6.13E-01
Xyloglucan-endotransglucosylase	VIT_06s0061g00550	2.32	4.71	174	**4.31E-04**
thaumatin-like protein	VIT_18s0001g14480	2.00	0.76	589	6.58E-01
	Pearson of LFCs, R =	0.75		**Binomial p-val†**	**0.05**

* Kallisto-sleuth method [84].

† Bionomial distribution probability of 13 successful LFC values being the right sign in 13 tests when DE up-regulated = 79% probability (true for 12 of 13 genes; a conservative estimate) based on results reported in [82].

Objectives 3a,b. Xylem Sap P_i and Polyphenolic Changes

We previously reported in the July 2017 Interim Progress Report results for mass spectrometric quantification of cyanin and malvin in xylem sap from the Temecula June 2017 field samples, and anthocyanins in leaves, showing significant differences between infected and control samples for the latter. These results are further substantiated by prior results for other grape cultivars[87, 88], supporting the working model. Spectrophotometric quantification of anthocyanins in leaves of 2018 field samples from Temecula are shown above in **Table 2** and consistent with prior results.

We also showed conclusively in the Final Report for 15-0214-SA higher anthocyanin concentrations in infected xylem sap, and previously P_i quantifications by two methods of fully expanded leaves and canes in 2016 and 2017 Temecula PD samples that support the hypothesis that *Xf* infection results in significantly lower [P_i] (about 60% decrease) in host leaves and xylem sap that correlate with elevated anthocyanins quantified in PD xylem sap by

mass spectrometry and leaves by spectrophotometry. Thus, we have accomplished Objective 3a and will publish the results in due course.

Table 5. Comparison of Gene Ontology over-represented terms metrics of project field sample PD RNA-Seq versus published greenhouse *Xf* challenge RNA-Seq results [82].

MapMan Gene Ontology term	**MapMan fold over-represented field RNA-Seq**	**Field expt MapMan pval**	**Greenhouse RNA-Seq PANTHER Gene Ontology term**	**Greenhouse PANTHER fold enrichment**	**Greenhouse expt PANTHER pval**
phenylpropanoid metabolism	576	0.00001	phenylpropanoid metabolic process	5.85	0.0012
flavonoid metabolism	2610	0.049	flavonoid biosynthetic process	5.23	0.0016
TCA/organic acid transformation	6.3	0.002	carboxylic acid transport	4.95	0.016
cell wall	124	0.15	cell wall organization or biogenesis	2.68	0.0035
glycolysis	5.4	0.002	carbohydrate catabolic process	3.56	0.034
UDP glucosyl and glucoronyl transferase	28	0.014	UDP-glucosyltransferase activity	4.73	0.037
transport -p and v-ATPase H+ exporting ATPase	20	0.009	transmembrane transporter activity	2.16	0.008

Table 6. Quantification of *Xf* titers by quantitative real-time (qRT) PCR and RNA-Seq in 2016 greenhouse replicated *Xf* challenge experiment with AtMYB90-overexpressing transgenic tobacco, and Temecula, CA 2017 field samples.

Sample	**Log_{10}, cfu/gfw qRT-PCR (± s.e.m.)**	**Leaf *Xf* RNA-Seq Reads/10^6 host reads**	***P*- value**
Control leaf petioles, 2017 Temecula	5.21 (0.17)	1.8 (n=2)	---
PD symptom leaf petioles, 2017 Temecula	6.82 (0.40)	10.2 (n = 1)	0.006*****
SRI non transgenic-Buffer control	7.30	3.0	
Hemizygous transgenic-Buffer	7.30	0	
Homozygous transgenic-Buffer	7.32	0.8	
SRI non transgenic-*Xf* infected	12.4	88	
Hemizygous transgenic-*Xf* infected	12.0	129	
Homozygous transgenic-*Xf* infected	12.1	299	0.03†

* Significantly different from qRT-PCR control, Student's two-sided t-test, n = 5, equal variance assumed.

† Significantly different from RNA-Seq buffer controls, Student's one-sided t-test.

We have obtained preliminary results for *Xf* titers by RT-PCR in concordant petiole samples from field leaf samples, as well as for the 2016 replicated greenhouse *Xf* tobacco MYB90 overexpression experiment. **Table 6** shows the results correlated with digital abundances of *Xf* transcriptome reads from Objective 2 new results quantified by bowtie[89]. These results together directly support the hypothesis that *Xf* infection results in accumulation of anthocyanins in xylem sap and leaves. Thus, we have accomplished Objective 3b and will publish the results in due course. 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[87, 88]. Phenolic levels in Merlot xylem sap correlate with PD severity compared to other cultivars[90].

Objective 3c. P_i Analogue Phi as a Protectant/Safener for PD

Supporting our previous results (March 2017 Progress Report) that *Xf* infection induces miR828 and *TAS4* expression in tobacco, **Figure 4** shows results of an RNA blot for samples extracted from the 2016 repeat greenhouse *Xf* challenge experiment, probed with the *PAP2/MYB90* transgene. The significance of this result is further evidence for the importance of the autoregulatory feedback loop responsive to P_i[38] in *Xf* host response based on two observations: (i) *PAP2/AtMYB90* induction upon *Xf* infection, and (ii) in the absence of the transgene (the SR1 non-transgenic control line) the endogenous PAP2/MYB90 orthologue *Nt-ANTHOCYANIN2* is inferred to hybridize with the homologous *PAP2/MYB90* probe, clearly showing that *AN2* is up-regulated several fold in the SR1-treated sample in response to *Xf* infection.

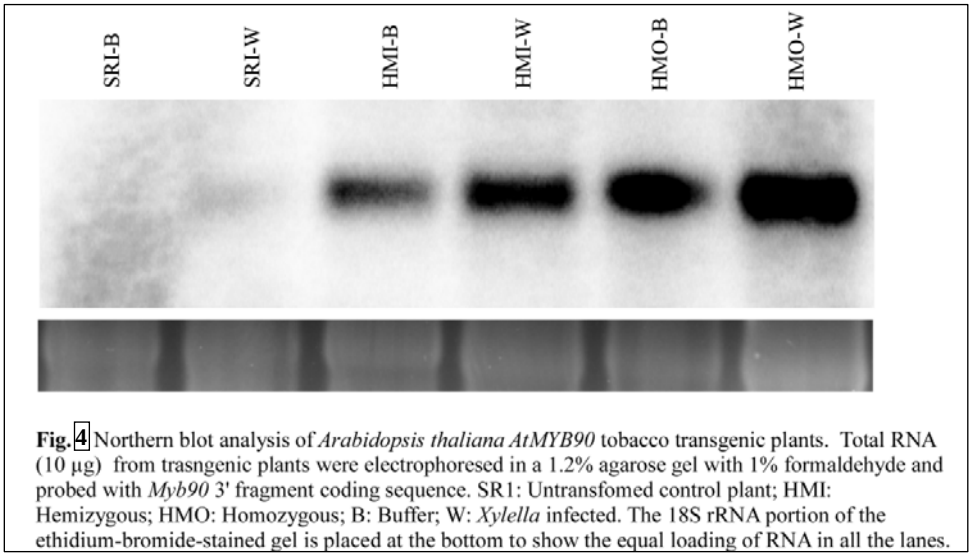

Fig. 4 Northern blot analysis of *Arabidopsis thaliana AtMYB90* tobacco transgenic plants. Total RNA (10 µg) from trasngenic plants were electrophoresed in a 1.2% agarose gel with 1% formaldehyde and probed with *Myb90* 3' fragment coding sequence. SR1: Untransfomed control plant; HMI: Hemizygous; HMO: Homozygous; B: Buffer; W: *Xylella* infected. The 18S rRNA portion of the ethidium-bromide-stained gel is placed at the bottom to show the equal loading of RNA in all the lanes.

Results presented in the 2017 Final Report provided additional validation of preliminary results showing that the LD_{50} < 3 mM [Phi] for inhibition of plate growth of *Xf*. Based on these pilot experiments we conducted a greenhouse *Xf* challenge experiment from April until July 2018 with phosphite treatments as test. We encountered technical problems with (1) plant growth in the absence of fertigation (we did not want phosphite effects to be confounded by excess nutrient conditions, and thus withheld application of NPK fertigation), and (2) with the third time point (experiment endpoint) RT-PCR *Xf* titer assay that requires us to repeat the experiment. **Table 7** shows the preliminary results for five tobacco plants of each genotype (SR1 non-transgenic, HMI heterozygous transgenic, and HMO homozygous transgenic overexpressing AtPAP2/MYB90[40, 43]) challenged with *Xf* in the greenhouse. The evidence of *Xf* titers demonstrates the technical methods and experimental procedures give reproducible results in our hands, because we validated and extended the prior results documented in the February 2016 Progress Report that the transgenic lines have lower *Xf* titers that correlate with transgene copy number, yet higher leaf scorch symptom severity in the homozygous transgenic line (data not shown). We will conduct a larger phosphite test for *Xf* antagonism going forward by bracketing the parameters of phosphite concentrations and interaction with amounts of fertigation supplement during post-inoculation growth and development.

Table 7. Results of *Xf* challenge of greenhouse-grown transgenic tobacco plants (n = 5) overexpressing AtPAP2/MYB90 assayed at two and seven weeks post infection (WPI) for bacterial titer by RT-PCR.

Genotype	2 WPI	7 WPI	p value† vs control, 2 WPI
	cfu/gfw		
SR1 non transgenic	2.3E+07	3.0E+09	--
HMI heterozygous transgenic	6.4E+06	2.1E+07	0.07
HMO homozygous transgenic	5.6E+06	9.4E+06	0.05

† Two-sided Student's t-test, unequal variance assumed.

CONCLUSIONS

Our novel results demonstrating that Phi impacts *Xf* growth underscore the practical value of the project to develop a durable management tool while generating new knowledge about PD etiology and engineered resistance. Research on knocking out genes involved in diffusible signals and host chemical specificity for PD etiology by CRISPR has been suggested, and this is what this project is pursuing. Knocking out any host gene (e.g., PD resistance or P stress effector) may result in increased susceptibility to infections. Thus, engineer-ing PD resistance is likely to be by incremental advances from characterizing hypothesized and modeled molecular mechanisms.

REFERENCES CITED

1. Karl DM. 2014. Microbially mediated transformations of phosphorus in the sea: New views of an old cycle. *Annu Rev Marine Sci* 6:279-337.
2. Patterson M, Alyokhin A. 2014. Survival and development of Colorado potato beetles on potatoes treated with phosphite. *Crop Protection* 61:38-42.
3. Collins JR. 1993. Control of arthropod pests with phosphorous acid and mono-esters and salts thereof. Edited by USPTO#5206228, vol. #5206228.
4. Krugner R, Hagler JR, Groves RL, Sisterson MS, Morse JG, Johnson MW. 2012. Plant water stress effects on the net dispersal rate of the insect vector *Homalodisca vitripennis* (Hemiptera: Cicadellidae) and movement of its egg parasitoid, *Gonatocerus ashmeadi* (Hymenoptera: Mymaridae). *Environ Entomol* 41:1279-1289.
5. Johnson ET, Berhow MA, Dowd PF. 2010. Constitutive expression of the maize genes B1 and C1 in transgenic Hi II maize results in differential tissue pigmentation and generates resistance to *Helicoverpa zea*. *J Agric Food Chem* 58:2403-2409.
6. Barbehenn RV, Constabel CP. 2011. Tannins in plant-herbivore interactions. *Phytochemistry* 72:1551-1565
7. Makoi J, Belane AK, Chimphango SBM, Dakora FD. 2010. Seed flavonoids and anthocyanins as markers of enhanced plant defence in nodulated cowpea (*Vigna unguiculata* L. Walp.). *Field Crops Res* 118:21-27.
8. Raju BC, Goheen AC. 1981. Relative sensitivity of selected grapevine cultivars to Pierce's disease bacterial inoculations. *Am J Enol Vitic* 32:155-158.
9. Krivanek AF, Walker MA. 2004. Vitis resistance to Pierce's disease is characterized by differential *Xylella fastidiosa* population in steams and leaves. *Phytopathology* 95:44- 52.
10. Fry SM, Milholland RD. 1990. Multiplication and translocation of *Xylella fastidiosa* in petioles and stems of grapevine resistant, tolerant, and susceptible to Pierce's disease. *Phytopathology* 80:61-65.
11. Cantos E, Espín JC, Tomás-Barberán FA. 2002. Varietal differences among the polyphenol profiles of seven table grape cultivars studied by LC−DAD−MS−MS. *J Agric Food Chem* 50:5691-5696.
12. Rashed A, Daugherty MP, Almeida RPP. 2011. Grapevine genotype susceptibility to *Xylella fastidiosa* does not predict vector transmission success. *Environ Entomol* 40:1192-1199.
13. Nascimento R, Gouran H, Chakraborty S, Gillespie HW, Almeida-Souza HO, Tu A, Rao BJ, Feldstein PA, Bruening G, Goulart LR, Dandekar AM. 2016. The type II secreted lipase/esterase LesA is a key virulence factor required for *Xylella fastidiosa* pathogenesis in grapevines. *Sci Rep* 6:e18598.
14. Morrell PL, Buckler ES, Ross-Ibarra J. 2012. Crop genomics: Advances and applications. *Nature Rev Genet* 13:85-96.
15. Langridge P, Fleury D. 2011. Making the most of 'omics' for crop breeding. *Trends Biotech* 29:33.
16. Varshney RK, Bansal KC, Aggarwal PK, Datta SK, Craufurd PQ. 2011. Agricultural biotechnology for crop improvement in a variable climate: Hope or hype? *Trends Plant Sci* 16:363-371.
17. Carbonell A, Fahlgren N, Garcia-Ruiz H, Gilbert KB, Montgomery TA, Nguyen T, Cuperus JT, Carrington JC. 2012. Functional analysis of three *Arabidopsis* ARGONAUTES using Slicer-defective mutants. *Plant Cell* 24:3613-3629.
18. Wang X-B, Jovel J, Udomporn P, Wang Y, Wu Q, Li W-X, Gasciolli V, Vaucheret H, Ding S-W. 2011. The 21-nucleotide, but not 22-nucleotide, viral secondary small interfering RNAs direct potent antiviral defense by two cooperative Argonautes in *Arabidopsis thaliana*. *Plant Cell* 23:1625-1638.
19. Pant BD, Buhtz A, Kehr J, Scheible W-R. 2008. MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. *Plant J* 53:731-738.
20. Molnar A, Melnyk CW, Bassett A, Hardcastle TJ, Dunn R, Baulcombe DC. 2010. Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. *Science* 328:872.

21. Bai S, Kasai A, Yamada K, Li T, Harada T. 2011. A mobile signal transported over a long distance induces systemic transcriptional gene silencing in a grafted partner. *J Exp Bot* 62:4561-4570.
22. Fahlgren N, Howell MD, Kasschau KD, Chapman EJ, Sullivan CM, Carrington JC. 2007. High-throughput sequencing of *Arabidopsis* microRNAs: Evidence for frequent birth and death of *MIRNA* genes. *PLoS ONE* 2:e219.
23. Li Y, Zhang QQ, Zhang JG, Wu L, Qi YJ, Zhou JM. 2010. Identification of microRNAs involved in pathogen-associated molecular pattern-triggered plant innate immunity. *Plant Physiol* 152:2222
24. Padmanabhan C, Zhang XM, Jin HL. 2009. Host small RNAs are big contributors to plant innate immunity. *Curr Opin Plant Biol* 12:465-472.
25. Zhang W, Gao S, Zhou X, Chellappan P, Chen Z, Zhou X, Zhang X, Fromuth N, Coutino G, Coffey M, Jin H. 2011. Bacteria-responsive microRNAs regulate plant innate immunity by modulating plant hormone networks. *Plant Mol Biol* 75:93-105.
26. Zhang XM, Zhao HW, Gao S, Wang WC, Katiyar-Agarwal S, Huang HD, Raikhel N, Jin HL. 2011. *Arabidopsis* Argonaute 2 regulates innate immunity via miRNA393*-mediated silencing of a Golgi-localized SNARE gene, MEMB12. *Mol Cell* 42:356-366.
27. Navarro L, Jay F, Nomura K, He SY, Voinnet O. 2008. Suppression of the microRNA pathway by bacterial effector proteins. *Science* 321:964-967.
28. Ellendorff U, Fradin EF, de Jonge R, Thomma BPHJ. 2009. RNA silencing is required for *Arabidopsis* defence against Verticillium wilt disease. *J Exp Bot* 60:591-602.
29. Hewezi T, Maier TR, Nettleton D, Baum TJ. 2012. The *Arabidopsis* microRNA396-GRF1/GRF3 regulatory module acts as a developmental regulator in the reprogramming of root cells during cyst nematode infection. *Plant Physiol* 159:321-335.
30. Sarris PF, Gao S, Karademiris K, Jin H, Kalantidis K, Panopoulos NJ. 2012. Phytobacterial type III effectors HopX1, HopAB1 and HopF2 enhance sense-post-transcriptional gene silencing independently of plant *R* gene-effector recognition. *Mol Plant-Microbe Interact* 24:907-917.
31. Li H, Deng Y, Wu T, Subramanian S, Yu O. 2010. Misexpression of miR482, miR1512, and miR1515 increases soybean nodulation. *Plant Physiol* 153:1759-1770.
32. Lopez A, Ramirez V, Garcia-Andrade J, Flors V, Vera P. 2011. The RNA silencing enzyme RNA Polymerase V Is required for plant immunity. *PLoS Genet* 7:e1002434.
33. Devers EA, Branscheid A, May P, Krajinski F. 2011. Stars and symbiosis: MicroRNA- and microRNA*-mediated transcript cleavage involved in arbuscular mycorrhizal symbiosis. *Plant Physiol* 156:1990-2010.
34. Xin M, Wang Y, Yao Y, Song N, Hu Z, Qin D, Xie C, Peng H, Ni Z, Sun Q. 2011. Identification and characterization of wheat long non-protein coding RNAs responsive to powdery mildew infection and heat stress by using microarray analysis and SBS sequencing. *BMC Plant Biol* 11:61.
35. Djami-Tchatchou AT, Dubery IA. 2015. Lipopolysaccharide perception leads to dynamic alterations in the microtranscriptome of *Arabidopsis thaliana* cells and leaf tissues. *BMC Plant Biol* 15:79.
36. Seifi A. 2011. Write 'systemic small RNAs': Read 'systemic immunity'. *Funct Plant Biol* 38:747-752.
37. Boyko A, Kovalchuk I. 2011. Genetic and epigenetic effects of plant-pathogen interactions: An evolutionary perspective. *Mol Plant* 4:1014-1023.
38. Hsieh LC, Lin SI, Shih ACC, Chen JW, Lin WY, Tseng CY, Li WH, Chiou TJ. 2009. Uncovering small RNA-mediated responses to phosphate deficiency in *Arabidopsis* by deep sequencing. *Plant Physiol* 151:2120-2132.
39. Luo Q-J, Mittal A, Jia F, Rock CD. 2012. An autoregulatory feedback loop involving *PAP1* and *TAS4* in response to sugars in *Arabidopsis*. *Plant Mol Biol* 80:117-129.
40. Velten J, Cakir C, Cazzonelli CI. 2010. A spontaneous dominant-negative mutation within a 35S::*AtMYB90* transgene inhibits flower pigment production in tobacco. *PLoS ONE* 5:e9917.
41. Lang Q, Jin C, Lai L, Feng J, Chen S, Chen J. 2011. Tobacco microRNAs prediction and their expression infected with *Cucumber mosaic virus* and *Potato virus X*. *Mol Biol Rep* 38:1523-1531.
42. Rock CD. 2013. *Trans-acting small interfering RNA4*: Key to nutraceutical synthesis in grape development? *Trends Plant Sci* 18:601-610.
43. Velten J, Cakir C, Youn E, Chen J, Cazzonelli CI. 2012. Transgene silencing and transgene-derived siRNA production in tobacco plants homozygous for an introduced *AtMYB90* construct. *PLoS ONE* 7:e30141.
44. Usadel B, Poree F, Nagel A, Lohse M, Czedik-Eysenberg A, Stitt M. 2009. A guide to using MapMan to visualize and compare Omics data in plants: A case study in the crop species, maize. *Plant Cell Environ* 32:1211-1229.

45. Di Gaspero G, Cipriani G, Adam-Blondon AF, Testolin R. 2007. Linkage maps of grapevine displaying the chromosomal locations of 420 microsatellite markers and 82 markers for R-gene candidates. *Theor Appl Genet* 114:1249-1263.
46. Song X, Li P, Zhai J, Zhou M, Ma L, Liu B, Jeong D-H, Nakano M, Cao S, Liu C, Chu C, Wang X-J, Green PJ, Meyers BC et al. 2012. Roles of DCL4 and DCL3b in rice phased small RNA biogenesis. *Plant J* 69:462-474.
47. Vogel JP, Garvin DF, Mockler TC, Schmutz J, Rokhsar D, Bevan MW, Barry K, Lucas S, Harmon-Smith M, Lail K, Tice H, Grimwood J, McKenzie N, Huo NX et al. 2010. Genome sequencing and analysis of the model grass *Brachypodium distachyon*. *Nature* 463:763-768.
48. Shivaprasad PV, Chen HM, Patel K, Bond DM, Santos B, Baulcombe DC. 2012. A microRNA superfamily regulates nucleotide binding site-leucine-rich repeats and other mRNAs. *Plant Cell* 24:859-874.
49. Johnson C, Kasprzewska A, Tennessen K, Fernandes J, Nan GL, Walbot V, Sundaresan V, Vance V, Bowman LH. 2009. Clusters and superclusters of phased small RNAs in the developing inflorescence of rice. *Genome Res* 19:1429-1440.
50. Klevebring D, Street N, Fahlgren N, Kasschau K, Carrington J, Lundeberg J, Jansson S. 2009. Genome-wide profiling of Populus small RNAs. *BMC Genomics* 10:620.
51. Lin J-S, Lin C-C, Lin H-H, Chen Y-C, Jeng S-T. 2012. MicroR828 regulates lignin and H_2O_2 accumulation in sweet potato on wounding. *New Phytol* 196:427-440.
52. Xia R, Zhu H, An Y-q, Beers E, Liu Z. 2012. Apple miRNAs and tasiRNAs with novel regulatory networks. *Genome Biol* 13:R47.
53. Zhu H, Xia R, Zhao BY, An YQ, Dardick CD, Callahan AM, Liu ZR. 2012. Unique expression, processing regulation, and regulatory network of peach (*Prunus persica*) miRNAs. *BMC Plant Biol* 12:18.
54. Zhai J, Jeong D-H, De Paoli E, Park S, Rosen BD, Li Y, Gonzalez AJ, Yan Z, Kitto SL, Grusak MA, Jackson SA, Stacey G, Cook DR, Green PJ et al. 2011. MicroRNAs as master regulators of the plant NB-LRR defense gene family via the production of phased, *trans*-acting siRNAs. *Genes Dev* 25:2540-2553
55. Zhang C, Li G, Wang J, Fang J. 2012. Identification of *trans*-acting siRNAs and their regulatory cascades in grapevine. *Bioinformatics* 28:2561-2568.
56. Guan X, Pang M, Nah G, Shi X, Ye W, Stelly DM, Chen ZJ. 2014. miR828 and miR858 regulate homoeologous MYB2 gene functions in Arabidopsis trichome and cotton fibre development. *Nat Commun* 5:3050.
57. Källman T, Chen J, Gyllenstrand N, Lagercrantz U. 2013. A significant fraction of 21 nt sRNA originates from phased degradation of resistance genes in several perennial species. *Plant Physiol* 162:741-754
58. Xia R, Xu J, Arikit S, Meyers BC. 2015. Extensive families of miRNAs and PHAS loci in Norway spruce demonstrate the origins of complex phasiRNA networks in seed plants. *Mol Biol Evol* 32:2905-2918.
59. Pantaleo V, Szittya G, Moxon S, Miozzi L, Moulton V, Dalmay T, Burgyan J. 2010. Identification of grapevine microRNAs and their targets using high-throughput sequencing and degradome analysis. *Plant J* 62:960-976.
60. Turner M, Yu O, Subramanian S. 2012. Genome organization and characteristics of soybean microRNAs. *BMC Genomics* 13:169.
61. Zhang C, Xian Z, Huang W, Li Z. 2015. Evidence for the biological function of miR403 in tomato development. *Sci Hort* 197:619-626.
62. Xia R, Meyers BC, Liu Z, Beers EP, Ye S, Liu Z. 2013. MicroRNA superfamilies descended from miR390 and their roles in secondary small interfering RNA biogenesis in Eudicots. *Plant Cell* 25:1555-1572.
63. Li F, Pignatta D, Bendix C, Brunkard JO, Cohn MM, Tung J, Sun H, Kumar P, Baker B. 2012. MicroRNA regulation of plant innate immune receptors. *Proc Natl Acad Sci U S A* 109:1790-1795.
64. Choi H-K, Iandolino A, da Silva FG, Cook DR. 2013. Water deficit modulates the response of *Vitis vinifera* to the Pierce's disease pathogen *Xylella fastidiosa*. *Mol Plant-Microbe Interact* 26:643-657.
65. Rogers EE. 2012. Evaluation of *Arabidopsis thaliana* as a model host for *Xylella fastidiosa*. *Mol Plant-Microbe Interact* 25:747-754.
66. Lin W-Y, Huang T-K, Chiou T-J. 2013. NITROGEN LIMITATION ADAPTATION, a target of microRNA827, mediates degradation of plasma membrane–localized phosphate transporters to maintain phosphate homeostasis in Arabidopsis. *Plant Cell* 25:4061–4074.
67. Lin S-I, Santi C, Jobet E, Lacut E, El Kholti N, Karlowski WM, Verdeil J-L, Breitler JC, Périn C, Ko S-S, Guiderdoni E, Chiou T-J, Echeverria M. 2010. Complex regulation of two target genes encoding SPX-MFS proteins by rice miR827 in response to phosphate starvation. *Plant Cell Physiol* 51:2119.

68. Park BS, Seo JS, Chua N-H. 2014. NITROGEN LIMITATION ADAPTATION recruits PHOSPHATE2 to target the phosphate transporter PT2 for degradation during the regulation of *Arabidopsis* phosphate homeostasis. *Plant Cell* 26:454-464.
69. Hackenberg M, Shi B-J, Gustafson P, Langridge P. 2013. Characterization of phosphorus-regulated miR399 and miR827 and their isomirs in barley under phosphorus-sufficient and phosphorus-deficient conditions. *BMC Plant Biol* 13:214.
70. Zhao H, Sun R, Albrecht U, Padmanabhan C, Wang A, Coffey MD, Girke T, Wang Z, Close TJ, Roose M, Yokomi RK, Folimonova S, Vidalakis G, Rouse R et al. 2013. Small RNA profiling reveals phosphorus deficiency as a contributing factor in symptom expression for citrus Huanglongbing disease. *Mol Plant* 6:301-310.
71. Lu Y-T, Li M-Y, Cheng K-T, Tan CM, Su L-W, Lin W-Y, Shih H-T, Chiou T-J, Yang J-Y. 2014. Transgenic plants that express the phytoplasma effector SAP11 show altered phosphate starvation and defense responses. *Plant Physiol* 164:1456-1469.
72. Nakajima I, Ban Y, Azuma A, Onoue N, Moriguchi T, Yamamoto T, Toki S, Endo M. 2017. CRISPR/Cas9-mediated targeted mutagenesis in grape. *PLoS ONE* 12:e0177966.
73. Wang X, Tu M, Wang D, Liu J, Li Y, Li Z, Wang Y, Wang X. 2018. CRISPR/Cas9-mediated efficient targeted mutagenesis in grape in the first generation. *Plant Biotech J* 16:844-855.
74. Ren C, Liu X, Zhang Z, Wang Y, Duan W, Li S, Liang Z. 2016. CRISPR/Cas9-mediated efficient targeted mutagenesis in Chardonnay (*Vitis vinifera* L.). *Sci Rep* 6:32289.
75. Zhu X, Xu Y, Yu S, Lu L, Ding M, Cheng J, Song G, Gao X, Yao L, Fan D, Meng S, Zhang X, Hu S, Tian Y. 2014. An efficient genotyping method for genome-modified animals and human cells generated with CRISPR/Cas9 system. *Sci Rep* 4:6420.
76. Kalvari I, Argasinska J, Quinones-Olvera N, Nawrocki EP, Rivas E, Eddy SR, Bateman A, Finn RD, Petrov AI. 2018. Rfam 13.0: Shifting to a genome-centric resource for non-coding RNA families. *Nucl Acids Res* 46:D335-D342.
77. Jaillon O, Aury J, Noel B, Policriti A, Clepet C, Casagrande A, Choisne N, Aubourg S, Vitulo N, Jubin C. 2007. The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. *Nature* 449:463-467.
78. Kozomara A, Griffiths-Jones S. 2014. miRBase: Annotating high confidence microRNAs using deep sequencing data. *Nucl Acids Res* 42:D68-D73.
79. Li F, Orban R, Baker B. 2012. SoMART: A web server for plant miRNA, tasiRNA and target gene analysis. *Plant J* 70:891-901.
80. Zhao MX, Cai CM, Zhai JX, Lin F, Li LH, Shreve J, Thimmapuram J, Hughes TJ, Meyers BC, Ma JX. 2015. Coordination of microRNAs, phasiRNAs, and NB-LRR genes in response to a plant pathogen: Insights from analyses of a set of soybean *Rps* gene near-isogenic lines. *Plant Genome* 8: doi: 10.3835/plantgenome2014.3809.0044.
81. Guo QL, Qu XF, Jin WB. 2015. PhaseTank: Genome-wide computational identification of phasiRNAs and their regulatory cascades. *Bioinformatics* 31:284-286.
82. Zaini PA, Nascimento R, Gouran H, Cantu D, Chakraborty S, Phu M, Goulart LR, Dandekar AM. 2018. Molecular profiling of Pierce's Disease outlines the response circuitry of *Vitis vinifera* to *Xylella fastidiosa* infection. *Front Plant Sci* 9: doi.org/10.3389/fpls.2018.00771.
83. Lin H, Doddapaneni H, Takahashi Y, Walker MA. 2007. Comparative analysis of ESTs involved in grape responses to *Xylella fastidiosa* infection. *BMC Plant Biol* 7:8.
84. Pimentel HJ, Bray N, Puente S, Melsted P, Pachter L. 2017. Differential analysis of RNA-Seq incorporating quantification uncertainty. *Nat Meth* 14:687-690.
85. Rapicavoli JN, Blanco-Ulate B, Muszyński A, Figueroa-Balderas R, Morales-Cruz A, Azadi P, Dobruchowska JM, Castro C, Cantu D, Roper MC. 2018. Lipopolysaccharide O-antigen delays plant innate immune recognition of *Xylella fastidiosa*. *Nat Commun* 9:390.
86. Kyrkou I, Pusa T, Ellegaard-Jensen L, Sagot M-F, Hansen LH. 2018. Pierce's disease of grapevines: A review of control strategies and an outline of an epidemiological model. *Front Microbiol* 9:2141.
87. Wallis CM, Chen J. 2012. Grapevine phenolic compounds in xylem sap and tissues are significantly altered during infection by *Xylella fastidiosa*. *Phytopathology* 102:816-826.
88. Wallis CM, Wallingford AK, Chen J. 2013. Grapevine rootstock effects on scion sap phenolic levels, resistance to *Xylella fastidiosa* infection, and progression of Pierce's disease. *Front Plant Sci* 4:502.

89. Langmead B, Trapnell C, Pop M, Salzberg S. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* 10:R25.
90. Wallis CM, Wallingford AK, Chen J. 2013. Effects of cultivar, phenology, and *Xylella fastidiosa* infection on grapevine xylem sap and tissue phenolic content. *Physiol Mol Plant Pathol* 84:28-35.

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

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ABSTRACT
Xylella fastidiosa (*Xf*) is a gram-negative, fastidious xylem-limited bacterium that causes scorching diseases in many economically important plant species like Pierce's disease of grapevine, the most valued fruit crop in the U.S. Lipopolysaccharide (LPS) covers most of the cell surface in Gram-negative bacteria and is a well-described pathogen-associated molecular pattern that elicits host basal defense responses plants. *Xf* LPS-mediated elicitation of the basal defense response in grapevine leads to systemic and prolonged activation of defense pathways related to *Xf* perception. In addition, this molecule can induce plant defense priming against *Xf* resulting in enhanced Pierce's disease tolerance. Our objectives explore the persistence of *Xf* LPS-mediated defense priming in grapevine and the molecular mechanisms underlying the defense priming phenomenon. Ultimately, our studies will result in fundamental knowledge about grapevine immune response genes that will be utilized to create Pierce's disease resistant grapevines.

LAYPERSON SUMMARY
Successful plant pathogens must overcome plant immune responses to establish themselves and cause disease. *Xylella fastidiosa* (*Xf*) utilizes the prominent O antigen surface carbohydrate found in the lipopolysaccharide (LPS) molecule to shield bacterial cell surface elicitors from the grapevine immune system, effectively delaying pathogen recognition. *Xf* LPS elicits strong immune responses in the grapevine and conditions grapevines for enhanced defense against *Xf*. We will employ this knowledge to better understand the mechanism of this enhanced response, test if we can maintain the primed state, and apply these results to create Pierce's disease resistant grapevines.

INTRODUCTION
Xylella fastidiosa (Xf), a Gram-negative fastidious bacterium, is the causal agent of Pierce's disease (PD) of grapevine (*Vitis vinifera*) and several other economically important diseases (Chatterjee et al., 2008). *Xf* is limited to the xylem 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. PD has devastated some viticulture areas in California, and research on devising effective control is an active area of research.

Our previous study confirmed that lipopolysaccharide (LPS) is a major virulence factor for *Xf*. LPS comprises approximately 70% of the Gram-negative bacterial cell surface, making it the most dominant macromolecule displayed on the cell surface (Caroff and Karibian, 2003). LPS is a tripartite glycolipid that is generally comprised of a highly-conserved lipid A, an oligosaccharide core, and a variable O antigen polysaccharide (Whitfield, 1995) (**Figure 1**). We demonstrated the *Xf* O antigen is a linear α1-2 linked rhamnan and compositional alterations to the O antigen significantly affected the adhesive properties of *Xf*, consequently affecting biofilm formation and virulence (Clifford et al., 2013). In addition, we demonstrated that truncation of the LPS molecule severely compromises insect acquisition of *Xf* (Rapicavoli et al., 2015). We coupled these studies with quantification of the electrostatic properties of the sharpshooter foregut to better understand the interface between the *Xf* cell and the insect. We then sought to test our additional hypothesis that the *Xf* LPS molecule acts as a pathogen-associated molecular pattern (PAMP), and the long chain O antigen serves to shield *Xf* from host recognition, thereby modulating the host's perception of *Xf* infection (Rapicavoli et al., 2018).

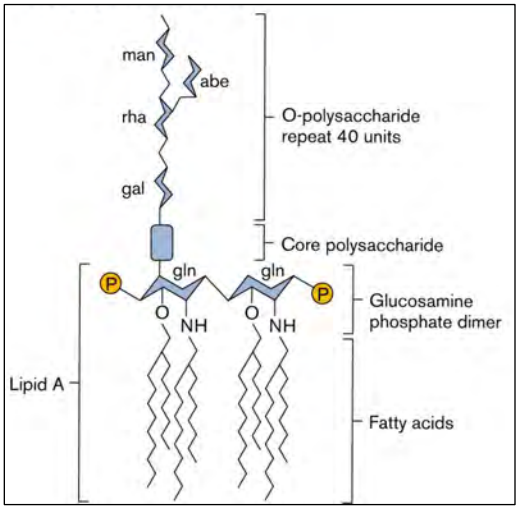

Figure 1. Schematic of a single LPS molecule containing lipid A, core polysaccharide, and the O-antigen (O-polysaccharide). Adapted from Microbiology, An Evolving Science.

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.). PAMPs are recognized by the host as "non-self" and can be potent elicitors of basal defense responses. This line of defense against invading pathogens is referred to as PAMP-triggered immunity (PTI) and represents the initial layer of defense against pathogen ingress (Nicaise et al., 2009). PTI is well studied in both mammalian and plant hosts. However, little is known about the mechanisms involved in perception of LPS in grapevine, particularly the *Xf* LPS PAMP. *Xf* is introduced by its insect vector directly into the xylem; a non-living tissue, which cannot mount a defense response on its own. However, we observe profound changes that occur in the xylem that are linked to the presence of *Xf*. These include an oxidative burst and suberin deposition, as well as tyloses production (Rapicavoli et al, 2018). Interestingly, we also observe significant defense response to *Xf* in the phloem tissue, a tissue historically overlooked in the context of this xylem dwelling pathogen that mainfest in the form of callose deposition. The plant immune system can recognize several regions of the LPS structure, including the conserved lipid A and core polysaccharide components (Newman et al., 2007; Silipo et al., 2005). Bacteria can also circumvent the host's immune system by altering the structure of their LPS molecule. Clearly, *Xf* has evolved a mechanism to circumvent the host basal defense response as it successfully colonizes and causes serious disease in grapevine. We tested our hypothesis that the bacterium's long chain, rhamnose-rich O antigen shields the conserved lipid A and core-oligosaccharide regions of the LPS molecule from being recognized by the grapevine immune system, providing an opportunity for it to subvert basal defense responses and establish itself in the host (Rapicavoli et al., 2018).

To explore the role of LPS as a shield against basal defense responses in grapevine, we investigated elicitation of an oxidative burst, an early marker of basal defense responses, *ex vivo* in *V. vinifera* Cabernet Sauvignon leaf disks exposed to either wild-type *Xf* or *wzy* mutant cells. When we examined reactive oxygen species (ROS) production in response to whole cells, *wzy* mutant cells (in which lipid A-core is exposed) induced a stronger and more prolonged oxidative burst in grapevine leaf disks than did wild-type *Xf*. 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 shown in Rapicavoli et al, 2018).

In addition to the role of LPS in promoting bacterial infection, pre-treatment of plants with LPS can prime the defense system resulting in an enhanced response to subsequent pathogen attack. This defense-related memory is called "priming" and stimulates the plant to initiate a faster and/or stronger response against future invading pathogens (Conrath, 2011; Newman et al., 2000). We demonstrate that pre-treatment with LPS isolated from *Xf* would result in an increase in the grapevine's tolerance to *Xf* by stimulating the host basal defense response. Our

ex vivo data showing that both wild-type *and wzy* mutant LPS elicit an oxidative burst, an early marker of defense that can potentiate into systemic resistance, in grapevine leaf disks support this hypothesis. To determine if the primed state affects the development of PD symptoms, we documented disease progress in plants that were pre-treated with either wild-type or *wzy* LPS and then challenged with *Xf* either 4 or 24 hours later. Notably, we observed a decrease in PD severity in vines pre-treated with *Xf* LPS and then challenged with *Xf* **(Figure 2) (**Rapicavoli et al, 2018).

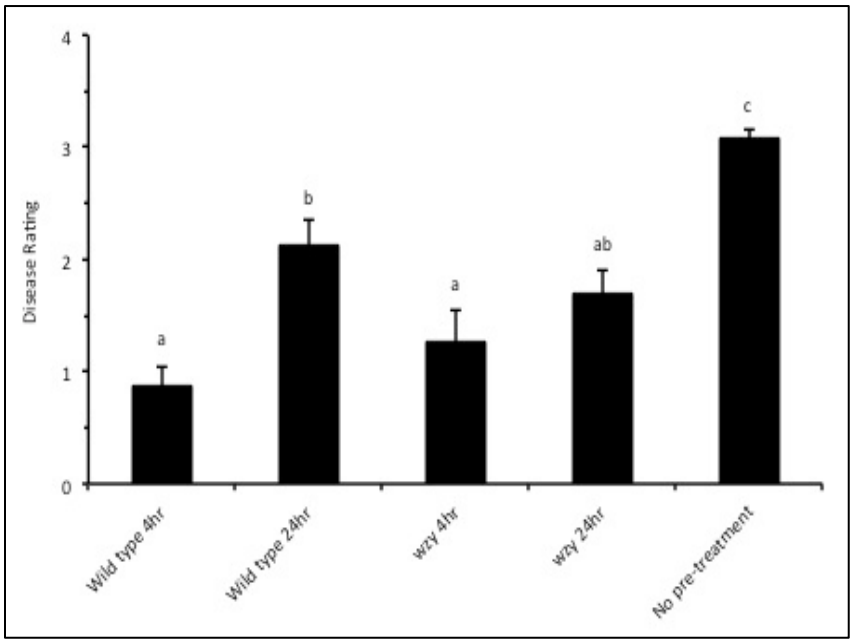

Figure 2. PD symptom severity in grapevines primed with purified *Xf* LPS. Average disease ratings of *V. vinifera* Cabernet Sauvignon grapevines pre-treated with wild-type or *wzy* mutant LPS (50 μg/mL), then challenged at 4 hours or 24 hours post-LPS treatment with live *Xf* cells. Disease ratings were taken at 12 weeks post-challenge. The LPS pre-treated plants are significantly attenuated in symptom development, compared with plants that did not receive pre-treatment ($P < 0.05$). Graph represents the mean of 24 samples per treatment. Bars indicate standard error of the mean.

Previously, we completed a global RNA-sequencing (RNA-seq)-based transcriptome profile where we sequenced the transcriptomes of grapevines treated with wild-type, *wzy* mutant cells, or 1 x phosphate buffered saline (PBS) buffer (Rapicavoli et al, 2018). The goal was to identify genes that are differentially expressed when plants are inoculated with either wild-type or the *wzy* mutant while using mock-inoculated plants as the controls. PTI usually causes major transcriptional reprogramming of the plant cells within hours after perception (Dow et al., 2000; Tao et al., 2003), so our initial experiments were targeted toward early time points during the infection process (0, 8, and 24 hours post-inoculation). The RNA-seq data demonstrate that the grapevine is activating defense responses that are distinct to each treatment and time point **(Figure 3A)**. For example, enrichment analysis of *wzy*-responsive genes at eight hours post-inoculation identified predominant biological processes associated with cellular responses to biotic stimulus and oxidative stress **(Figure 3B)**. This included a significant increase in the production of thioredoxins, glutaredoxins, and other ROS-scavenging enzymes involved in antioxidant defense. In addition, there was high expression of genes involved in the production of phytoalexins (e.g., stilbene synthase), antimicrobial peptides (e.g., thaumatin), and pathogenesis-related (PR) genes. In contrast, wild-type responsive genes in this time point were enriched primarily in responses to abiotic or general stresses (i.e., drought, oxidative, temperature, and wounding stresses) and were not directly related to immune responses **(Figure 3B)**. Notably, by 24 hours post-inoculation, overall transcriptional profiles of both *wzy* and wild-type-inoculated vines shifted dramatically. Grape genes in *wzy* mutant-inoculated vines were no longer enriched for immune-specific responses, and we speculate that this is due to the effective O antigen-modulated oxidative burst. In contrast, genes of wild-type-inoculated plants were strongly enriched for immune responses **(Figure 3C)**. We hypothesize that at 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., 2018). Complete RNA-seq data can be found in the supplementary information in Rapicavoli et al, 2018.

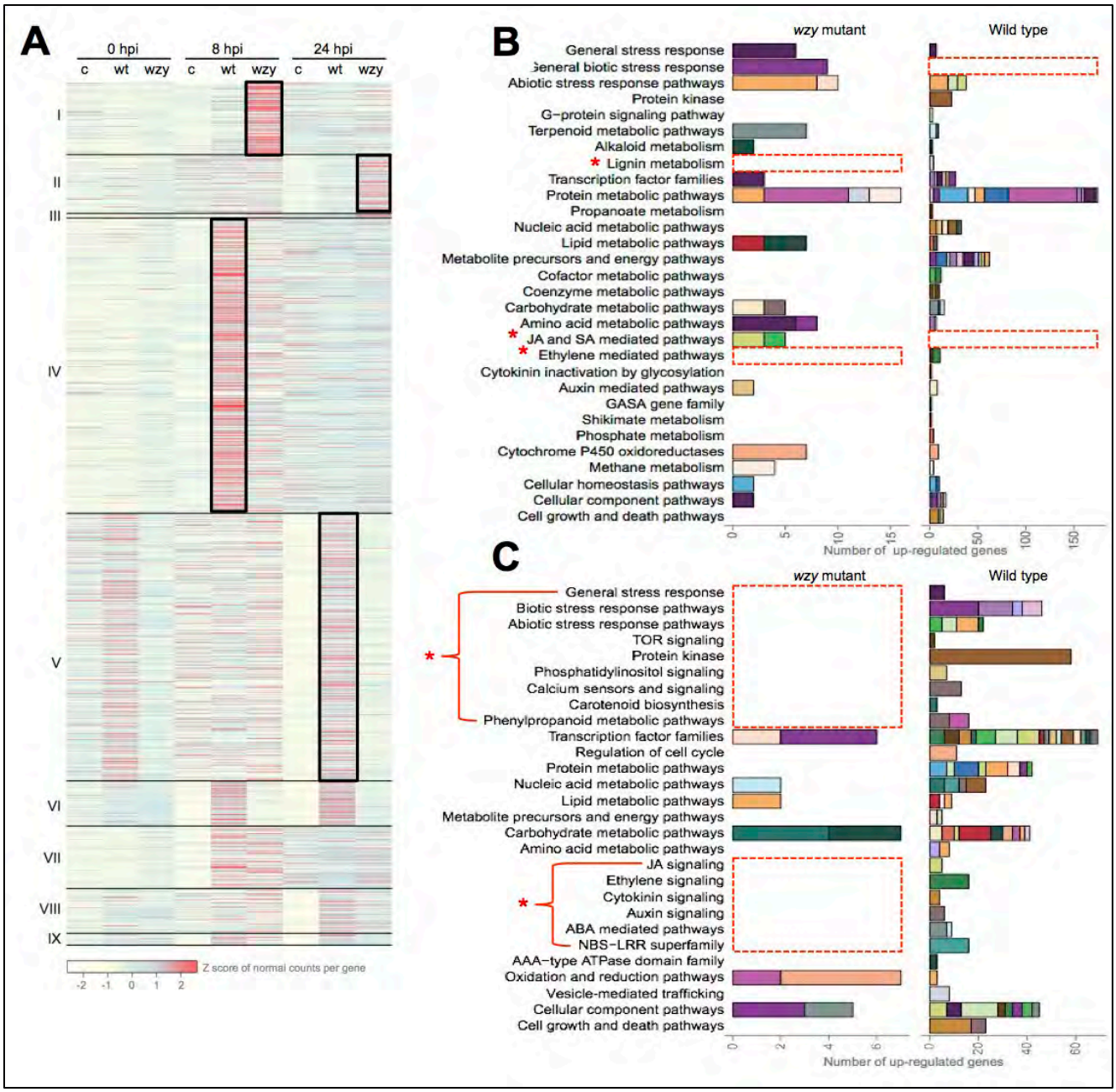

Figure 3. Grapevine responses to early infections by *wzy* mutant and wild-type *Xf*. (A) Up-regulated grape genes ($P < 0.05$) in response to *wzy* mutant or wild-type bacteria at eight and 24 hours post-inoculation when compared to the wounded control (c). Genes are classified into nine groups (I - 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 hours post-inoculation. (C) Enriched grape functional subcategories ($P < 0.05$) among genes up-regulated during wzy (Group II) or wild-type (Group V) infections at 24 hours post-inoculation. Colored stacked bars represent individual pathways. Red boxes highlight functions of interest (*) that are enriched in one treatment, but not enriched in the other at each time point.

In addition to exploring early defense response, we also characterized the transcriptional response at systemic locations distal to the point of inoculation and at longer time points: 48 hours, one week, and four weeks. This tested our hypotheses that (i) truncated *Xf* O antigen is more readily perceived by the grapevine immune system, allowing the plant to mount an effective defense response to *Xf*, and (ii) that the initial perception of the truncated LPS, belonging to the *wzy* mutant, is propagated into a prolonged and systemic response. Local tissue of *wzy*-infected plants induced genes enriched in cell wall metabolism pathways, specifically pectin modification, at four weeks post-inoculation **(Figure 4A)**. This is a stark contrast with wild-type-inoculated vines, in which these pathways were up-regulated as early as eight hours post-inoculation. This likely explains why this pathway is not enriched in local tissue of wild-type-inoculated vines at these later time points. The induction of salicylic acid (SA)-mediated signaling pathways in *wzy*-inoculated vines was further supported by the presence of four genes, including two enhanced disease susceptibility 1 (EDS1) genes. EDS genes are known defense genes associated with the SA pathway and have been implicated in grapevine defenses against powdery mildew. The consistent enrichment and up-regulation of SA-associated genes (and thus, the maintenance of the signal), including the presence of PR-1 and other SA-responsive genes at eight hours post-inoculation, strongly suggests that the plant is preventing the development of infections by *wzy* cells via an SA-dependent pathway. In wild-type vines, consistent enrichment of jasmonic acid (JA)-associated genes was further supported by the presence of nine genes functioning in the metabolism of alpha-linolenic acid, which serves as an important precursor in the biosynthesis of JA **(Figure 4A)**.

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

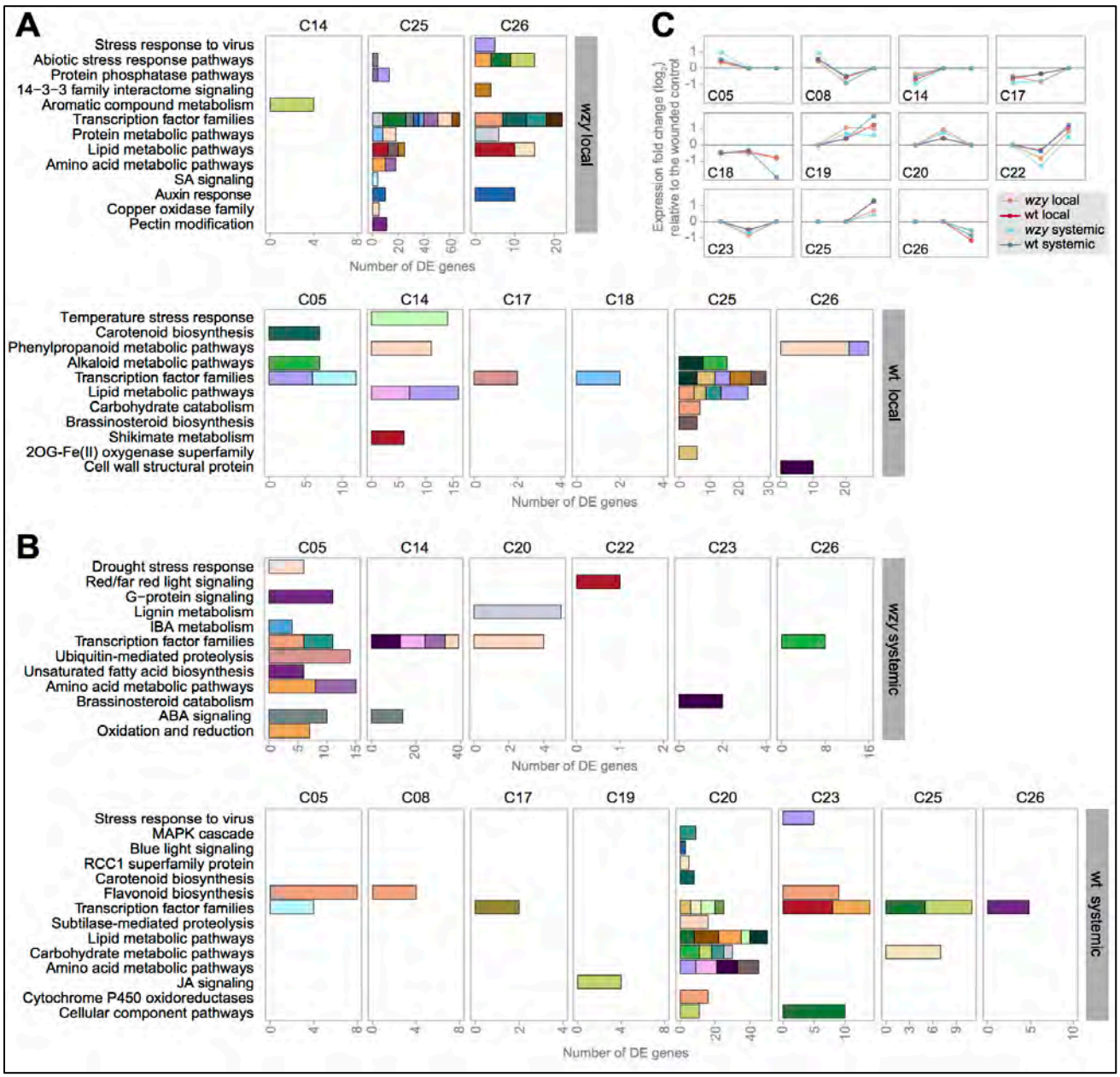

Figure 4. Transcriptomic analysis of late grapevine responses to *Xf* wild-type and *wzy* mutant strains in local and systemic tissue. Enriched grape functional pathways ($P < 0.05$) in differentially expressed (DE) gene clusters representing local (A) or systemic (B) responses to *Xf* inoculation. Only enriched pathways related to grapevine immune responses and that were unique to wild-type (wt) or *wzy* mutant inoculations are depicted. Colored stacked bars represent individual pathways. (C) Patterns of expression of gene clusters enriched in functional pathways with biological relevance. Lines represent the medoids for each cluster. Dots represent expression fold changes of each medoid (log2) at a given time point post-inoculation (in order: 48 hours, one week, and four weeks) when compared to the wounded control.

OBJECTIVES

1. Characterization of the temporal aspects of the primed state in grapevine.
2. Characterization of the molecular mechanisms underlying the grapevine immune response to *Xf.*
3. Functional genomics of grapevine immunity to *Xf.*

RESULTS AND DISCUSSION

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

We have previously shown pre-treatment of plants with LPS can induce plant defense priming against *Xf* resulting in enhanced PD tolerance **(Figure 2)** (Rapicavoli et al, 2018). To explore if the primed state can be extended over

time, we have tested if additional LPS applications following elicitation of the plant defense priming can increase PD tolerance. Grapevines were treated with wild-type LPS (50 µg/ml) and challenged with *Xf* four hours later. After 48 hours or one week, grapevines received an additional LPS treatment (50 µg/ml). Appropriate controls received diH_2O instead of LPS and 1 x PBS instead of *Xf* cells. All plants are currently under examination for PD symptom development using a disease rating scale of 0 to 5, where 0 is a healthy and 5 is a dead vine (Guilhabert and Kirkpatrick, 2005). Thus far at 12 weeks post-inoculation, average disease scores for plants that received an additional LPS dose, 'LPS-Xf-LPS (48h)' and 'LPS-Xf-LPS (1w),' are lower than the scores of plants that did not receive an additional dose, 'LPS-Xf-H2O (48h)' and 'LPS-Xf-H20 (1w)' **(Figure 5)**. We will continue to monitor these plants until 20 weeks post-inoculation and determine values for 'area under the disease progress curve' for all treatments and perform statistical analyses to determine any significant difference between the treatments. In addition to observing disease progression, we will collect petioles at the point of inoculation and 20 nodes above the point of inoculation to quantify bacterial titer.

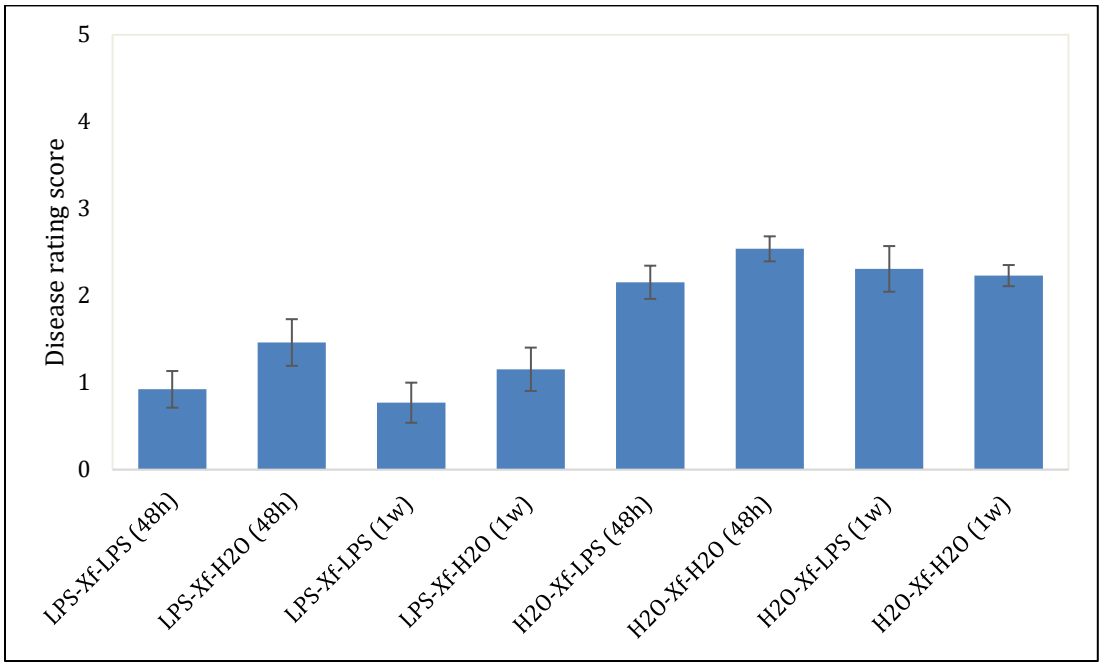

Figure 5. PD symptom severity in LPS-primed grapevines treated with an additional dose of LPS. Average disease ratings of *V. vinifera* Cabernet Sauvignon grapevines primed with wild-type LPS (50 µg/mL) and challenged with *Xf* cells following an additional LPS treatment. Disease ratings were taken at 12 weeks post-challenge. Graph represents the mean of 13 samples per treatment. Bars indicate standard error of the mean.

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

The molecular mechanisms underlying defense priming and its importance in enabling heightened immunity to pathogen ingress are poorly understood. To better understand the changes occurring in gene expression patterns that potentiate the priming phenotype in grapevine, we will perform a series of RNA-Seq experiments that will highlight genes and pathways induced during priming in both local and systemic tissue. For this objective, we repeated the LPS priming experiment in our previous study (Rapicavoli *et al*, 2018) and harvested petioles for RNA-Seq. Grapevines were treated with wild type LPS (50 µg/ml) and challenged with *Xf* cells 4 hours later. Petioles for RNA-Seq were harvested at 4 h, 24 h, and 48 h post-*Xf* challenge from the point of inoculation and 20 nodes above the point of inoculation. RNA has been extracted from the samples and sequencing libraries are under preparation. In addition to collecting plant tissue for transcriptome analysis, we monitored plants for disease progression and collected petioles for quantification of bacterial titer.

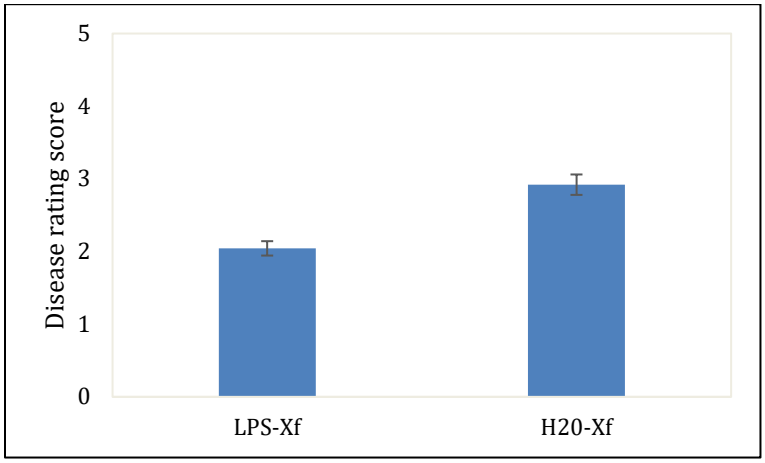

Figure 6. PD symptom severity in LPS-primed grapevines used to harvest petioles for RNA-seq. Average disease ratings of *V. vinifera* Cabernet Sauvignon grapevines primed with wild-type LPS (50 μg/mL) and challenged with *Xf* cells four hours post-LPS treatment. Disease ratings were taken at 12 weeks post-challenge. Graph represents the mean of 27 samples per treatment. Bars indicate standard error of the mean.

Objective 3. Functional Genomics of Grapevine Immunity to *Xf*

In our previous study, we determined that LPS-mediated early elicitation of the basal defense response leads to systemic and prolonged activation of defense pathways related to *Xf* perception in grapevine. Our experiments identified several genes involved in plant defense that were enriched in response to *wzy* cells (Rapicavoli et al, 2018). For Objective 3, we will create transgenic grapevines overexpressing these genes and test resistance to *Xf*. We will also incorporate candidate genes from our transcriptome analysis results in Objective 2.

CONCLUSIONS

Our ongoing work demonstrates that pre-treatment with purified LPS primes the grapevine immune system and this immune activation results in reduced disease severity when these primed plants are challenged with *Xf* cells. We plan to characterize the temporal persistence of *Xf* LPS-mediated defense priming in grapevine. We will also conduct in-depth transcriptome analyses of grapevines treated with the LPS molecule. The overall outcome will result in fundamental knowledge about grapevine immune responses at the molecular level that we will utilize to test novel gene targets for creating PD-resistant grapevines.

REFERENCES CITED

Caroff M, et al. 2003. *Carbohyd Res* 338:2431-2447.
Chatterjee S, et al. 2008. *Annual Review of Phytopathology* 46:243-271.
Clifford JC, et al. 2013. *Mol Plant Microbe Interact* 26:676-85.
Conrath U. 2011. *Trends Plant Sci* 16:524-531.
Dow M, et al. 2000. *Annual Review of Phytopathology* 38: 241-261.
Guilhabert MR, Kirkpatrick BC. 2005. *Mol Plant Microbe Interact* 18:856-868.
Newman KL, et al. 2004. *P Natl Acad Sci USA* 101:1737-1742.
Newman MA, et al. 2000. *Mol Plant Pathol* 1:25-31.
Newman MA, et al. 2002. *Plant J* 29:487-495.
Newman MA, et al. 2007. *J Endotoxin Res* 13:69-84.
Nicaise V, et al. 2009. *Plant Physiology* 150:1638-1647.
Rapicavoli JN, et al. 2018. *Nature Communications* 9:390.
Rapicavoli JN, et al. 2015. *Appl Environ Microbiol* 81:8145-8154.
Silipo A, et al. 2005. *Journal of Biological Chemistry* 280:33660-33668.
Sun Q, et al. 2013. *Plant Physiol* 161:1529-1541.
Tao Y, et al. 2003. *The Plant Cell* 15:317-330.
Whitfield C. 1995. *Trends in Microbiol* 3:178-185.

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

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ABSTRACT
Xylella fastidiosa (*Xf*) is the causal agent of Pierce's disease of grapevine. This xylem-limited bacterial pathogen systemically colonizes the xylem by using cell wall degrading enzymes (CWDEs) to dismantle the pit membrane barriers that separate xylem vessels. Tylose formation is the predominant vascular occlusion associated with *Xf* infection, and excessive tylose development has been linked to the extreme susceptibility of *Vitis vinifera* winegrapes to Pierce's disease. Thus, we sought out to better understand this host defense response in the context of *Xf*-mediated cell wall degradation. By using visual evidence (scanning electron microscopy and micro-computed tomography), coupled with transcriptome analyses of inoculated grapevines, we determined that endoglucanase-deficient *Xf* mutants differentially induce tylose production relative to the wild-type *Xf* strain. These findings indicate that *Xf* endoglucanases play a role in facilitating host tylose production. Given these findings and that *Xf* CWDEs are important for the degradation of pit membranes (thus allowing systemic colonization), it is imperative that these virulence factors are targeted for inhibition. However, inhibiting each CWDE individually as a commercial strategy for controlling *Xf* is both impractical and costly. As these CWDEs are predicted to be secreted by the type II secretion system, we are currently searching for natural products that block the type II secretion system, thus preventing the secretion of CWDEs, and subsequently minimizing both *Xf* systemic colonization and excessive host tylose production.

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 determine the mechanisms that lead to disassembly of the plant cell wall that eventually leads to systemic colonization of *Xf* in grapevines. Here we have performed 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 are designing experiments to 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 (PD) of grapevine, a serious and often lethal disease (Hopkins and Purcell, 2002; Chatterjee et al., 2008; Purcell and Hopkins, 1996). This xylem-limited bacterial pathogen colonizes the xylem, and in doing so must be able to move efficiently from one xylem vessel element to adjacent vessels (Roper et al., 2007). Xylem conduits are separated by pit membranes (PMs) that are composed of cellulose microfibrils embedded in a meshwork of pectin and hemicellulose and prevent the movement of air embolisms and pathogens within the xylem (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 and Hopkins, 1974). Based on functional genomics and *in planta* experimental

evidence, *Xf* utilizes cell wall degrading enzymes (CWDEs) to actively digest the polymers within the PMs, thereby facilitating its movement throughout the xylem network (Simpson et al., 2000; Roper et al., 2007; Perez-Donoso et al., 2010). It is known that polygalacturonase (PG) is a major pathogenicity factor for *Xf* (Roper et al., 2007) and that it acts in concert with at least one EGase to breach the PM barrier (Perez-Donoso et al., 2010). EGases are implicated in virulence and colonization of the xylem in other bacterial phytopathogens, such as *Pantoea stewartii* subsp. *stewartii*, *Ralstonia solanacearum*, and *Xanthomonas campestris* pv. *campestris* (Gough, 1988; Roberts et al., 1988; Saile et al., 1997; Mohammadi et al., 2012). In our previous study (project # 14-0144-SA), we tested the role of the *Xf* EGases *in planta* by constructing deletion mutants in two of the EGases (Δ*engXCA1* and Δ*engXCA2*) and mechanically inoculating the modified *Xf* lines into *Vitis vinifera* cv. Cabernet Sauvignon grapevines. Interestingly, both Δ*engXCA1* and Δ*engXCA2* achieved the same titers (data not shown) in the Cabernet Sauvignon vines as wild-type *Xf*, yet they were less virulent and elicited fewer PD symptoms (**Figure 1**).

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

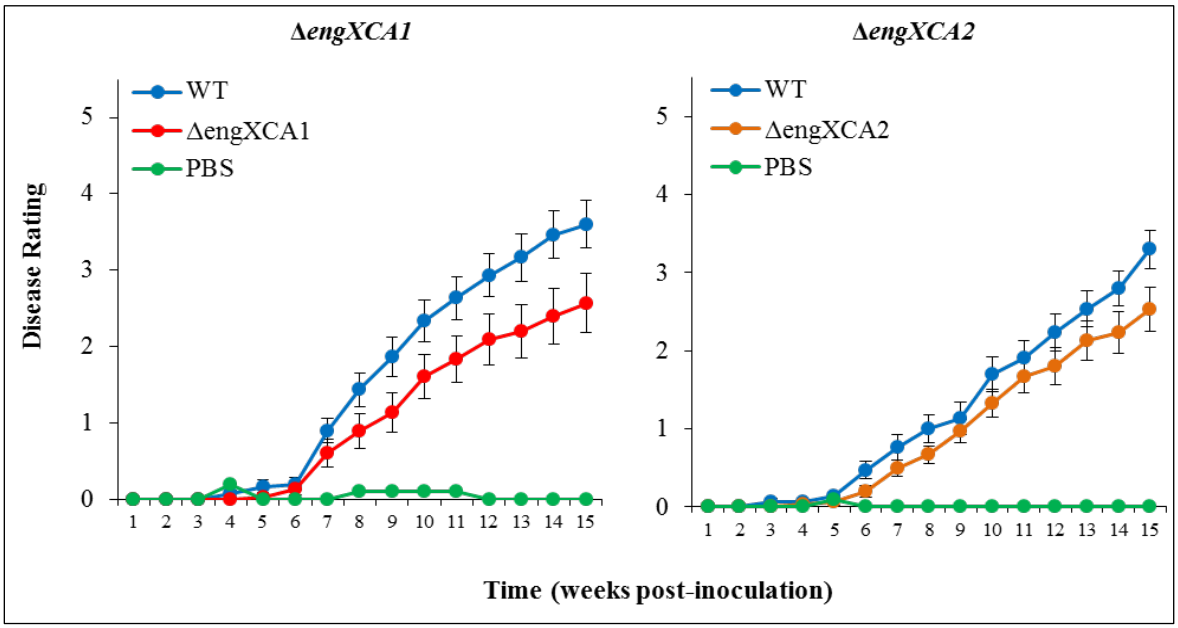

Figure 1. Pierce's disease development over 15 weeks in Cabernet Sauvignon grapevines after inoculation with wild-type Temecula 1 (blue), and the Δ*engXCA1* (red) or Δ*engXCA2* (orange) mutant strains. 1X phosphate buffered saline (green) served as the negative control. All vines were rated on a disease scale of 0-5, where 0 = healthy, 1-4 = increasing degrees of scorching, and 5 = vine death. Data are the means of three independent assays with ten replicates each. Bars represent the standard error of the mean.

Tyloses are outgrowths of parenchyma cells that emerge through vessel-parenchyma pits into vessel lumen, and are common in a wide range of species (Bonsen and Kučera, 1990; Esau, 1977; Tyree and Zimmermann, 2002). Tyloses impede fluid penetration (Parameswaran et al., 1985) and induce a permanent state of reduced hydraulic conductivity, and are triggered by abiotic and biotic stresses, such as pathogen infection (Aleemullah and Walsh,

1996; Collins et al., 2009; Dimond, 1955; Parke et al., 2007). Tylose formation is the predominant vascular occlusion associated with *Xf* infection (**Figure 2A, B**), and excessive tylose development has been linked to the extreme susceptibility of *V. vinifera* winegrapes to PD (Fritschi et al., 2008; Sun et al., 2013).

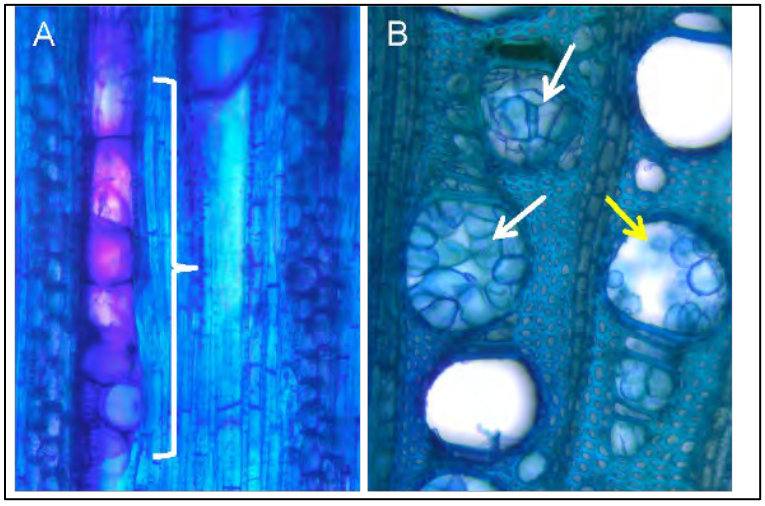

Figure 2. Xylem vessels of *V. vinifera* grapevines inoculated with *Xf*. **A.** Longitudinal section **B.** Cross-section. Grapevine petiole sections were stained with toluidine blue O (0.05%). White arrows and bracket indicate vessels that are completely occluded with tyloses, and yellow arrow indicates a partially occluded vessel. Images taken by J. Rapicavoli (Roper Lab).

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 sought out 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 Δ*engXCA1* mutant using a high resolution micro-computed tomography (microCT) technique (a kind of CAT scan) by the McElrone laboratory determined that these vines exhibited fewer tyloses than those inoculated with wild-type *Xf* (data not shown). 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 patterns (DAMPs). DAMP signaling defense pathways, which leads to downstream tylose production and PD symptom development in certain grape cultivars.

Given that *Xf* CWDEs are important for the degradation of PMs (thus allowing systemic colonization), it is imperative that these virulence factors are targeted for inhibition. However, inhibiting each CWDE individually as a commercial strategy for controlling *Xf* is both impractical and costly. Interestingly, these CWDEs are predicted (using SignalP software) to be secreted via the type II secretion system (T2SS). The T2SS is a molecular nanomachine that transports pre-folded proteins from the periplasm across a dedicated channel in the outer membrane (Cianciotto, 2005; Korotkov et al., 2012). The T2SS 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). 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 *Xf* 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 PD symptoms and remained healthy, a phenotype similar to the grapevine response to the *Xf* Δ*pglA* mutant (**Figure 3**).

We hypothesize that this is due to the pathogen's inability to secrete the CWDEs necessary for xylem colonization. In addition, we have indirect experimental evidence that *Xf* utilizes the T2SS to secrete PG. We observed that the Δ*xpsE* mutant produces visibly less extracellular polymeric substances (EPS) on XFM minimal

medium containing pectin as the sole carbon source, resulting in a much less mucoid phenotype (data not shown). However, when wild-type *Xf* and Δ*xpsE* are grown on XFM+galacturonic acid (i.e., the monomeric sugar that makes up the pectin polymer) or on XFM+glucose, both strains produce similar amounts of EPS. We infer from this that the breakdown of the pectin substrate is necessary to produce EPS, and when the T2SS is disrupted, this prevents secretion of PG and the subsequent breakdown of pectin.

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

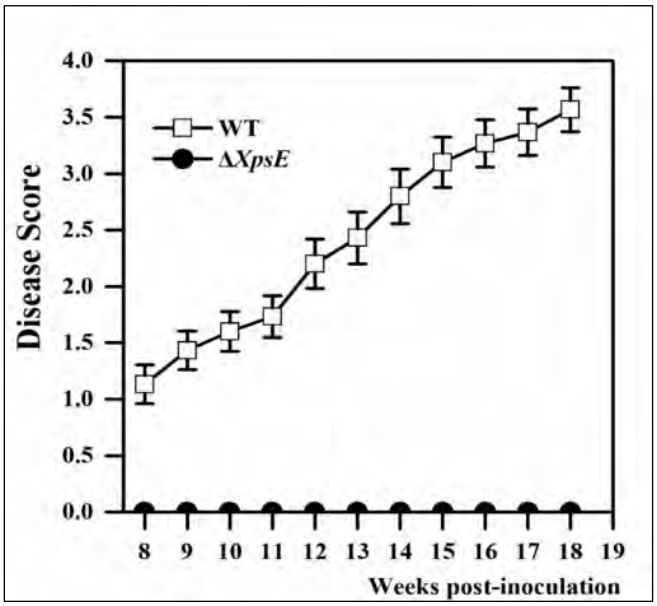

Figure 3. The *Xf* T2SS is necessary for PD development in grapevine. The Δ*xpsE* mutant does not induce PD symptoms in *V. vinifera* grapevines. Disease severity was based on a visual disease scale from 0 (no disease) to 5 (dead). Vines inoculated with 1X phosphate-buffered saline (negative control) did not develop PD symptoms.

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* CWDE activity: Biochemical and transcriptional studies.
3. Inhibition of the T2SS using natural products produced by grapevine microbial endophytes.

RESULTS AND DISCUSSION

Objective 1. Qualitative Analysis of the Effect of Cell Wall Degradation on the Grapevine Response to *Xf*

In the context of plant cell wall degradation, we examined the effects that different *Xf* endoglucanase mutants (Δ*engXCA1*, Δ*engXCA2*, and Δ*engXCA1*/Δ*engXCA2*) have on the integrity and carbohydrate composition of grapevine PMs using both microscopic and immunological techniques coupled with fluorescence (Sun et al., 2011) and/or electron (Sun et al., unpublished) microscopy. We coupled these microscopic observations with macroscopic studies of the spatial distribution of tyloses and other vascular occlusions, such as plant-derived gels and bacterial aggregates, using high resolution microCT. This non-destructive method/technique uses x-rays to create cross-sections of an object that can be used to re-create 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.

Wild-type *Xf* (Temecula 1) and *Xf* endoglucanase mutant strains have been used to inoculate Cabernet Sauvignon grapevines in the greenhouse. Phosphate-buffered saline (PBS)-inoculated vines were used as negative controls. Each *Xf* strain was inoculated into 27 plants (three biological replicates with nine technical replicates each) and PD symptoms were rated each week using the 0-5 PD rating index (Guilhabert and Kirkpatrick, 2005). Vine samples (stem and petiole) were collected at three time-points covering early-, mid-, and late-infection based on the PD rating index (early infection = 1-2, mid-infection = 2-3, late-infection = 3-4). Each sampling consisted of three biological replications (each with three technical replications) per treatment. All stem samples were analyzed using RNA sequencing, microCT, and electron microscopy to determine host response when challenged with either wild-type *Xf* or the *Xf* mutant strains.

Modifications of Different *Xf* Strains on Xylem Structures of Cabernet Sauvignon Vines**.** Late time-point stem samples of Cabernet Sauvignon vines that were inoculated with PBS, wild-type *Xf*, or the *Xf* endoglucanase mutant strains were analyzed using scanning electron microscopy. We found that at the late time-point of PD symptom development, certain *Xf* strains display differences in vascular occlusion, intervessel PM integrity, and *Xf* cell presence.

In the vines inoculated with PBS, vascular occlusion and *Xf* cells were not observed, and intervessel PMs remained mostly intact at the late time-point (**Figure 4**). Vines inoculated with wild-type *Xf* also displayed significant xylem structural modifications at the late time-point. Over 50% of the vessels in the transverse section of a stem were occluded by tyloses, *Xf* cells occurred as large clusters in addition to individual occurrence or small clusters, and intervessel PMs were completely degraded (**Figure 5**). In late time-point samples from Δ*engXCA2*-inoculated vines, 30% of the total vessels were occluded with tyloses (**Figure 6A, B**). Several broken intervessel PMs were present, and clusters of Δ*engXCA2* cells were seen near these broken PMs (**Figure 6C, D**). However, late time-point samples from Δ*engXCA1*-inoculated vines showed relatively few tyloses despite several instances of significant intervessel PM degradation (**Figure 7**). Interestingly, in the late time-point samples inoculated with the Δ*engXCA1*/Δ*engXCA2* double mutant, tyloses occurred in very few vessels (**Figure 8A, B**), intervessel PMs were mostly intact, and Δ*engXCA1*/Δ*engXCA2* cells were not observed (**Figure 8C, D**).

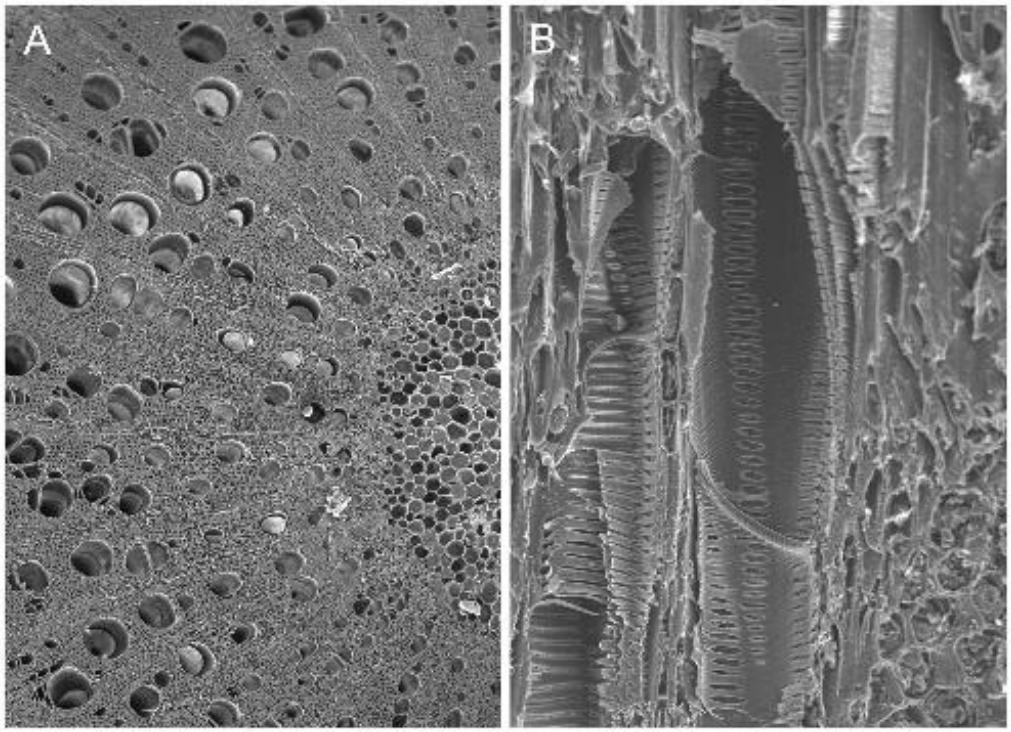

Figure 4. Xylem structural features in PBS-inoculated Cabernet Sauvignon vine at the late time-point. **A.** Transverse section of stem secondary xylem, showing absence of occluded vessels. **B.** Longitudinal section of stem secondary xylem, showing vessels free of tyloses.

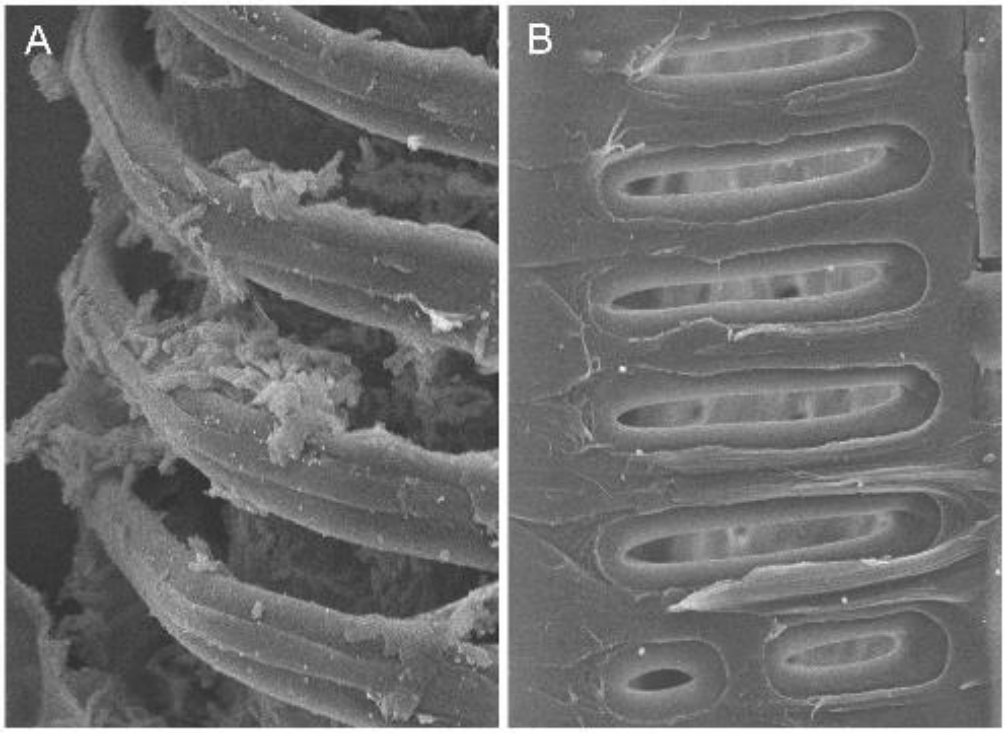

Figure 5. Xylem structural features in wild-type Temecula 1-inoculated Cabernet Sauvignon vine at the late time-point. **A.** Longitudinal section of stem secondary xylem, showing abundant presence of wild-type cells in a vessel. **B.** A longitudinally transected vessels, showing that intervessel PMs have completely disappeared.

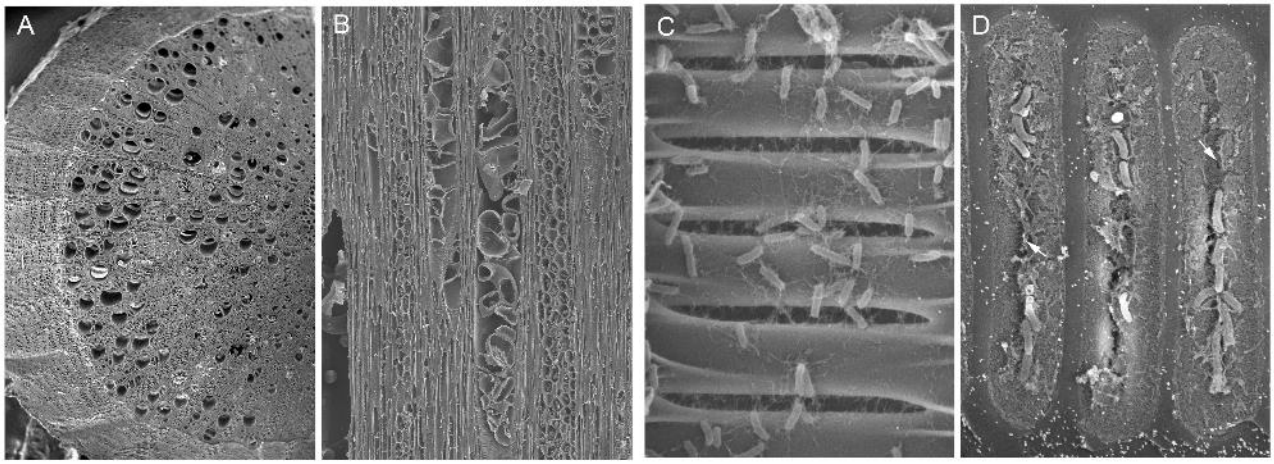

Figure 6. Xylem structural features in Δ*engXCA2*-inoculated Cabernet Sauvignon vine at the late time-point of PD symptom development. **A.** Transverse section of secondary xylem, showing occlusion in some vessels. **B.** Longitudinal section of secondary xylem, showing two transected vessels fully occluded by tyloses. **C.** A longitudinally transected vessel, showing an abundant presence of Δ*engXCA2* cells. **D.** Δ*engXCA2* cells on some partially degraded intervessel PMs (arrows indicate pores or cracks in the PMs).

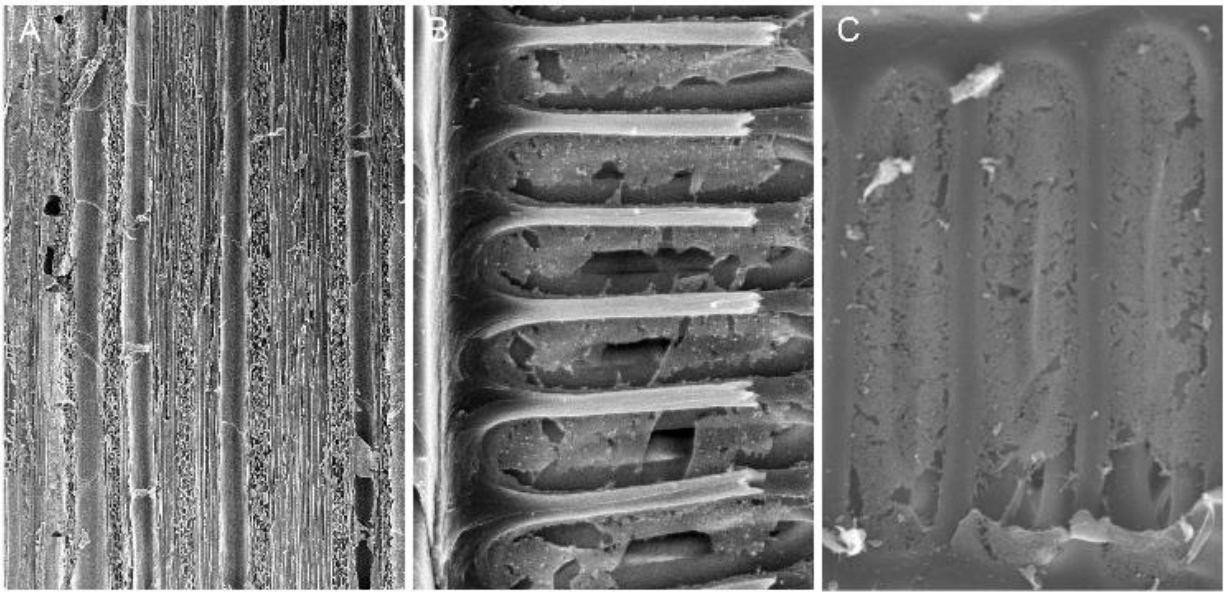

Figure 7. Xylem structural features in Δ*engXCA1*-inoculated Cabernet Sauvignon vine at the late time-point of PD symptom development. **A.** Longitudinal section of stem secondary xylem, showing open vessels. **B** and **C.** Longitudinally transected vessels, showing intervessel PM degradation.

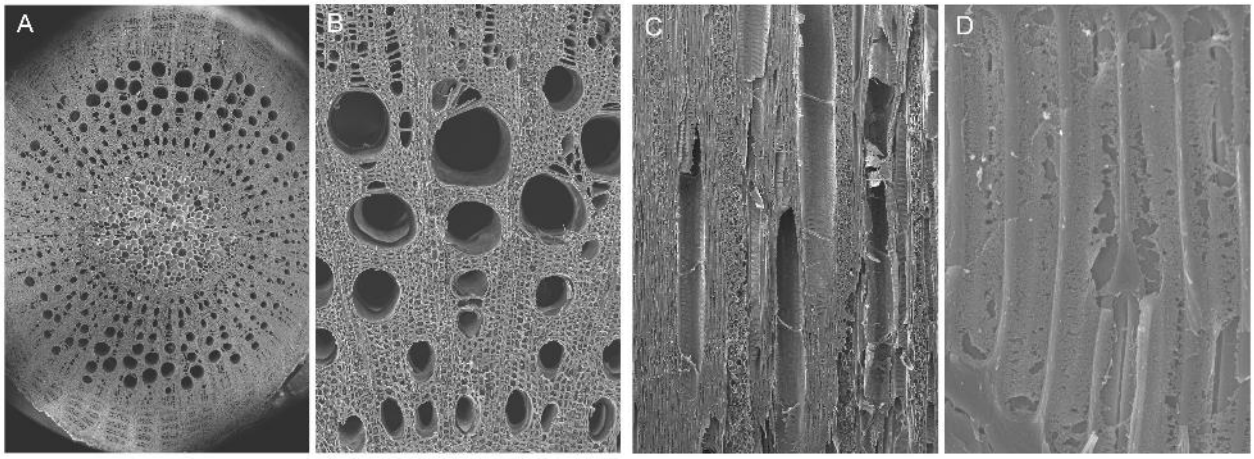

Figure 8. Xylem structural features in Δ*engXCA1*/Δ*engXCA2*-inoculated Cabernet Sauvignon vine at the late time-point of PD symptom development. **A** and **B.** Transverse section of stem secondary xylem, showing vessels free of occlusions. **C.** Longitudinal section of secondary xylem, showing empty vessels with mostly intact PMs. **D.** A longitudinally transected vessel, showing pores of different sizes in intervessel PMs.

In addition to samples imaged via electron microscopy, samples from inoculated Cabernet Sauvignon have also been analyzed by microCT for all time-points. Singular midslice images were analyzed to determine if tyloses formed in the xylem in response to *Xf* infection (**Figure 9A, B, C**). Cabernet Sauvignon vines inoculated with wild-type *Xf*, Δ*engXCA1*, or Δ*engXCA2* exhibited a similar number of vessels containing tyloses and both early and middle time-points. However, at the late time-point, Δ*engXCA2*-inoculated vines had more vessels with tyloses than vines inoculated with wild-type *Xf*, and vines inoculated with Δ*engXCA1* had relatively few vessels with tyloses. Vines inoculated with the Δ*engXCA1*/Δ*engXCA2* double mutant had fewer vessels with tyloses relative to all other treatments across all time-points (**Figure 9D**).

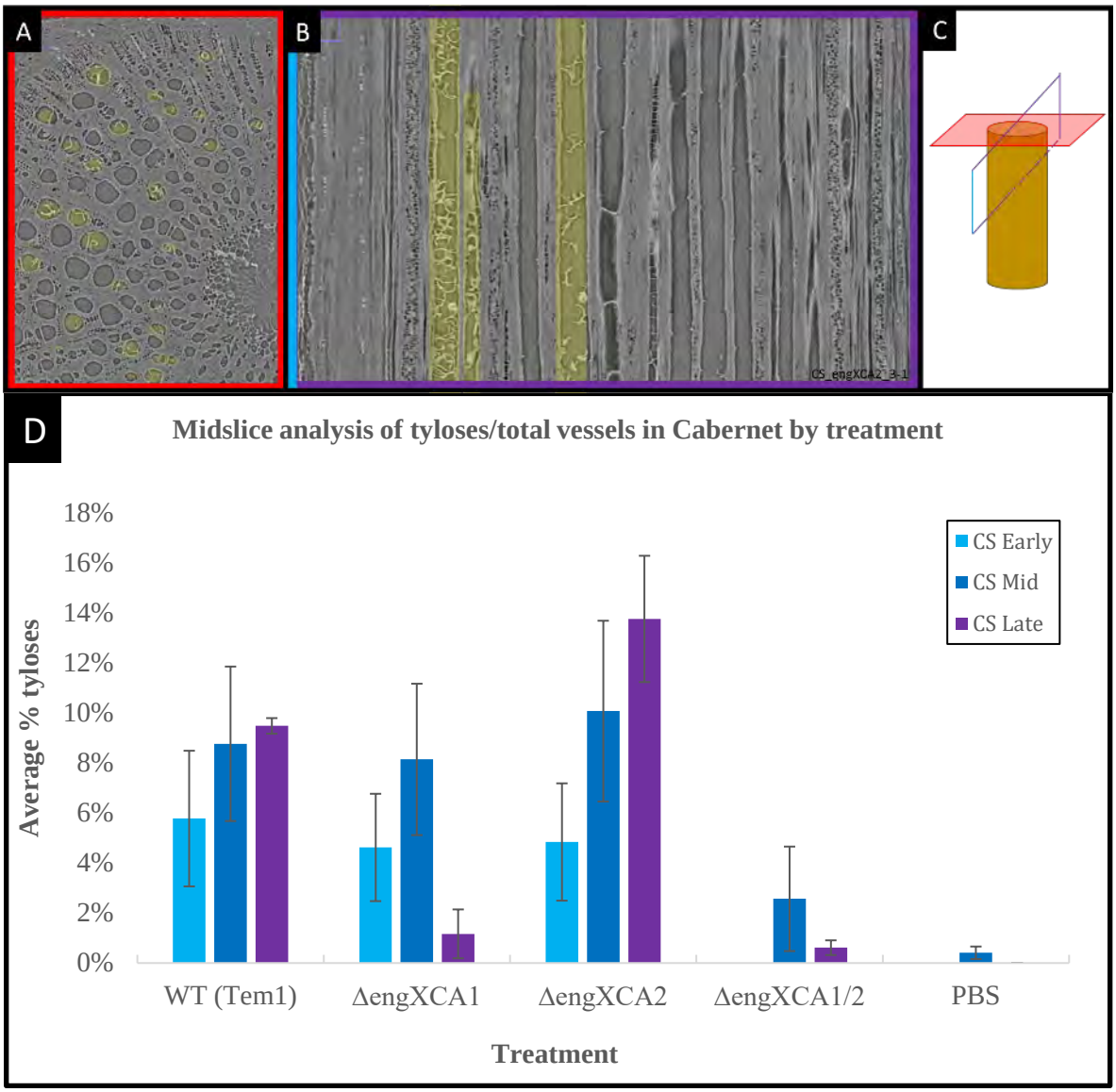

Figure 9. Improved tyloses detection/quantification. Colored outlines in **A** (xy-axis) and **B** (yz-axis) correspond with **C** to help orient the viewer. Tyloses (highlighted in yellow) are small and rare features relative to empty vessels on the xy-axis, and can easily be confused with interconnected vessels, yet appear more distinctly in the yz-axis. **D.** Manual midslice analysis of %-tyloses (occluded vessels/total vessels) per treatment in Cabernet Sauvignon. Vessels with tyloses were manually counted on midslices of microCT scans.

The McElrone lab recently developed a method to measure starch content in ray and axial parenchyma (RAP) *in vivo* using microCT and machine learning algorithms (Earles, 2018). In microCT images, x-ray absorption corresponds to the distinct molecular structure of air, water, starch, and cell wall material, which enables the visualization of RAP, which are located in xylem tissue between radial files of vessels. While microCT images pictured here are of dried stems, patterns of full/empty RAP reflect those found *in vivo* in grapevine rootstocks, and the method has implications for tracking starch utilization over the course of *Xf* infection. RAP in Cabernet Sauvignon vines inoculated with wild-type *Xf* show patterns of starch depletion at the early time-point, with significant depletion at the late time-point. RAP in Δ*engXCA1*-inoculated vines are full of starch at the early time-point and moderately depleted at the late time-point (**Figure 10**). RAP in PBS-inoculated vines remain full of starch at all time-points.

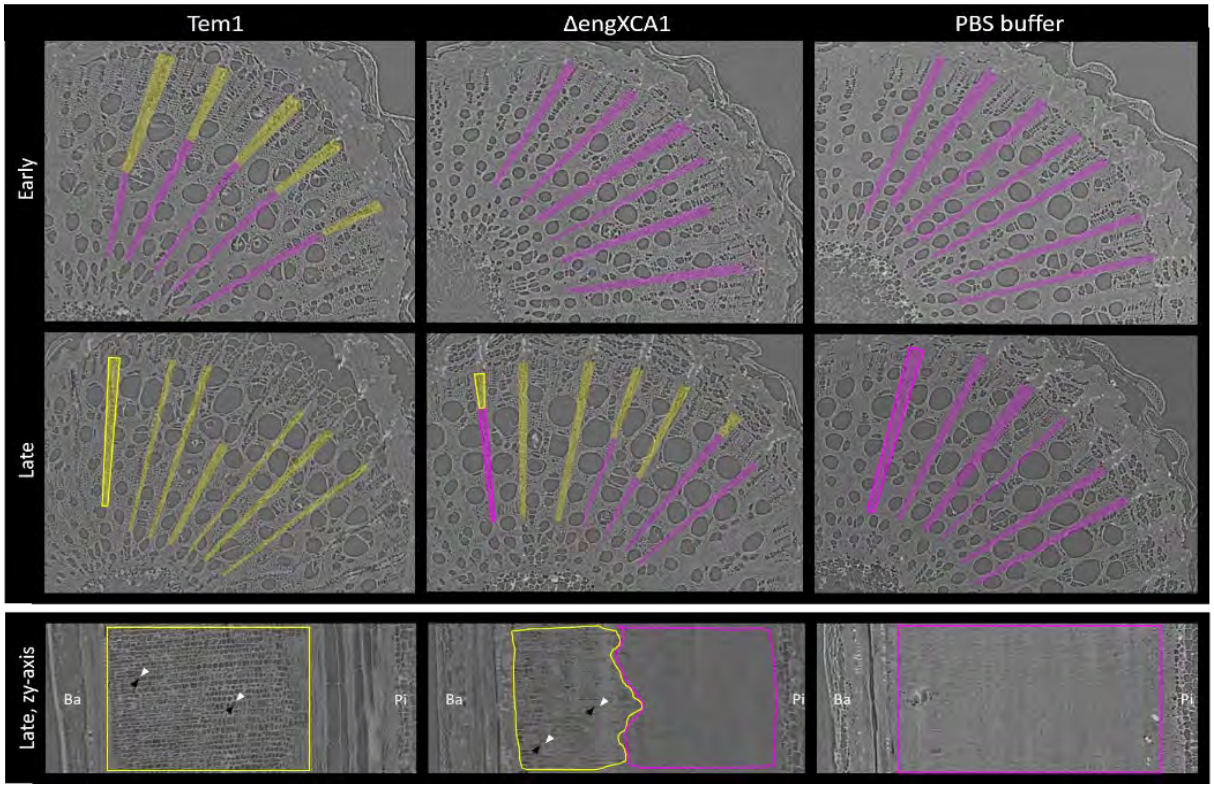

Figure 10. Visual classification of RAP regions as full (magenta) or empty (yellow) in Cabernet Sauvignon vines inoculated with either wild-type Temecula 1, Δ*engXCA1*, or PBS (negative control). Longitudinal slices of outlined, late-timepoint RAP emphasize a spatial pattern of starch depletion, with empty cells (dark airspace and light cell walls indicated with corresponding triangles) near the periphery bark (Ba) layer progressing towards the pith (Pi).

Objective 2. Quantitative Analysis of Plant Defense Pathways Induced by *Xf* CWDE Activity: Biochemical and Transcriptional Studies

PM 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., DAMPs). It is possible that oligosaccharides released from PM degradation are being recognized by associated parenchyma cells, triggering defense responses such as tylose production. To test this hypothesis, we used RNA sequencing to analyze the Cabernet Sauvignon transcriptome to determine if PM degradation products act as elicitors of plant immunity and trigger tylose production. So far, we have counts of differentially expressed genes (DEGs, p-value < 0.05) from the early and middle time-points in 2016 and the early time-point in 2017. When compared to PBS-inoculated vines, the transcriptomes of vines inoculated with either wild-type *Xf* or any of the endoglucanase mutant strains differed significantly (**Table 1**). When compared to wild-type *Xf*-inoculated vines, the transcriptomes of all vines inoculated with any of the *Xf* endoglucanase mutant strains differed significantly, though there were less DEGs in Δ*engXCA1*- and Δ*engXCA2*-inoculated vines and more in Δ*engXCA1*/Δ*engXCA2*-inoculated vines (**Table 2**).

Table 1. Summary of the DEGs (*P*-value < 0.05) between the Cabernet Sauvignon vines inoculated with *Xf* strains (wild-type, Δ*engXCA1*, Δ*engXCA2*, or Δ*engXCA1*/Δ*engXCA2*) and PBS.

Year	Time-point	Number of DEGs	Wild-type vs. PBS	Δ*engXCA1* vs. PBS	Δ*engXCA2* vs. PBS	Δ*engXCA1*/Δ*engXCA2* vs. PBS
2016	Early	Up-regulated	2,831	2,335	469	-
		Down-regulated	1,805	1,446	240	-
		Total	4,636	3,781	709	-
	Middle	Up-regulated	1,791	4,495	1,263	-
		Down-regulated	471	2,566	325	-
		Total	2,262	7,061	1,588	-
2017	Early	Up-regulated	4,567	1,356	3,272	449
		Down-regulated	3,114	638	1,789	259
		Total	7,681	1,994	5,061	708

Table 2. Summary of the DEGs (*P*-value < 0.05) between the Cabernet Sauvignon vines inoculated with the endoglucanase mutant strains and the wild-type *Xf* strain.

Year	Time-point	Number of DEGs	Δ*engXCA1* vs. WT	Δ*engXCA2* vs. WT	Δ*engXCA1*/ Δ*engXCA2* vs. WT
2016	Early	Up-regulated	215	1,214	-
		Down-regulated	260	1,695	-
		Total	475	2,909	-
	Middle	Up-regulated	486	29	-
		Down-regulated	255	89	-
		Total	741	118	-
2017	Early	Up-regulated	1,717	300	2,866
		Down-regulated	2,965	507	4,068
		Total	4,682	807	6,934

CONCLUSIONS

Excessive tylose production has been well-documented in grapevines displaying PD symptoms, and is likely one of the factors causing these symptoms. However, the mechanism by which *Xf* triggers tyloses has not been elucidated. Our scanning electron microscopy and microCT data indicate that tylose production differs in vines inoculated with *Xf* endoglucanase mutants when compared to vines inoculated with the wild-type *Xf* strain. Tylose production increases in vines inoculated with Δ*engXCA2*, while it decreases in vines inoculated with Δ*engXCA1*. Interestingly, tylose production is severely reduced in vines inoculated with the endoglucanase double mutant, Δ*engXCA1*/Δ*engXCA2*. The DEG counts from our RNA sequencing analysis also show that vines inoculated with either Δ*engXCA1* or Δ*engXCA2* behave somewhat similarly to vines inoculated with wild-type *Xf*. Conversely, vines inoculated with the Δ*engXCA1*/Δ*engXCA2* double mutant behave similarly to vines inoculated with PBS. Therefore, we propose that *Xf* endoglucanases play a role in facilitating tylose production in grapevines. How they facilitate tylose production remains unclear, though we hypothesize that the oligosaccharide byproducts of PM degradation trigger a DAMPs response that culminates in the sealing of xylem vessels. We are currently testing this hypothesis by analyzing the specific genes that are differentially expressed in vines inoculated with wild-type *Xf* and vines inoculated with the *Xf* endoglucanase mutants, and we suspect that several of these genes will be linked to DAMPs signaling pathways. Additionally, we are analyzing the xylem sap of vines inoculated with all *Xf* strains to determine if oligosaccharide profiles differ in vines inoculated with wild-type *Xf* and vines inoculated with the *Xf* endoglucanase mutants.

In light of these findings, it appears that *Xf* CWDEs may be triggering host defense responses that exacerbate PD symptoms. For this reason the inhibition of these CWDEs may alleviate excessive tylose production, allowing more xylem vessels to remain open and minimize drought-stress symptoms. However, the inhibition of each individual CWDE is neither practical nor economical. As these CWDEs are predicted to be T2SS-secreted, inhibition of this secretion system will likely prevent both PM degradation (and subsequent systemic colonization) and minimize excessive host defense responses. Therefore, we will continue on into the final phase of this project, looking for natural products that can inhibit the T2SS and block the proliferation of *Xf* CWDEs.

REFERENCES CITED

Aleemullah M, Walsh KB. 1996. Australian papaya dieback: Evidence against the calcium deficiency hypothesis and observations on the significance of laticifer autofluorescence. *Australian Journal of Agricultural Research* 47:371-385.

Benedetti M, Pontiggia D, Raggi S, et al. 2015. Plant immunity triggered by engineered *in vivo* release of oligogalacturonides, damage-associated molecular patterns. *Proc Natl Acad Sci USA* 112:5533-8.

Boller T, Felix G. 2009. A renaissance of elicitors: Perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu Rev Plant Biol* 60:379-406.

Bonsen KJM, Kučera LJ. 1990. Vessel occlusions in plants: Morphological, functional, and evolutionary aspects. *International Association of Wood Anatomists Bulletin* 11:393-399.

Buchanan BB, Gruissem W, Jones RL. 2000. The Cell Wall. Chapter 2, pp. 52-100 in *Biochemistry and Molecular Biology of Plants*. American Society of Plant Physiologists. Maryland.

Chatterjee S, Almeida RPP, Lindow S. 2008. Living in two worlds: The plant and insect lifestyles of *Xylella fastidiosa*. *Annual Review of Phytopathology* 46:243-71.
Cianciotto NP. 2005. Type II secretion: A protein secretion system for all seasons. *Trends Microbiol* 13:581-8.
Collins BR, Parke JL, Lachenbruch B, Hansen EM. 2009. The effects of *Phytophthora ramorum* infection on hydraulic conductivity and tylosis formation in tanoak sapwood. *Canadian Journal of Forest Research / Revue Canadienne De Recherche Forestiere* 39:1766-1776.
Dimond AE. 1955. Pathogenesis in the wilt diseases. *Annual Review of Plant Physiology and Plant Molecular Biology* 6:329-350.
Earles JM, Knipfer T, Tixier A, Orozco J, Reyes C, Zwieniecki MA, Brodersen CR, McElrone AJ. 2018. *In vivo* quantification of plant starch reserves at micrometer resolution using X-ray microCT imaging and machine learning. *New Phytologist*.
Esau K. 1977. *Anatomy of Seed Plants*. Second Edition. Wiley, New York, NY.
Fritschi FB, Lin H, Walker MA. 2008. Scanning electron microscopy reveals different response pattern of four *Vitis* genotypes to *Xylella fastidiosa* infection. *Plant Disease* 92:276-286.
Gough CL, Dow MJ, Barber CE, Daniels MJ. 1988. Cloning of two endoglucanase genes of *Xanthomonas campestris* pv. *campestris*: Analysis of the role of the major endoglucanase in pathogenesis. *Molecular Plant Microbe Interactions* 1:275-81.
Guilhabert MR, Kirkpatrick BC. 2005. Identification of *Xylella fastidiosa* antivirulence genes: Hemagglutinin adhesins contribute to *X. fastidiosa* biofilm maturation and colonization and attenuate virulence. *Molecular Plant-Microbe Interactions* 18:856-868.
Hopkins DL, Purcell AH. 2002. *Xylella fastidiosa*: Cause of Pierce's disease of grapevine and other emergent diseases. *Plant Disease* 86:1056-66.
Jha G, Rajeshwari R, Sonti RV. 2005. Bacterial type two secretion system secreted proteins: Double-edged swords for plant pathogens. *Molecular Plant Microbe Interactions* 18:891-8.
Korotkov KV, Sandkvist M, Hol WG. 2012. The type II secretion system: Biogenesis, molecular architecture, and mechanism. *Nature Reviews Microbiology* 10:336-51.
Mohammadi M, Burbank L, Roper MC. 2012. *Pantoea stewartii* subsp. *stewartii* produces an endoglucanase that is required for full virulence in sweet corn. *Molecular Plant Microbe Interactions* 25:463-70.
Mollenhauer HH, Hopkins DL. 1974. Ultrastructural study of Pierce's disease bacterium in grape xylem tissue. *J Bacteriol* 119:612-8.
Parameswaran N, Knigge H, Liese W. 1985. Electron microscopic demonstration of a suberized layer in the tylosis wall of beech *Fagus sylvatica* and oak *Quercus robur*. *Intl. Assoc. Wood Anatomists Bull.* 6:269-271.
Parke JL, Oh E, Voelker S, Hansen EM, Buckles G, Lachenbruch B. 2007. *Phytophthora ramorum* colonizes tanoak xylem and is associated with reduced stem water transport. *Phytopathology* 97: 1558-1567.
Perez-Donoso AG, Sun Q, Roper MC, Greve LC, Kirkpatrick B, Labavitch JM. 2010. Cell wall-degrading enzymes enlarge the pore size of intervessel pit membranes in healthy and *Xylella fastidiosa*-infected grapevines. *Plant Physiology* 152:1748-59.
Purcell AH, Hopkins DL. 1996. Fastidious xylem-limited bacterial plant pathogens. *Annu Rev Phytopathol* 34:131-51.
Roberts DP, Denny TP, Schell MA. 1988. Cloning of the egl gene of *Pseudomonas solanacearum* and analysis of its role in phytopathogenicity. *J Bacteriol* 170:1445-51.
Roper MC, Greve LC, Warren JG, Labavitch JM, Kirkpatrick BC. 2007. *Xylella fastidiosa* requires polygalacturonase for colonization and pathogenicity in *Vitis vinifera* grapevines. *Molecular Plant Microbe Interactions* 20:411-9.
Saile E, McGarvey JA, Schell MA, Denny TP. 1997. Role of extracellular polysaccharide and endoglucanase in root invasion and colonization of tomato plants by *Ralstonia solanacearum*. *Phytopathology* 87:1264-71.
Simpson AJ, Reinach FC, Arruda P, et al. 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. *Nature* 406:151-9.
Slonczewski JL, Foster JW. 2014. *Microbiology: An Evolving Science*. W.W. Norton & Company, NY, NY.
Sun Q, Sun Y, Walker MA, Labavitch JM. 2013. Vascular occlusions in grapevines with Pierce's disease make disease symptom development worse. *Plant Physiol* 161:1529-41.
Tyree MT, Zimmermann MH. 2002. *Xylem Structure and the Ascent of Sap*. 2nd Ed. Springer, Berlin, Germany.

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

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Reporting Period: The results reported here are from work conducted September 6, 2018 to October 4, 2018.

ABSTRACT
The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas gene editing technology allows for precise alterations in plant genomes. Given the revolution occurring in gene editing technology, protoplast culture provides one of the best avenues for producing non-chimeric gene edited plants for clonally propagated species. Although non-protoplast-based gene editing techniques are being developed for many crops, recovery of non-chimeric gene edited plants is still problematic. In seed propagated crops, gene editing technology can be introduced via *Agrobacterium tumefaciens* or biolistic-mediated DNA delivery systems. Once gene editing has been accomplished, the CRISPR-Cas insert can be segregated out of the population in the next generation with the null segregant, containing only the desired gene edit and advanced using traditional plant breeding. However, for clonally propagated plants like wine grapes, it is not possible to use breeding to eliminate the CRISPR-Cas insert and still maintain the fidelity of the clonal germplasm. CRISPR-Cas has been introduced into plant protoplasts using polyethylene glycol or electroporation and expressed transiently without integration of the CRISPR-Cas DNA. Cell walls re-form on the protoplast in 48 to 72 hours and the edited cells can be stimulated to form mini callus colonies and subsequently regenerated into whole plants. We plan to utilize advances we have made in grape cell biology to develop a method to isolate plant protoplasts from grape suspension cultures, generate mini callus colonies from the protoplast and regenerating whole plants from the callus. The UC Davis Plant Transformation Facility has developed cell biology capability in grape culture, which includes the establishment of suspension cultures, formation of somatic embryos from those cultures, and regeneration of whole plants from the somatic embryos. This project will explore whether these advances in grape cell biology can be utilized to facilitate the recovery of whole plants from suspension-derived grape protoplasts.

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

INTRODUCTION
The development of a system that allows the isolation of grape protoplast, formation of mini calli, and the ultimate regeneration of protoplast-derived plants has significant relevance to the Pierce's disease research community and the winegrape industry. It provides an excellent vehicle for deploying non-*Agrobacterium*-mediated non-integrating gene editing technology for fundamental research and product development. Even if the goal of regeneration of plants from protoplasts is not achieved, efficient formation of protoplast-derived mini calli can be used for high throughput testing of potential gene editing guide RNAs. If regeneration of whole plants can be achieved, it will allow for the production of non-chimeric gene edited plants, which is critical for clonally

propagated crops such as grape. Recently, the United States Department of Agriculture announced that it does not regulate or have any plans to regulate plants generated using gene editing techniques that could otherwise have been developed through traditional breeding techniques as long as they are not plant pests or developed using plant pests.

Protoplast technology was actively researched in the 1980s and early 1990s, but the advent of transgenic technology resulted in this cell culture technique falling out of favor. Although there are published reports in the literature demonstrating successful isolation of protoplasts from grapes, production of mini calli from grape protoplasts has historically proven to be inefficient, with less than 5% of the isolated protoplasts forming calli (Xu et al., 2007). In addition, to my knowledge, regeneration of grape plants from protoplasts has not yet been achieved. We believe that utilizing rapidly dividing grape suspension cultures may provide advantages over other tissue sources. Encapsulating protoplasts in alginate beads and culturing in conditioned medium or nurse cultures may enhance the frequency of protoplast division. It will also allow us to test many different media components by culturing beads in a 24-well plate format. Given that our suspension cultures are highly efficient in regenerating embryos and plants, and given that the protoplasts will be produced directly from these suspensions, we believe these suspensions give us the best possibility of regenerating embryos and plants from protoplast-derived callus. However, it should be mentioned that protoplast technology has a number of challenges that will affect its utility for broad-based use in gene editing technology. The major challenges include the low transfection rates of DNA into protoplasts by polyethylene glycol (PEG) or electroporation, poor survival of PEG or electroporated protoplasts, low plating efficiency of protoplasts, and low frequency of regeneration of plants from protoplast-derived mini calli. In addition, even for systems that are amenable to protoplast culture like lettuce, regeneration of plants from protoplast can be highly genotype dependent. This project is focused on the isolation of grape protoplasts, reformation of their cell walls, development of mini calli, and regeneration of whole plants.

OBJECTIVES

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

RESULTS AND DISCUSSION

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

For 2018, we have established new somatic embryogenic cultures for Merlot, 1103P, and Thompson Seedless from anther filaments harvested from immature flowers collected from the UC Davis Foundation Plant Service's vineyards **(Figure 1).**

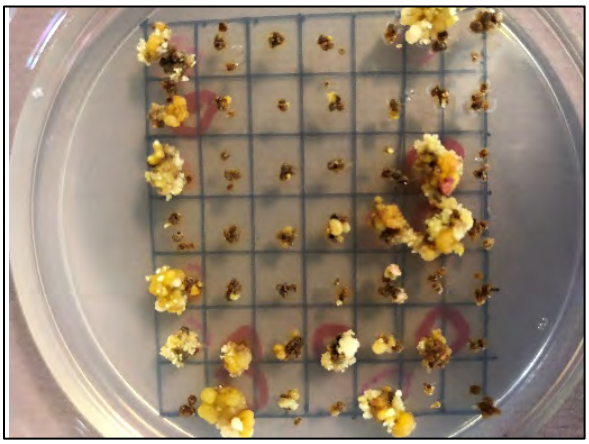

Figure 1. Somatic embryos of Merlot clone 3 generated from anther filaments and increased *in vitro*.

We have used these somatic embryo cultures to establish new 2018 embryogenic suspension cultures. Somatic embryos from anther filaments were transferred from agar-solidified plates to liquid woody plant medium (WPM; Lloyd and McCown, 1981) supplemented with 20 g/l sucrose, 1 g/l casein hydrolysate, 10.0 mg/l Picloram, 2.0 mg/l metatopolin, 2 g/l activated charcoal, 100 mg/l ascorbic acid, and 120 mg/l reduced glutathione (Pic/MTag)

and grown in 125 ml shake flasks on a gyratory shaker at 90 rpms in the dark. (**Figure 2**). These suspensions are being used as a source of embryogenic cells for protoplast isolation.

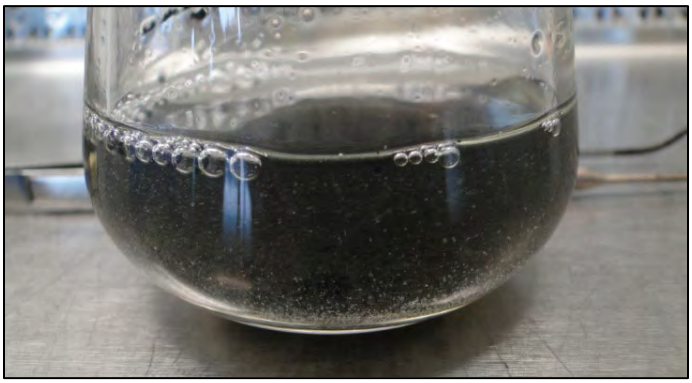

Figure 2. Fine suspension cultures of 1103P growing in WPM, 1 g/l casein, 1 mM MES, 1,000 mg/l activated charcoal, 10 mg/l picloram, and 2 mg/l meta-topolin.

When aliquots (0.5ml) of these suspensions are plated onto the appropriate agar-solidified medium, (WPM supplemented with 20 g/l sucrose, 1 g/l casein, 1 mM 2-(N-morpholino)ethanesulfonic acid (MES), 500 mg/l activated charcoal, 0.1 mg/l BAP and 8 g/l agar) and cultured in the light, these suspensions exhibit a high frequency of regeneration into whole plants. These suspension cultures should serve as an excellent potential source of tissue for protoplast isolation and plant regeneration **(Figure 3)**.

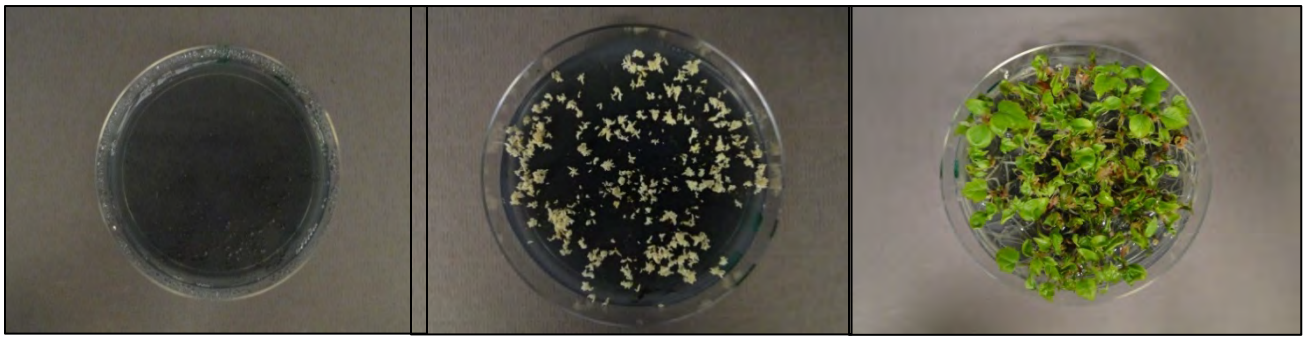

Figure 3. Chardonnay culture after plating 0.2 ml of suspensions onto grape embryogenesis medium (left). Somatic embryos of Chardonnay developing from plated cell suspensions (middle). Regeneration of whole plants of Chardonnay after transferring embryos to regeneration medium and culturing in the light (right).

Protoplast Isolation. Aliquots of rapidly dividing embryogenic grape suspension cultures of 1103P were collected and centrifuged to harvest 3-5 ml packed cell volume. The supernatant was removed and replaced with 10 ml of protoplast isolation solution and transferred to a 60 x 15 mm petri dish. The enzyme solution consisted of filter sterilized 0.5% Onozuka Cellulase R10, 0.25% pectinase, 0.25% macerozyme R10, 0.4 M mannitol, 5 mM $CaCl_2$, 10 g/l bovine serum albumin (BSA), and 5 mM MES. The enzyme solution containing the suspension culture was subjected to vacuum infiltration under house vacuum for three, two-minute exposures, incubated in the dark at 25ºC, and agitated at 50 rpms. After approximately 16 hours incubation, the protoplast containing solutions were filtered through a 40 µm screen and the protoplasts collected by pelleting under centrifugation at 2,000 rpm for 10 minutes. The protoplasts were washed twice in an osmotically adjusted wash solution containing 0.4 M mannitol, 2 mM $CaCl_2$, 1 g/l BSA and 1,191 mg/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). We initially tried to purify the protoplast using a dextran gradient consisting of 4 ml of a 13% dextran solution, overlaid with 2 ml of a 9.1% dextran solution, overlaid with 1 ml of 0.4 M wash solution. We have successfully used this dextran gradient to isolate lettuce and soybean mesophyll protoplast. However, when this gradient was used for grape suspension cultures and centrifuged at 2,000 rpm for eight minutes we found that the activated charcoal that is used in the suspension culture layers at the same band as the protoplast; the interface of the 9.1%

dextran and the wash solution. We therefore modified the dextran gradient by adding a third dextran layer. The new gradient consists of 4 ml of a 13% dextran solution, overlaid with 3 ml of a 9.1% dextran solution, overlaid with 2 ml 4.05% dextran solution, overlaid with 1 ml of wash solution. When centrifuged at 2,000 rpm for eight minutes the protoplast layer at the interface of the 4.05% dextran layer and the wash solution effectively separate the protoplast with minimal contamination from the activated charcoal. Protoplasts were readily harvested from this layer with a sterile Pasteur pipette, and transferred to 60 x 15 mm petri dishes. Yields of protoplasts from 5 ml packed cell volume ranged from 2.5-6 x10^6 cells per ml **(Figure 4)**.

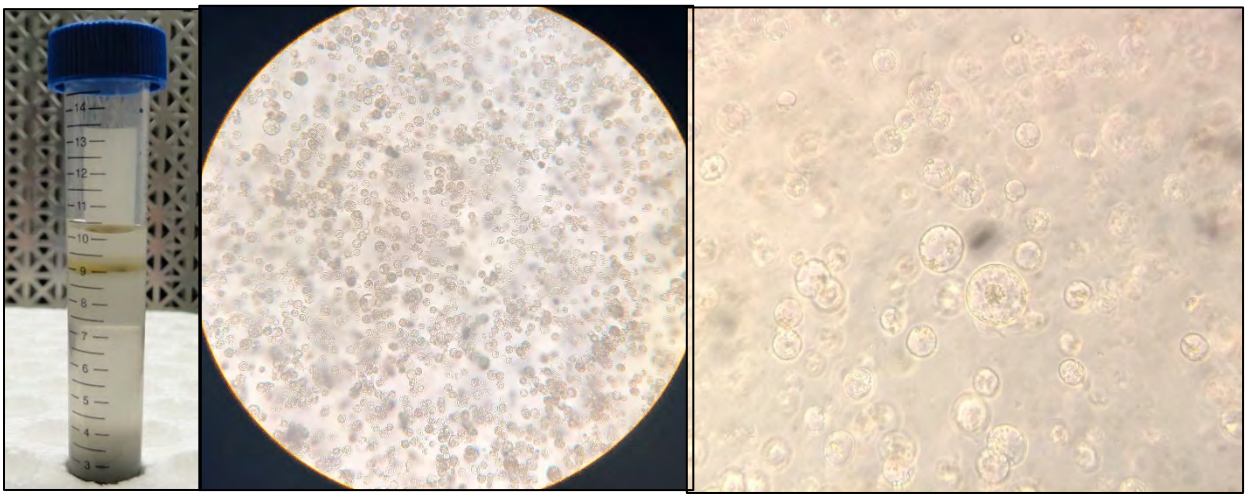

Figure 4. Dextran gradient separates protoplast from charcoal and debris (left). Harvested grape protoplast prior to encapsulation in calcium alginate beads (middle and right).

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

The Plant Transformation Facility has developed a method for encapsulating and culturing protoplasts in alginate beads with or without an osmotically conditioned feeder suspension culture. The feeder suspension is used to stimulate the protoplast to divide to form mini-calli even at low protoplast culture density. This system was demonstrated to be efficacious in soybean (Tricoli et al., 1986) and lettuce protoplasts (Tricoli, unpublished). Osmotically adjusted grape feeder suspensions are being generated by gradually increasing the osmotic potential of the suspension medium over time. During the biweekly subcultures of the suspension cultures, one-half of the medium is removed and replaced with grape suspension medium containing 72.87 g/l mannitol, 1,191 mg/l HEPES, and 1 g/l BSA, pH 5.7 along with the appropriate plant growth regulators. During subsequent biweekly subcultures, one-half of the old medium is again removed and replaced with an equal volume of medium containing 72.87 g/l mannitol, 1,191 mg/l HEPES, and 1g/L BSA, pH 5.7. This process is repeated biweekly allowing cells to gradually acclimate to the high osmotic medium over time. Once suspensions are actively growing on high osmoticum medium, conditioned medium can be harvested on a biweekly basis by centrifugation at 2,000 rpm for 10 minutes. The supernatant is collected and stored at 4ºC or used immediately to culture the bead-encapsulated protoplasts. Alternatively, encapsulated protoplasts are cultured in conditioned suspension cultures as opposed to conditioned medium lacking cells, but great care must be taken to ensure that suspension cells are completely removed prior to the alginate bead being dissolved.

We have begun creating cell suspension cultures acclimated to growing under high osmoticum conditions. The media we are using are formulations used to stimulate somatic embryo development from isolated grape anther filaments. These include:

- Nitsch and Nitsch minimal organics medium (1969) supplemented with 60 g/l sucrose, 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), and 2.0 mg/l benzylaminopurine (BAP) (PIV);
- MS minimal organics medium supplemented with 20 g/l sucrose 1.0 mg/l 2,4-D and 0.2 mg/l BAP (MSE);
- MS minimal organics medium supplemented with 30 g/l sucrose 1.0 mg/l 2,4-D and 1.0 mg/l BAP (MSI); or
- WPM medium supplemented with 20 g/l sucrose 10 mg/l Picloram and 2.0 mg/l thidiazuron (TDZ) (Pic/TDZ).

In order to generate the protoplast containing alginate beads, the protoplast density is adjusted to two times the desired final density with 0.4 M mannitol/buffer solution. The protoplast solution is mixed with an equal volume of 6.4% Na alginate solution (adjusted to pH 5.7). Beads are formed by drawing up the solution into a 12 ml sterile syringe and expelling the solution dropwise through a 23-gauge needle into an osmotically adjusted 50 mM $CaCl_2$ solution. After 30 minutes in the $CaCl_2$ solution, beads are rinsed one time in 0.4 M mannitol/buffer wash solution **(Figure 5).** The size of the beads can be increased or decreased depending on the gauge of the needle.

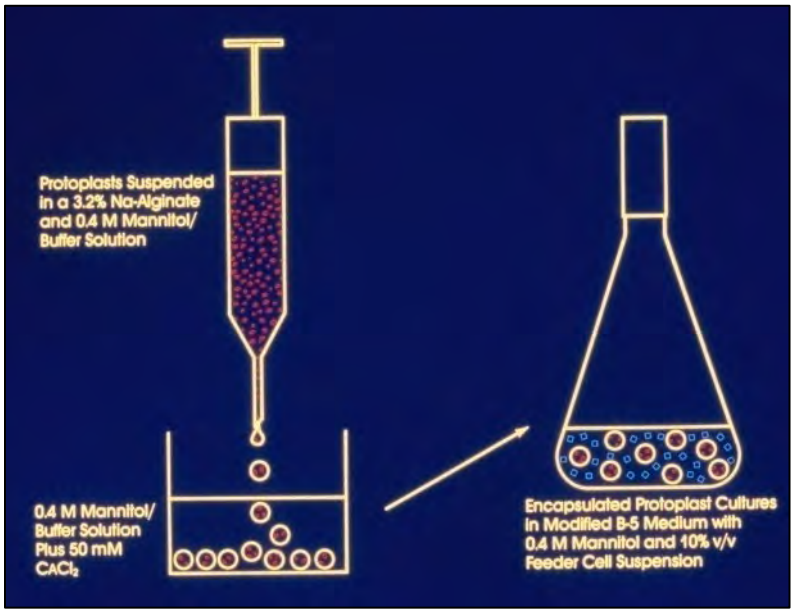

Figure 5. Diagram of the production of protoplast encapsulated in alginate beads and cultured in conditioned medium.

In addition to allowing one to test various media formulation, embedding protoplasts in calcium alginate beads also insures that each protoplast derived callus colony is from single cell descent. This will be important for gene editing experiments since if protoplasts are not fixed in a matrix they will rapidly clump together, making determining single cell descent impossible. Normally when cultured at low density, protoplasts fail to divide. However, culturing embedded protoplast in conditioned medium or with feeder suspensions has been shown to stimulate protoplasts division in other species even at very low cell densities. Since the alginate matrix is permeable to nutrients, the conditioned medium serves as a nurse culture. Previously, we have demonstrated that a single protoplast encapsulated in a 3-4 mm alginate bead could be stimulated to divide using this nurse culture system for both soybean and lettuce **(Figure 6).** Recently, we have successfully embedded grape protoplast in calcium alginate beads and they have survived the embedding process **(Figure 7).**

We will begin testing various conditioned media for their ability to stimulate cell division of the grape protoplasts. Embedded protoplasts are re-suspended in the conditioned osmotically adjusted grape suspension culture medium with or without cells, transferred to shake flasks, and incubated at 100 rpm and 25ºC. Viable protoplasts are expected to begin dividing in four to seven days. After twelve days, the 0.4 M mannitol conditioned medium is replaced with grape suspension culture medium without mannitol, thereby reducing the starting mannitol concentration to 0.2 M.

We will also culture encapsulated protoplasts in 24-well culture plates, which will allow us to test multiple hormone and media formulations using a factorial design. This system of alginate bead encapsulated protoplasts and the use of 24-well culture dishes will allow us to test a wide range of media and hormone combinations for their ability to stimulate cell division.

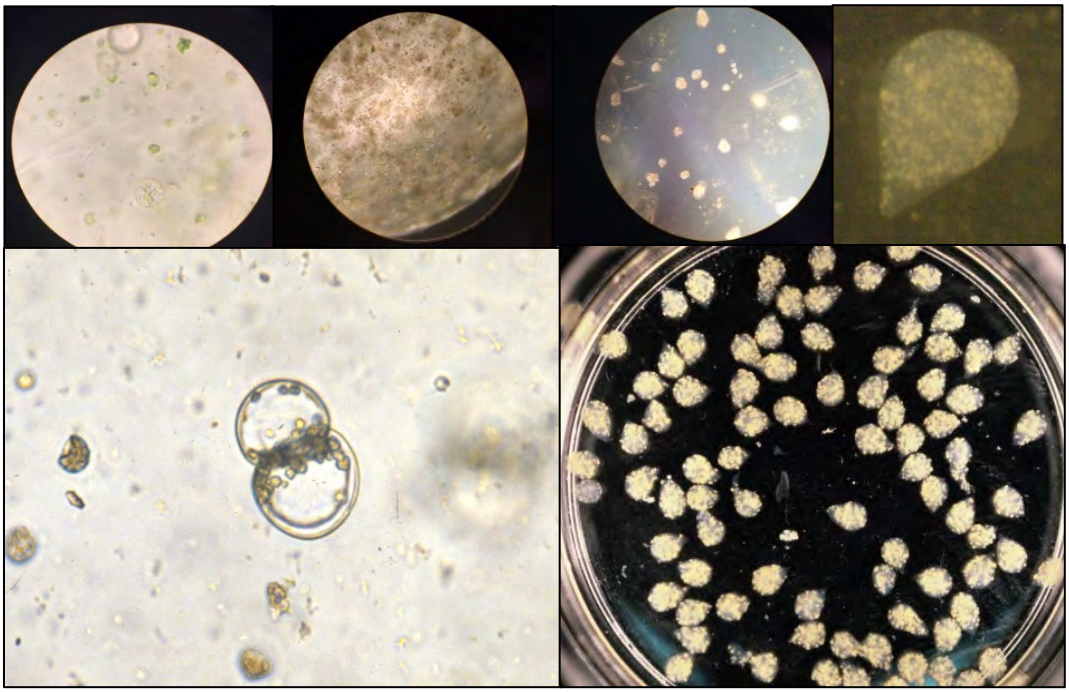

Figure 6. Alginate encapsulated mesophyll protoplast of lettuce (top) and soybean (bottom) dividing to form mini callus colonies when grown in conditioned medium.

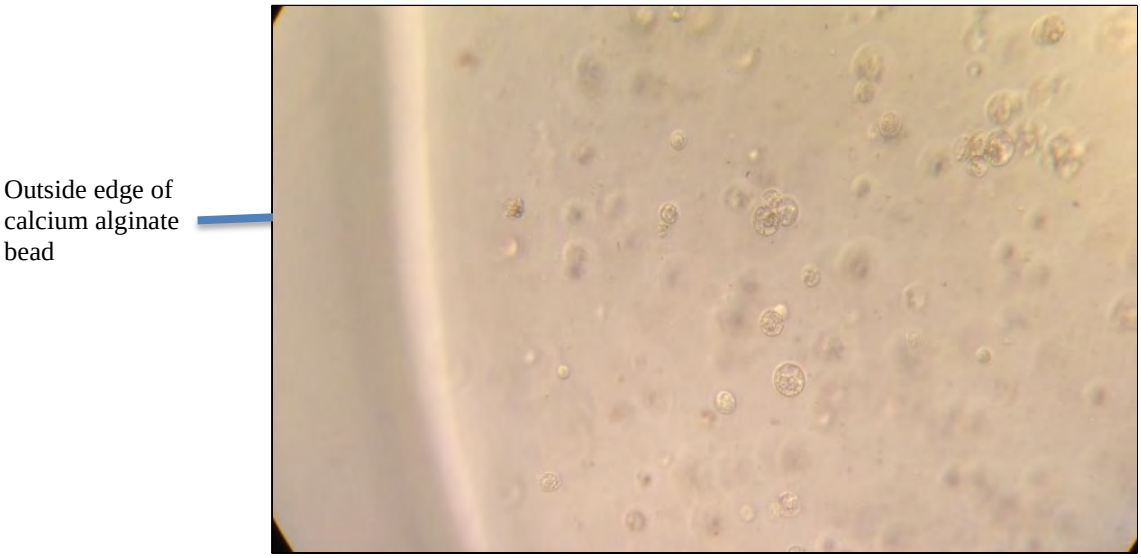

Figure 7. Grape protoplast encapsulated in calcium alginate bead.

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

Once mini callus colonies develop, the calcium alginate matrix is dissolved by transferring the beads to an osmotically adjusted 0.05 M potassium phosphate solution and agitating at 125 rpm for 120 minutes. The resulting solution is diluted and plated onto agar-solidified media of various formulations for further callus development and ultimately plant regeneration. For lettuce, we have previously demonstrated protoplast isolation, encapsulation, division, and mini-calli formation in calcium alginate beads and have dissolved the alginate matrix and regenerated whole plants **(Figure 8).**

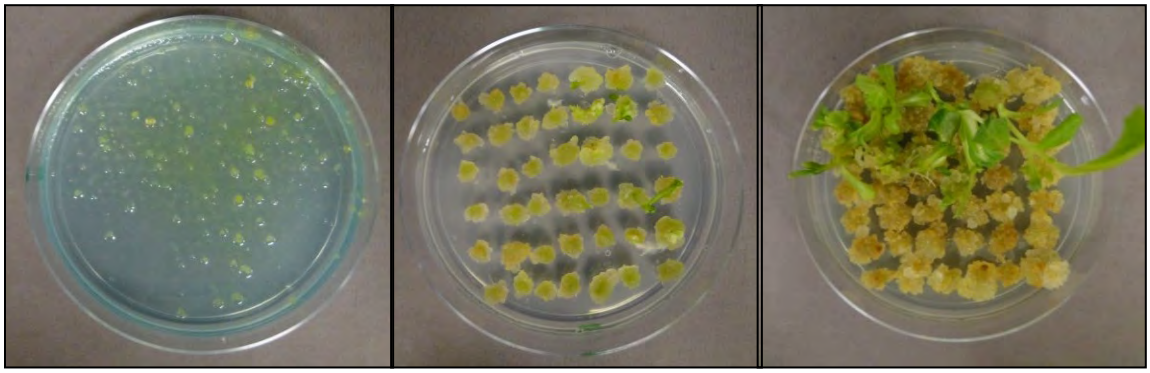

Figure 8. Lettuce protoplast derived mini-callus colonies plated onto agar-solidified medium after dissolving the alginate bead in an osmotically adjusted 0.05 M potassium phosphate solution (left). Early shoot regeneration from protoplast-derived callus (middle), and regeneration of whole plants (right).

REFERENCES CITED

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

Lloyd G, McCown BH. 1981. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by shoot tip culture. *Proc. Int. Plant Prop. Soc*. 30:421-427.

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

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

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

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

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BREEDING PIERCE'S DISEASE RESISTANT WINEGRAPES

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Reporting Period: The results reported here are from work conducted October 2017 to October 2018.

ABSTRACT
Breeding Pierce's disease (PD) resistant winegrapes continues to advance, accelerated by aggressive vine training and selection for precocious flowering that has resulted in a seed-to-seed cycle of two years. To further expedite breeding progress, we are using marker-assisted selection (MAS) for PD resistance genes to select resistant progeny as soon as seeds germinate. These two practices have allowed us to produce four backcross generations with elite *Vitis vinifera* winegrape cultivars in 10 years. We have screened through about 2,000 progeny from the 2009, 2010, and 2011 crosses that are 97% *V. vinifera* with the *PdR1b* resistance gene from *V. arizonica* b43-17. We select for fruit and vine quality and then move the best to greenhouse testing, where only those with the highest resistance to *Xylella fastidiosa*, after multiple greenhouse tests, are advanced to multi-vine wine testing at Davis and other test sites. The best of these have been advanced to field testing with commercial-scale wine production, the first of which was planted in Napa in June 2013. To date 20 scion and three PD resistant rootstocks have been advanced to Foundation Plant Services at UC Davis for certification. Five of these selections are now in pre-release to nurseries. Stacking of *PdR1b* with PD resistance from b42-26 (an alternative form of PD resistance controlled by multiple genes) has been advanced to the 96% *V. vinifera* level using MAS to confirm the presence of *PdR1*, as well as the recently discovered (see companion report) PD resistance locus on Chromosome 8 from b42-26, *PdR2*. 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), Healdsburg (Dry Creek Valley and Sonoma Grape Growers and Winemakers), and UC Davis.

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 (PD) resistance from wild grape species into a diverse selection of high quality winegrapes. To date we have identified two different chromosome regions that house very strong sources of PD 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., 2018) - we have been able to use marker-assisted selection (MAS) to screen for DNA markers associated with both *PdR1* and *PdR2*, allowing us to select resistant progeny shortly after seeds germinate. MAS and aggressive training of the selected seedling vines have allowed us to produce new PD 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 PD hot spots around California. The best of these are advanced to field plots where commercial-scale wines can be produced. We have sent 20 advanced selections to Foundation Plant Services (FPS) over the past six winters to verify their virus-free status. Five of these selections are now in pre-release to nurseries. Three PD resistant rootstocks were also sent to FPS for certification. Other wild grape species are being studied and the resistance of some will be genetically mapped for future efforts to combine multiple resistance sources and ensure durable PD resistance. Very small-scale wines made from our advanced *PdR1* selections have been very good and received well at professional tastings throughout California.

INTRODUCTION

We continue to make rapid progress breeding Pierce's disease (PD) resistant winegrapes. Aggressive vine training and selection for precocious flowering have allowed us to reduce the seed-to-seed cycle to two years. To further expedite breeding progress we are using marker-assisted selection (MAS) for the PD resistance loci *PdR1* and *PdR2* to select resistant progeny as soon as seeds germinate. These two practices have greatly accelerated the breeding program and allowed us to produce four backcross (BC) 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 20 scion and three PD resistant rootstocks have been advanced to Foundation Plant Services (FPS) at UC Davis for certification. Five of these have been pre-released to grapevine nurseries to build up the amounts available for grafting. Stacking of *PdR1b* with b42-26 Pierce's disease resistance has been advanced to the 96% *V. vinifera* level using MAS to confirm the presence of *PdR1* as well as the recently discovered (see companion report) PD resistance locus on LG8 from b42-26, *PdR2*. Initial selections for release will begin in 2018. Greenhouse screening is used to advance only genotypes with the highest possible levels of PD 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. PD 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 positioned to undertake this important breeding effort, having developed rapid screening techniques for *Xf* resistance (Buzkan et al., 2003; Buzkan et al., 2005; Krivanek et al., 2005a, 2005b; Krivanek and Walker, 2005; Baumgartel, 2009), and having unique and highly resistant *V. rupestris* x *V. arizonica* selections, as well as an extensive collection of southwestern grape species, which allows the introduction of extremely high levels of *Xf* resistance into commercial grapes. We genetically mapped and identified what seems to be a single dominant gene for *Xf* resistance in *V. arizonica/candicans* b43- 17 and named it *PdR1*. This resistance has been backcrossed through four generations to elite *V. vinifera* cultivars (BC4) and we now have 97% *V. vinifera* PD resistant material to select from. Individuals with the best fruit and vine characteristics are then tested for resistance to *Xf* under our greenhouse screen. Only those with the highest levels of resistance are advanced to small-scale winemaking trials by grafting them onto resistant rootstocks and planting six to eight vine sets on commercial spacing and trellising at PD 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 ten years and from the 97% *V. vinifera* level for seven 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 PD. 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 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 PD resistance locus from *V. arizonica* b40-14 (*PdR1c*) that maps to the same region of Chromosome (Chr) 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 PD symptom expressions, we have continued to advance the b40-14 (*PdR1c*) resistance line as a future breeding resource. Our companion research project is pursuing the genetic basis of these differences between *PdR1b* and *PdR1c*. In 2005, we started a PD resistant breeding line from another Mexican accession, b42-26. Markers linked to this resistance proved elusive but strong resistance 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 MAS to select for *PdR1b* and a higher than usual resistance in our greenhouse screen to

move the b42-26 resistance forward. Late in 2016 our companion project identified the location of a significant PD resistance locus from b42-26 on Chr 8, which we have called *PdR2*. In 2014, we advanced our *PdR1* x *PdR2* line to the 92% *V. vinifera* level and in spring 2016 made crosses to advance it to the 96% *V. vinifera* level. MAS was used to advance only genotypes with both *PdR1b* and *PdR2* for the first time on these crosses. The resistance from southeastern United States species 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 PD resistance with a focus on accessions collected from the southwestern United States and northern Mexico. Develop F1 and BC1 populations from the most promising new sources of resistance. Evaluate the inheritance of resistance and utilize populations from the most resistant sources to create mapping populations.
2. Provide support to the companion mapping/genetics program by establishing and maintaining mapping populations, and using the greenhouse screen to evaluate populations and selections for PD resistance.
3. Develop advanced lines of PD resistant winegrapes from unique resistance sources through four backcross generations to elite *V. vinifera* cultivars. Evaluate and select on fruit quality traits such as color, tannin content, flavor, and productivity. Complete wine and fruit sensory analysis of advanced selections.
4. Utilize MAS to stack (combine) different resistance loci from the BC4 generation with advanced selections containing *PdR1*. Screen for genotypes with combined resistances, to produce new PD resistant grapes with multiple sources of PD resistance and high-quality fruit and wine.

RESULTS AND DISCUSSION

We reached the 97% *V. vinifera* level in the *PdR1b* line in 2009 and finished planting out additional crosses at that level in 2011. A total of 2,911 genotypes were planted in the 2010-12 period. Subsequently, thousands of plants were subjected to our rapid greenhouse screen and rigorous field evaluations, and a select few chosen for small-scale winemaking. At this time, five selections at this level are in pre-release to California grapevine nurseries for expansion of graftable material and sale to growers as early as 2020. A dozen others are also at FPS and completing late stage winemaking evaluations for possible future release.

Another area of focus, and one that should produce our next line of PD resistant winegrape selections for release, are those that stack *PdR1b* resistance from b43-17 and *PdR2* resistance from b42-26. In 2017 we planted 126 seedlings from four different crosses that are 96% *V. vinifera* and have both resistance loci. **Table 1** shows the distribution of greenhouse resistance ratings for each cross for the first 77 genotypes tested. Although promising in that we see some genotypes with R-ratings above their parental means, we don't see genotypes scoring in the most resistant 10 category. Scores of five are adequate for release as they have enzyme-linked immunosorbent assay (ELISA) titer values statistically the same as uninoculated Chardonnay. However, genotypes in this category do have more PD symptoms in our greenhouse screen than we like to see but the greenhouse screen is much more severe than what the plants experience in the field, and plants scoring five should perform well there.

Table 1. Greenhouse screen results from the first screening of 77 genotypes at the 96% *V. vinifera* level with both *PdR1b* and *PdR2*.

Female Parent	Male Parent	Parental mean R-rating	PD R-rating category				Count of Genotypes tested
			-1 = S	1 = R	5 = Very R	10 = Immune	
14309-111	Primitivo	2.2	9	12	1		22
14309-111	Cabernet Sauvignon	2.2	1	11	3		15
14388-029	Chardonnay	3.6	1	13	2		16
F2-35	14309-016	3.3	3	19	2		24
R-rating totals			14	55	7	0	77

Fruit evaluations were conducted this fall and three of the seven most resistant also demonstrated promising fruit and horticultural characteristics. Results of these are shown in **Tables 2a-c** and **Figure 1**. These and other selections are currently being retested (**Table 8g**) in the greenhouse to verify the high level of PD resistance.

Table 2a. Three promising 96% *V. vinifera PdR1b* x *PdR2* PD resistant selections: Background and fruit characteristics.

Genotype	Parentage	2018 Bloom Date	2018 Harvest Date	Berry Color	Berry Size (g)	Ave Cluster Wt. (g)	Prod 1 = v low, 9 = v high
16353-072	14388-029 x Chardonnay	05/25/2018	08/30/2018	W	1.0	160	6
16329-015	14309-111 x Primitivo	05/29/2018	08/30/2018	B	1.3	199	8
16333-022	14309-111 x Cab Sauvignon	05/22/2018	08/30/2018	B	1.3	286	4

Table 2b. Juice analysis of three promising 96% *V. vinifera PdR1b* x *PdR2* PD resistant selections.

Genotype	°Brix	TA (g/L)	pH	L-malic acid (g/L)	potassium (mg/L)	YAN (mg/L, as N)	catechin (mg/L)	tannin (mg/L)	Total antho-cyanins (mg/L)
16353-072	25.4	8.2	3.28	2.4	1,780	167			
16329-015	25.6	6.4	3.64	3.3	2,060	260	97	649	2,344
16333-022	23.4	6.6	3.53	3.5	2,020	223	146	589	1,618

Table 2c. Three promising 96% *V. vinifera PdR1b* x *PdR2* resistant selections: 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)
16353-072	Green tech yellow	Med-	Green apple, pear, slight spice	spicy, slight green hay	2	4	Spicy, woody, warm	3
16329-015	Red	Dark-	Strawberry jam, sweet spices	Berry, fruity	1	3	Spicy, hot	2
16333-022	Red-orange	Light+	Fruity, like PN	spicy, slight grass	2	2	Woody, spicy	3

Figure 1. Three promising 96% *V. vinifera PdR1b* x *PdR2* PD resistant selections (l – r): 16353-072, 16329-015, and 16333-022.

In 2017 we expanded the diversity of elite *V. vinifera* parents used in the 96% *V. vinifera PdR1* x *PdR2* breeding line (**Table 3**). These will give us varieties with a wide range of fruit and horticultural characteristics to present to the industry. A total of 328 MAS tested seedlings were planted from 1,095 seedlings tested. This may appear low relative to previous MAS efficiencies but is the result of screening for two dominant resistance loci rather than our more typical single locus. The expected seedlings retained should be about 25%. Overall for this group we averaged approximately 30% retained, with a range among the crosses from 5% to 43%. Clearly some crosses experienced significant segregation distortion, both positive and negative. Initial greenhouse screening will begin over the winter. These should fruit for the first time in 2019.

Table 3. 2017 Crosses of elite *V. vinifera* cultivars to three PD resistant genotypes that have both the *PdR1b* and *PdR2* loci. Progeny are 96% *V. vinifera.*

Resistant Parent	***V. vinifera* Parent**	**Seeds Planted**	**Seedlings Saved**	**Seedlings MAS Tested**	**Seedlings Planted**
14309-002	Alvarelhao	119	56	50	16
	Dolcetto	201	56	50	11
	Mataro	111	32	30	10
	Montepulciano	169	80	75	10
	Pinot noir FPS32	156	56	50	13
	Pinot noir FPS77	199	56	50	9
	Refosco	150	56	50	12
	Touriga Nacional	431	80	75	26
14309-111	Dolcetto	200	80	75	32
	Mataro	337	128	125	49
	Morrastel	80	56	50	13
	Refosco	223	128	120	48
14388-029	Arneis	173	56	50	9
	Morrastel	271	80	75	25
	Pedro Ximenez	316	56	50	16
	Pinot noir FPS32	75	32	25	2
	Refosco	48	24	20	1
	Sauvignon vert	296	80	75	26

Over the duration of our PD breeding program, more than 322 wild accessions have been tested for PD resistance with the greenhouse screen, most of which were collected from the southwestern United States and Mexico. Our goal is to identify accessions with the most unique PD resistance mechanisms. Thus, we evaluate the genetic diversity of these accessions and test them for genetic markers from Chr 14 (where *PdR1* resides) to ensure that we are choosing genetically diverse resistance sources for population development and greenhouse screening efforts. Fifteen of the most unique accessions were used to develop F1 populations with *V. vinifera* to investigate the inheritance of PD resistance in their F1 progeny and the degree to which they resist *Xf*. Most of the resistance lines we have explored from the southwestern United States have PD resistance associated with Chr 14, the same region as our primary resistance line *PdR1b* (Riaz, 2016*)*. Our mapping project identified *PdR2* on Chr 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 Chr 8. Until we better understand the nature of Chr 8 PD resistance and explore additional resistance loci in these lines, it is important to continue advancing multiple sources of Chr 8 resistance.

Three particularly promising and diverse accessions of the 15 were chosen for more extensive testing. **Table 4** presents the greenhouse screen resistance distribution of the F1 progeny of these three possible new PD resistant sources. In contrast to our LG14 resistance sources, few genotypes are seen to manifest the highest levels of

resistance. With *PdR1* lines we breed with genotypes in the 10 and occasionally 5 categories. For the b42-26 lines we have typically had to use genotypes in the 5 category as parents. Should further testing in these F1 populations fail to yield satisfactory parental material, we will approach the problem either by adding an intercross generation to regain resistance, cross to a wide range of *V. vinifera* parents looking for fortuitous combinations, or expand populations and look for transgressive segregants.

Table 4. Greenhouse screen results on 328 F1 genotypes from three new PD resistance sources. PD rating categories are based on both *Xf* titer by ELISA and degree of PD symptom expression: -1 = *Xf* titer statistically higher than our U0505-01 88% *PdR1b* resistant biocontrol; 1 = R with *Xf* titer statistically the same as our U0505-01 biocontrol; 5 = Very R with *Xf* titer the same as the uninoculated Chardonnay control but having some phenotypic symptoms of PD; 10 = Immune - *Xf* titer below ELISA detection threshold and no PD symptoms.

PDR Source	PD Rating Category				PDR Source Totals
	-1 = S	1 = R	5 = Very R	10 = Immune	
ANU67	21	9			30
b41-13	68	56	21	7	152
T 03-16	58	79	9		146
Category Total	147	144	30	7	328

Early on we noticed the very limited number of highly resistant progeny in the T 03-16 line. Thus, in 2016 and 2017 we made small trial populations comprised of nine intercrosses and three selfs using eight of the more resistant T 03-16 F1 progeny as parents (**Table 5**). We have only completed greenhouse screens on 27 genotypes from three different crosses. Results are shown in **Table 6**. Admittedly, the numbers tested are small but the fact that the self of 13336-018 didn't increase resistance in the progeny while the cross of 13336-046 x 13336-018 did appears promising and warrants a more complete testing of these and the rest of the cross combinations. Should further greenhouse testing validate these results and reveal other crosses that have progeny in the 5 and 10 categories, larger mapping populations can be created to identify resistance loci for future MAS. Crosses were made in 2018 to further expand these F1 populations as well as the ANU14 line with an estimated total of 940 seeds.

Table 5. Small test populations of the T 03-16 resistance source made by intercrosses and selfs. Decimal numbers are mean parental PD R-rating, while whole numbers are number of genotypes in the field for that cross combination made in 2016 and 2017. Highlight colors correspond to the same cross in **Table 6**.

Female x Male	Female Ave R-Rating	13336-018		13336-025		13336-034		13336-068	
M Ave R-Rating		1.0		1.0		2.3		3.0	
13302-10	3.0					2.7	30	3.0	8
13302-19	2.0					2.3	50		
13336-018	1.0	1.0	30						
13336-034	2.3					2.3	35		
13336-046	1.7	1.3	19	1.3	11	2.0	30	1.7	12
13336-068	3.0							2.3	12
13336-108	5.3					5.3	50	3.8	12

Table 6. Greenhouse PD R-rating for 27 genotypes tested from two intercross and one selfed population in the T 03-16 resistance background. Highlight colors correspond to the same cross in **Table 5**.

Female	Male	Parental Mean R-Rating	PD R-rating Category				Count of Genotypes Tested
			-1 = S	1 = R	5 = Very R	10 = Immune	
13336-046	13336-018	1.3	8	3	1	1	13
13336-046	13336-025	1.3	2	4			6
13336-018	13336-018	1.0	5	3			8
PD R-rating Category Total			15	10	1	1	27

A focus of our PD breeding efforts in 2018 was to stack PD resistance, either from *PdR1b* alone or in combination with b42-26 resistance, with one or more powdery mildew (PM) resistance sources in elite *V. vinifera* backgrounds. We have genetic markers for PM resistance derived from *V. vinifera* (*Ren1*), *V. romanetii* (*Ren4*), *V. piasezkii* (*Ren6, Ren7*), and two forms from *Muscadinia rotundifolia (Run1 and Run2.1)*. As usual we use MAS to advance only those progeny with resistance markers, the greenhouse screen to select only the most PD resistant, and field and *in vitro* testing for PM resistance. Crosses in the 91-93% *V. vinifera* range were made with the goal of creating highly resistant breeding lines stacked with multiple resistances to cross one last time to a final elite *V. vinifera* cultivar resulting in progeny between 96-98% *V. vinifera*. Those in the 95-97% *V. vinifera* range would be candidates for release. With the exception of 7d where crosses were made directly to elite *V. vinifera* cultivars, the challenge of the other crosses in **Table 7** are both practical, as required for rapid advance of stacking and for inheritance of typical *V. vinifera* characteristics, and perceptual, in terms of easier market acceptance, since they, unlike those in **Table 7d**, don't have a most recent elite *V. vinifera* parent to differentiate them. These factors will require a longer period of horticultural and enological evaluation than has been our experience to date with the crosses bred for PD resistance alone, where the most recent parent has always been a *V. vinifera* cultivar.

Table 7. Estimated number of seeds produced from PD x PM crosses made in 2018. *PdR1b* (F8909-08) is from Monterrey *V. arizonica/candicans* PD resistance b43-17; b42-26 is the Baja California *V. arizonica/girdiana* PD resistance source. *Ren1* and *Ren4* are PM resistance loci from *V. vinifera* and *V. romanetii* respectively. *Run1* and *Run2.1* are PMR loci derived from *Muscadinia rotundifolia*.

Resistances	Recent *V. vinifera* Parents in Background	Percent *V. vinifera*				Total
		91%	93%	95%	97%	
7a. PD - *PdR1b*. PM - *Run1*	Cabernet Sauvignon, Nero d'Avola, Zinfandel, 4 UCD *PdR1b* releases				445	445
7b. PD - *PdR1b*. PM - *Ren1 & Run2.1*	Airen, Cabernet Sauvignon, Riesling, 2 UCD *PdR1b* releases			550		550
7c. PD - *PdR1b*. PM - *Ren1, Ren4 & Run1*	Cabernet Sauvignon, Riesling, 2 UCD *PdR1b* releases			325		325
7d. PD - *PdR1b* with b42-26. PM - *Ren4*	Alvarelhao, Bonarda, Carmenere, Cortese, Fiano, Gouveio, Melon, Pinot blanc, Teroldego, Tinta Amarella, Tinta Cao, 3 UCD *PdR1b* releases		575	1,241		1,816
7e. PD - *PdR1b* with b42-26. PM - *Run1* with either *Ren1* or *Ren4*	Cabernet Sauvignon, Grenache, Touriga Nacional, Zinfandel, 1 UCD *PdR1b* release	100	295			395
7f. PD - *PdR1b* with b42-26. PM - Ren1, Ren4 & Run1	Cabernet Sauvignon, F2-35, Grenache, Zinfandel		256			256

Our rapid greenhouse screen is critical to our evaluation of PD resistance in wild accessions, new F1 and BC1 mapping populations, and for selection of advanced late generation backcrosses for release. **Table 8** provides a list of the PD greenhouse screens analyzed, initiated, and/or completed over the reporting period.

The first 79 genotypes from the 96% *V. vinifera PdR1* x *PdR2* stack line were tested in Group 8a, and represent the first multiple gene more broadly PD resistant candidates for release (**Table 1**). This group also included 50 PD x PM resistant genotypes from 2015 and 2016 crosses, which have *PdR1b* and various combinations of three powdery mildew resistance genes (*Ren1*, *Ren4* or *Run1*). From this we identified some of the parents used in 2018 PD x PM crosses (**Table 7**). Tested for the first time were 28 intercross selections at the 50% *V. vinifera* level in the T 03-16 line to check for possible complementary loci (see **Tables 5 and 6)**. Results of testing 63 BC1 selections in the b41-13 and T 03-16 lines showed an R/S ratio of 25/17 for the former and 9/11 for the latter. No genotypes tested as immune and only three of the b41-13 line were scored very R. We also have another 29 BC1 genotypes crossed to a third b41-13 resistant F1 genotype and following greenhouse screening of those we'll consider further testing or whether to wait for marker results before pursuing this line any further into BC generations.

In Group 8b we retested for the second or third time 93 promising selections that have scored well in previous greenhouse tests to confirm marker efficacy and PD resistance. Twenty-two were identified as very resistant and nine as immune. *V. vinifera* percentage in the latter group ranged from 86 to 94%; two were parents of one of the 8h groups and another was used as a parent in the **Table 7** crosses. We also retested six of the nine double homozygous potential breeding parents mentioned in previous reports. Only one of the six was highly resistant and unfortunately it didn't fruit in 2018.

Table 8. Greenhouse PD screens analyzed, completed, and/or initiated during 2017-18 (projected in italics).

Group	Test Groups	No. of Genotypes	Inoculation Date	ELISA Sample Date	PD Resistance Source(s)
8a	2017 Parents, 96% vin PD Stack, 2015-16 PD x PM crosses; T 03-16 F1, Intercross and BC1; b41-13 BC1	254	12/19/2017	3/16/2018	*PdR1b*, *PdR2*, T 03-16, b41-13
8b	2016 PD crosses, SWUS BC1s, homozygous PD Stack test 2	114	2/8/2018	5/10/2018	*PdR1b*, *PdR2*, b42-26
8c	*PdR1b*x*PdR2*^2, b41-13 F1s	171	3/22/2018	6/19/2018	*PdR1b*, *PdR2*, b46-43, b41-13
8d	*Xf* strain trial (3 strains, 7 BC genotypes, 3 time points)	7	5/24/2018	7/19/2018, 8/2/2018, 8/21/2018	b43-17, SEUS, *PdR1b*
8e	SWUS PD species, b41-13, 2017 parents	133	5/24/2018	8/21/2018	Species, b41-13, *PdR1b*
8f	Mapping Pops, 2015 PD x PM untested	115	6/23/2018	9/25/2018	T 03-16, b41-13, *PdR1b*
8g	92 & 96% PD stack, retest of recent promising	170	8/23/2018	*11/22/2018*	*PdR1xPdR2*
8h	2017 PD x PM, PD Species, 2018 parents	241	*10/9/2018*	*1/8/2019*	Species, *PdR1b* x b42-26

In Group 8c we tested 30 selections that carry *PdR1b* and are homozygous carriers of *PdR2* to identify alternate potential parents that will, when backcrossed to elite *V. vinifera*, result in half the progeny having both *PdR1* and *PdR2*. Eight were identified as very resistant and two as immune. They will be retested to confirm these promising findings. In this same screen we tested 32 BC1 selections in the b46-43 line looking at a different resistant parent to see if inheritance of the resistant phenotype is similar to the 14-399 line being studied by one of the graduate students. The 14-399 results are still being analyzed. To facilitate marker discovery in our companion mapping project, an additional 74 F1 genotypes in the b41-13 line were tested.

The trial in **Table 8d** was a three x three factor matrix testing genotype, *Xf* isolate, and sample date. The genotypes tested were our standard seven southeastern United States (SEUS) and *PdR1b* biocontrols. The *Xf* isolates came from the SEUS cultivar Blanc du Bois, U0505-35 (our intermediate *PdR1b* biocontrol), and Chardonnay, our standard culture source. These were sampled at 8, 9, and 13 weeks to see how *Xf* titer and phenotype scores compare across genotype, strain, and sample date. The goals are twofold: to see if pathogenicity

increases when the culture comes from a resistant plant, and to see if our screen can be shortened to allow us to conduct more screens in a set period of time. ELISA is complete and data analysis is underway.

In Group 8e we tested 81 untested PD species accessions to better characterize our collection and elucidate PD resistance performance by geographical provenance and species. Also tested were twenty-six more F1 genotypes in the b41-13 mapping populations for marker discovery, five promising PD x PM accessions from crosses made in 2015, and the second testing of 2017 PD parents. ELISA results were just completed and analysis is underway. Group 8f continued testing F1 mapping populations with 50 and 27 genotypes, respectively, in the b41-13 and T 03-16 populations. Also tested were 11 untested genotypes from 2015 PD x PM crosses and retests on 20 genotypes identified as highly promising in recent greenhouse screens. Results are expected soon.

In 8g, thirty 96% *PdR1b* x *PdR2* hermaphrodite genotypes are being tested for resistance. Should these have sufficient resistance and have adequate fruit and wine quality, they would be candidates for release similar to the selections presented above in **Tables 1 and 2** and **Figure 1**. An additional 55 genotypes homozygous at either *PdR1* or *PdR2* and having the other resistance source are being tested to see if there is a pattern to high levels of resistance inheritance. Second or third screens are being conducted on 54 genotypes with PD or PD x PM to validate previous results. Confirming screens are being conducted on five 2018 genotypes used as parents that didn't already have three completed screens.

In 8h two main groups are being examined: 78 untested species to better characterize our collection and further elucidate PD resistance performance by geographical provenance and species; and 148 PD x PM crosses from 2017. The latter is of interest as the lines involved have, in the previous two generations, conferred an exceptionally high level of resistance on an exceptionally large percentage of their progeny. Resistance comes from *PdR1b* and b42-26 but with genotypes not having *PdR2*.

Tables 9a through 9c detail the vine, fruit, and juice characteristics for the 16 PD resistant selections used to make wine lots in 2017. 03182-084 is 75% *V. vinifera* with multigenic resistance from the Florida cultivar BD5-117 crossed with a pure *V. vinifera* Cabernet Sauvignon x Carignane genotype. 07355-075 is 94% and thirteen 97% (starting with 09311-160 and ending with 10317-035) *V. vinifera PdR1b* selections represented the majority of wines made. Selection 12351-03 is our most advanced *PdR1a* selection and is also 97% *V. vinifera* most recently crossed to a selfed Zinfandel selection 08319-62. In addition, we made wines from a number of *V. vinifera* controls and Blanc du Bois and Lenoir as reference PD resistant cultivars. Except for the two lots with Napa designations, the wines were made from Davis grown fruit. The Napa lots were brought in to compare analytically and sensorially these two accessions grown in the two locations made at the same scale in the same winery.

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 20 advanced scion selections to Foundation Plant Services (FPS) over the past five 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, Georgia, and Virginia.

Table 9a. The 16 PD resistant selections used in small-scale winemaking in 2018. Background and fruit characteristics. Those with turquoise highlight were pre-released to nurseries in winter/spring 2017.

Genotype	Parentage	2018 Bloom Date	2018 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	05/22/2018	09/18/2018	B	1.5	136	6
07355-075	U0505-01 x Petite Syrah	05/05/2018	08/28/2018	B	1.4	279	7
07355-075 N	U0505-01 x Petite Syrah	05/11/2018	09/18/2018	B	1.3	250	7
09311-160	07371-20 x Cab Sauvignon	05/12/2018	09/11/2018	B	1.2	253	5
09314-102	07370-028 x Cab Sauvignon	05/25/2018	08/23/2018	W	1.6	315	9
09330-07	07370-039 x Zinfandel	05/29/2018	09/04/2018	B	1.6	375	8
09331-047	07355-020 x Zinfandel	05/22/2018	08/28/2018	B	1.6	208	5
09331-047 N	07355-020 x Zinfandel	05/29/2018	09/18/2018	B	1.8	280	5
09331-133	07355-020 x Zinfandel	05/19/2018	09/04/2018	B	1.5	211	6
09333-370	07355-020 x Chardonnay	05/22/2018	09/11/2018	B	1.2	318	6
09338-016	07371-20 x Cab Sauvignon	05/29/2018	09/04/2018	W	1.2	255	6
09356-235	07371-19 x Sylvaner	05/29/2018	08/30/2018	B	1.2	467	7
10302-178	07370-028 x Riesling	05/16/2018	08/14/2018	W	1.0	184	4
10302-238	07370-028 x Riesling	05/04/2018	08/28/2018	W	1.4	210	7
10302-293	07370-028 x Riesling	05/04/2018	08/09/2018	W	0.9	110	4
10302-309	07370-028 x Riesling	05/02/2018	08/21/2018	W	1.2	124	6
10317-035	07370-028 x Riesling	05/15/2018	08/21/2018	W	1.0	134	5
12351-03	08319-62 x 10312-064	05/25/2018	09/06/2018	B	1.3	237	7

Table 9b. Juice analysis of PD resistant selections used in small-scale winemaking in 2018.

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	22.0	4.6	3.48	1.2	1,520	133	48	312	671
07355-075	27.3	5	3.67	2.0	2,340	111	12	621	1,285
07355-075 N	26.4	6.8	3.51	3.3	1,980	154	5	468	1,632
09311-160	23.9	5.8	3.58	2.5	2,100	171	36	205	815
09314-102	24.0	6.1	3.74	4.2	2,440	191			
09330-07	24.2	5.1	3.86	2.4	2,440	231	40	536	1,248
09331-047	26.3	4.9	3.67	1.4	2,080	248	18	540	1,530
09331-047 N	25.9	5.9	3.62	2.0	1,820	325	8	386	1,287
09331-133	24.2	5	3.62	1.5	1,900	137	6	764	1,413
09333-370	22.8	4.5	3.56	1.3	1,760	118	3	459	948
09338-016	21.5	5.8	3.41	1.5	1,740	84			
09356-235	24.2	7	3.53	3.9	2,040	166	120	450	2,074
10302-178	23.2	7.2	3.27	1.7	1,700	149			
10302-238	21.7	4.5	3.53	1.9	1,640	66			
10302-293	23.2	7.5	3.22	1.8	1,720	57			
10302-309	21.6	5.4	3.42	1.8	1,480	58			
10317-035	22.8	3.9	3.67	1.2	1,620	57			
12351-03	22.2	5.5	3.46	1.2	1,760	92	33	342	1,078

Table 9c. Berry sensory analysis of PD resistant selections used in small-scale winemaking in 2018.

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	Brown	Light	Strawberry jam, sweet spices	Slight hay, plum	1	4	Woody	4
07355-075	Red	Med	Cherry, cinnamon, white pepper	Cherry, slight plum	3	4	Woody, nutty, slightly bitter	2
07355-075 N	Red	Dark-	Spice, jam	jam, hay, plum	2	3	warm, woody, buttery	3
09311-160	pink	Light	Cherry, berry	neutral, slight fruit	2	4	Smokey, woody, warm	4
09314-102	Green	Light	Green apple, spice	neutral, apple, hay	2	3	Woody, spicy	1
09330-07	Red	Med+	Plum, spice, dark cherry	jam, hay, plum	2	4	woody, bitter, salty	3
09331-047	Red	Med+	Plum, cherry, black pepper	Plum, slight strawberry	3	4	Hot, bitter, terribly drying	4
09331-047 N	Red	Med+	Plum, jam, dried fruit	Berry, fruity	3	3	Woody, nutty, spicy	2
09331-133	Red	Med-	Raspberry, spice, cherry	Fruity, slight hay	3	4	buttery, woody	3
09333-370	Red	Med	Plum, jam, dried fruit	Hay, fruity	2	4	Woody, nutty, warm	4
09338-016	Green touch yellow	Light+	Green apple, spice	neutral, slight hay	1	4	Woody, nutty, spicy	3
09356-235	Red	Med+	Cherry, berry, dried cranberry	Fruity, plum	3	4	Woody, bitter	1
10302-178	White-touch brown	Med	Green apple, spice	Neutral	2	4	Clay, green, hot	2
10302-238	Yellow-touch brown	Med	Pear, spice	Slight grass	1	3	Acrid, bitter	1
10302-293	Green-white	Pale	pear, green apple	neutral, apple, hay	1	3	Woody, spicy	3
10302-309	Green, some brown	Med	Green apple, spice	spicy, neutral	2	3	warm, woody, buttery	3
10317-035	Yellow-brown	Med+	Pear, spice	Veg, hay	3	3	Warm, bitter	1
12351-03	Pink, touch orange	Light	Cherry, hint of herbs	Hay, spice, dandelion?	2	4	woody, slightly bitter	4

REFERENCES CITED

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

Buzkan N, Krivanek AF, Eskalen A, Walker MA. 2003. Improvements in sample preparation and polymerase chain reaction detection techniques for *Xylella fastidiosa* in grapevine tissue. *Am. J. Enol. Vitic*. 54:307-312.

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

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

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

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

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

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

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

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Reporting Period: The results reported here are from work conducted July 2014 to October 2018.

ABSTRACT
Greenhouse screening, marker testing, and quantitative trail locus analysis of breeding populations was completed for 15 new resistance sources, including b46-43 and T03-16. Pierce's disease resistance in T03-16 and b41-13 were both identified as having resistance on a different region than *PdR1* on chromosome 14. Crosses were made to expand seedling populations from these new resistances for framework map development in order to identify where their resistance resides. Given that the incorporation of multiple resistances should make resistance more durable, our goal is to identify new sources of resistance that do not reside on chromosome 14 and facilitate stacking of these resistance sources with *PdR1* from b43-17 using genetic markers. A new resistance locus *PdR2* from the b42-26 background was located and closely linked markers are being used in marker-assisted selection to stack resistance loci from these different backgrounds. The genetic and physical mapping of the Pierce's disease resistance from b40-14 was also completed. This resistance source maps within the *PdR1b* locus, but it may be an alternative gene within this complex replicated locus. In addition, we verified the sequence of two candidate genes from the *PdR1b* locus, completed transformations with resistance gene analog (RGA)18 and RGA14, and obtained transgenic lines for complementation tests in the greenhouse. This effort was undertaken to verify that these potential resistance genes provide resistance to Pierce's disease. Although some transgenic lines responded better than untransformed plants to *Xylella infection*, none reached the level of the resistant biocontrols. Testing of RGA14 and RGA18 in a genetic background other than *Vitis vinifera*, as well as more information about RGA15, RGA16, and RGA17, will help to clarify the meaning and importance of these results.

LAYPERSON SUMMARY
Our main focus is to identify and genetically characterize unique Pierce's disease resistance sources from the southwestern United States and Mexican *Vitis* species collections. In order to carry out this task, we create targeted 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 germinate. Markers developed from different sources of resistance allow us to combine multiple forms of resistance and therefore produce offspring with the likelihood of having more durable resistance. These markers also allow us to identify candidate resistance genes and study how they function by engineering them into susceptible grape varieties to better understand the genes and the resistance.

INTRODUCTION
This project provides molecular support to the Pierce's disease (PD) resistance 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 the 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 PD resistant accessions. Analysis using population genetics tools has allowed us to better understand gene flow among resistant species and their taxonomic and evolutionary relationships. Fourteen promising resistant accessions were identified from

this germplasm. Markers were used to determine their genetic diversity and relationships to each other. Small breeding populations were developed and more than 700 seedlings were marker tested to ensure that they had the correct parentage and identity. We used a limited mapping strategy by utilizing markers from chromosome (Chr) 14 in conjunction with greenhouse screen data of small breeding populations to determine if the resistance to PD in these 14 accessions is different from the previously identified locus *PdR1* (Riaz et al., 2018**)**. Three unique resistance sources (T03-16, ANU67, and b41-13) were identified as having a different resistance region than Chr14. More crosses were made in spring 2016-2017 to expand these breeding populations for map-based identification of genomic regions that contribute to resistance.

The identification and characterization of resistance genes and their regulatory sequences will help determine the basis of resistance/susceptibility in grape germplasm. In addition, these genes and their promoters could be employed in production of 'cisgenic' plants. Cisgenesis is the transformation of a host plant with its own genes and promoters (Holmes et al., 2013). We have completed the physical map of *PdR1*a and *PdR1*b locus for b43-17 to clone and characterize resistance genes (see earlier reports). The physical map of the *PdR1*c locus (from b40-14) is also complete. Development of *V. vinifera* plants transformed with our PD resistance genes and grape promoters might work more effectively and allow us to better understand how the *PdR1* resistant gene functions.

Upstream and downstream sequences, as well as the gene sequences of two candidate genes, open reading frame (ORF) 14 and ORF18, from *PdR1*b were verified, and constructs were developed to test their function. Transformation experiments with the *PdR1* resistance gene with a native grape promoter were completed with ORF18, and transgenic lines are being developed and maintained for later resistance verification. A 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 (qPCR) 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 to test the function of gene sequences. These efforts will help us identify candidate resistance genes by complementation, and better understand how they function.

OBJECTIVES

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

RESULTS AND DISCUSSION

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

We completed screening of over 250 southwestern United States and northern Mexico *Vitis*, which included accessions, collected from multiple collection trips from 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*. A total of 704 individuals obtained from these breeding populations were greenhouse screened and a limited mapping strategy with markers from Chr14 that are linked to the *PdR1* locus (see previous reports for details of the *PdR1* locus) were used. This strategy allowed us to identify resistance sources whose resistance is similar to *PdR1*, and sources that are different. 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 Chr14 (**Table 1**). These three accessions were identified as candidates

for further work and the development of framework maps with larger populations to detect new unique loci for PD resistance breeding. The major findings of this work were recently published (Riaz et al., 2018).

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*. In order to identify the genomic regions housing PD resistance in these two accessions, crosses were made in spring 2016 and 2017 to expand population sizes. A total of 295 seedling plants of the F1 population from b41-13 are established in the field. We have completed the greenhouse screening of 122 F1 seedling plants. An additional 150 seedlings from a 2016 cross are in different stages of greenhouse screening and the results will be available by November 2018. SSR markers from Chr8 and Chr14 were tested on a small set of parents and progeny, and 35 polymorphic markers were run on the entire population of 295 seedlings. Further marker testing is in progress to develop a framework map and quantitative trait locus (QTL) analysis to identify genomic regions linked to PD resistance. Thus far we have completed marker screening with 320 SSR markers. Fifty-three percent of the markers are polymorphic, and they will be added to the set of 295 seedling plants to develop a framework map. Crosses were also made with T03-16 and 285 seedling plants from the F1 population were established in the field. Multiple replicates of seedling plants were propagated for greenhouse screening, and 173 seedling plants are now in different stages of greenhouse testing, with results expected by the end of 2018.

Table 1. The 14 resistant accessions used to create 23 breeding populations in an effort to identify PD resistance sources that differ from *PdR1*. Resistant accessions with different sources of resistance are marked as Not 14 in the last column. Accessions marked as LG14 possess the *PdR1* locus. Resistance affinity to Chr14 could not be determined for the accessions that are marked as "Inconclusive" due to small population size and less informative markers.

Resistance Source	Species Description	Populations Tested	Number of Screened Genotypes	PD Resistance
ANU5	*V. girdiana*	12-314	60	LG14
b40-29	*V. arizonica*, brushy	12-340, 12-341, 14-367, 14-368	29	LG14
b46-43	*V. arizonica*, glabrous with *V. monticola*	12-305, 14-308, 14-321, 14-322, 14-324, 14-336	159	LG14
b41-13	*V. arizonica-mustangensis* and *champinii* hybrid,	13-355	47	Inconclusive
b47-32	*V. arizonica* glabrous with *monticola*,	13-344	13	Inconclusive
SC36	*V. girdiana*	13-348	35	LG14
T03-16	*V. arizonica* glabrous	13-336	62	Inconclusive
A14	*V. arizonica*	14-313	25	Inconclusive
A28	*V. arizonica*	14-347, 14-364	42	LG14
ANU67	*V. arizonica* glabrous	14-362	28	Inconclusive
ANU71	*V. arizonica-riparia* hybrid	14-340	30	LG14
C23-94	*V. arizonica* glabrous and brushy	14-303	44	LG14
DVIT 2236.2	*V. cinerea* like,	14-360	30	LG14
SAZ 7	*V. arizonica*	14-363	52	LG14

We 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 202 markers, which grouped to 18 chromosomes. Chr19 was not represented. We tested more than 50 SSR markers that have been mapped on Chr19 in other breeding populations, and none of them were polymorphic for b42-26. **Table 2** represents the genetic maps of both susceptible *vinifera* F2-35 and accession b42-26, and the consensus map. We carried out QTL analysis with this map and identified resistance on Chr8 and Chr10 (**Figure 1**). The resistance from Chr8 was also

verified on the basis of linked alleles in the pBC1 and pBC2 populations. Using the Cabernet Sauvignon genome sequence, we developed nine new SSR markers for Chr8 and added them to the genetic map. In spring 2017 and 2018 we began using closely linked markers to assist the breeding program with marker-assisted selection to stack the *PdR1*b and *PdR2* loci together. A manuscript detailing genetic mapping in b42-26 and b40-14 is approaching publication.

This project provides molecular support to the companion PD resistance winegrape breeding project by conducting marker-assisted selection on seedling populations. In spring 2018 we extracted DNA and marker tested 3,102 seedling plants from 59 different crosses for the *PdR1* (b and c) and *PdR2* loci (**Table 3**). Marker screening is a time intensive process, but extremely important, and makes our breeding program extremely efficient and successful. Each year we spend 12 to 14 weeks in the spring to support the breeding program. Planting only resistant plants saves us huge amounts of time and resources in the field.

Table 2. Description of the genetic maps of susceptible *vinifera* parent, resistant accession b42-26 and consensus map.

	F2-35		b42-26		Consensus	
Chromosome	**Mapped Markers**	**Map Length (cM)**	**Mapped Markers**	**Map Length (cM)**	**Mapped Markers**	**Map Length (cM)**
Chr1	11	37.70	16	43.50	19	59.90
Chr2	0	0.00	4	9.50	4	9.50
Chr3	6	28.10	7	43.40	9	44.90
Chr4	12	53.70	12	53.30	14	53.10
Chr5	6	22.20	10	29.20	12	33.10
Chr6	7	45.90	9	54.20	9	45.00
Chr7	3	15.80	9	37.80	9	37.30
Chr8	12	54.90	22	74.10	20	74.40
Chr9	3	21.40	6	27.50	7	28.20
Chr10	9	48.20	10	55.20	10	51.80
Chr11	4	33.90	5	34.80	5	34.40
Chr12	4	5.40	12	40.00	12	39.10
Chr13	8	30.20	13	33.00	15	32.20
Chr14	12	66.20	22	61.50	23	61.80
Chr15	2	31.80	4	48.90	4	51.10
Chr16	8	46.80	9	55.50	10	53.90
Chr17	5	28.90	6	29.70	6	30.10
Chr18	8	61.50	13	94.40	14	95.00
Chr19	0	0.00	0	0.00	0	0.00
Total	120	632.60	189	825.50	202	834.80
Avg. marker distance	2.92		4.37		4.13	

Objective 2. Complete a Physical Map of the PdR2 Region from the b42-26 Background and Carry Out Comparative Sequence Analysis with b43-17 (*PdR1a* and *b*) and b40-14 (*PdR1c*)

We completed the physical maps of the *PdR1*a, *PdR1*b, and *PdR1*c loci from the b40-14 and b43-17 backgrounds. In summary, 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, Chr14-56 and Chr14-58. BAC clones that represent *PdR1c* were separated from the other haplotype and four overlapping BAC clones, VA29E9, VA57F4, VA30F14, and VA16J22, were selected for sequencing. Common probes between the *PdR1c* and *PdR1b* region were used to align the sequences. The assembly of four BAC clones is presented in **Figure 2 and 3**

and represents the sequence analysis of *PdR1b* and the reference grape genome PN40024 region. A manuscript titled "The Physical Map of the PD Resistance Locus, *PdR1c*" is in preparation.

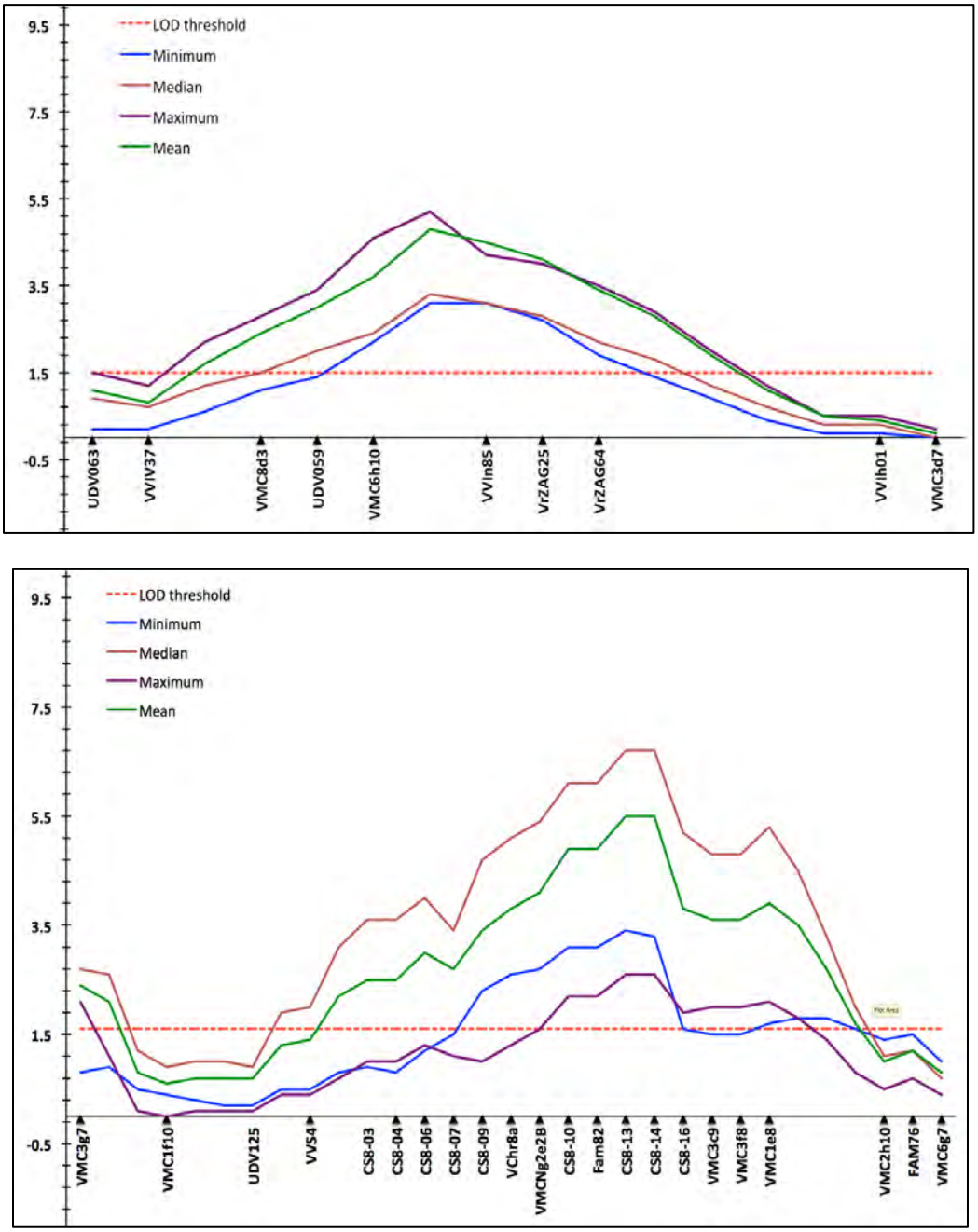

Figure 1. QTL analysis results for Chr8 and Chr10 using the greenhouse screening results for the 05347 population, which segregates for PD resistance from *V. arizonica* b42-26.

Table 3. Summary of marker testing completed in spring 2018 to support our PD resistance breeding program.

PD Locus	Number of Crosses	Number of Seedlings
PdR1b	24	1,350
PdR1b x *PdR2*	28	1,450
PdR1c	7	302
Total	59	3,102

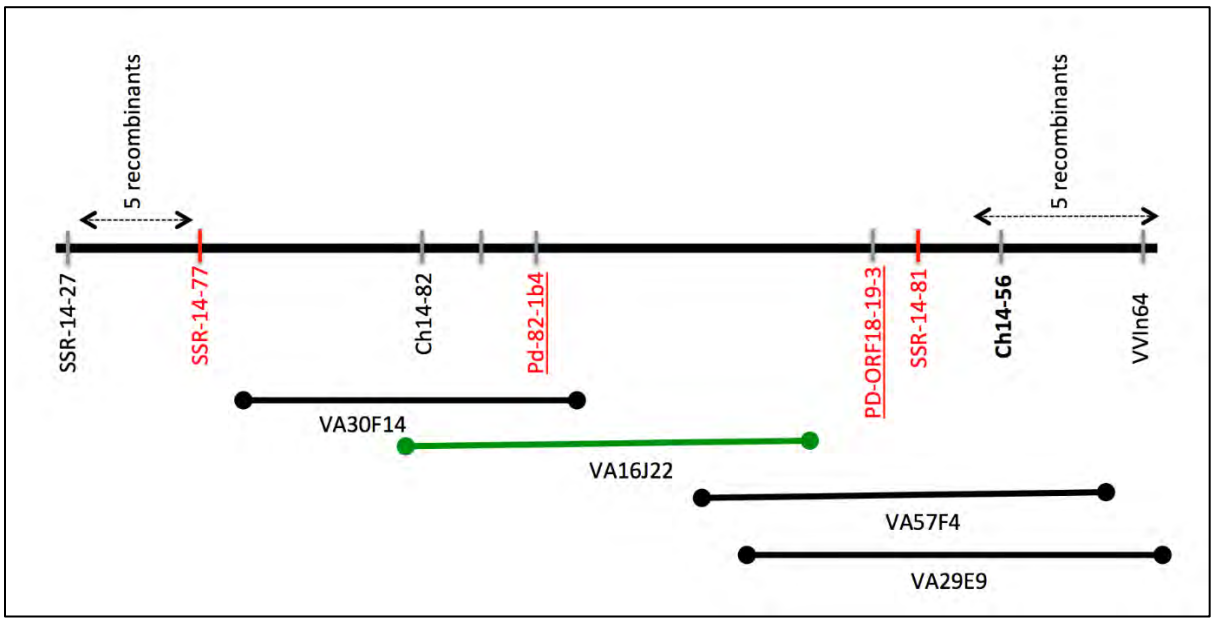

Figure 2. A BAC library was developed from genomic DNA of b40-14 and screened with probes. Four over-lapping clones were selected for sequencing the complete region. Marker names in red were developed from the sequence of accession b43-17.

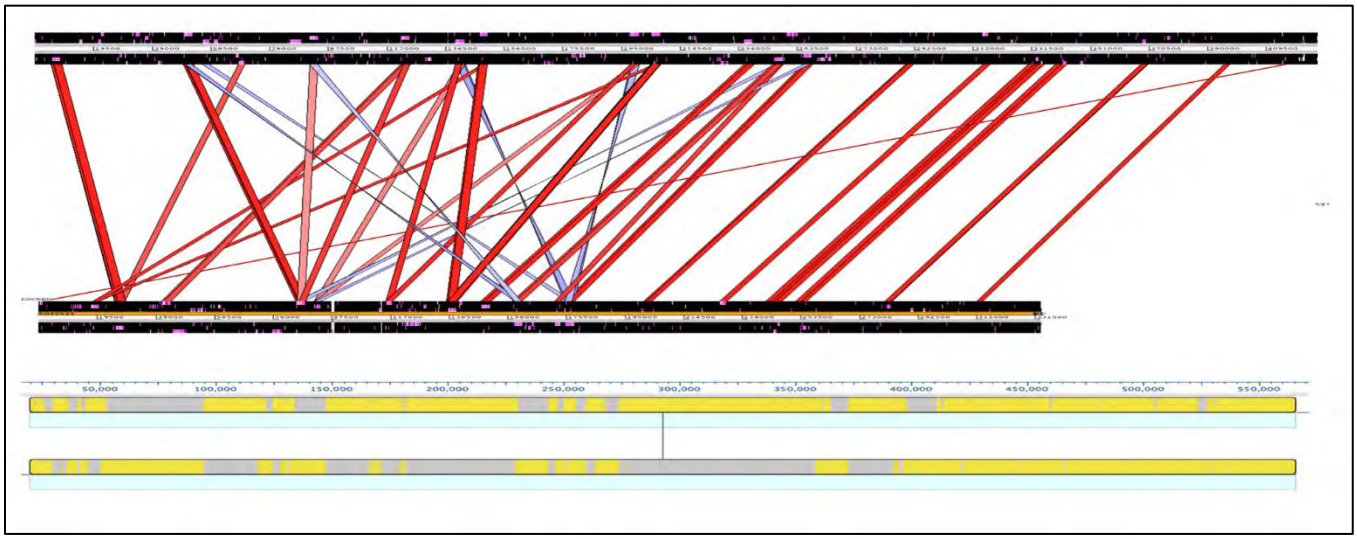

Figure 3. The sequences of four BAC clones were assembled and full-length ORFs were identified. Sequences were compared with the reference genome and checked for synteny in that region. Currently, analysis is being carried out with the Cabernet Sauvignon genome sequence.

The assembly of H43-I23 from the b43-17 BAC library that represents the *PdR1a* haplotype (F8909-17) was also completed. The length of assembled sequence was 206 Kb. The ORFs of the *PdR1b* region and the BAC clone H69J14 were used to make comparisons. There was complete homology between the over-lapping BAC clone sequences that reflect two different haplotypes. The BAC clone H43I23 has ORF16 to ORF20 and all five ORFs have identical sequences compared to the *PdR1b* haplotype. Based on these results we concluded that there is complete sequence homology between haplotypes *PdR1a* and *PdR1b* 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.

In regards to the physical map of b42-26, first we need to refine the position of the *PdR2* locus and narrow the region to less than 1 cM. For this purpose we developed new markers using the Cabernet Sauvignon genome sequence (described in Objective 1) and added them to the genetic map. We are developing more markers to fill gaps around the resistance QTL on Chr10 as well, so that library screening can be used to identify BAC clones that represent both resistance regions.

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

RNA sequencing is a powerful approach to identity transcripts and quantify gene expression while combined with a single high-throughput sequencing assay. A good RNA sequencing study relies on experimental design (library type, sequencing depth, and number of replicates) and a careful execution of the sequencing experiment to ensure that data acquisition is not contaminated with unnecessary biases. We completed a time course experiment to monitor the bacterial level in control and inoculated resistant and susceptible plants to design an experiment capable of answering our biological questions with the maximum statistical power. For this purpose, three resistant and three susceptible plants from the 07744 population with resistance from b40-14 PD (*PdR1c*) were used. Plants were propagated and a time course experiment was carried out in growth chambers with temperature and humidity control to reduce the variance. Stem samples were collected from positions 10 cm, 20 cm, 30 cm, and 40 cm above the point of inoculation and weekly RNA extractions were performed. Samples were also collected from 30 cm above the point of inoculation for enzyme-linked immunosorbent assay (ELISA) screening. We have completed RNA extractions of 400 samples, and the remaining 96 samples are in the pipeline. Analysis is underway to determine when and where gene expression is optimized.

Objective 4. Cloning PD Resistance Genes with Native Promoters

We employed a PAC BIO RSII sequencing approach to sequence H69J14 and three other overlapping BAC clones containing both markers that flank 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 within this region. 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 analog (RGA) 14 and 18 have a very similar sequence profile except that RGA18 is 2,946 bp in size and lacks the first 252 bp of sequence that is part of RGA14. Functional analysis of both RGAs revealed that RGA14 lacks a signal peptide in the amino terminal of the protein. 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 messenger RNA (mRNA) does not contain an N-terminal signal peptide necessary for proper membrane localization, thus leaving RGA18 as the strongest candidate. However, this could result from a lack of effect of 35S on splicing. In addition, sequencing of complementary DNA (cDNA) from b43-17, the original source of resistance, inoculated with *Xylella fastidiosa* resulted in the amplification of fragments that comprise sequences identical to RGA14 but different from RGA18. *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.

Sequence verification for RGA14 and RGA18 and flanking sequences was completed and fragments that contain the entire coding region plus ~3 Kb upstream and ~1 Kb downstream sequences were synthesized and cloned into pCLB2301NK at Genewiz Inc. 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 (NOS) promoter.

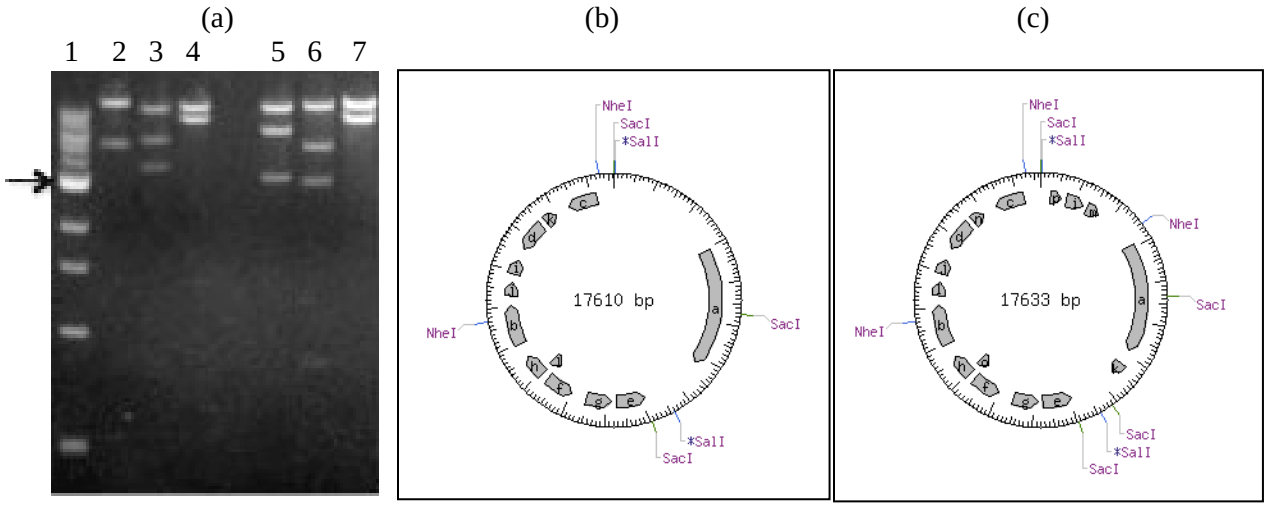

Figure 4. (a) Restriction analysis of plasmids pCLB2301NK-14 (lanes 2, 3, 4) and pCLB2301NK-18 (lanes 5, 6, 7) after digestion with Nhe1 (lanes 2, 5), Sac1 (lanes 3, 6) and Sal1 (lanes 4, 7). Gel image includes a 1Kb ladder (lane 1) with the 3 Kb fragment having increased intensity to serve as a reference 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.

Objective 5. Comparing the PD 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 (TS), Cabernet Sauvignon, Chardonnay (CH), and the rootstock *V. rupestris* St. George (SG) (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 PD resistance or tolerance. These results are in accordance with the latest assembly obtained using the 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.

We have also developed meristematic bulks (MB) (Xie et al., 2016), which we are using as an alternative explant for genetic transformation of PD 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 and transformation experiments with *Agrobacterium* have been initiated.

Transformations with *Agrobacterium tumefaciens* carrying binary plasmids that contain hygromicin (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 when selecting with kanamycin, confirming our previous observation that meristematic bulks are highly sensitive to hygromicin. 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* is being used to transform meristematic bulks of PD susceptible genotypes selected from the 04-191 population. **Table 4** shows the number of independent lines regenerated so far. Transformation was checked through PCR, and transformed plants were transferred to the greenhouse. Primers that bind the promoter and coding regions of RGA14 or RGA18 were used for amplification. DNA fragments amplified successfully in all the lines tested (**Figure 5)**. Transformation was also verified by fluorescence

microscopy to visualize green fluorescent protein (GFP), since pCLB2301NK-18 and pCLB2301NK-14 also contain a 35S:GFP5-ER cassette (**Figure 6**).

Table 4. Number of independent lines regenerated after transformation with *Agrobacterium* carrying pCLB2301NK-18 or pCLB2301NK-14.

Genotype	**No. Lines *in vitro***	**No. Lines in Greenhouse**
pCLB2301NK-18		
Chardonnay	13	11
Thompson Seedless	30	11
St. George	4	4
29-42	1	-
pCLB2301NK-14		
Chardonnay	20	11
Thompson Seedless	18	10
St. George	4	4

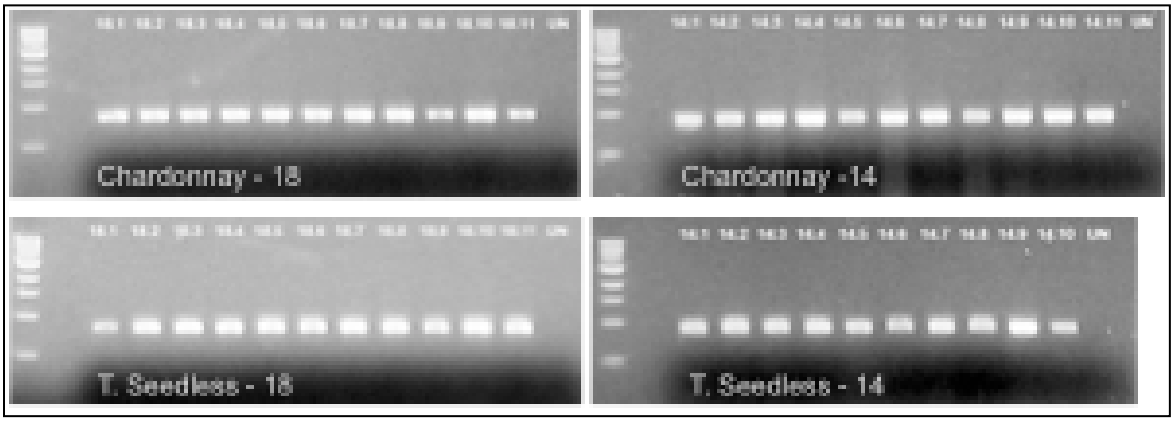

Figure 5. Transgene detection of RGA18 and 14 through PCR (UN: untransformed Chardonnay or Thompson Seedless, 1-10 or 11: transgenic lines). Gel image includes a 1 Kb ladder (left lane) with the 3 Kb fragment having increased intensity to serve as a reference band. The results on the gel match the predicted sizes inferred from the sequence information.

Screening of Transgenic Lines**.** Chardonnay and Thompson Seedless transgenic lines in the greenhouse were multiplied from green cuttings and were inoculated with the Beringer strain of *Xylella fastidiosa* in August 2017 (RGA18 lines) and March 2018 (RGA14 lines). Twelve weeks after inoculation, PD symptoms were evaluated using a 0-5 score for leaf scorch-leaf loss (LS-LL) and a 0-6 score for cane maturation index (CMI). For ELISA, plants were sampled by taking 0.5 g sections of stem tissue from 30 cm above the point of inoculation (Krivanek and Walker, 2005; Krivanek et al., 2005). For gene expression analysis, plants were sampled by taking 0.5 g sections of stem tissue from 50 cm above the point of inoculation. Testing of St. George started in August 2018.

Tables 5 and 6 demonstrate that transgenic lines displayed disease symptoms with different degrees of intensity. On the one hand, lines CH 18-2 and CH 18-7 showed low cane maturation index. On the other, most CH 14 lines, especially CH 14-1 and CH 14-2, exhibited low leaf scorching (**Figure 7**). Bacteria concentration in CH 18-2, CH 18-7, and CH 18-10 was lower than in the untransformed control, but not as low as the resistant biocontrols (**Table 3**). However, significant differences were found between CH 18-2 and CH 18-7 and untransformed CH. Thompson Seedless was considerably more susceptible than Chardonnay.

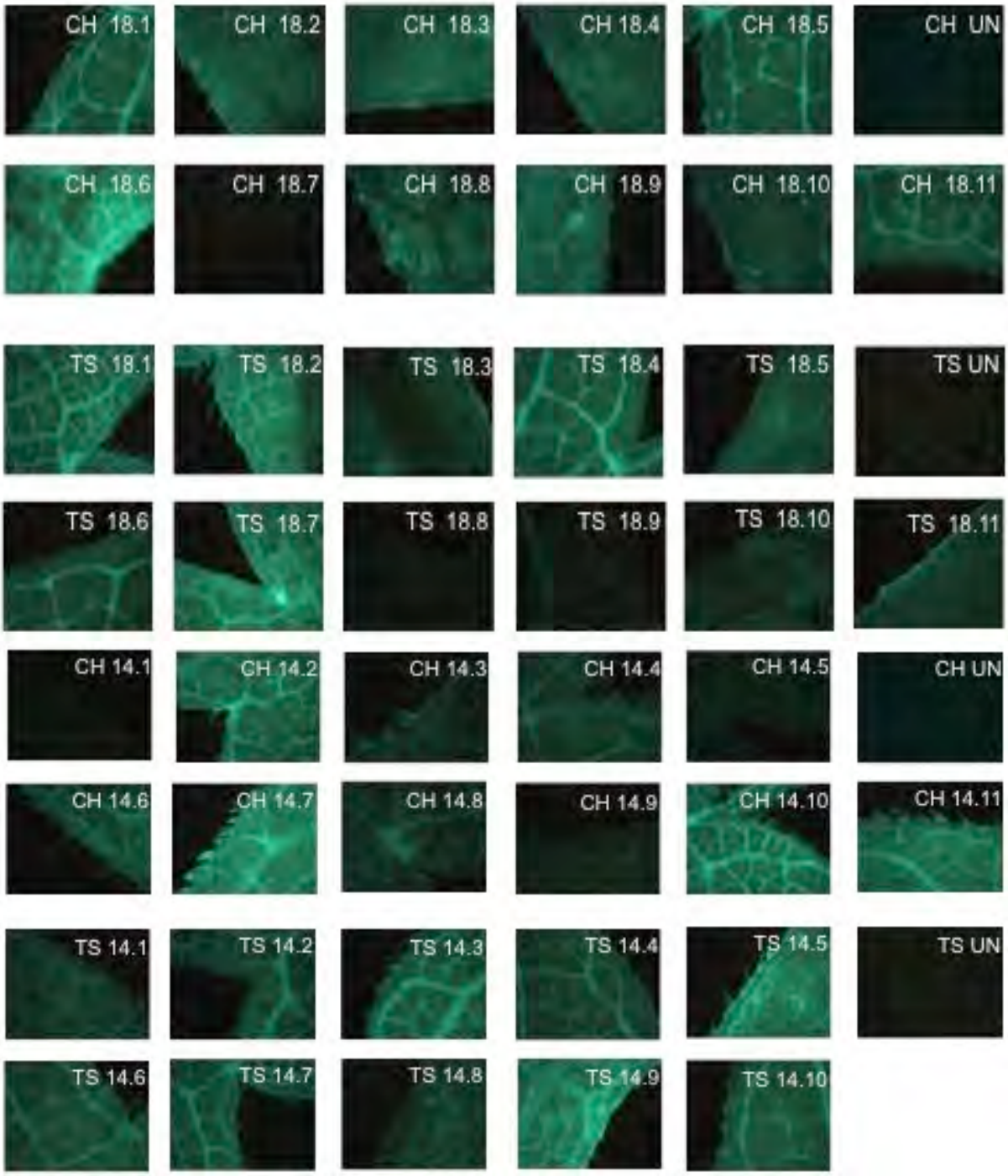

Figure 6. GFP fluorescence in untransformed Chardonnay (CH UN), Thompson Seedless (TS UN), and RGA18 and RGA14 transgenic lines (1-10 or 11).

qPCR analysis to determine the correlation between the level of transgene expression and GFP fluorescence/PD symptoms/bacteria concentrations has been inconclusive. Untransformed Chardonnay infected with *Xylella fastidiosa* also exhibits low cycle threshold (Ct) numbers (**Figure 8**, primers 14.3 and 18.5) and cDNA sequencing has revealed that genes with high homology with RGA14 and RGA18 are being expressed.

Table 5. Greenhouse screen results for Chardonnay and Thompson Seedless transformed with V.ari-RGA18. Top six genotypes correspond to negative control and resistant biocontrols. CH 0 and TS 0 are untransformed Chardonnay and Thompson Seedless, respectively.

Genotype	cfu/ml	ln cfu/ml	Std Error (cfu/ml)	CMI Mean	CMI Std Err	LS-LL Mean	LS-LL Std Err
CH uninoc	10,034	9.2	0.00	0.0	0.00	0.0	0.00
b43-17	23,416	10.1	0.32	2.1	0.48	2.9	0.48
U0505-01	37,499	10.5	0.62	0.3	0.15	1.8	0.41
U0505-35	100,798	11.5	0.83	0.3	0.33	1.5	0.43
Blanc du Bois	194,385	12.2	0.46	2.1	0.49	1.9	0.49
Roucaneuf	245,426	12.4	0.78	0.3	0.33	0.8	0.40
U0505-22	760,190	13.5	0.27	2.3	0.71	2.6	0.43
CH 0	6,119,118	15.6	0.42	3.6	0.60	1.6	0.53
CH 18-1	4,636,369	15.3	0.18	1.0	0.63	1.8	0.20
CH 18-2	2,078,921	14.5	0.42	0.8	0.58	2.4	0.51
CH 18-3	6,152,629	15.6	0.04	2.6	0.81	2.4	0.40
CH 18-4	4,686,410	15.4	0.06	3.4	0.82	0.9	0.11
CH 18-5	5,562,260	15.5	0.10	1.6	0.24	2.8	0.58
CH 18-6	4,888,719	15.4	0.18	1.0	0.55	3.0	0.32
CH 18-7	1,786,455	14.4	0.60	0.0	0.00	1.8	0.58
CH 18-8	6,500,000	15.7	0.00	2.2	0.58	3.2	0.37
TS 0	6,500,000	15.7	0.00	4.2	0.20	5.0	0.00
TS 18-1	6,500,000	15.7	0.00	4.6	0.40	4.6	0.40
TS 18-2	6,500,000	15.7	0.00	4.0	0.32	5.0	0.00
TS 18-3	6,500,000	15.7	0.00	4.0	0.32	4.4	0.24
TS 18-4	6,500,000	15.7	0.00	5.0	0.45	4.8	0.20
TS 18-5	6,500,000	15.7	0.00	4.0	0.00	4.6	0.24
TS 18-6	6,500,000	15.7	0.00	4.2	0.66	4.2	0.20
TS 18-7	6,500,000	15.7	0.00	3.6	0.75	4.6	0.24
TS 18-8	6,500,000	15.7	0.00	5.0	0.45	5.0	0.00
TS 18-9	6,500,000	15.7	0.00	4.2	0.37	4.8	0.20
TS 18-10	6,500,000	15.7	0.00	4.6	0.40	5.0	0.00
TS 18-11	6,500,000	15.7	0.00	4.2	0.73	4.8	0.20

Table 6. Greenhouse screen results for Chardonnay and Thompson Seedless transformed with V.ari-RGA14. Top three genotypes correspond to resistant biocontrols. CH-0 and TS-0 are untransformed Chardonnay and Thompson Seedless, respectively.

Genotype	cfu/ml	ln cfu/ml	Std Error (cfu/ml)	CMI Mean	CMI Std Err	LS-LL Mean	LS-LL Std Err
b43-17	29,269	10.3	1.5	1.5	0.5	2.0	1.0
Blanc du Bois	239,720	12.4	1.8	0.5	0.5	2.0	1.0
Roucaneuf	412,439	12.9	3.2	1.4	1.0	0.7	0.5
CH 0	3,927,940	15.2	0.8	1.9	0.46	3.1	0.29
CH 18-9	3,283,644	15.0	0.9	2.4	0.51	3.6	0.37
CH 18-10	2,034,341	14.5	0.8	0.7	0.70	2.7	0.41
CH 18-11	4,463,678	15.3	0.5	1.7	0.62	2.1	0.37
CH 14-1	2,840,069	14.9	0.8	1.7	0.37	1.3	0.25
CH 14-2	2,996,840	14.9	1.1	1.6	0.64	2.0	0.16

Genotype	cfu/ml	ln cfu/ml	Std Error (cfu/ml)	CMI Mean	CMI Std Err	LS-LL Mean	LS-LL Std Err
CH 14-3	2,516,781	14.7	1.0	1.2	0.72	2.5	0.59
CH 14-4	3,293,318	15.0	0.8	3.2	0.58	2.3	0.34
CH 14-5	4,278,523	15.3	0.6	3.7	0.60	3.1	0.29
CH 14-6	3,183,492	15.0	0.7	2.5	0.94	2.2	0.25
CH 14-7	3,785,181	15.1	0.6	2.7	0.80	1.4	0.46
CH 14-8	2,726,498	14.8	0.8	1.4	0.48	1.8	0.30
CH 14-9	3,480,812	15.1	0.6	2.5	1.04	2.0	0.16
CH 14-10	3,656,052	15.1	0.8	3.3	0.46	1.7	0.60
CH 14-11	5,029,740	15.4	0.4	1.8	0.54	1.8	0.41
TS 0	4,549,700	15.3	0.5	4.8	0.20	4.7	0.20
TS 14-1	5,588,848	15.5	0.3	4.7	0.30	4.8	0.20
TS 14-2	6,500,000	15.7	0.0	4.4	0.10	4.5	0.27
TS 14-3	6,500,000	15.7	0.0	4.3	0.20	5.0	0.00
TS 14-4	6,500,000	15.7	0.0	4.7	0.20	4.9	0.10
TS 14-5	4,085,304	15.2	1.0	3.8	0.40	4.8	0.20
TS 14-6	5,846,462	15.6	0.2	4.1	0.33	4.9	0.10
TS 14-7	6,027,879	15.6	0.2	4.2	0.37	4.1	0.37
TS 14-8	6,103,502	15.6	0.1	5.1	0.24	4.8	0.20
TS 14-9	4,418,184	15.3	0.7	4.7	0.20	4.7	0.20
TS 14-10	5,430,851	15.5	0.3	4.4	0.24	5.0	0.00

Figure 7. Left: lignification observed in nodes collected 40 cm above the point of inoculation, three months after inoculations in transgenic lines CH 18.2, CH 18.7, and untransformed Chardonnay (CH UN). Right: scorching observed in basal leaves, three months after inoculations in transgenic lines CH 14.1, CH 14.7, and untransformed Chardonnay (CH UN).

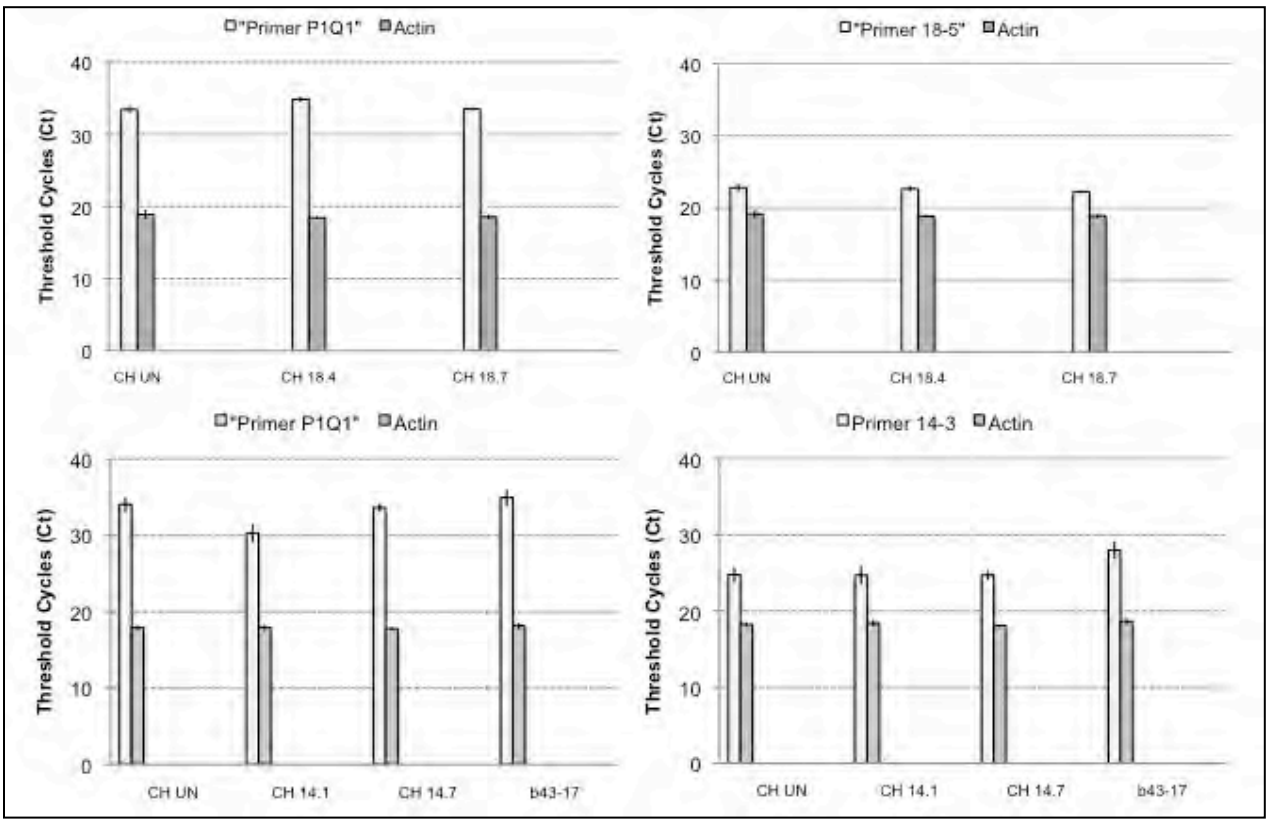

Figure 8. RGA18 and RGA14 expression in transgenic lines CH 18.4, CH 18.7, CH 14.1, CH 14.7, untransformed CH, and b43-17. Error bars represent standard deviation of the mean (n = 3). UN: Untransformed. Primers 18-5 and 14-3 amplify a fragment in the 3' region of the RGA18 and RGA14 respectively, while P1Q1 amplifies a fragment in the first 252 bp of RGA14. Higher Ct values correspond to lower expression levels. Actin is used as reference gene.

CONCLUSIONS

We completed greenhouse screening, marker testing, and quantitative trait locus 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 Chr14. Crosses were made to expand these breeding populations for framework map development in order to identify other genomic regions of resistance. Given that the incorporation of multiple resistances should make resistance more durable, our goal is to identify new sources of resistance that do not reside on Chr14 and facilitate stacking of these resistance sources with *PdR1* from b43-17 using genetic markers. We have 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 PD 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 RGA18 and RGA14, and obtained transgenic lines for complementation tests in the greenhouse. Although some transgenic lines responded better than untransformed plants to *Xylella* infection, none reached the level of resistant biocontrols. Testing of RGA14 and 18 in a genetic background other than *vinifera*, as well as more information about RGA15, 16, and 17, will help to clarify the meaning and importance of these results.

REFERENCES CITED

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

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

Krivanek AF, Walker MA. 2005. *Vitis* resistance to Pierce's disease is characterized by differential *Xylella fastidiosa* populations in stems and leaves. *Phytopathology* 95:44-52.
Krivanek AF, Stevenson JF, Walker MA. 2005. Development and comparison of symptom indices for quantifying grapevine resistance to Pierce's disease. *Phytopathology* 95:36-43.
Holmes IB, Wendt T, Holm PB. 2013. Intragenesis and cisgenesis as alternatives to transgenic crop development. *Plant Biotech. J.* 11:395-407.
Riaz S, Walker MA. 2013. Phylogeographic analysis of resistance to Pierce's disease in North American and Mexican species. *64th Annual Meeting of the American Society for Enology and Viticulture*, Monterey, CA.
Riaz S, Huerta-Acosta K, Tenscher AC, Walker MA. 2018. Genetic characterization of *Vitis* germplasm collected from the southwestern US and Mexico to expedite Pierce's disease resistance breeding. *Theoretical and Applied Genetics* 131:1589-1602.

FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board. Additional support from the Louise Rossi Endowed Chair in Viticulture supporting work with grape species is also gratefully acknowledged.

NOVEL AMPLIFICATION TARGETS FOR RAPID DETECTION AND DIFFERENTIATION OF *XYLELLA FASTIDIOSA* SUBSPECIES *FASTIDIOSA* AND *MULTIPLEX* IN PLANT AND INSECT TISSUE

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ABSTRACT
Several different subspecies of *Xylella fastidiosa* (*Xf*) have been described worldwide, causing disease in a variety of economically important crops. Numerous molecular detection protocols are available for quarantine screening, surveillance, and research applications, but most cannot differentiate between strains or subspecies of the pathogen. In areas such as California where more than one subspecies is present, it is important to be able to determine subspecies affiliation. This study describes quantitative polymerase chain reaction (qPCR) and loop-mediated isothermal amplification assays (LAMP) which can rapidly identify *Xf* isolates belonging to the *fastidiosa* and *multiplex* subspecies. The TaqMan qPCR primers described here are used to differentiate *Xf* strains by subspecies in plant and insect tissue in a single reaction, with the inclusion of a general amplification control probe to identify potential false negative samples. Sensitivity of the TaqMan qPCR protocol is between 10^3 and 10^4 colony-forming units (cfu) per ml concentrations of *Xf* in a variety of sample types. Additionally, LAMP targets were designed for faster detection of *Xf* subspecies *fastidiosa* and *multiplex*, which could be modified to use in a field setting. These assays are effective for strain differentiation in artificially and naturally inoculated plant material, and in field collected insect vectors.

FUNDING AGENCIES
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ENHANCING GRAPEVINE IMMUNITY BY DESIGN AND DELIVERY OF ANTI-*XYLELLA FASTIDIOSA* PEPTIDES AND PROTEINS FACILITATES TREATMENT AND PREVENTION OF PIERCE'S DISEASE

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ABSTRACT

Innate Immunity LLC, established in 2016, focuses on developing and marketing peptide and protein therapy for the treatment and prevention of human and plant diseases. Peptides and proteins enhance immunity and enable the host to clear deadly pathogens that otherwise compromise host immunity. In this project, we focus on the application of peptide and protein therapy for clearance of *Xylella fastidiosa* (*Xf*), the causative agent of Pierce's disease (PD) in grapevines.

The peptide therapy is applied for treatment of grapevines infected with *Xf*, which will suppress or cure PD and increase the productive years of grapevines. The therapeutic peptides are helix-turn-helix (HTH) scaffolds. The single helices in the scaffold are derived from the grape proteome and they show bactericidal activity (albeit low). We have performed molecular dynamics simulations in model membrane to predict that the HTH scaffolds should possess higher bactericidal activity than the single helix because they are more efficient in attaching to, inserting into, and rupturing the bacterial membrane. Indeed, bactericidal assays validate our prediction in that the HTH peptides are more active on *Xf* than the single endogenous helix. We have performed *in vitro* toxicity assays to select HTH peptides that are nontoxic to grape protoplasts and human lung cells, red blood cells, and neutrophils. We have also evolved *in vitro* bacterial strains resistant to a single endogenous helix, performed comparative genomics/transcriptomics of the two strains susceptible and resistant to a single endogenous helix, and shown that multiple mutations in the resistant strain decrease attachment, insertion, and rupture of the bacterial membrane by the single endogenous helix. We have shown that our design strategy increases attachment, insertion, and rupture of the bacterial membrane by the HTH peptides and consequently enables them to overcome bacterial resistance. Interestingly the HTH peptides also reduce *Xf* biofilm formation, a critical process in PD pathogenesis. Finally, we have selected HTH peptides based on anti-*Xf* activity, lack of human/plant toxicity, and the ability to overcome bacterial resistance, and shown that these HTH peptides are capable of clearing *Xf* from the infected leaves collected from the field. We are currently planning and implementing field efficacy studies on the topical delivery of the anti-*Xf* HTH peptides in collaboration with the wine and grape industry.

The protein therapy is designed for prevention of PD by the generation of transgenic grape rootstocks expressing protein chimeras with *Xf* recognition and lysis domains, both of which are derived from grape proteome. The recognition domains are selected respectively from grape subtilisin and the bacterial permeability increasing/lipid binding protein (BPI/LBP) family. Specific subtilisins are selected/engineered to respectively cleave the *Xf* outer-membrane protein MopB, whereas specific BPI/LBPs are selected/engineered to bind the *Xf* lipopolysaccharide (LPS). The lysis domain is chosen and engineered from the grape thionin family to lyse the *Xf* membrane. We have used SR1 tobacco, which can be infected by *Xf*, as a model plant. We have constructed transgenic SR1 tobacco lines expressing various chimeras. Also, we have been able to screen the chimeras from SR1 tobacco transgenic lines that are the most effective in clearing *Xf* and blocking PD. We will construct transgenic grape rootstocks expressing the most efficient anti-*Xf* chimeras, graft on them scions from white and red wine cultivars, propagate them, and perform greenhouse and field efficacy studies.

Innate Immunity LLC is working on both short-term solutions (i.e., PD treatment by anti-*Xf* peptides) and long-term solutions (i.e., PD prevention by transgenic grape expressing anti-*Xf* protein chimeras). Not only have we developed PD therapies that are viable, but also formulated strategies to turn our inventions into marketable products that would be beneficial to the wine and grape industries.

FUNDING

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PIERCE'S DISEASE MONITORING PROGRAM IN KERN COUNTY, 2017

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ABSTRACT

For more than a decade the University of California (UC) and the Consolidated Central Valley Table Grape Pest and Disease Control District have partnered to monitor the development and spread of Pierce's disease (PD) in Kern County. These efforts have complemented work by the California Department of Food and Agriculture to monitor populations of glassy-winged sharpshooter (GWSS) during the same period of time. The goal of these monitoring programs has been to use trapping and monitoring data to coordinate growers' efforts to remove PD-infected vines and control GWSS. The success of these programs is based on the premise that in the absence of the disease, the vector is not a concern, yet in the absence of the vector, the disease can't spread. Therefore, by controlling both the disease and the vector there can be a synergistic benefit to protect the San Joaquin Valley table grape industry.

In the 2000s, highly effective insecticides for the vector coupled with aggressive roguing programs for the disease led to very low levels of PD in the General Beale region. As a reference, PD surveys within 15 vineyards during 2011 found a total of 44 PD-positive vines out of more than 115,000 vines surveyed (three positives per 10,000 vines surveyed). In contrast, during 2017 we found more than 9,000 PD-positive vines out of approximately 183,000 vines surveyed (496 positives per 10,000 vines surveyed). However, 90.6% of those vines were located within the eight most infected sites, whereas the remaining 9.4% were spread out over the other 21 vineyards surveyed. The increase in PD-positive vines has been attributed to a period from 2012 to 2015 where insecticide programs for GWSS had reduced efficacy, presumably due to neonicotinoid resistance, during a period where not all table grape growers were diligent at roguing infected vines.

Following 2017 surveys table grape growers made a significant effort to remove infected vines. With minor exceptions, all vines marked as PD-positive in UC surveys were either removed individually or by removing the entire vineyard prior to 2018. UC employees also trained representatives of table grape growers in the region to identify and remove PD-positive vines beyond the scope of UC efforts. The year 2018 was also a record low year for GWSS due to the adoption of dormant pyrethroid treatments to citrus. In previous years pyrethroid use was discouraged by citrus growers in their efforts to preserve natural enemies. This attitude has changed since the introduction of Asian citrus psyllid.

At the time of writing, 2018 PD surveys are underway. Initial indications are that roguing efforts in 2016 and 2017, coupled with improved GWSS control, have led to a significant reduction in the number of PD-positive vines in 2018. A full report of 2018 survey efforts should be available by mid-December.

FUNDING AGENCIES

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

SCREENING AND IDENTIFICATION OF PIERCE'S DISEASE AND POWDERY MILDEW DUAL RESISTANCE GRAPEVINES FROM AN ADVANCED BACKCROSS BREEDING POPULATION

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ABSTRACT

Backcrosses (BC) made in 2015 combined Pierce's disease resistance from *Vitis arizonica* with powdery mildew (PM) resistance from *V. romanetii*. This advanced BC population was derived from BC3 population (F8909-08 x b42-26) previously developed by the USDA Agricultural Research Service, Parlier, CA. Seeds were obtained and germinated. Of those, 120 seedlings were selected and grown in a greenhouse. Pathogenicity tests identified 46 resistant progeny with no foliar PM symptoms, while 44 progeny showed severe PM symptoms. Thirty plants were moderately susceptible to PM. Dormant cuttings have been prepared from 46 PM resistant progeny for Pierce's disease resistance screening. To further identify the molecular basis of dual resistance, functional genomic and genotyping by sequence approaches have been implemented to facilitate mapping resistance loci linked to resistant traits.

FUNDING AGENCIES

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

ASSESSMENT OF TRANSMISSION, ADAPTATION, AND COMPETITION OF MUTANT STRAINS OF *XYLELLA FASTIDIOSA* BY THE GLASSY-WINGED SHARPSHOOTER

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ABSTRACT

Pierce's disease of grapevines is caused by *Xylella fastidiosa* (*Xf*) and transmitted by glassy-winged sharpshooters (*Homalodisca vitripennis*; GWSS). *Xf* is a foregut-borne, xylem-limited and non-flagellated, gram-negative bacterium. This bacterium is propagative and noncirculative in its insect vectors. The putative functions of virulence genes of *Xf* have been characterized by creating deletion mutants and complemented strains via mechanical inoculation of grapevines followed by *in planta* pathogenicity assays. However, information regarding the functional roles of virulence genes involved in transmission of *Xf* by GWSS is very limited. To further understand their roles involved in transmission by GWSS, several mutant strains including Xf-Δ*rpfA*, Xf-Δ*gacA*, Xf-Δ*pilG*, Xf-Δ*popP*, and *Xf-ΔpilH* were investigated via artificial diet acquisition and subsequent inoculation to grapevines. Thirty plants were inoculated for each mutant and were maintained in the greenhouse for symptom development and further analyses. Functional confirmation of key virulence genes responsible for transmission through GWSS will facilitate the development of target basis for therapeutic control of Pierce's disease.

FUNDING AGENCIES

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EPIDEMIOLOGY OF PIERCE'S DISEASE IN THE GENERAL BEALE AREA OF KERN COUNTY

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ABSTRACT
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 (Kern County) in the late 1990s and early 2000s, respectively. In response, an area-wide suppression program was implemented that primarily relied on application of insecticides. Analysis of trapping data from seven vineyards, two conventional citrus orchards, and two organic citrus orchards located in Kern County indicated that the area-wide program suppressed sharpshooter populations from 2002 to 2008. However, GWSS abundance increased in 2009 and peaked in 2015. In association, incidence of Pierce's disease rose. GWSS populations persisted in monitored vineyards and conventional citrus orchards through much of 2016, declining to undetectable levels in 2017 and 2018. Sharpshooter populations persisted in monitored organic citrus orchards in 2017 and 2018. From spring of 2016 to fall of 2018, GWSS adults were collected (if present) every three weeks from four vineyards, two conventional citrus orchards, and two organic citrus orchards and subjected to quantitative polymerase chain reaction to determine if *Xylella fastidiosa* (*Xf*) was present in sharpshooter mouthparts. In conjunction, petiole samples were collected from systemically infected grapevines every three weeks from two vineyards to quantify seasonal changes in *Xf* abundance in plants. In 2016, GWSS testing positive for *Xf* were first observed in July and the percentage of sharpshooters positive for *Xf* increased through the summer. A low percentage of petiole samples collected from chronically infected grapevine tested positive for *Xf* in May, with the percentage testing positive peaking in August/September. No sharpshooters were collected from vineyards in 2017 and 2018. In vineyards, the period during which GWSS appear most likely to transmit *Xf* is between July and September.

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

GENOTYPING *XYLELLA FASTIDIOSA* PRESENT IN THE GLASSY-WINGED SHARPSHOOTER IN THE GENERAL BEALE AREA OF KERN COUNTY, CALIFORNIA

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ABSTRACT

During 2016 and 2017, glassy-winged sharpshooters (*Homalodisca vitripennis*; GWSS) collected from vineyards or nearby citrus orchards in the General Beale area of Kern County were assessed for *Xylella fastidiosa* (*Xf*) infection by real-time polymerase chain reaction (PCR) using total DNA samples extracted from GWSS heads. Of 1,031 insects, 154 (15%) tested positive for *Xf*. A subset of *Xf*-positive GWSS DNA samples were subjected to multi-locus sequence typing (MLST) to determine *Xf* genotypes associated with GWSS. Conventional PCR products for three *Xf* genes (*petC, leuA*, and *holC*) were cloned and sequenced. Cloned sequences were assigned to *Xf* subspecies based on single-nucleotide polymorphism (SNP) signatures (7-12 polymorphic sites per gene that differentiate reference genomes of subspecies *fastidiosa* and *multiplex*). Of 1,914 MLST clones sequenced, 1,412 were genotyped as subspecies *fastidiosa* and 491 as subspecies *multiplex*. Of cloned subspecies *fastidiosa* sequences, 98% had SNP signatures identical to the corresponding gene present in 24 *Xf* subspecies *fastidiosa* strains cultured from Pierce's disease affected vines sampled in 2016 and 2017. Presence of SNP signatures representing both subspecies in one or more cloned genes was commonly observed within individual GWSS. This observation indicates that genetic complexity of *Xf* in many insects was greater than one due to mixtures of two subspecies or mixtures of two genotypes of one subspecies (in which a proportion of the population was derived from a lineage with a history of horizontal gene transfer and homologous recombination). Inferences drawn from these conclusions suggest that (1) individual GWSS visit multiple host species (inoculum sources); (2) sequential acquisition events may lead to co-infection of insect vectors; (3) competitive exclusion of *Xf* in the foregut is weak or not operating; and (4) the vector foregut represents a potential arena for exchange of genetic material among sympatric *Xf* subspecies.

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PLANT MICROBIAL COMMUNITY ASSOCIATED WITH INFECTION OF *XYLELLA FASTIDIOSA* REVEALED BY NEXT GENERATION SEQUENCING ANALYSES

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ABSTRACT
Xylella fastidiosa (*Xf*) causing Pierce's disease (PD) of grapevine is known to interact with microorganisms in the plant endosphere during the course of infection and pathogenicity development. Yet, *in planta* study of *Xf* has been highly challenging due to the lack of efficient technology. The increase of *Xf* genetic resources in online databases, along with improved sequencing and genetic analysis technology, has provided a framework to study this economically important bacterium within the host plant endosphere. A particular interest in this study was the composition and variation of microbial community *in planta*. DNA was extracted from three samples: 1) PD symptomatic grapevine inoculated with *Xf* strain Stag's Leap in greenhouse (Greenhouse Grape - GG); 2) a non-symptomatic ragweed (*Ambrosia trifida*) inoculated with *Xf* strain Temecula in greenhouse (Greenhouse Ragweed - GR); and 3) PD symptomatic grapevine from a vineyard in Bakersfield, California in July of 2017 (Field Grape - FG). All three samples were subjected to next generation sequencing (NGS). Illumina HiSeq was used on samples GG and FG and generated 190 M and 316 M 100-base pair (bp) short sequence reads, respectively. Illumina MiSeq was used on sample GR and generated 39 M 250-bp short sequence reads. Percentages of *Xf* reads were 1.7 for GG, 0.5 for FG, and 4.0 for GR. These *in planta Xf* genome data were analyzed for similarities to the available sequenced *Xf* genomes as well as for plasmids and other unique genetic content. Genomic variations of *Xf* under different conditions (field, greenhouse, sampling time, plant host) were studied. Additionally, the microbial community of each plant sample was analyzed to determine the major taxonomic groups of bacteria and fungi through metagenomic approach with the help of Kaiju software. The most abundant bacterial genera present in the greenhouse plant samples were *Xylella*, followed by *Enterococcus* and *Staphylococcus* for both GG and GR. The most abundant bacterial genera present in the field plant sample was *Paenarthrobacter*, with *Xylella* ranking-the third in abundance. The most abundant fungal genera present in all three plant samples was *Rhizophagus*. These results provide new information on the microbial community of plants associated with *Xf* infection under different environments.

FUNDING AGENCIES
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Section 2:

Glassy-winged Sharpshooter

SHARPSHOOTER ELECTROPENETROGRAPHY X WAVE REPRESENTS THE *XYLELLA FASTIDIOSA* INOCULATION BEHAVIORS: UPDATE ON EVIDENCE FROM SYSTEMIC, SYMPTOMATIC PIERCE'S DISEASE INFECTIONS INDUCED AFTER X WAVES

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ABSTRACT
The mechanism of *Xylella fastidiosa* (*Xf*) inoculation by sharpshooter vectors has been hypothesized for nearly 50 years to be due to egestion from the functional foregut. Recently, combined egestion plus salivation was demonstrated conclusively as the mechanism of bacterial ejection from the vector's stylets. However, bacteria were ejected into/onto artificial diets, not plants. It has been hypothesized that (1) egestion plus salivation inject *Xf* bacterial cells into grape xylem cells, and 2) electropenetrography (EPG) can be used to observe and quantify vector behaviors in real time because the sharpshooter X wave represents combined salivation and egestion. Once these hypotheses are definitively supported, EPG can be used to search for novel avenues of disease resistance. This report updates progress in analyzing data from a four-year project to conclusively test these hypotheses. Results to date support that the XN portion of the X wave represents the *Xf* inoculation behaviors, and that (under certain circumstances), a single stylet probe with at least two XN events can initiate a quantitative polymerase chain reaction (qPCR)-positive, systemic (both close to and distant from the site of probing), symptomatic Pierce's disease infection.

LAYPERSON SUMMARY
Electropenetrography (EPG) passes a tiny amount of electricity through an insect to visualize the insect's feeding as electrical waveforms. Research to biologically define the EPG waveforms from sharpshooter feeding detected a waveform called the X wave, which is associated with the insect's mouth parts in the xylem. Evidence supports that part of the X wave occurs when a mixture of fluid food and insect saliva previously secreted into the food is taken up into the insect's mouth cavity, swished around, then spit back out (egested). It has been hypothesized that these actions chemically and mechanically loosen *Xylella fastidiosa* (*Xf*) cells from the walls of the vector's mouth cavity and then inject them into a xylem cell. This report updates the latest findings from a four-year project to conclusively test whether part of the X wave, performed by vectors carrying *Xf* bacteria in their mouth cavities, represents *Xf* inoculation into xylem sufficient to cause a systemic Pierce's disease infection in grapevines. Results to date conclusively supported this idea, by showing that vectors performing X waves had a much greater likelihood of inoculating *Xf* that was later detectable by polymerase chain reaction. If future analysis of completed experiments continues to support this idea, it will make possible research using the EPG X wave as a diagnostic tool to identify grape accessions resistant to the vector's *Xf* inoculation behaviors. This would be a new and novel mechanism of Pierce's disease resistance for grape, which can be combined with other sources of resistance for improved durability.

INTRODUCTION
Xylella fastidiosa (*Xf*) cells colonize the cuticular walls of a sharpshooter's functional foregut or mouth (buccal) cavity. Recently, it was demonstrated that *Xf* bacteria are ejected from vector stylets by combined egestion and salivation during stylet probing [1]. In other words, a mixture of fluid food and insect saliva is taken up into the insect's mouth cavity, swished around, then spit back out (egested). It has been hypothesized that these actions

cause *Xf* cells to be loosened from the cuticle of the mouth cavity and inoculated into a xylem cell [2]. This hypothesis has yet to be proven. Correlational evidence supports that the XN portion of the sharpshooter X wave, detected by electropenetrography (EPG), represents the *Xf* inoculation behaviors [2]. This report updates progress in analyzing data from a four-year project to definitively test these hypotheses.

OBJECTIVES

1. To test whether the XN portion of the EPG X wave represents *Xf* inoculation behaviors leading to systemic infection, by detecting the presence or absence of bacteria in stylet-probed grapevines.

RESULTS AND DISCUSSION

Experimental Design

Blue-green sharpshooters (*Graphocephala atropunctata*) were caged on young Chardonnay grapes for 24 hours to acclimate them to feeding on grape. Sharpshooters then were wired and individually fed, each on its own artificial diet sachet (containing approximately 10^7 *Xf* colony-forming units [cfu]) for 2.5 to seven hours. Each insect then was allowed to make a single, EPG-recorded stylet probe on the petiole of the second leaf on a small (three to four inch), vegetatively propagated, two-leaf grape plantlet (one plantlet per insect). Probing was artificially stopped either (1) before XN (termed 'pathway treatment') or (2) after performance of two to four XN events in the same xylem cell (termed 'X wave treatment'). Four to 11 insects were recorded per day for eight to 25 days per year. *Xf* strains used and total number of insects recorded per year were: green fluorescent protein (GFP)-expressing Temecula, 67 (2015), Stag's Leap, 75 (2016), and wild-type Temecula for both 2017 and 2018, 140 and 65, respectively. Each probe site was marked, then the petiole was cut ~2 mm above the mark. The remaining petiole and its leaf (termed the 'probed leaf') was frozen at -20 ºC. The rest of the grape plantlet was held in the greenhouse for five to six months for growth and symptom development. Multiple leaves that were not probed yet apparently symptomatic (termed 'held' leaves) were sampled (at 14, 16, 18, and 20 weeks post-EPG) per plant and frozen (-20 ºC). Frozen leaves were later lyophilized, DNA-extracted, then quantitative polymerase chain reaction (qPCR) tested.

qPCR

In the past two years, many steps have been taken to optimize qPCR methods to remove suspected non-specific binding that could have accounted for uncertain, preliminary findings reported at the 2016 Pierce's Disease Research Symposium. Optimizations included improved standards (plant + *Xf* DNA) and comparisons of various extraction methods (DNEasy kit, cetyl trimethylammonium bromide (CTAB), and lyophilized tissue extraction modified from [3]). For the 2015-2016 probed leaf samples, primers targeting the *Xf* 16S rRNA gene plus SYBR green were used, with quantification of standards using either cfu/ml (mostly probed leaves) or ng/µl DNA (mostly held leaves). PCR runs had a mean R^2 of 99.8% for standards, mean efficiency of 88.1%. A C_T of $\leq$ 30.4 was considered qPCR positive. Further optimization is underway to use HL5/6 primers plus TaqMan for the 2017-2018 samples, to further improve sensitivity and support credibility of results.

Findings

Preliminary results from the 2015-2016 experiments were reported at the 2016 Pierce's Disease Research Symposium and are updated herein. Partial results from 2017-18 experiments may be presented at the 2018 Pierce's Disease Research Symposium.

For the 2015 Experiment. qPCR revealed that none of the negative (healthy) control plants (not insect-probed but reared alongside experimental plants; N = 11) or insect-acclimation plants (fed upon by clean colony insects; N = 18) had detectable *Xf*. In contrast, out of the 67 insect-probed plants, 19 (28%) were positive for *Xf* (**Figure 1**). Of these 19 plants, 14 positives were held leaves, four were probed leaves, and one plant had both held and probed leaves positive (**Figure 2**). The mean C_T value for positive samples was 27.7, with a range of 20.4 to 30.4. While *Xf* copy numbers of 2232 and 109 cfu/ml were detected in two samples (both held leaves), all others had <100 *Xf* cfu/ml (range 6.5 to 91.6) detected. Of the X wave-exposed plants, 18/44 (41%) were PCR-positive, from both held (14/18) and probed (4/18) leaves. In contrast, only one plant (out of 10) exposed to the pathway treatment was PCR-positive (a probed leaf); thus, 95% of positive plants were exposed to X waves.

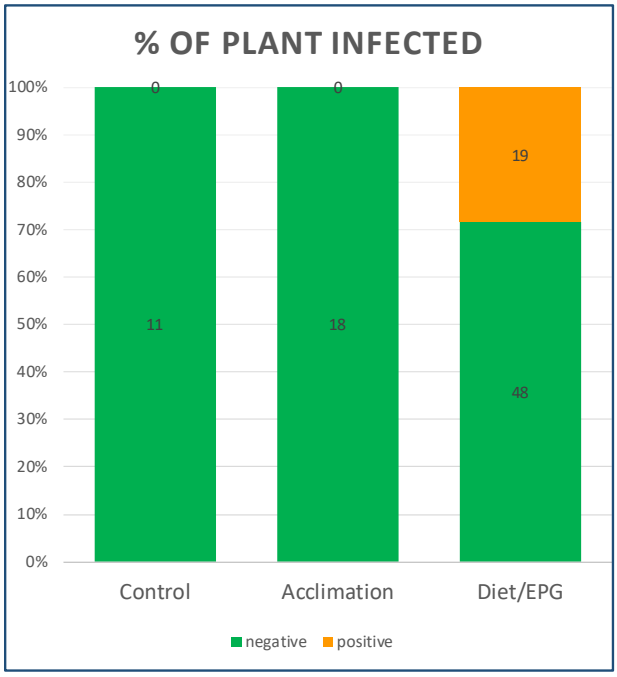

Figure. 1. Percentage of 2015 plants that were insect-inoculated with and subsequently became infected with qPCR-detectable titers of *Xf*.

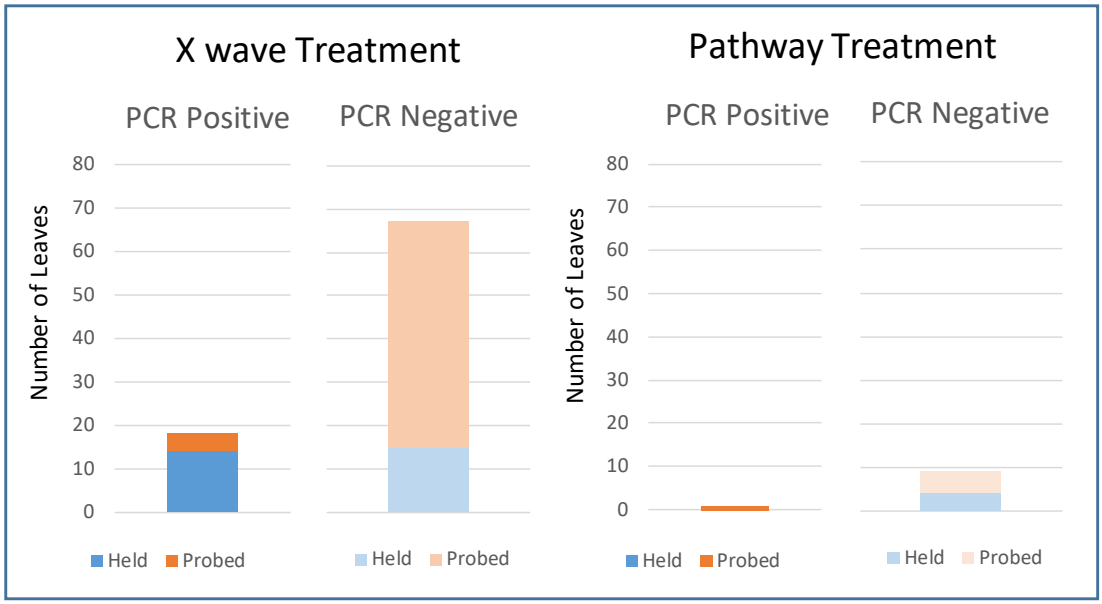

Figure 2. Numbers of 2015 held versus probed leaves from PCR-positive versus PCR-negative plants for each of the two experimental treatments.

It is likely that many more held leaves would have been positive except for a problem with the 2015 protocol. In an attempt to examine the salivary sheath via confocal microscopy, 34/67 plants were cut under paraformaldehyde fixative, rather than in air. All probed leaves survived this treatment, but all 34 plantlets died within three weeks of cutting under fixative. Apparently, paraformaldehyde was pulled into the remaining plant tissues by cavitation (see below), killing the plants. Unfortunately, the fixed tissues were too fragile to survive processing, so fixation was not used for subsequent experimental years; all other plants were cut in air.

EPG waveforms have not yet been analyzed for all 2015 insects; however, preliminary EPG findings probably explain the lone positive pathway-treated plant. Durations of pathway phase varied greatly in probes terminated before the X wave. Some insects made atypically long (>1 minute) pathway phases, especially after being on the

diets for >4 hours. In those cases, insects often performed one or more events of the B2 pathway waveform. B2 has been correlated with salivary sheath branching after the insect has briefly encountered a small, immature xylem cell that was tasted/tested (i.e., egested/spit up into) but rejected for sustained sap ingestion [4]. Behavior of the insect on the PCR-positive, pathway-only plant was extremely unusual; it performed pathway activities for nearly five minutes (292 seconds), especially an extraordinary 19 B2 events. It is likely this insect tasted/egested into numerous immature xylem cells before its probe was artificially terminated. Even only a few bacteria egested into so many (although immature) xylem cells could eventually develop a sufficient titer to be detected via PCR of a probed leaf; no bacteria were detected in held leaves distant from the site of probing. Therefore, under very rare circumstances, the X wave might not be the sole indicator of *Xf* inoculation. Egestion and salivation are known to occur during pathway waveforms also, but usually occur in non-xylem or immature xylem cells. This hypothesis will be tested further in 2017-2018 samples.

For the 2016 Experiment. We attempted to use Stag's Leap *Xf* to shorten the holding time in the greenhouse. This strain of *Xf*, however, clumped severely in the artificial diet, making it difficult for the insects to suck up bacteria. We also attempted to pull more bacteria into the probed leaf by putting stylet-probed plantlets under bright, hot sodium vapor lamps for 30 minutes before cutting in air. This may have worked because only 3/75 held leaves were PCR-positive. However, the 2016 probed leaves have not yet been PCR-tested.

Additional Thoughts. Bacteria detected in any 2015 probed leaf were those injected into a xylem cell at the probe site and then were transported systemically further than two mm above the insect's probe site within two minutes after the termination of the probe. Bacteria detected in the held leaf were injected into xylem during probing but not transported above the site of the cut. When the petiole cut was made, instantaneous cavitation would pull bacteria above the cut further into the petiole-leaf intersection; bacteria below the cut would be pulled into the stem and, ultimately, into the remainder of the tiny plantlet. These cells then initiated an infection that was later detectable in leaves many cm away, five to six months later. The more numerous detections of bacteria in held leaves suggests that most cells remained close to the probe site; fewer were pulled above the cut site into the probed leaf. Bacteria injected into immature xylem cells during pathway treatment were pulled >2 mm above the probe site.

CONCLUSIONS

Results to date continue to support the hypothesis that the XN portion of the X wave represents egestion and salivation behaviors sufficient to inoculate *Xf* into xylem and initiate a systemic infection. If this finding continues to be supported in future analyses, the EPG X wave can be used to detect grape resistance to the vector inoculation behavior, a new *Xf* resistance trait.

REFERENCES CITED

1. Backus EA, Shugart HJ, Rogers EE, Morgan JK, Shatters R. 2015. Direct evidence of egestion and salivation of *Xylella fastidiosa* suggests sharpshooters can be "flying syringes." *Phytopathology* 105:608-620.
2. Backus EA. 2016. Sharpshooter feeding behavior in relation to transmission of *Xylella fastidiosa*: A model for foregut-borne transmission mechanisms. *Vector-Mediated Transmission of Plant Pathogens*. Brown JK (ed.). American Phytopathological Society Press, pp. 175-194.
3. Lee SB, Milgroom M, Taylor J. 1988. A rapid, high yield mini-prep method for isolation of total genomic DNA from fungi. *Fungal Genetics Newsletter* 35:23-24.
4. Backus EA, Holmes WJ, Schreiber F, Reardon BJ, Walker GP. 2009. Sharpshooter X wave: Correlation of an electrical penetration graph waveform with xylem penetration supports a hypothesized mechanism for *Xylella fastidiosa* inoculation. *Annals of the Entomological Society of America* 102(5):847-867.

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RIVERSIDE COUNTY GLASSY-WINGED SHARPSHOOTER PROGRAM IN THE TEMECULA VALLEY

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ABSTRACT
For more than 15 years Temecula Valley has been part of an area-wide program for an invasive vector, the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS). The goal of this program is to limit Pierce's disease spread by suppressing vector populations in commercial citrus, an important reproductive host for this insect, before they move out into vineyards. The area-wide program originally consisted of applications of typically systemic insecticides to citrus groves along with monitoring of GWSS populations – both to evaluate the effectiveness of the treatments and to guide grape grower treatment decisions. The treatment element of the program was halted in 2013, though it is likely that similar chemical control is occurring to target important citrus pests – particularly the Asian citrus psyllid (*Diaphorina citri*). Monitoring of GWSS populations continues to occur, with 176 yellow sticky traps placed throughout Temecula citrus and select vineyards being inspected on a biweekly basis. Compared to 2017, which saw the highest GWSS catch since at least 2003, overall GWSS catch in 2018 is down to more typical levels. 2018 trapping results exhibit typical seasonal patterns for this pest in the region, with a modest winter peak in catch, and a total of approximately 812 GWSS caught during the summer peak (July through September). Thus far, there is no apparent late summer peak, which occurs in some years.

LAYPERSON SUMMARY
The glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) constitutes one of the primary threats to the wine, table grape, and raisin industries in California owing to its ability to spread the pathogen that causes Pierce's disease. In the Temecula Valley, an area-wide control program has been in place for more than 15 years, which until recently relied on insecticide applications in citrus groves to control GWSS before they move into vineyards and still entails regular monitoring of GWSS populations throughout the region. This program is important for guiding management decisions for vineyards in the area. Last year's extremely high trap catch warned of a resurgence in GWSS populations, as has occurred in some parts of the Central Valley. However, results for 2018 don't support that conclusion in that GWSS catch was substantially lower and more in line with what has been observed in years past.

INTRODUCTION
The wine grape industry and its associated tourism in Temecula Valley generate an estimated $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 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 (*Cosmocomoidea* spp.) 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 advisers, 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 Temecula Valley. This change was motivated, in part, by the expectation that citrus growers would likely be treating already for the Asian citrus psyllid (*Diaphorina citri*), an invasive vector of the pathogen associated with huanglongbing or citrus greening disease. Sharpshooter and psyllid integrated pest management rely on largely the same insecticides. However the timing of applications differs slightly depending on the focal pest. Therefore, monitoring of sharpshooter populations continues to be important, to identify for grape growers those vineyards most at risk to GWSS and Pierce's disease.

OBJECTIVES

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

Double-sided yellow-sticky cards (14x22 cm; Seabright Laboratories, Emeryville, CA) are being used to monitor for adult sharpshooters in citrus. 176 such sticky traps have been placed in citrus groves (primarily), vineyards, and select residential areas with citrus trees throughout the Temecula Valley. All traps are labeled, numbered, and bar coded to identify the site within the management program. Each trap is then georeferenced with a handheld global positioning system (GPS) monitor. Most traps are placed at the edge of the groves at the rate of approximately one per 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. Sharpshooters found on the traps are counted and then removed from the trap.

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

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

RESULTS AND DISCUSSION

The results of the 2018 monitoring are shown in **Figure 1**, relative to prior years. These data consist of monthly censuses of GWSS through April, then biweekly censuses from May through October. Results for the remainder of 2018 are pending. Thus far, 2018 trapping shows seasonal patterns of GWSS activity or abundance that are typical for the region. GWSS catch was low for much of the year, then increased dramatically at the beginning of the summer before dropping off through August and September. Thus far, no late summer peak in GWSS activity has been observed, which occurs in some years. Notably, despite a greater number of traps being deployed this year (176) compared to the prior six years (~140), the overall 2018 catch appears to be intermediate compared to past years. Although GWSS were more abundant than in the lowest census years (e.g., 2010, 2011), peak count in July 2018 was just 20% of that observed in 2017, which was the highest observed in at least 15 years.

This year we began monitoring GWSS in select vineyards that are nearby citrus groves that had shown high GWSS activity in past years. GWSS counts on citrus traps were slightly higher than on vineyard traps, but the seasonal patterns of GWSS abundance appear to closely track each other in the two habitats (**Figure 2**).

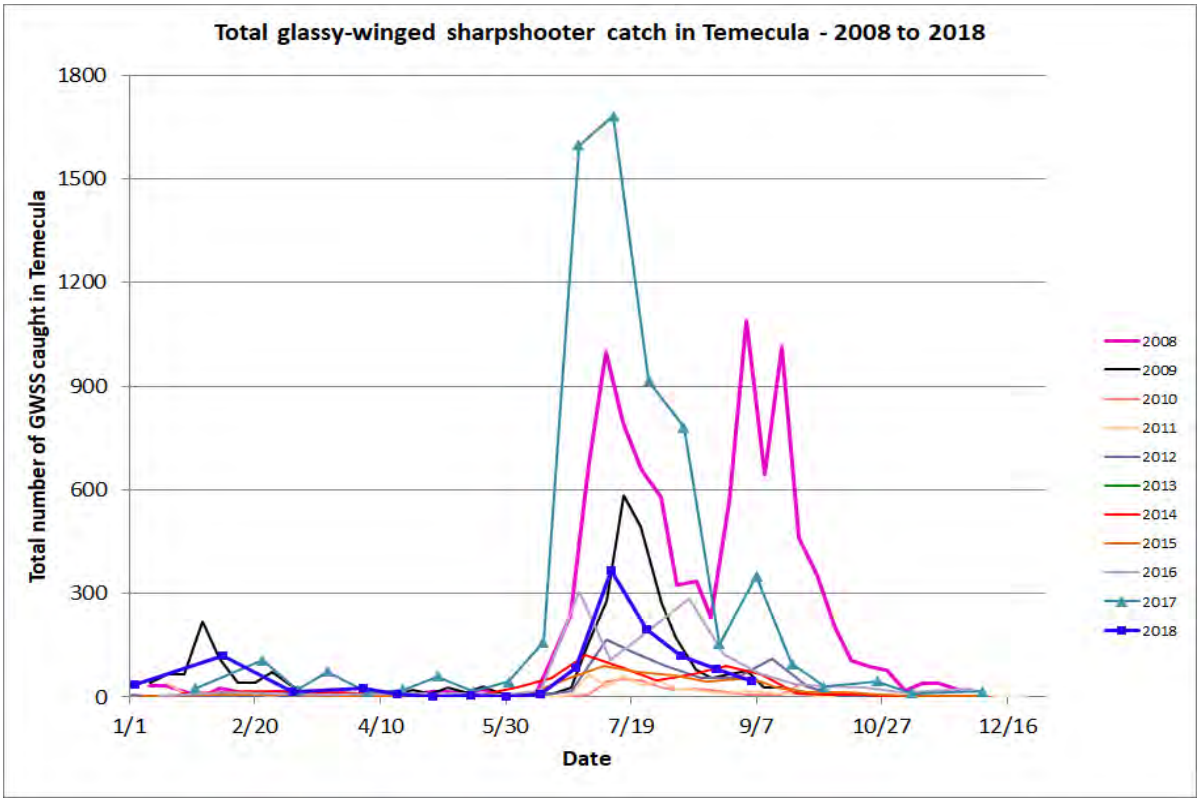

Figure 1. Seasonal total GWSS catch in 2018 compared to prior years.

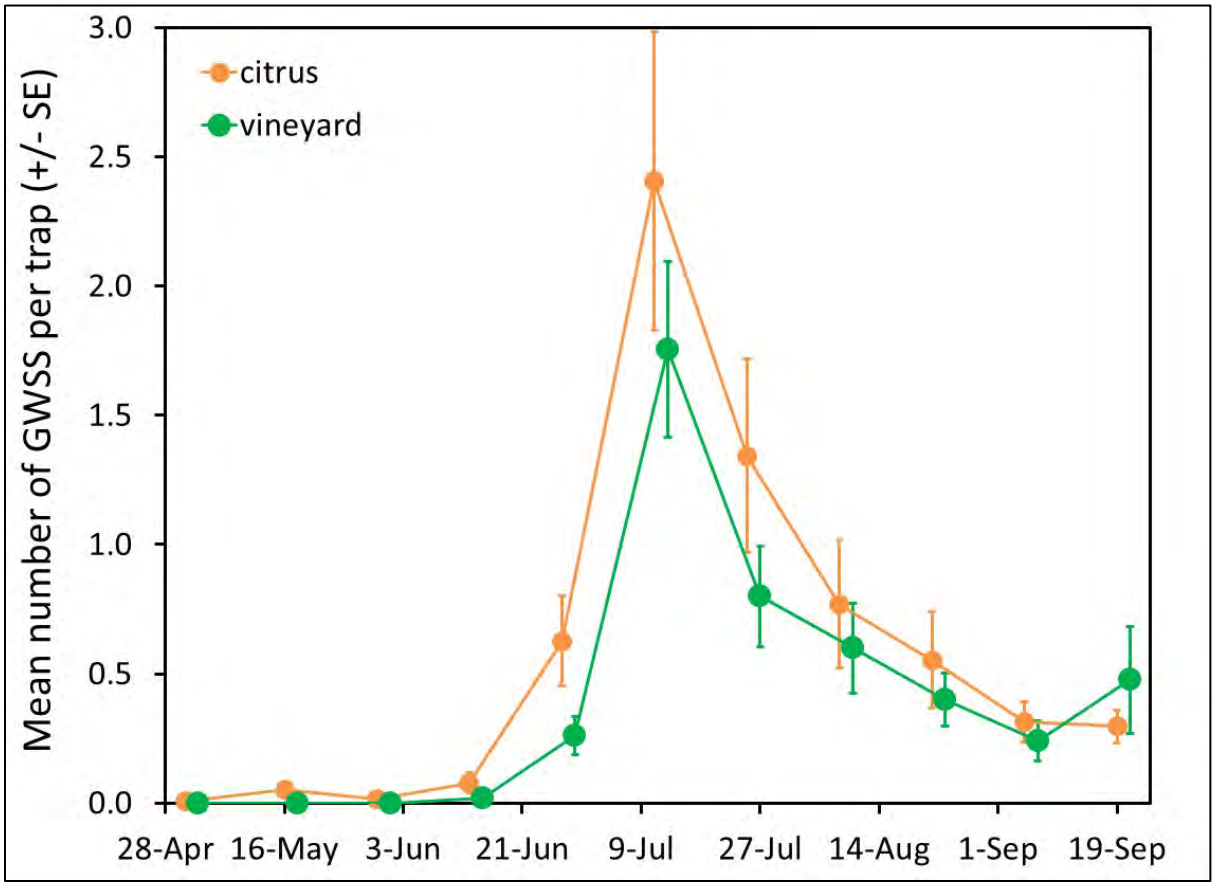

Figure 2. Seasonal GWSS catch in Temecula Valley citrus versus vineyards.

CONCLUSIONS

The sizeable increase in GWSS activity seen in Temecula in 2017 prompted concerns of a chronic resurgence in GWSS populations as has occurred in other areas of California over the last few years. Results for 2018 seem to suggest last year's results could represent an acute spike in activity. Even so, the driver of last year's high numbers is not known definitively and there is clearly potential for substantial interannual variability in GWSS abundance in the region. As a result, Temecula grape growers are encouraged to remain vigilant with respect to the monitoring and management activities for GWSS and Pierce's disease in their vineyards.

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INSECTICIDE RESISTANCE IN THE GLASSY-WINGED SHARPSHOOTER: USING HISTORICAL USE PATTERNS TO INFORM FUTURE MANAGEMENT STRATEGIES

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Reporting Period: The results reported here are from work conducted July 2017 to October 2018.

ABSTRACT

Populations of the glassy-winged sharpshooter (*Homalodisca vitripennis*: GWSS), a vector of *Xylella fastidiosa*, causal agent of Pierce's disease, sharply increased in 2012 in Kern County, California. Despite continued management with insecticides, persistently high populations over the next few years suggested that resistance had developed in the region. Tests of susceptibility to eight different chemical insecticides began in the 2014 growing season and were repeated through 2016. These early bioassays revealed significantly lowered susceptibility of GWSS to acetamiprid (7-fold) and bifenthrin (152-fold) and a trend of decreasing susceptibility to the neonicotinoids from early to late season. In 2017, bioassays of imidacloprid susceptibility levels in four different populations over the growing season were conducted. These four locations, with unique patterns of proximate imidacloprid field applications, were monitored monthly from July through October. Two of the sites maintained high populations of GWSS from early (July) to late (September/October) season months and demonstrated significantly decreased imidacloprid susceptibility over that time. GWSS collected at the South Highway 65 site, which had fewer imidacloprid applications within a 1.5-mile radius, demonstrated an 11-fold decrease in susceptibility, while those at the North Highway 65 site, with earlier and more frequent applications, demonstrated a 29-fold decrease in susceptibility to imidacloprid. With access to 17 years of insecticide treatment records and GWSS trap counts in Kern County, current work is focused on evaluating GWSS population dynamics in response to pesticide applications. Applications of all formulations of acetamiprid, bifenthrin, chlorpyrifos, dimethoate, fenpropathrin, flupyradifurone, and thiamethoxam to GWSS hosts have been identified for Kern County CDFA zones 1 and 3, the regions with high GWSS populations currently and historically. Pairing tests of current versus past insecticide susceptibility levels with historical application records and GWSS trap counts can contribute to a better understanding of population dynamics and resistance development corresponding to insecticide use for the management of GWSS.

LAYPERSON SUMMARY

Insecticides remain the most frequently used tool for the management of glassy-winged sharpshooter (*Homalodisca vitripennis*: GWSS) and Pierce's disease. Our interest in this project was due to the high GWSS numbers from 2012-2015, despite continued monitoring and treatments. This suggested that the populations may be changing with respect to their susceptibility to commonly used products. Our studies in 2015, 2016, and 2017 showed varying levels of resistance to insecticides in Kern County populations of GWSS. Compared to similar studies conducted in 2001 and 2002, we found resistance to the chemicals acetamiprid and bifenthrin. We also found that as the season progressed, the insects were less susceptible to one of the most widely used materials, imidacloprid. In 2017, we documented that insects collected near fields that had been treated early and often with imidacloprid were less susceptible later in the season than insects collected near fields that had not been treated early and often. This suggests that timing and frequency of imidacloprid impacts the season-long susceptibility of GWSS to this material.

INTRODUCTION

Initiated in July 2016, this project is an extension of a pilot study that was conducted in 2014 and 2015 with support from the Consolidated Central Valley Table Grape Pest and Disease Control District and the CDFA Pierce's Disease and Glassy-winged Sharpshooter (PD/GWSS) Board. Despite continued efforts in the Area-wide GWSS Management Program, numbers of sharpshooters sharply increased after 2011 and remained high through 2015, causing concern among the industry. At the same time, surveys of PD-infected vines indicated an increase in disease incidence in the General Beale region of Kern County (Haviland 2015).

In the 2015 study, we evaluated eight commonly used compounds (**Table 1**), in both systemic uptake and foliar bioassays. We collected GWSS on three dates in July and August from an organic citrus grove in the Edison area and three dates in September and October from the General Beale area. These studies showed that GWSS collected in 2015 were much less susceptible to some of the tested insecticides than they were in 2001 and 2002 (Prabhaker et al., 2006) when the Area-wide GWSS Management Program was initiated (Perring et al., 2015). For some insecticides, the studies showed LC_{50} values to be significantly 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.

Table 1. Insecticides tested on adult GWSS bioassays in 2015.

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

These high levels of resistance may explain the upsurge in GWSS number in the region. At the same time, 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 LC_{50} value 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.

In 2016, these studies were repeated. Despite a reduced number of sharpshooters compared to 2016, we evaluated two pyrethroids and three neonicotinoids on two dates from table grapes and one date from citrus. The data from 2016 showed susceptibility levels similar to those in 2015 for all five chemicals (Perring et al., 2016), demonstrating that resistance levels in 2015 and 2016 were higher than in 2001 and 2002, again indicating that susceptibility had declined over the years. The data also showed declining susceptibility to the systemic neonicotinoids imidacloprid and thiamethoxam over the course of the season, revealing a trend repeated from the 2015 bioassays and similar to Redak et al. (2016).

With limited numbers of GWSS available for collection in 2016 and because imidacloprid has been used extensively in citrus (Grafton-Cardwell et al., 2008) and grapes (Daane et al., 2006), our 2017 bioassays focused on imidacloprid testing. As previously mentioned, bioassays with imidacloprid in 2015 and 2016 demonstrated a trend of declining susceptibility from early season (June/July) bioassays to late season (September/October) bioassays. We followed up on this discovery by choosing four different sites with unique patterns of nearby field applications of imidacloprid (Admire® Pro) and similar mode of action compounds, acetamiprid (Assail® 70 WP) and thiamethoxam (Actara®) for monthly testing to determine if seasonal reduced susceptibility occurred within different Kern County regions (**Figure 1**). We called two sites 'organic' and two sites 'treated' based on distance,

greater than 1 mile and less than 0.5 miles, respectively, from imidacloprid applications during the season. We found that seasonal reduced susceptibility did occur in both the 'organic' and 'treated' sites and that the degree of reduction was likely due to nearby field applications.

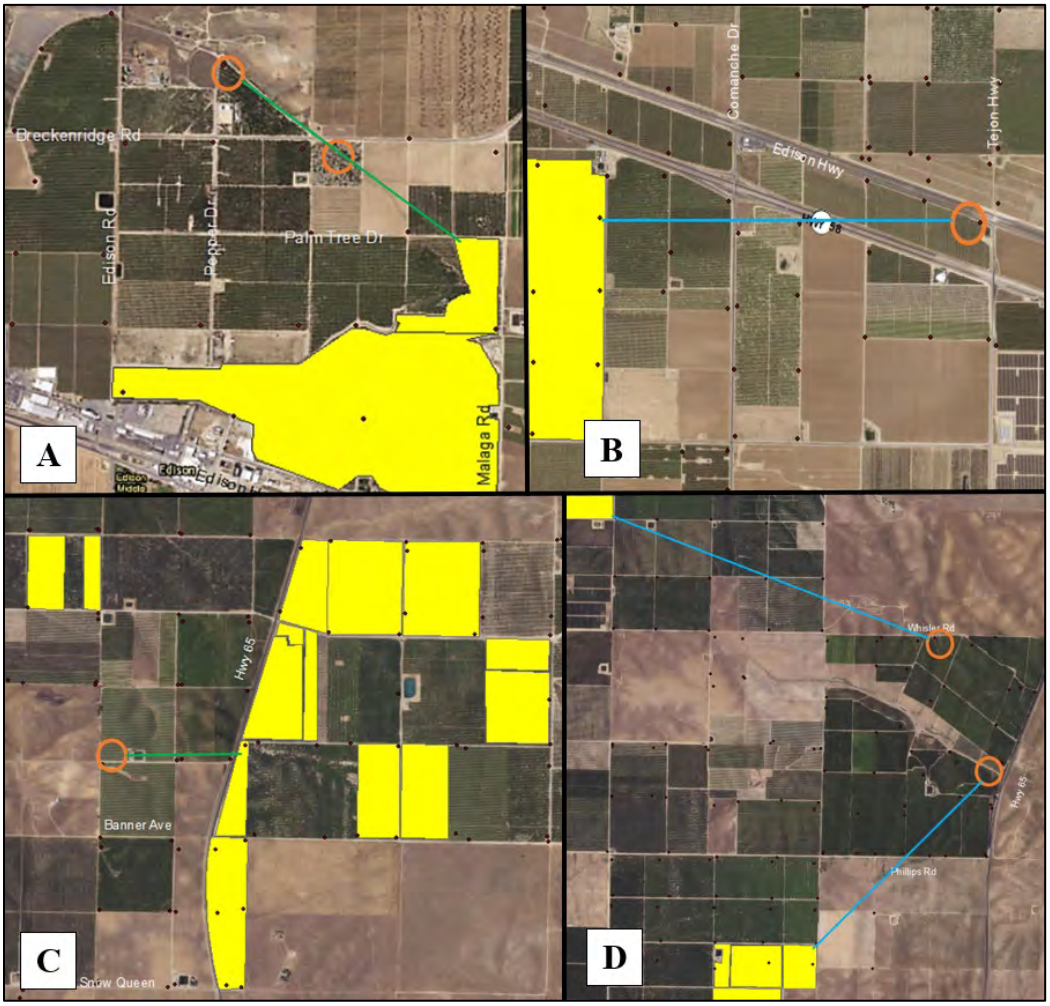

Figure 1. Four Kern County locations chosen for GWSS collection and imidacloprid bioassays. (A) Treated Site 1 (T1), (B) Organic Site 1 (O1), (C) Treated Site 2 (T2), and (D) Organic Site (O2). Citrus or grapes treated with imidacloprid in 2017 are represented by the yellow areas. Orange circles indicate collection sites. Green lines represent distances between collection sites and treated areas that are less than 0.5 mile. Blue lines represent distances between collection sites and treated areas of over 1 mile.

The purpose of this project was to determine if GWSS has become less susceptible to various insecticides over the last 15 years and if resistance development possibly contributed to the recent resurgence of GWSS in Kern County. Additionally, we aimed to determine how patterns of GWSS resurgence (areas and timing) were related to historical insecticide applications. Increasing our understanding of the factors contributing to reduced resistance, both seasonal and over the years, may help growers in their selection of GWSS management materials and application timings in their areas.

OBJECTIVES

1. Conduct laboratory bioassays on field-collected GWSS from Kern County to document the levels of susceptibility at the beginning of the 2017 field season and document changes in susceptibility as the season progresses.
2. Document differences in insecticide susceptibility in GWSS collected from organic versus 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. Conduct Lab Bioassays on Field-Collected GWSS and Document Differences in GWSS Insecticide Susceptibility from Organic vs. Non-Organic Vineyards and Orchards

The 2017 bioassays with imidacloprid were conducted on GWSS collected monthly from four different Kern County sites from July through October. Initially our bioassays were grouped and analyzed according to the 'organic' versus 'treated' site designations as reported in Perring et al. (2017). We have since analyzed each site individually to determine if susceptibility reduction over the season was related to the distance of the collection sites from field applications of imidacloprid. The previously named 'organic' sites included the East (E) Edison (O1) and South Highway (S Hwy) 65 (O2) locations, and the 'treated' sites included the West (W) Edison (T1) and North Highway (N Hwy) 65 (T2) locations (**Figure 1**). We created a new map of our four sites which includes the timing of nearby imidacloprid applications (all formulations) applied to surrounding perennial hosts (grape, grapefruit, lemon, orange, pistachio, tangelo, and tangerine; listed in CDFA Plant Quarantine Manual, Section 454; http://pi.cdfa.ca.gov/pqm/manual/pdf/454.pdf) from January 1 through October 9, 2017 (**Figure 2**).

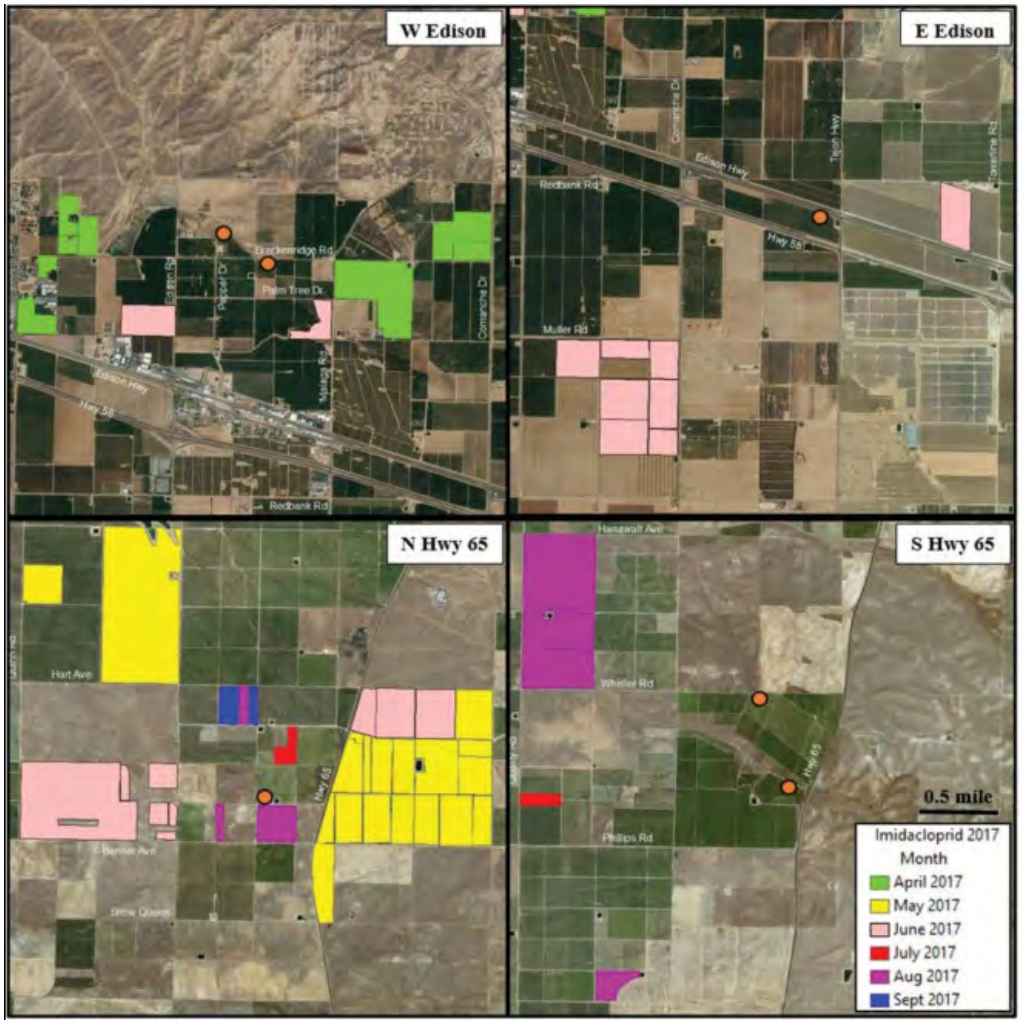

Figure 2. Locations of GWSS collections in 2017. Orange dots represent the exact collection sites. In the upper left quadrant is site W Edison; upper right is E Edison; lower left is N Hwy 65; and lower right is S Hwy 65. Each quadrant contains the approximately 3 mi^2 region surrounding each site. The legend indicates the months in which imidacloprid applications were made to field near the collection sites (from Andreason et al., 2018).

Each GWSS collection site had a unique situation of proximate imidacloprid applications and treatment timings. The two 'treated' sites, W Edison and N Hwy 65, had applications early in the growing season (April and May, respectively) whereas the previous 'organic' sites,' E Edison and S Hwy 65, had the earliest applications in June and July, respectively. There also were more frequent applications within a 1.5-mile radius around the W Edison and N Hwy 65 collection sites as well as applications closer to these sites. Collections from citrus orchards and subsequent bioassays began in July and were repeated at each site in August (**Table 2**). Resulting LC_{50} values were similar to those determined at the beginning of 2016 and 2015 tests, indicating that the reduced susceptibility levels at the end of the previous year do not continue into the next year and that LC_{50} values revert back to previous years' early season levels. The LC_{50} values also were not significantly different among sites nor were they different from July to August. Unfortunately, E and W Edison could not be tested into late season as GWSS numbers were significantly lower in September. N and S Hwy 65 collections were assayed in mid-September, but then only S Hwy 65 could be tested in October. Analyzing these sites individually, we found that susceptibility of the GWSS collected at the N and S Hwy 65 sites decreased significantly from July to September and July to October, respectively (**Table 2**). At N Hwy 65, where imidacloprid was applied early and often, susceptibility dropped 29-fold. At S Hwy 65, with applications later and less frequent, susceptibility decreased 11-fold. These results suggest that seasonal reductions in susceptibility to imidacloprid occur and that differential proximity to field applications likely contributes to the degree of reduction.

Table 2. Toxicities of imidacloprid to GWSS determined in uptake bioassays in multiple locations in Kern County, California, USA in 2017.

Year	Date	Location	*n*	LC_{50} µg/ml (95% FL)	Slope ± SE	χ^2 (df)
2017	Jul. 24	E Edison	270	4.01 (0.63-11.31)	1.26 ± 0.23	3.15 (3)
		W Edison	140	*0.38 (0.02-12.49)	0.88 ± 0.13	9.12 (3)
		S Hwy 65	150	0.80 (0.13-2.07)	1.29 ± 0.36	2.46 (3)
		N Hwy 65	150	1.79 (0.54-3.98)	1.50 ± 0.37	1.73 (3)
	Aug. 8	E Edison	238	1.27 (0.26-4.73)	0.95 ± 0.12	4.71 (3)
		W Edison	50	*1.12 (0.03-22.72)	0.90 ± 0.20	3.57 (3)
		S Hwy 65	237	0.56 (0.09-2.09)	1.11 ± 0.15	5.48 (3)
		N Hwy 65	59	*0.13 (0.08-0.18)	1.37 ± 0.58	0.09 (3)
	Sep. 12	S Hwy 65	150	*8.99 (1.00-47.78^{+})	1.15 ± 0.25	6.48 (3)
		N Hwy 65	150	51.53 (21.33-204.99)	1.02 ± 0.27	2.50 (3)
	Oct. 9	S Hwy 65	504	8.71 (2.93-27.28)	0.89 ± 0.09	5.62 (3)

* LC_{50} determined by probit analysis using PoloSuite because of high variability in dose responses.
$^{+}$ 90% fiducial limit (FL) reported in place of indeterminable 95% FL.

Further analysis of our bioassay results using a generalized linear mixed model (GLMM) corroborated the significance of the observed seasonal decreases. With all sites combined, there was a significant decrease from an average 50.5% mortality in July to 23.7% and 29.6% in September and October, respectively (**Table 3**). When the sites were analyzed separately, mortalities at S Hwy 65 significantly decreased from 61.3% to 29.6%, while mortalities at N Hwy 65 significantly decreased from 53.3% to 20.0%.

Table 3. Imidacloprid-induced mortality of GWSS collected in 2017 at different locations in Kern County, CA analyzed by a generalized linear mixed model (GLMM) (from Andreason et al. 2018).

Year	Date	Combined Mortality (%)	S Hwy 65 Mortality (%)	N Hwy 65 Mortality (%)
2017	Jul. 24	50.5 (147) a	61.3 (30) a	53.3 (30) a
	Aug. 8	46.4 (120) b	47.5 (48) b	62.1 (12) a
	Sep. 12	23.7 (60) c	27.3 (30) c	20.0 (30) b
	Oct. 9	29.6 (101) c	29.6 (101) c	--------

Values within the same column followed by the same letter are not significantly different, Tukey's test ($P < 0.05$). The number of replicates (clip cages containing five insects) on each date are given in parentheses.

Comparing this study's results to the baseline susceptibility levels determined in 2001 and 2002 (Prabhaker et al., 2006), all data from the yearly bioassays conducted on imidacloprid, thiamethoxam, acetamiprid, bifenthrin, and fenpropathrin were used to calculate an overall LC_{50} value for each chemical (**Table 4**). We did not include data from some previously tested compounds (flupyradifurone, chlorpyrifos, and dimethoate) because of a lack of adequate bioassay replicates resulting from few GWSS in 2016, and because of high variation in the responses of the tests we were able to conduct. For each of the neonicotinoid and pyrethroid compounds, the annual LC_{50} values were not significantly different from 2015 to 2016.

Table 4. Toxicities of various insecticides to GWSS collected from multiple locations in Kern County, CA from 2015 through 2017 as determined by uptake and leaf dip bioassays. Average 2001, 2002, 2003 values calculated from Prabhaker et al. (2006).

Compound	Year	*n*	LC_{50} µg/ml (95% FL)	Slope ± SE	χ^2 (df)
Imidacloprid	2015	1,171	2.51 (0.98-5.29)	0.77 ± 0.06	53.68 (13)
	2016	575	3.43 (0.61-17.76)	0.74 ± 0.07	10.02 (3)
	2017	2,098	2.90 (1.05-6.45)	0.88 ± 0.05	11.59 (3)
	Overall	3,844	2.91 (1.93-4.21)	0.82 ± 0.04	47.27 (15)
	2001	312	1.27 (0.68-2.54)	1.1 ± 0.30	6.24 (4)
	2002	295	0.36 (0.09-0.51)	1.2 ± 0.35	4.76 (4)
Thiamethoxam	2015	775	0.74 (0.35-1.50)	0.93 ± 0.07	15.53 (6)
	2016	563	1.48 (0.35-4.94)	1.02 ± 0.08	11.33 (3)
	Overall	1,338	1.03 (0.54-1.87)	0.97 ± 0.05	20.67 (6)
Acetamiprid	2015	450	2.88 (1.06-8.13)	0.77 ± 0.07	4.41 (3)
	2016	450	0.94 (0.15-3.59)	0.59 ± 0.07	4.23 (3)
	Overall	900	1.78 (1.11-2.75)	0.67 ± 0.05	2.36 (3)
	2001	315	0.44 (0.18-0.56)	2.0 ± 0.14	4.85 (4)
	2002	320	0.08 (0.02-0.14)	1.4 ± 0.11	3.87 (3)
Bifenthrin	2015	746	0.54 (0.21-1.15)	0.74 ± 0.06	3.15 (3)
	2016	302	1.03 (0.29-3.72)	1.09 ± 0.11	6.73 (3)
	Overall	1,048	0.67 (0.30-1.29)	0.82 ± 0.06	4.00 (3)
	2001	312	0.0005 (0.0002-0.0038)	1.4 ± 0.24	3.76 (4)
	2002	320	0.0126 (0.0085-0.0347)	1.7 ± 0.32	2.88 (4)
	2003	285	0.0001 (0.00009-0.0004)	2.9 ± 0.27	2.64 (4)
Fenpropathrin	2015	735	0.33 (0.19-0.54)	0.60 ± 0.05	3.46 (4)
	2016	150	0.80 (0.32-1.70)	1.13 ± 0.20	1.13 (3)
	Overall	885	0.40 (0.19-0.77)	0.66 ± 0.05	4.45 (4)
	2001	306	0.064 (0.045-0.205)	1.2 ±0.21	5.82 (4)
	2002	215	0.020 (0.007-0.060)	1.1 ± 0.25	4.76 (4)

For imidacloprid, the overall LC_{50} value of 2.91 µg/ml represented a 3.5-fold decrease in susceptibility compared to the average values from 2001 and 2002 (average LC_{50} = 0.82 µg/ml). However, with a 95% FL overlapping with one of the previous years (2001), this decrease was not significant. The thiamethoxam LC_{50} value determined in 2001/2002 could not be compared to the current value because the compound was previously tested as a foliar insecticide and we used a systemic bioassay in our studies. Thus, the present study establishes the baseline susceptibility level of GWSS to thiamethoxam applied systemically. For acetamiprid, the present overall LC_{50} of 1.78 µg/ml showed a seven-fold decrease in susceptibility from the previous assays (2001/2002 average LC_{50} = 0.26 µg/ml). With no overlap in 95% FL between the earlier and present bioassays, this was a significant decrease in susceptibility. GWSS susceptibility to bifenthrin significantly decreased as well. The current 2015/2016 overall LC_{50} was 0.67 µg/ml which is a 152-fold decrease from the 2001/2002/2003 average LC_{50} of 0.0044 µg/ml. The lack of overlapping 95% FLs indicate that these values are significantly different. Finally, for fenpropathrin the 2015/2016 overall LC_{50} of 0.40 µg/ml was 9.5 times higher than the average 2001/2002 LC_{50} value of 0.042 µg/ml, but the overlap in 95% FLs indicates that this was not a significant increase. Overall, of the five compounds tested, acetamiprid and bifenthrin were determined to be significantly less toxic to GWSS, indicating that resistance to these compounds has likely developed over the last 15 years.

Objectives 3 and 4. Obtain and Organize Historic GWSS Densities and Treatment Records into a GIS and Determine the Relationship Between GWSS Insecticide Susceptibility and Treatment History

After the recent publication of our findings related to Objectives 1 and 2 (Andreason et al., 2018), we have shifted our focus to Objectives 3 and 4. To explore the relationships between historical pesticide applications and GWSS resurgence in different areas, we have obtained the Kern County pesticide application records and identified all applications of the eight compounds of interest to GWSS hosts from 2001 through 2017. These filtered records include applications of every formulation of each compound to all reported hosts of GWSS, both annual and perennial, within Kern County zones 1 and 3 over the last 17 years. These data have been compiled into an excel spreadsheet which can be imported into our GIS. Currently, we are determining patterns of GWSS abundance as determined by CDFA trap data relative to application dates of the various chemicals.

CONCLUSIONS

Repeated bioassays in 2015 and 2016 with three neonicotinoids, imidacloprid, acetamiprid, and thiamethoxam, as well as two pyrethroids, bifenthrin and fenpropathrin, were conducted to determine if GWSS susceptibility levels had shifted since 2001 and 2002. We found the toxicity of acetamiprid and bifenthrin to GWSS in Kern County was significantly reduced, suggesting resistance development to these insecticides. The toxicity of the three other materials was not significantly changed from past studies. In the third year of our bioassays, we focused on the observed reduction of susceptibility to imidacloprid from early summer to early fall, when GWSS are at their peak populations in Kern County. These tests demonstrated that in at least two separate locations, susceptibility significantly drops from July to September and October. This understanding of current versus past toxicity levels, and seasonal changes in susceptibility, combined with our present work on GWSS population dynamics in relation to insecticide use can be helpful to growers in their selection of materials for GWSS management.

REFERENCES CITED

Andreason SA, Prabhaker N, Castle SJ, Ganjisaffar F, Haviland DR, Stone-Smith B, Perring TM. 2018. Reduced susceptibility of *Homalodisca vitripennis* (Hemiptera: Cicadellidae) to commonly applied insecticides. *J. Econ. Entomol*. 111:2340-2348.

Daane KM, Bentley WJ, Walton VM, Malakar-Kuenen R, Millar JG, Ingels C, Weber E, Gispert C. 2006. New controls investigated for vine mealybug. *Calif. Agric*. 60:31-38.

Grafton-Cardwell EE, Lee JE, Robillard SM, Gorden JM. 2008. Role of imidacloprid in integrated pest management of California citrus. *J. Econ. Entomol*. 101:451-460.

Haviland D. 2015. Monitoring the resurgence of Pierce's disease in Kern County vineyards. *Consolidated Central Valley Table Grape Pest and Disease Control District Newsletter: Fall 2015*, pp. 1-2.

Perring TM, Prabhaker N, Castle S. 2015. Monitoring for insecticide resistance in the glassy-winged sharpshooter in California. *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. 142-146.

Perring TM, Prabhaker N, Castle S, Haviland D, Stone-Smith B. 2016. Monitoring for insecticide resistance in the glassy-winged sharpshooter in California. *Proceedings of the 2016 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA, pp. 221-229

Perring TM, Prabhaker N, Andreason S, Castle S, Haviland D, Stone-Smith B. 2017. Monitoring for insecticide resistance in the glassy-winged sharpshooter in California. *Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes*. December 2017. California Department of Food and Agriculture, Sacramento, CA, pp. 157-162.

Prabhaker N, Castle SJ, Byrne FJ, Toscano NC, Henneberry TJ. 2006. Establishment of baseline susceptibility to various insecticides for glassy-winged sharpshooter, *Homalodisca coagulata*, by comparative bioassays. *J. Econ. Entomol*. 99:141-154.

Redak R, White B, Byrne FJ. 2015. Management of insecticide resistance in glassy-winged sharpshooter populations using toxicological, biochemical, and genomic tools. *Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes*. December 2015. California Department of Food and Agriculture, Sacramento, CA, pp. 157-163.

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

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

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

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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 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. Synergism bioassays with piperonyl butoxide suggest that the causal mechanism of imidacloprid resistance is due to metabolism by cytochrome P450 enzymes.

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 insensitivity, indicating that there is no target site resistance to OPs (or carbamates, which share the same acetylcholinesterase 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 RNA sequencing 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 complementary DNA 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. Our current data indicate that the basis for the imidacloprid resistance appears to be metabolic.

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 was introduced to the management program to suppress overwintering adults and reduce the first early season cohort of egg-laying adults. The combination of early season foliar treatments combined with the more persistent systemic treatments has effectively managed GWSS populations in Kern County for many years.

In Kern County, GWSS populations have been monitored since the area-wide treatment program was 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_{50} 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 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

2018 Monitoring Data. In 2018, bioassays were conducted on insects collected from four locations in Kern and Tulare Counties. Resistance to imidacloprid was confirmed in populations in the General Beale Road (GBR) area (**Figure 1**). While the resistance in the GBR region is not new, in 2018 we detected a highly resistant population in Tulare County. In previous work, we tested insects from an organic grove where GWSS numbers are consistently high (using the CDFA maps as indicators of numbers) during the summer season. During our monitoring work in 2018, we sampled insects from a grove under conventional management located three miles from the organic site, and detected levels of imidacloprid resistance that were comparable with those measured in the Edison and GBR populations. Full details are provided in the caption to **Figure 1**.

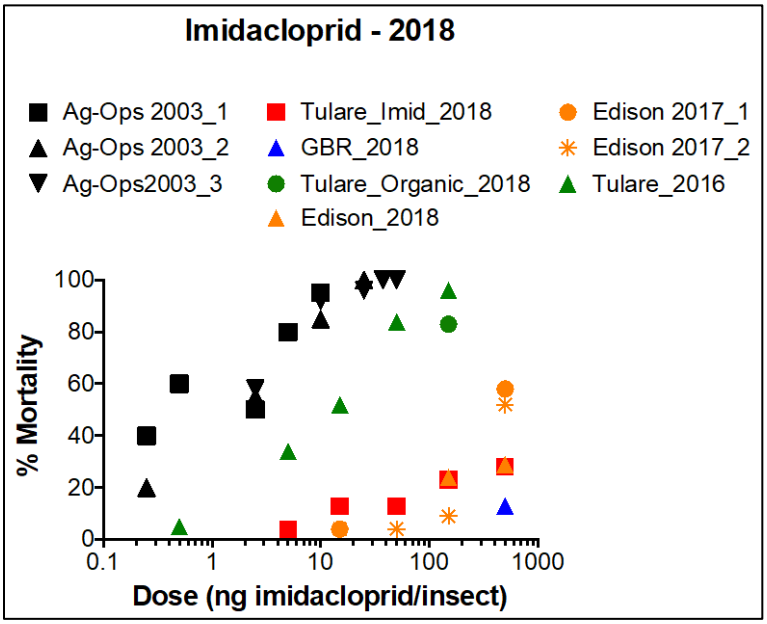

Figure 1. Toxicological response of GWSS adults to the neonicotinoid imidacloprid applied topically to the abdomen. Mortality was assessed at 48 hours post-treatment. Data for Ag-Ops at UC Riverside (black symbols) were generated in 2003 and are included for comparison. Tulare 2016 (green triangles) was collected from an organic grove in Tulare County and tested during the 2016 monitoring program. A discriminating dose bioassay with insects from this same location (green circle) was conducted in 2018, and confirmed that the population was still susceptible. Bioassays were also conducted with insects from the Edison (orange symbols) and General Beale Road (blue triangle) areas, located east of Bakersfield, and confirmed a high degree of resistance at both sites. The Tulare_Imid_2018 population (red squares) was collected from a conventional grove within three miles of the Tulare_Organic_2018 site, and exhibited levels of resistance close to those of the GBR and Edison insects.

Synergism of Imidacloprid Toxicity with Piperonyl Butoxide. Bioassays with synergists can assist with the elucidation of potential resistance mechanisms that occur in insects. In an attempt to identify the mechanism conferring insecticide resistance to imidacloprid in the Central Valley populations, we conducted discriminating dose bioassays on the Tulare resistant strain (Tulare_Imid_2018). Pre-treatment of insects with piperonyl butoxide, a known inhibitor of cytochrome P450 oxidase activity, had a significant effect on the efficacy of imidacloprid (**Figure 2**). We are conducting further synergism studies on this strain, to determine the extent of the synergistic effect. Thus far, the synergist effect was evaluated with a dose of 0.5 µg piperonyl butoxide applied at one hour prior to treatment with imidacloprid.

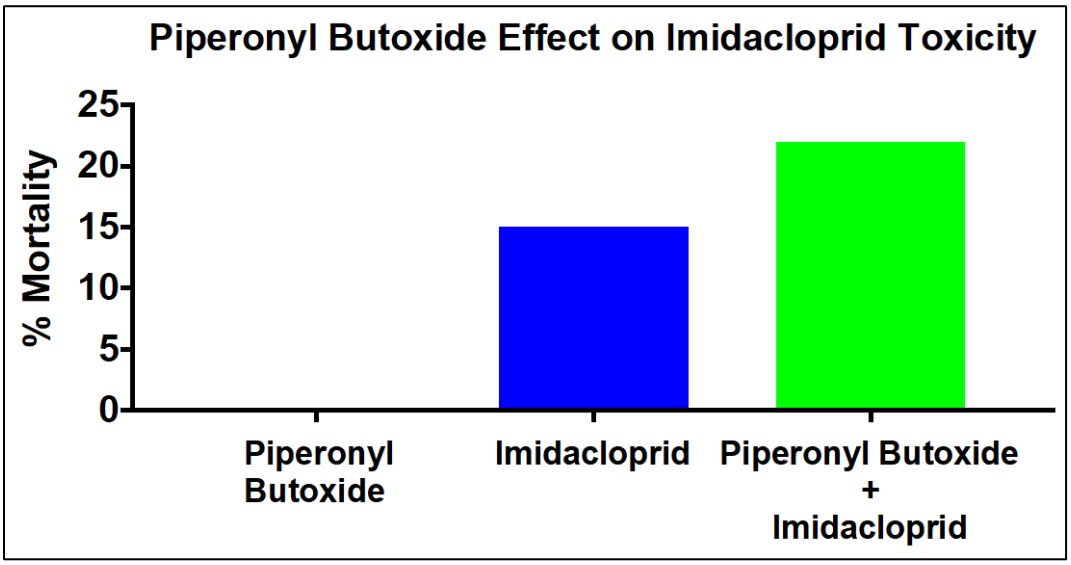

Figure 2. Synergism of imidacloprid toxicity with piperonyl butoxide. The Tulare_Imid_2018 population was pre-treated with 0.5 µg piperonyl butoxide/insect at one hour before treatment with imidacloprid at a discriminating dose of 50 ng/insect. Treatment with piperonyl butoxide did not result in any mortality. Fifty insects were treated at each dose.

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 confirmed that resistance to imidacloprid conferred cross-resistance to acetamiprid. However, datasets were not completed for several populations during the 2017 monitoring program, due to dwindling insect numbers late in September. The priority for 2018 was to complete the toxicological profiles for the Edison and Highway (HWY) 65 populations.

A discriminating dose bioassay with insects from the Tulare organic grove (Tulare_Organic_2018) showed it had the same response as the Ag-Ops data from 2003 (**Figure 3**). Both the Edison and GBR populations exhibited cross-resistance between imidacloprid and acetamiprid; however, acetamiprid was more toxic than imidacloprid to these insects, and enabled us to derive complete dose-response curves. As with imidacloprid, the response of the HWY65 insects to acetamiprid was intermediate between the Tulare/Ag-Ops insects and the GBR/Edison insects.

Genetic Analysis

The analysis of RNA sequencing data generated for the Tulare, HWY65, and GBR populations is underway. The bioassay data show the likely involvement of cytochrome P450s as a potential mechanism conferring imidacloprid resistance. This information will help guide our interpretation of the genetic data, and shows the advantage of using a multi-disciplinary approach to addressing resistance issues.

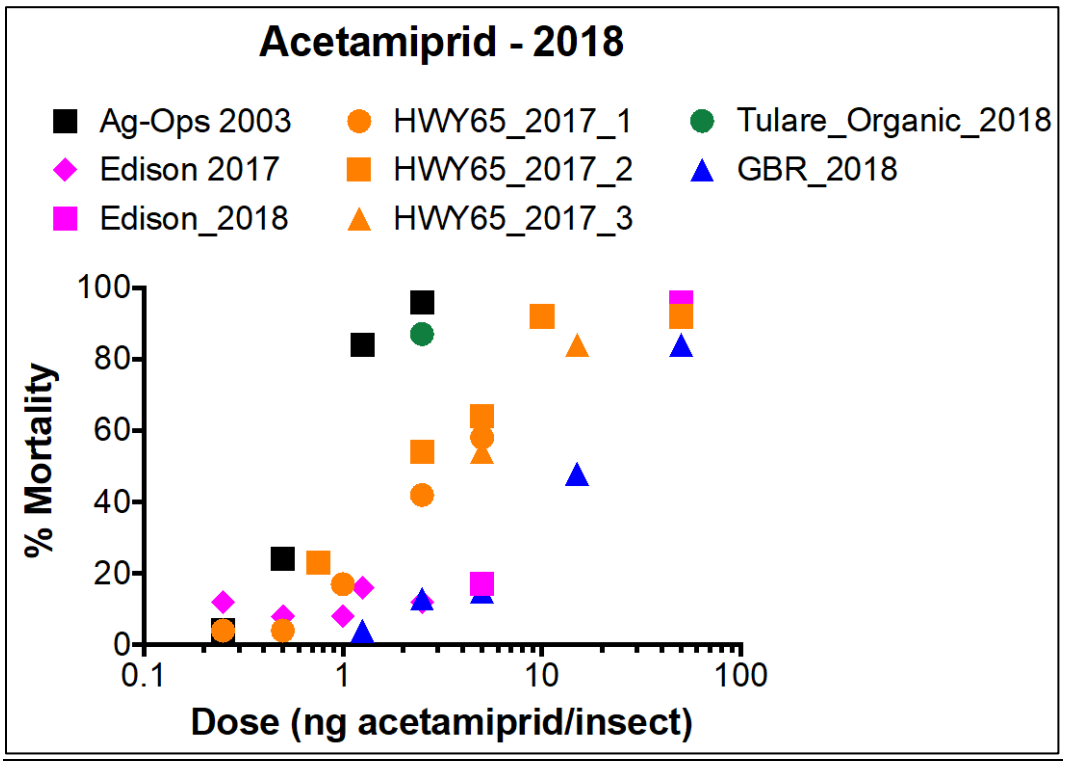

Figure 3. Toxicological response of GWSS adults to the neonicotinoid acetamiprid applied topically to the abdomen. Mortality was assessed at 48 hours post-treatment. Data for Ag-Ops (black symbols) were generated in 2003 and are included for comparison. A discriminating dose bioassay with insects from the Tulare organic site (green circle) confirmed that the population was susceptible. Bioassays conducted with insects from the Edison (pink symbols) and GBR (blue triangle) areas, located east of Bakersfield, confirmed a high degree of resistance at both sites. Insects from HWY65 (orange symbols) expressed resistance levels that were intermediate between the Tulare and GBR locations.

CONCLUSIONS

We have confirmed the variable levels of resistance to imidacloprid in Central Valley populations of the GWSS, and confirmed with the most recent monitoring data that the resistance extends into Tulare County. The dramatic shift in susceptibility is based on a comparison with bioassay data generated in 2003 for a population in Riverside County that we regard as a reliable reference susceptible, and a comparison with 2016 and 2017 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 continue to work effectively against imidacloprid resistant GWSS. However, continued monitoring for pyrethroid resistance should be a high priority if this important insecticide class is to remain effective.

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

REFERENCES CITED

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

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

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

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

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

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

FUNDING AGENCIES

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PRELIMINARY FINDINGS SUPPORT THAT VECTOR FEEDING BEHAVIORS CONTROLLING INOCULATION OF *XYLELLA FASTIDIOSA* ARE PERFORMED LESS ON THREE BACKCROSS SIBLINGS OF *PDR1* PARENTS THAN ON CHARDONNAY

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ABSTRACT

The most successful example of classical grapevine breeding for resistance to *Xylella fastidiosa* (*Xf*) (to date) is the *PdR1* gene, which mediates resistance to *Xf* multiplication and spread in the host. *PdR1* originated from wild grapes such as *Vitis arizonica* and was introgressed into *V. vinifera* cultivated genotypes. Electropenetrography (EPG) makes it possible to study whether vector feeding behaviors that control *Xf* transmission (acquisition, retention, and inoculation) could be affected by wild grapes or their *PdR1*-containing offspring. If so, then *PdR1* might confer resistance to *Xf* transmission, in addition to bacterial spread. The sharpshooter EPG X wave is diagnostic for such a behavioral resistance mechanism because it likely represents *Xf* inoculation. The X wave represents mixed plant fluid and insect saliva being taken up into the insect's mouth cavity, swished around, then spit back out into a xylem cell (thereby injecting any *Xf* loosened from the cuticle of the mouth cavity).

Last year, it was reported that X waves of blue-green sharpshooters (*Graphocephala atropunctata*) were strikingly different for inoculative versus clean insects, and those on resistant versus susceptible grape. Stylet probing behaviors of 80 sharpshooters were EPG-recorded; 20 insects on each of four treatments in a two by two factorial experimental design. Host plants were either wild, *V. arizonica* b43-17 or *V. vinifera* Chardonnay. Sharpshooters had putatively acquired *Xf* strain Stag's Leap (i.e., were inoculative) or had not acquired *Xf* (were clean). 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 (to remove clogging deposits of biofilm) than clean insects do. In addition, one of the most important X wave components, C1, was performed for shorter overall durations by inoculative insects on *V. arizonica* than on Chardonnay, despite X waves being attempted more frequently. Some feature of *V. arizonica* xylem may present an impediment to fluid injection, such as a structural (narrow cell diameter?), physical (low xylem tension?), or chemical (bad taste?) feature.

This year, preliminary findings from a second study of blue-green sharpshooter feeding continue to support a behavioral component to *PdR1* resistance. Stylet probing behaviors of 80 sharpshooters were EPG-recorded; 20 each on four, non-factorial treatments. All sharpshooters had putatively acquired *Xf* Stag's Leap. Host plants were either Chardonnay or one of three *PdR1* accessions (8909-8, 8909-17, and A81-139) resulting from offspring of *V. arizonica* b43-17 x *V. vinifera*, then backcrossed to *V. vinifera*. Preliminary results from four out of 20 insects per host plant were statistically compared ($\alpha = 0.05$). Sharpshooters on Chardonnay made more frequent but shorter probes, thus more X waves, than did insects on all three *PdR1* siblings. In addition, xylem sap ingestion was significantly longer on all three *PdR1* siblings than on Chardonnay. It is possible that, while sharpshooters might acquire more bacteria (during xylem sap ingestion, if *Xf* were present) on the *PdR1* siblings, they would be less likely to inoculate it to clean *PdR1* plants than to susceptible Chardonnay. If completed analysis of feeding continues to support these findings, then EPG can be used to demonstrate previously unknown mechanisms of resistance to *Xf* inoculation by its sharpshooter vectors. Such novel resistance traits could be pyramided with the *PdR1* traits, for more durable field resistance to *Xf* in the future.

FUNDING AGENCIES

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PROKARYOTIC COMMUNITY OF A GLASSY-WINGED SHARPSHOOTER INFECTED WITH *XYLELLA FASTIDIOSA*

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Reporting Period: The results reported here are from work conducted October 2017 to September 2018.

ABSTRACT

Glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) transmits *Xylella fastidiosa* (*Xf*) that causes Pierce's disease of grapevine. Insects are known to harbor endosymbionts/microorganisms that can confer various fitness advantages on the host including nutritional upgrading and enhancement of pathogen resistance. Knowledge on GWSS microbial communities may help elucidate many biological processes in GWSS and provide the baseline information needed to develop new control strategies. However, research on GWSS microbial communities remains limited, particularly in characterization of insect prokaryotic communities through next generation sequencing (NGS) technology. Adult GWSS from a laboratory colony established in 2018 with individuals originated from Bakersfield, California, were allowed a two-month acquisition access period (AAP) on a grapevine infected with *Xf* (strain Stag's Leap). After the AAP, total DNA was extracted and subjected to NGS (Illumina HiSeq3000, 2x100). A total of 316,193,544 short sequence reads (101 base pairs (bp) per read), or 31,935,547,944 bp, were generated. *De novo* assembling was performed and generated 550,712 contigs ranging from 500 to 341,870 bp. The circular GWSS mitochondrial genome (15,301 bp) was identified. The whole genome sequence of *Xf* was assembled through reference mapping. Besides *Xf*, preliminary metagenomic analysis (BLASTn against GenBank nr database and Kaiju software) confirmed the presence of "*Candidatus* Baumannia cicadellinicola" and "*Candidatus* Sulcia muelleri" in high abundance. In addition, other prokaryotic bacteria (tentatively at genus level) supported by >100,000 sequence reads were: *Wolbachia*, *Acinetobacter*, *Chryseobacterium*, *Comamonas*, *Sphingobacterium*, and *Vibrio*. Further research will refine the taxonomy nature of these bacteria and possibly more previously unknown bacteria, along with the generation of a draft genome sequence of GWSS from California.

FUNDING AGENCIES

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Section 3:

Other Pests and Diseases of Winegrapes

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

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ABSTRACT

Three economically important viruses – leafroll, red blotch, and fanleaf – are devastating the winegrape industry by decreasing yields, lowering fruit quality, inhibiting cluster ripening, and decreasing the lifespan of vineyards. The Lodi Grapevine Virus Research Focus Group (Virus Focus Group), formed in October 2017, has begun to provide detailed, real-world advice on virus management topics such as how to rogue, how to economically test for viruses, how to replant after leafroll, and how to order clean grapevines. By taking into consideration a thorough review of virus management in the literature (previous studies), current virus research projects, regional perceptions of viruses, and management of viruses internationally (especially in South Africa and New Zealand), the Virus Focus Group is producing practical advice for growers while demonstrating why it is of utmost importance financially to manage viruses now. Additionally, the Virus Focus Group will serve as a communication network between growers, pest control advisors, nurseries, laboratories, extension personnel, County Agricultural Commissioners, the California Department of Food and Agriculture, Foundation Plant Services, and researchers, ensuring a long-term sustainable strategy for virus management in California. The overall objective is to learn how to best manage and prevent grapevine virus disease in the 110,000 acres of Crush District 11, providing outreach tools and strategies to be shared with other regions across California.

LAYPERSON SUMMARY

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

INTRODUCTION

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

In fact, it will take a joint effort by all sectors of the industry to find a sustainable solution which will allow growers to continue profitably farming winegrapes. Growers need more education to make responsible virus management decisions. Even when responsible growers plan ahead and pay extra for CDFA-certified material, viruses and/or mealybug vectors are too often slipping through registered nursery doors. Preliminary case study collections are uncovering a lack in formal reporting procedures for when this scenario occurs, making it difficult for the industry to know there is need for improvement in virus prevention protocols. When 300-acre vineyards must be ripped out due to a virus infection after being in the ground for less than four years, there is a problem. The best way to learn is by doing, and Lodi growers are learning the hard way that ignoring grapevine viruses – either individually or as an industry – is one expensive mistake.

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

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

OBJECTIVES

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

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

RESULTS AND DISCUSSION

Grapevine virus management has been established as a top outreach and research priority for Lodi, due to severe economic losses from region-wide virus infections and a general lack of knowledge about viruses. Lodi's winegrowing community is fully committed to learning more about viruses in general and to discovering sustainable, economically viable management options to allow for profitable grape growing.

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

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

We've collected scientific articles, textbooks, online information, and a grower workbook on leafroll virus from sources in the USA and internationally. All information is shared within the Virus Focus Group and discussed at length during the monthly meeting, trying to understand how each piece of information applies locally in California. Information concerning leafroll from South Africa and New Zealand has been extremely useful and has allowed us to develop an overall virus strategy (**Figure 1**) at a faster pace. We've been able to share and discuss our findings and materials with other interested regional grower groups (including The Vineyard Team, the Monterey County Vintners & Growers Association, the Contra Costa Winegrowers Association, the California Association of Winegrape Growers, and the Washington Winegrowers Association) so that we all may learn and work together.

Case studies regarding the economics of virus management and individual virus-related situations are being collected and used in research and outreach. The financial losses experienced due to viruses are much greater than our local winegrowing community had realized. For example, one 70-acre block planted in 2012 was infected with leafroll virus and had to be removed in 2018, at a total loss (including revenues) of at least $2.5 million. The collection and sharing of local case studies is helping influence growers towards learning more about viruses and how to manage them.

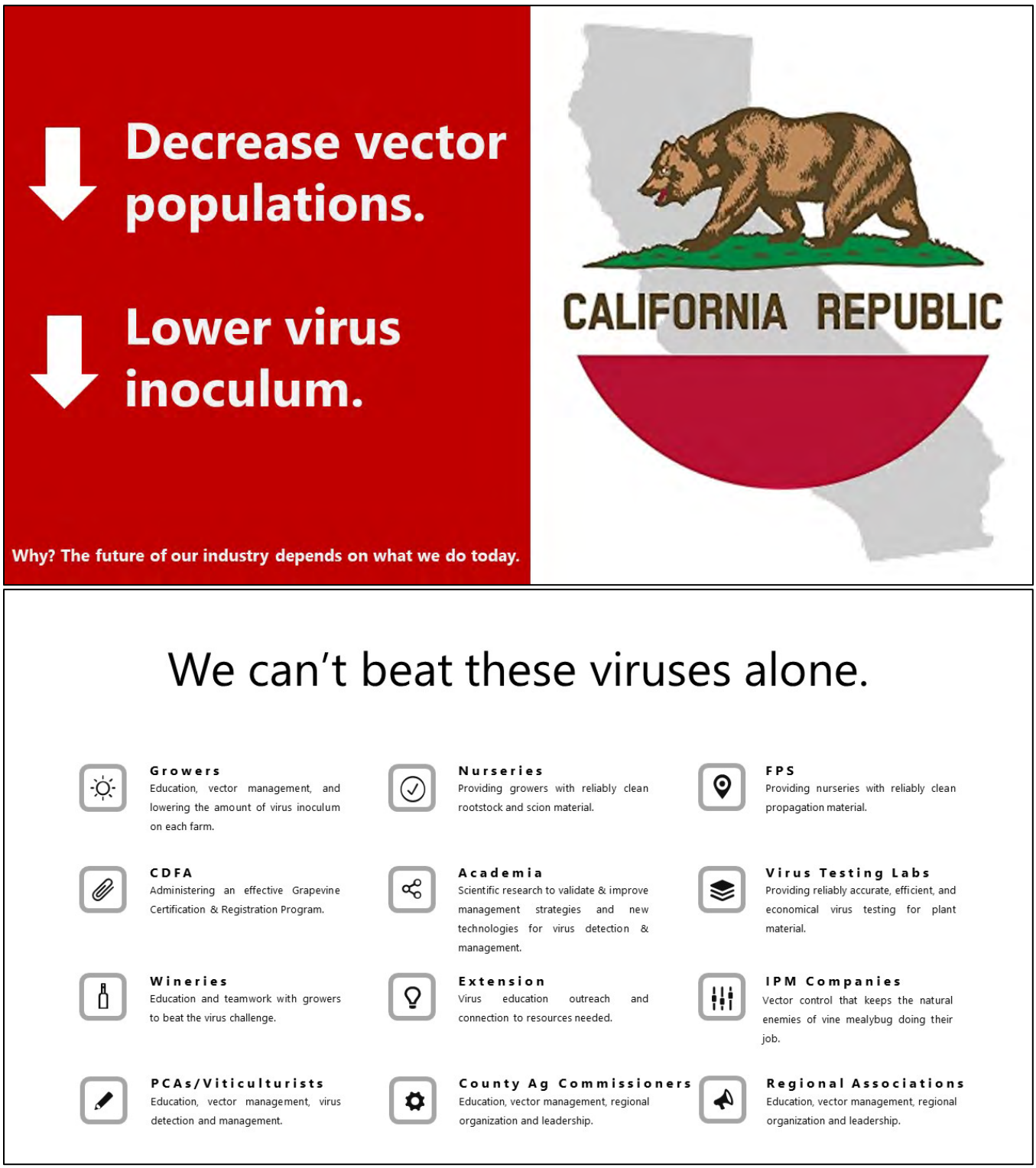

Figure 1. A visual representation of the overall virus strategy for California to guide the outreach initiatives of the Virus Focus Group. Created by Bolton for the 2018 Mealybug & Virus Outreach Meeting.

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

Research into virus testing procedures revealed that there is no standard protocol for virus testing in California, nor is there a virus-specific accreditation available for laboratories. Virus testing is expensive (in the range of $150-300 per sample or vine) and directions for sample collection need to be followed carefully for the most accurate results. On April 4, 2018, the Lodi Winegrape Commission hosted the first meeting where all seven

grapevine testing laboratories came together with growers, nurseries, and pest control advisors (PCAs). At this meeting it was decided that a third-party ring test would help improve the accuracy and reliability of California virus testing. Dr. Bob Martin at the USDA Agricultural Research Service in Oregon is orchestrating the ring test for fall 2018 with samples donated by Dr. Maher Al Rwahnih from Foundation Plant Services (FPS) at UC Davis. All seven California laboratories will be invited to participate. The Virus Focus Group will also work with FPS to help teach growers how to properly take virus testing samples for each laboratory.

Efficient use of $22,000 worth of CDFA grant money awarded for regional virus testing will allow us to meet the following goals:

1. To experience virus testing with all seven laboratories as a grower would for improved, real-world educational materials on "how to test."
2. To determine if leafroll 3, red blotch, and/or fanleaf virus play a role in a regional mystery vine collapse disease.
3. To create a Leafroll Virus Demonstration Vineyard with scouting and training opportunities.
4. To gather virus case studies and photographs from across the Lodi American Viticultural Area (AVA) which are verified by testing and can be used in educational materials.
5. To teach growers and PCAs how to sample and test for viruses (each sampling is an opportunity to teach the grower and/or his or her PCA how to test).
6. To show growers, especially those in virus denial, how widespread grapevine viruses are across the Lodi AVA.
7. To determine which leafroll 3 virus strains exist in the Lodi AVA (M. Al Rwahnih will be testing selected samples to the strain level).

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

Grapevine root remnants remain alive for several years after the removal of a vineyard, and the results below show that these root pieces can test positive for leafroll virus (**Figure 2**). Cooperators are experimenting with methods to kill grapevines, to remove as much root material as possible, and to best prepare the soil following the removal of a leafroll-infected vineyard. Methods used in New Zealand with imidacloprid drenches are being evaluated.

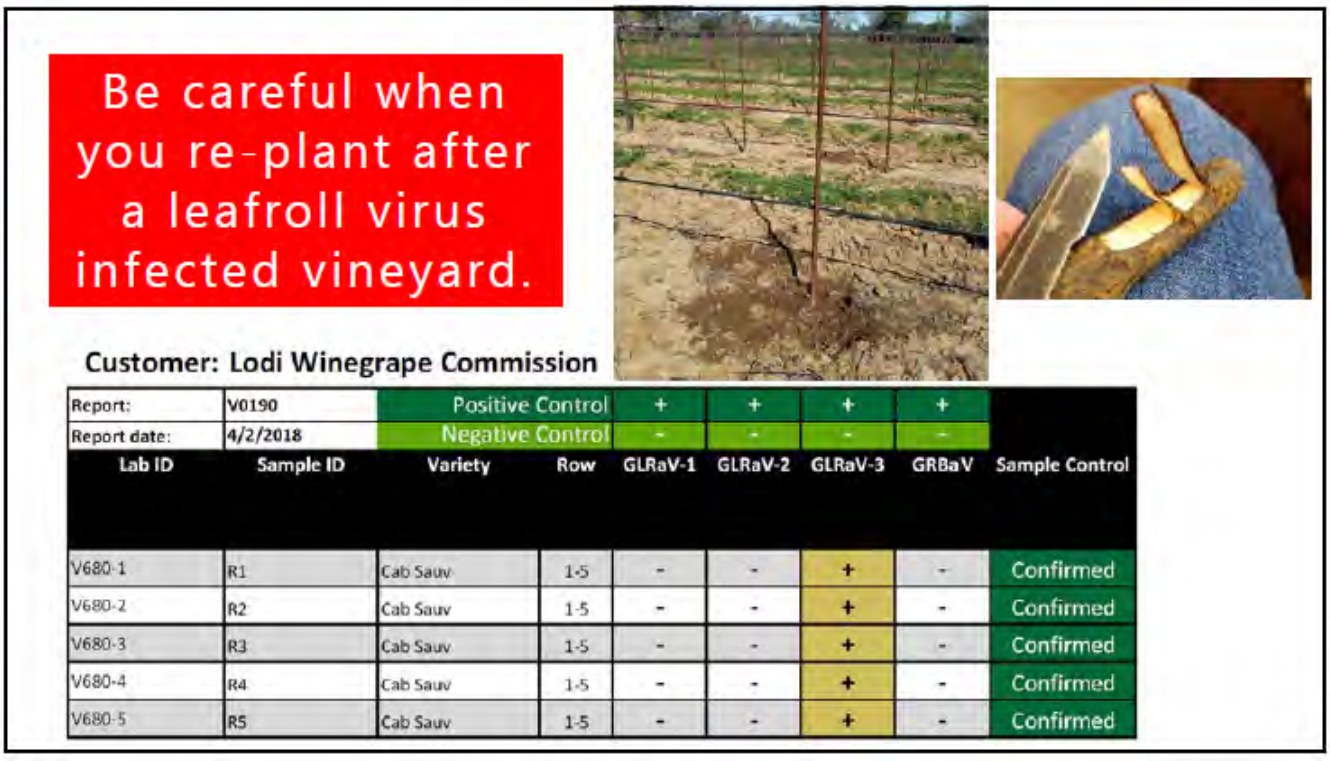

Figure 2. Wonderful Nurseries performed a complimentary virus test on root remnants to show growers that leftover root pieces can be an inconspicuous source of leafroll 3 virus inoculum. (Slide created by Bolton for 2018 Mealybug & Virus Outreach Meeting.)

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

As viruses are costing everyone a good deal of money, people have been more than willing to work together to find long-term strategies for virus control statewide. The first step is to get all the entities (nurseries, laboratories, extension personnel, County Agricultural Commissioners, scientists, the National Clean Plant Network, and the CDFA) talking to each other, with informed growers as part of these conversations. Thus far, we have had discussions with every group listed. All entities are being invited and encouraged to work with the Virus Focus Group, and as mentioned earlier, teamwork with other grower groups has begun as well.

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

The Lodi Winegrape Commission has multiple established channels for communicating with growers and the industry. The 750 growers and 200 supporting members of the winegrowing community (as well as the additional LODI RULES community, reaching ten other Crush Districts, and a network of Lodi wineries) receive information about virus educational workshops via mailings (postcards advertising events and a newsletter), email (a list-serve of over 800 people), twitter (@LodiGrower), a website (lodigrowers.com), and a blog (lodigrowers.com). Each method of communication listed provides an opportunity not only for educational outreach, but also for a conversation to begin between the recipient and the Virus Focus Group.

The Integrated Outreach Strategy

Open Communication Virus Meetings for Growers. The Lodi Winegrape Commission hosts monthly pest management network breakfast meetings where anyone in the Commission network (growers, PCAs, winemakers, etc.) can stop in and ask questions about grapevine pests and diseases. Beginning in April 2018, we devoted a portion of these roundtable meetings to viruses and their vectors so that the community has a consistent, approachable place to come with virus questions. At least three members of the Virus Focus Group are always in attendance. (Impact: 1,000+ industry members invited to meetings; free and open to the public.)

Virus Management Demonstration Vineyards. Two Virus Management Demonstration Vineyards are being established in Lodi, where growers can observe virus management in practice, learning symptom identification and how to mark and rogue vines during annual tailgate talks. The financials of the vineyards in terms of virus management, along with successes and failures, will be discussed openly. Demo Vineyard #1 will be an example of moving from >60% leafroll infection to effective leafroll control. Demo Vineyard #2 will be an example of <25% leafroll infection managed with roguing. Virus testing will be conducted to show results over time and to aid in a hands-on virus symptom identification workshop. In addition, red delta mealybug traps will be demonstrated as a tool for vector monitoring. Every fall, there will be a tailgate talk at each vineyard to discuss virus management. (Impact: 1,000+ industry members invited to annual tailgate talks; free and open to the public.)

Annual Virus Workshop (in spring of every year). Every year, the Lodi Winegrape Commission hosts a Virus Workshop with updated information and case studies from growers. This workshop provides timely, relevant information on nursery ordering, the CDFA Grapevine Registration & Certification Program, red blotch virus, leafroll virus, fanleaf virus, virus management, mealybugs, ants, and replanting after a virus infection. The first Workshop hosted by the Virus Focus Group (along with a similar team, the Mealybug Focus Group) was held on April 4, 2018 and over 150 people from all over California attended the half-day meeting (**Figure 3**). Attendees received two complimentary mealybug traps (courtesy of Suterra) along with instructions on how to use them. Several attendees also received a draft version of a Nursery Ordering 101: Viruses booklet (see Grapevine Virus Workbook section). A follow-up workshop with Suterra helped people learn how to identify the male mealybugs in their traps, and the Lodi Winegrape Commission has since served as a resource for male mealybug identification. All seven virus testing laboratories in California attended the outreach meeting and six stayed in the afternoon for a break-out session on how to improve virus testing. This was the first time that all virus testing laboratories came together. Another afternoon break-out session discussed mealybug biocontrol trials.

Figure 3. The 2018 Mealybug & Virus Outreach Meeting in Stockton, CA.

In 2019, the Workshop will include roundtable meetings for nurseries, laboratories, Agricultural Commissioners, regional grower associations, and extension personnel to discuss short- and long-term strategies. Also in 2019, South African Gerhard Pietersen (leafroll expert) and Cornell University's Dr. Marc Fuchs (red blotch expert) will be invited as keynote speakers and to consult with nurseries, CDFA, and growers. (Impact: 1,200+ industry members invited to annual workshops; free and open to the public.)

Grapevine Virus Grower Workbook. A Grapevine Virus Grower Workbook is being created which teaches growers why they need to care about viruses (using financial examples and case studies), where to start if their vineyard is sparsely or completely infected, how to identify/sample/test vines, how to rogue, the differences between red blotch, leafroll, and fanleaf viruses, and how to order CDFA-certified virus tested rootstock and scion from a nursery – plus why that is financially and socially important. The Workbook will include plentiful, recent photographs, case studies, myth-busters, question & answer sections, industry interviews, and very importantly sections where the grower can record pertinent virus management information for each vineyard. Instead of waiting until the entire Workbook is ready for publishing (which will be too late), we will publish four to five small booklets as the information is verified and available, starting with a nursery ordering instructional booklet (Nursery Ordering 101: Viruses; draft released in April 2018). Other booklet topics will potentially be how to tell if you have grapevine viruses, what to do if you have an infected vineyard (both sporadic and total infection), how to replant after a total leafroll infection, and how to manage a vineyard for viruses in general. These small booklets will undergo a grower test run in Lodi, then we will publish all the improved small booklets together after grower input as a Virus Workbook. The Workbook will be worked through with growers at neighborhood "kitchen table" meetings with members of the Virus Focus Group. The Workbook will also be available electronically on flash drives along with further virus educational materials, including a 15-20-minute virus video featuring growers speaking candidly about virus management. (Impact: distributed through Lodi Winegrape Commission to 800+ community members; available to the public and other winegrowing regions.)

LODI RULES Sustainability Standards: Viruses. The Virus Focus Group along with the Lodi Winegrape Commission's LODI RULES Committee will write new grapevine virus management standards for consideration in the LODI RULES Sustainable Winegrowing Program. (Impact: 46,000+ acres in California/Israel and 200+ growers.)

These new standards will be shared with the Sustainability in Practice (SIP) and the California Sustainable Winegrowing Alliance (CSWA) certification programs.

Objective 6. To Establish Priorities for Further Grapevine Virus Research Projects

Thus far, it appears that research on the following topics is much needed:

1. An effective and efficient ant bait for use on large (50+ acre) blocks to control ants which tend mealybugs.
2. The depth that mealybugs can be found on vine roots during the overwintering period (research is planned with Dr. Kent Daane).
3. A prevention strategy for leafroll replants (both individual vines and entire vineyard blocks).
4. The role of viruses in complexes with other biotic and abiotic stresses.
5. Rootstock and scion combinations which are more or less prone to virus disease symptoms.
6. How to determine the percent of a vineyard which is infected with virus in a cost-effective manner.
7. Cost-effective methods of virus testing.

CONCLUSIONS

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

These coordinated efforts directed by the Lodi Winegrape Commission, a trusted source for real-world grower education, will reach over 1,000 winegrape growers and PCAs to quickly and effectively implement virus management initiatives while establishing priorities for future research. Cooperators are willing to invest their time and money into discovering virus management strategies for the greater good, and they are very capable of comparing management techniques due to the large number of acres they cover. Demonstration vineyards will be managed by experienced growers in the LODI RULES sustainable winegrowing program, ensuring farming practices which are environmentally responsible and economically feasible. Outreach materials created, workshops and meetings hosted, and the communication channels which are opening between industry sectors will be of utmost importance for the winegrape industry across the state of California, as we collectively develop a long-term strategy for lowering the state's inoculum and reducing the spread of viruses.

REFERENCES CITED

Atallah SS, Gómez MI, Fuchs MF, Martinson TE. 2012. Economic impact of grapevine leafroll disease on *Vitis vinifera* cv. Cabernet franc in Finger Lakes vineyards of New York. *Am. J. Enol. Vitic.* 63:73-79.

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

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

Martelli GP, ed. 2014. Directory of Virus and Virus-Like Diseases of the Grapevine and Their Agents. *J. of Plant Path.* 96(1S):1-4.

Pietersen G. 2016. Leafroll: Replacing whole, highly leafroll infected vineyards with new healthy vineyards. *IGWS Factsheets*. University of Pretoria, April 28, 2016.

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

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

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

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

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

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

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Reporting Period: The results reported here are from work conducted July 1, 2017 to October 3, 2018.

ABSTRACT
This ongoing study used RNA sequencing and metabolite profiling to explore the effects of individual and mixed infections of grapevine leafroll-associated viruses (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 consists of Cabernet Franc grapevines grafted to Kober 5BB or MGT 101-14 rootstocks and carrying consequential GLRaVs. Cabernet Franc was used because it produces clear symptoms to GLRaVs. Among the treatments established in the vineyard, vines carrying GLRaV-1, GLRaV-3, GLRaV-5, GLRaV-1 + GLRaV-2, and GLRaV-1 + GLRaV-3 were included because infections with one or more of these viruses are associated with a range of symptoms of varying severities. The data generated may be used in the future to develop strategies to mitigate the detrimental effects of these viruses on ripening.

LAYPERSON SUMMARY
Our previous reports described that the 2017 samples had been crushed, total soluble solids measured, hormone detection and quantification methods were developed, the RNA sequencing libraries were sequenced, and the initial statistics were generated to determine which genes were impacted by each individual or dual infection and how rootstock might influence these effects. We also confirmed the infection status of the experimental vines for the 2018 sampling season. Currently, more detailed analyses of the 2017 RNA sequencing data are ongoing, as are the hormone and metabolite extractions and analyses from that year. We also completed the sampling for the 2018 sampling year and the samples are currently being crushed.

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

OBJECTIVES

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

RESULTS AND DISCUSSION

Pre-Objectives

Sampling and Sample Preparation 2017. 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) prior to sampling. Photographs were taken and berries were collected at four distinct developmental stages (pre-véraison, véraison, post-véraison, and harvest) from Cabernet Franc grapevines grafted to MGT 101-14 and Kober 5BB rootstocks. Twenty berries were picked from each of six vines at each sampling date and from each viral treatment. Berries were sampled evenly throughout the plant. Following their sampling, berries were crushed and their total soluble solids (TSS) were measured.

Measurement of Brix 2017. Differences in TSS were observed at each time point in the experiment that were dependent on the combination of infections and rootstock. These results were reported previously.

Sampling and Sample Preparation 2018. The Golino group oversaw re-testing of the experimental vines for viruses to ensure the same conditions in 2018 as in 2017. The grapevines were monitored throughout June in order to best estimate the beginning of samplings in 2018. Fruits were sampled at the same four developmental stages as in 2017. As in 2017, plants were photographed to monitor the onset of leafroll symptoms. Berries were deseeded and frozen at -80°C; these samples are currently being crushed and their TSS are being measured.

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

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

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

Library Preparation and Sequencing 2017. RNA extractions, library preparation, and sequencing are complete. Libraries with fewer than 12 million reads were re-sequenced. Following resequencing, the median number of reads sequenced for the 192 libraries was 17,256,960. The minimum and maximum number of reads sequenced among the 192 libraries were 12,007,531 and 34,591,412, respectively.

Statistical Analysis and Differential Expression 2017. The library normalization and differential gene expression analysis is complete and we are exploring the results. Among these results were as many as approximately 5,000 genes differentially expressed in berries given identical virus infections but from plants grafted to different rootstocks.

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

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

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

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

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

Hormone Identification by Liquid Chromatography - Mass Spectrometry (LC-MS) Using an In-House Dataset. Pre-existing datasets were used by the Ebeler group to identify the correct signatures of several hormones of interest. The same samples used for RNA sequencing are being used for the measurement of hormones and other metabolites. We optimized our extraction method and these extractions are ongoing, as is analysis of the samples by LC-MS. We are also preparing extracts for the targeted measurement of commercially important phenolic metabolites, including anthocyanins and other flavonoids.

CONCLUSIONS

Leafroll viruses are among the most consequential pathogens affecting grapevines. In 2017 and 2018, berries were sampled from Cabernet Franc grapevines grafted to different rootstocks and infected with individual or combinations of leafroll viruses. RNA sequencing and LC-MS were used to better understand the impact of infections on hormones, secondary metabolites, and signaling pathways during ripening. Preliminary results indicate significant differences in the impact of infections related to rootstock and results of the metabolite analyses are forthcoming.

REFERENCES CITED

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

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

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

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

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

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

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

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

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

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

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

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

Maree HJ. et al. 2013. Grapevine leafroll-associated virus 3. *Frontiers in Microbiology* 4(82). http://doi.org/10.3389/fmicb.2013.00082.
Moon JY, Park JM. 2016. Cross-talk in viral defense signaling in plants. *Frontiers in Microbiology* 7(307):904. http://doi.org/10.3389/fmicb.2016.02068.
Naidu RA, Maree HJ, Burger JT. 2015. Grapevine leafroll disease and associated viruses: A unique pathosystem. *Annual Review of Phytopathology* 53(1):613–634. http://doi.org/10.1146/annurev-phyto-102313-045946.
Prosser SW, Goszczynski DE, Meng B. 2007. Molecular analysis of double-stranded RNAs reveals complex infection of grapevines with multiple viruses. *Virus Research* 124(1-2):151–159. http://doi.org/10.1016/j.virusres.2006.10.014.
Vega A, Gutiérrez RA, Peña-Neira A, Cramer GR, Arce-Johnson P. 2011. Compatible GLRaV-3 viral infections affect berry ripening decreasing sugar accumulation and anthocyanin biosynthesis in *Vitis vinifera*. *Plant Molecular Biology* 77(3):261–274.

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

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ABSTRACT
Grapevine red blotch virus (GRBV) is a circular, single-stranded DNA virus (Geminiviridae: Grablovirus) associated with red blotch disease in winegrapes (*Vitis vinifera*) which negatively impacts crop vigor, yield, and quality. Surveys over the past five years have identified cultivated and wild grape (*Vitis* spp.) as the only known host plant reservoirs of this virus. While insect surveys in vineyards have shown that a limited number of insects can carry the virus (primarily Cicadellidae and Membracidae), so far only the three-cornered alfalfa hopper (*Spissistilus festinus*; TCAH) has been shown to transmit GRBV between grapevines. We are now entering a second phase of research about this virus and its insect vectors, in which knowledge of the ecology and transmission efficiency of the known vector TCAH will be refined and a short list of remaining candidate vectors will be evaluated. The goal of this newly-funded research program will be to not only improve our understanding of GRBV epidemiology, but to translate this knowledge into actionable management strategies for growers to adopt.

LAYPERSON SUMMARY
Grapevine red blotch virus (GRBV) is associated with red blotch disease in winegrapes (*Vitis vinifera*) and negatively impacts crop vigor, yield, and quality. Surveys have revealed that the virus only infects grapes (*Vitis* spp.). While multiple insects have tested positive for GRBV, only the three-cornered alfalfa hopper (*Spissistilus festinus*; TCAH) has been shown to actually transmit the virus between grapevines. We are now in the process of developing a better understanding of the seasonal ecology and transmission efficiency of TCAH in vineyards. Additionally, we plan to test the ability of any remaining candidate insect vectors to transmit GRBV. Our goal is to use this information to develop actionable management strategies for commercial grape growers to help reduce the incidence and spread of GRBV in vineyards.

INTRODUCTION
Grapevine red blotch virus (GRBV) is a circular, single-stranded DNA virus (Geminiviridae: Grablovirus) and is associated with red blotch disease in winegrapes (*Vitis vinifera*) (Krenz et al., 2012; Varsani et al., 2017). Symptoms of red blotch include reddening of leaf veins and the appearance of blotchy red areas on the leaf surface and/or at the leaf margin. Red blotch disease negatively impacts crop vigor, yield, and quality. Diseased vines typically exhibit reduced photosynthesis and stomatal conductance, delayed fruit maturation, decreased accumulation of sugars and anthocyanins, and lower pruning and berry weights (Al Rwahnih et al., 2013; Sudarshana et al., 2015; Blanco-Ulate et al., 2017).

While this disease was first reported in 2008 in a Napa County vineyard, subsequent surveys found GRBV to be widespread throughout North America (Krenz et al., 2014) and testing of archival plant material revealed the virus has been present in California since at least 1940 (Al Rwahnih et al., 2015). The wide geographic distribution of GRBV implicates that this virus was likely distributed via infected nursery material, although many have also reported in-field spread of red blotch disease. While increased incidence of red blotch disease over time within

vineyards and/or clustering of symptomatic vines gave reason to believe in the existence of one or more vectors, it could be argued that such trends were the result of environmental factors leading to latent expression of symptoms in some GRBV-positive vines. Yet the argument for an insect vector was strengthened by surveys that revealed the presence of GRBV in wild *Vitis* spp. naturally established outside of vineyards (Bahder et al., 2016a; Perry et al., 2016) and shortly thereafter it was shown that a treehopper, the three-cornered alfalfa hopper (*Spissistilus festinus*; TCAH) could successfully transmit GRBV between grapevines (Bahder et al., 2016b).

All characterizations of GRBV to date have placed it within the Geminiviridae (Krenz et al., 2012; Al Rwahnih et al., 2013; Sudarshana et al., 2015; Varsani et al., 2017). The only known vectors of viruses in this family are hemipterans, in particular leafhoppers, treehoppers, and whiteflies (Briddon and Stanley, 2015; Bahder et al., 2016b). Key vineyard hemipterans that are known to regularly feed on grapevines include *Erythroneura* leafhoppers (Cicadellidae: *E. elegantula*, *E. variabilis*, and *E. ziczac*), mealybugs (Pseudococcidae: *Planococcus ficus, Pseudococcus maritimus*, *Ps. viburni*, and *Ferrisia gilli*), blue-green sharpshooter (Cicadellidae: *Graphocephala atropunctata*), and to a lesser extent phylloxera (Phylloxeridae: *Daktulosphaira vitifoliae*), grape whitefly (Aleyrodidae: *Trialeurodes vittatas*), and lecanium scale (Coccidae: *Parthenolecanium corni*). While many of these candidate vectors are frequently encountered and/or in high abundance in vineyards, so far experiments have shown that only TCAH can successfully transmit GRBV between grapevines (Daane et al., 2017).

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

In addition to TCAH, broad testing of numerous non-economic insects in vineyards has revealed a number of potentially novel candidate vectors, including *Melaniolarus* sp. (Cixiidae), *Osbornellus borealis* (Cicadellidae), and *Colladonus reductus* (Cicadellidae) (Cieniewicz et al., 2017; Fuchs et al., 2017). Like TCAH, these organisms are typically found in low abundance in vineyards but are none-the-less present in and around these systems (Wilson et al., 2016; Daane et al., 2017).

While we know that TCAH can reproduce on certain leguminous annual ground covers found in vineyards (Zalom et al., 2017), the role of perennial non-crop plants found outside of or adjacent to vineyards is less clear.

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

OBJECTIVES

1. Identify TCAH overwintering and reproduction sites.
2. Determine timing of vineyard colonization by TCAH, including movement into the vine canopy and cane girdling.
3. Evaluate novel insect vector candidates.
4. Quantify TCAH transmission efficiency.

RESULTS AND DISCUSSION

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

Initial efforts to identify TCAH overwintering sites and reproductive hosts outside of vineyards have been inconclusive (Daane et al., 2017). A previous survey of non-crop plants conducted between March and November 2015/2016 recovered TCAH adults on toyon (*Heteromeles arbutifolia*), wild grape (*Vitis* spp.), and various

ground covers, primarily legumes. While TCAH is known to reproduce on legumes, it is unclear, but probably unlikely, that toyon and wild grape play any role in their reproductive cycle. A similar survey of non-crop plants was conducted between December and February 2017/2018 that yielded no TCAH. This effort will be expanded to include more sample sites and increased diversity of habitats in winter 2018/2019 and beyond.

Objective 2. Timing of TCAH Colonization, Movement into the Vine Canopy, and Cane Girdling
In February 2017 we established a study in Napa County and Sonoma County vineyards to evaluate the activity of TCAH populations along transects that extend out from large patches of natural habitat into vineyards. Field sites consist of vineyard blocks >2 acres adjacent to riparian and/or oak woodland habitat. There are five total study sites. All vineyard blocks are red varietals that are at least five years old and located on level ground with similar trellis and irrigation systems. All plots are maintained insecticide free throughout the course of the study.

At each site insects are sampled along five parallel transects (positioned 20 meters apart) that extend out from the riparian or oak woodland habitat (i.e., "natural habitat") into the vineyard. Each transect is 160 meters long, going 10 meters into the natural habitat and 150 meters into the vineyard. Along each transect samples are taken at the interior of the natural habitat (10 meters into the habitat) as well as at the edge and interior of the vineyard (10 and 150 meters into the vineyard, respectively). The edge of the vineyard and natural habitat are typically separated by a roadway or path that is about five meters wide. Densities of TCAH, *Erythroneura* leafhoppers, and other hemipterans are being monitored along the transects approximately every two weeks using a combination of yellow sticky traps, sweep nets and beat sheet sampling. Two yellow sticky traps (16 x 10 cm, Seabright Laboratories, Emeryville, CA) are placed at each transect point. In the vineyard, one trap is placed in the vine canopy (approximately four feet above the ground surface) and another trap is hung from irrigation lines (approximately one foot above the ground surface). In the natural habitat, two sticky traps are hung from a pole at each transect point at a height equal to those in the vineyard (i.e., one trap four feet and the other one foot above the ground surface). Traps are replaced approximately every two weeks between March 2017 and March 2019. Sweep nets are used to sample ground covers. At each transect point, a set of 30 unidirectional sweeps are collected from the ground covers using a 30.5 cm diameter sweep net (BioQuip Products, Rancho Dominguez, CA). Proportion of ground cover to bare soil is recorded, along with species composition and ground cover status (i.e., proportion of cover that was still green/healthy). A modified beat sheet is used at each transect point to sample the canopy of grapevines (in the vineyard) and non-crop species (in the natural habitat). The beat sheet consists of a one meter^2 nylon funnel that feeds into a detachable one-gallon plastic bag. For each sample, the funnel is held beneath the canopy while vigorously shaking the plant (or vine) for 30 seconds to dislodge insects into the funnel and plastic collection bag. Each month, vines along each vineyard transect point are evaluated for signs of TCAH feeding damage (i.e., girdling of leaf petioles). At each vineyard transect point, one cane from each of 10 randomly selected vines is visually inspected for leaf girdling. Total leaf nodes and leaf girdles per cane were recorded for each vine.

Here, we are reporting preliminary findings on TCAH adult densities observed in this study to date. TCAH activity showed a strong temporal trend, with densities generally increased between June and August along with some activity in March and October/November. Comparing the different sampling techniques, the highest TCAH densities were recorded on yellow sticky traps, followed by sweep nets and then beat sheets. While there was no clear gradient of TCAH activity across the transect points, densities on the yellow sticky traps and in the sweep samples were slightly elevated in natural habitats in early June just prior to increases observed in the vine canopy at both the vineyard edge and interior in the following round of sampling. Changes in TCAH densities between the ground covers and vine canopy were not always clearly reflected in the data. While densities in the vine canopy did increase as the proportion of healthy/green ground covers diminished, some TCAH could still be found on the little bit of ground cover that remained later in the season. Surprisingly these late season TCAH were most frequently encountered on ground covers in the vineyard interior. Finally, petiole girdling became apparent in August, with a higher proportion of girdles located at the vineyard interior. This increase in girdling in August follows increased TCAH densities observed in the vine canopy between June and August.

Objective 3. Evaluation of Novel Insect Vector Candidates
Candidate vectors are those insects collected in a previous survey that tested positive for GRBV, which includes *Melaniolarus* sp. (Cixiidae), *Osbornellus borealis* (Cicadellidae), *Colladonus* spp. (Cicadellidae), and *Scaphytopius* spp. (Cicadellidae). While these species can be found in vineyards, they are generally very low in

abundance. As such, robust colonies of each species will need to be established in order to conduct adequate transmission experiments, and this will be the focus of our efforts in 2019.

Objective 4. TCAH Transmission Efficiency

Previous transmission experiments (2015 to 2017) were conducted under greenhouse conditions using potted grapevines. Candidate vectors evaluated included western grape leafhopper (*Erythroneura elegantula*), Virginia creeper leafhopper (*Erythroneura ziczac*), grape whitefly (*Trialeurodes vittatas*), vine mealybug (*Planococcus ficus*), blue-green sharpshooter (*Graphocephala atropunctata*), and foliar form grape phylloxera (*Daktulosphaira vitifoliae*). To date, none of these candidates have been able to move GRBV between potted vines.

While Bahder et al. (2016b) demonstrated that TCAH can transmit GRBV between potted grapevines in a greenhouse, it remains unclear how well TCAH can move this virus under field conditions. As such, we are currently evaluating TCAH transmission using field vines for virus acquisition. That is, TCAH are caged on known positive vines in commercial vineyards for a 48-hour period and then moved to clean potted vines in the greenhouse. We would eventually like to place infected TCAH onto uninfected field vines >5 years old, pending that a field site can be located for this.

CONCLUSIONS

Over the past five years we have drastically improved our understanding of GRBV epidemiology, host plants, and insect vectors. We have effectively defined a narrow list of non-crop reservoirs for this virus and whittled down the range of candidate insect vectors. While it has been demonstrated that TCAH can transmit GRBV between vines, many questions remain about transmission efficiency, especially under field conditions and, more generally, TCAH seasonal ecology in vineyards. Additional candidate vectors remain to be tested as well, including *Colladonus* spp. and *Scaphytopius* spp. As we enter this second phase of research, our goal is to better characterize TCAH activity in vineyards and adjacent natural habitats, quantify transmission efficiency, and test any remaining candidate vectors.

REFERENCES CITED

Al Rwahnih M, Rowhani A, Golino D. 2015. First report of grapevine red blotch-associated virus in archival grapevine material from Sonoma County, California. *Plant Dis*. 99:895.

Al Rwahnih M, Dave A, Anderson MM, Rowhani A, Uyemoto JK, Sudarshana MR. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology* 103:1069-1076.

Bahder BW, Zalom FG, Sudarshana MR. 2016a. An evaluation of the flora adjacent to wine grape vineyards for the presence of alternative host plants of grapevine red blotch-associated virus. *Plant Dis.*: PDIS-02-16-0153-RE.

Bahder BW, Zalom F, Jayanth M, Sudarshana MR. 2016b. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* (Say) as a vector of grapevine red blotch-associated virus. *Phytopathology* 106(10):1223-1230.

Beyer BA, Srinivasan R, Roberts PM, Abney MR. 2017. Biology and management of the three-cornered alfalfa hopper (Hemiptera: Membracidae) in alfalfa, soybean, and peanut. *J. Integr. Pest Manage*. 8:10.

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

Briddon RW, Stanley J. 2015. *Geminiviridae, Encyclopedia of Life Sciences* (eLS). John Wiley & Sons, Chichester.

Cieniewicz EJ, Pethybridge SJ, Loeb G, Perry K, Fuchs M. 2017. Insights into the ecology of grapevine red blotch virus in a diseased vineyard. *Phytopathology* 108:94-102.

Daane KM, Almeida R, Cooper M, Golino D, Wilson H, Anderson J. 2017. Searching for potential vectors of grapevine red blotch-associated virus. *Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes*. December 2017. California Department of Food and Agriculture, Sacramento, CA, pp. 202-214.

Fuchs M, Perry K, Golino D. 2017. Ecology of grapevine red blotch virus,. Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes. December 2017. California Department of Food and Agriculture, Sacramento, CA, pp. 219-227.

Johnson M, Mueller A. 1989. Flight activity of the three-cornered alfalfa hopper (Homoptera: Membracidae) in soybean. *J. Econ. Entomol*. 82:1101-1105.
Krenz B, Thompson JR, Fuchs M, Perry KL. 2012. Complete genome sequence of a new circular DNA virus from grapevine. *Journal of Virology* 86:7715-7715.
Krenz B, Thompson J, McLane H, Fuchs M, Perry K. 2014. Grapevine red blotch-associated virus is widespread in the United States. *Phytopathology* 104:1232-1240.
Meisch M, Randolph N. 1965. Life-history studies and rearing techniques for the three-cornered alfalfa hopper. *J. Econ. Entomol*. 58:1057-1059.
Mitchell PL, Newsom L. 1984. Seasonal history of the three-cornered alfalfa hopper (Homoptera: Membracidae) in Louisiana. *J. Econ. Entomol*. 77:906-914.
Moore G, Mueller A. 1976. Biological observations of the three-cornered alfalfa hopper on soybean and three weed species. *J. Econ. Entomol*. 69:14-16.
Mueller A, Dumas B. 1975. Effects of stem girdling by the three-cornered alfalfa hopper on soybean yields. *J. Econ. Entomol*. 68:511-512.
Perry KL, McLane H, Hyder MZ, Dangl GS, Thompson JR, Fuchs M. 2016. Grapevine red blotch-associated virus is present in free-living *Vitis* spp. proximal to cultivated grapevines. *Phytopathology* 106:663-670.
Sudarshana MR, Perry KL, Fuchs M. 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Phytopathology* 105:1026-1032.
Varsani A, Roumagnac P, Fuchs M, Navas-Castillo J, Moriones E, Idris A, Briddon RW, Rivera-Bustamante R, Zerbini FM, Martin DP. 2017. Capulavirus and Grablovirus: two new genera in the family Geminiviridae. *Arch. Virol*. 162:1819-1831.
Wilson H, Quisenberry S. 1987. Impact of feeding by three-cornered alfalfa hopper (Homoptera: Membracidae): Greenhouse and field study. *J. Econ. Entomol*. 80:185-189.
Wilson H, Miles AF, Daane KM, Altieri MA. 2016. Host plant associations of *Anagrus* spp. (Hymenoptera: Mymaridae) and *Erythroneura elegantula* (Hemiptera: Cicadellidae) in northern California. *Environ. Entomol*. 45:602–615.
Wistrom C, Sisterson MS, Pryor MP, Hashim-Buckey JM, Daane KM. 2010. Distribution of glassy-winged sharpshooter and three-cornered alfalfa hopper on plant hosts in the San Joaquin Valley, California. *J. Econ. Entomol*. 103:1051-1059.
Zalom F, Sudarshana MR, Daane KM, Wunderlich LR, Smith RJ, Cooper ML. 2017. Biology and role of treehoppers in grapevine red blotch disease. *Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes*. December 2017. California Department of Food and Agriculture, Sacramento, CA, pp. 260-268.

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QUANTIFYING VINE MEALYBUG SPATIO-TEMPORAL DYNAMICS: ASSESSING INVASION RISK TO REFINE MANAGEMENT STRATEGIES

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ABSTRACT
The vine mealybug (*Planococcus ficus*; VMB) is a severe vineyard pest that contaminates fruit, debilitates vines, and transmits plant pathogens such as grapevine leafroll-associated virus-3. First reported in California from vines in the Coachella Valley, VMB soon spread throughout much of the state, likely on infested nursery stock. It is currently found in most California grape-growing regions and its range continues to expand, making this pest a serious threat to other grape-growing regions of the United States. The ongoing expansion of VMB in California and continued risk of its introduction into new areas necessitate better understanding of the factors driving its invasion. Here we use survey data on 2012-17 VMB occurrence to characterize the factors associated with VMB establishment and spread in Napa County, California. This work also identifies factors underlying hotspots in VMB activity, quantifies spatiotemporal patterns in VMB occurrence, and clarifies pathways that contribute to VMB spread. All analyses are ongoing or pending. Ultimately, results of this investigation can improve understanding of the educational and regulatory steps needed to mitigate VMB impact in Napa vineyards.

LAYPERSON SUMMARY
The invasive vine mealybug (*Planococcus ficus*; VMB) is an aggressive pest in California vineyards, where it reduces vine health and contaminates fruit. VMB management is challenging and costly - $300 to $500 per acre per year. Since VMB has proven difficult to eradicate once established, these costs are often incurred yearly for the life of the vineyard. VMB distribution is still expanding within California, and there is continued risk of introduction to other grape-growing regions of the United States. Although VMB biology and management have been intensively studied, the factors governing its invasion and spread are poorly characterized. Analyzing the patterns of VMB occurrence in surveys conducted in Napa County from 2012 to 2017 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 strategic response to this important pest.

INTRODUCTION
Geospatial analyses and niche-based/species distribution modeling have previously been used to characterize plant, aquatic invertebrate, amphibian, and insect invasions. Results of these and similar investigations have been applied, with varying degrees of success, to develop early detection strategies, identify and prioritize management in high risk areas, and minimize monitoring expenditures (Thuiller et al. 2005, Bradley et al. 2010, Venette et al. 2010, Jiménez-Valverde et al. 2011, 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, the vine mealybug (*Planococcus ficus*; VMB).

VMB is a severe vineyard pest that contaminates fruit, debilitates vines, and transmits plant pathogens such as grapevine leafroll-associated virus-3 (Daane et al. 2012; Almeida et al. 2013). Management of VMB has proven challenging and often requires the use of multiple tactics, including biological control, mating disruption and insecticides (Daane et al. 2008). Management can be particularly complicated in coastal wine grape-growing regions where climatic conditions are favorable and Argentine ants (*Linepithema humile*) disrupt biological control

(Daane et al. 2007; Gutierrez et al. 2008). Management costs may range from $300 to $500 per acre, per year, and due to the aggressive nature of VMB populations, these practices cannot be neglected.

VMB was first reported in California from vines in the Coachella Valley (Gill 1994) and soon spread throughout much of the state, likely on infested nursery stock (Haviland et al. 2005). It is currently found in most California grape-growing regions (Godfrey et al. 2002; Daane et al. 2004a, 2004b). However, despite the continued expansion of VMB in California, its current distribution in Napa County and areas at risk of VMB introduction in this region are not well characterized.

OBJECTIVES

Given the ongoing expansion of VMB in California and continued risk of its introduction into new areas, a better understanding is needed of what is driving its invasion. The overall goal of this research is to characterize the factors associated with VMB establishment and spread in Northern California vineyards, which will be addressed via the following objectives:

1. Quantify the spatiotemporal patterns in VMB occurrence to identify invasion hot spots and patterns of spread.
2. Characterize the landscape, climatic, and anthropogenic factors associated with current VMB occurrence to predict areas at risk to invasion.
3. Validate and update predictions of VMB risk via in-field monitoring.

RESULTS AND DISCUSSION

Survey data on 2012-17 VMB occurrence were acquired from the Napa County Agricultural Commissioner's Office and cleaned (i.e., removal of duplicate records, filling in missing information, correction of data inconsistencies, etc.). Traps in each year were georeferenced relative to grid cells in the CDFA Statewide Grid System. Both the greatest number of traps recording captures and number of male VMB captured were recorded in 2017, but the number of male VMB caught varied considerably among trapping years (**Table 1**). The reported total number of male VMB caught in 2017 is likely a conservative reflection of actual abundance as all individuals were not counted in traps that captured more than 100 individuals. A total of 4,148 traps were deployed in 2017; VMB captures in this year appear to be highly clustered in grid cells located in the south-central region of Napa (**Figure 1**).

Table 1. Summary of 2012-17 cumulative trapping effort for VMB in Napa County, California.

Year	# traps deployed	# traps recording VMB captures	Total # male VMB captured
2012	4,021	577	49,327
2013	3,437	327	16,488
2014	3,580	296	43,444
2015	3,479	841	26,577
2016	4,004	1,415	49,785
2017	4,148	1,602	>55,723

Objective 1. Quantify the Spatiotemporal Patterns in VMB Occurrence to Identify Invasion Hot Spots and Patterns of Spread

Analyses of spatiotemporal trends in VMB occurrence in 2012-17 are complete. More specifically, we evaluated the strength of spatial autocorrelation (SAC) among VMB trap detections, identified hotspots in VMB occurrence, and quantified both the directionality and rate of VMB spread in Napa for each study year.

We first analyzed the strength of SAC among traps that recorded VMB captures in an effort to characterize the scale of VMB movement. Pair correlative functions were used to estimate the strength of SAC for each study year; larger values indicate stronger SAC. In all years, SAC was greatest at the distance between traps (~250 m). This trend in the spatial scale and strength of SAC is attributable to the regular spacing between traps in each year, and because of this regular spacing, we are unable to quantify SAC at finer spatial scales. However, the strength of SAC varied across study years; SAC was greatest in 2013 and weakest in 2016 (**Figure 2**).

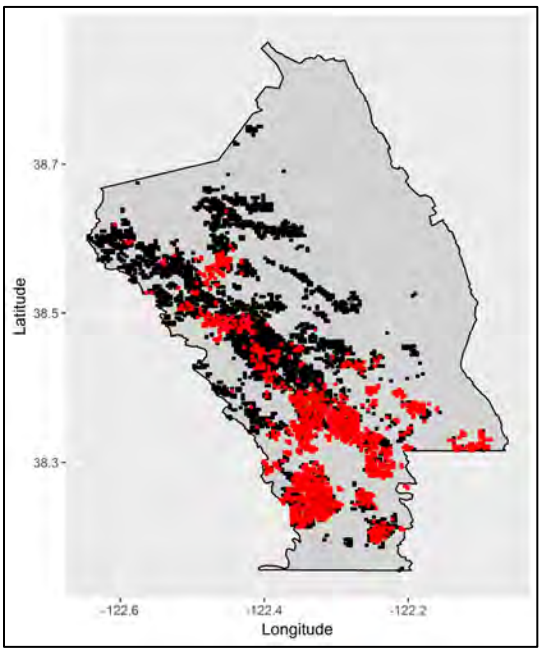

Figure 1. 2017 trapping effort for VMB in Napa County, with red cells denoting locations where VMB was detected and black cells denoting where traps did not detect VMB.

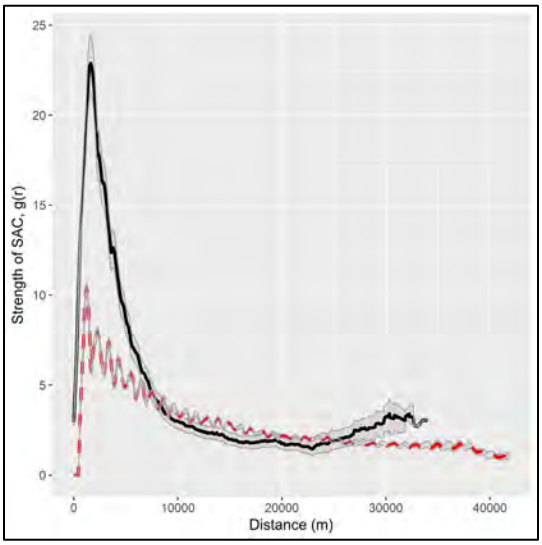

Figure 2. Estimates of the strength of spatial autocorrelation (SAC) as a function of distance. The strength of SAC was greatest in 2013 (solid black line) and weakest in 2016 (dashed red line). Grey shaded areas around each estimate represent 95% confidence intervals.

Hotspots in VMB occurrence are areas where a statistically greater numbers of traps recorded VMB captures relative to the rest of Napa County. Hotspots were identified by aggregating all traps recording VMB captures in each year within 1 km^2 grid cells. A Getis-Ord statistic was then used to compare the total number of traps recording captures in each grid cell to all other grid cells within Napa County. Hotspots of VMB occurrence were identified in each study year (**Figure 3**). Generally, the locations of hotspots were consistent between years, though there was also a greater amount of area that qualified as a hotspot in later years.

The rate of VMB spread was quantified via distance regression, square-root area regression, and boundary displacement methods (Tobin et al. 2015). For each method, traps recording VMB captures in each year were subset by three different thresholds (presence-only, 10, or 100 VMB). Mean estimates of yearly VMB spread, and their associated errors, varied substantially among the three methods used (**Table 2**). The distance regression and

boundary displacement methods produced the most conservative and liberal estimates, respectively. The boundary displacement method also quantifies the directionality of VMB spread. In general, there was considerable variation in the magnitude and directionality of VMB spread within and between study years, as well as the imposed thresholds of VMB abundances.

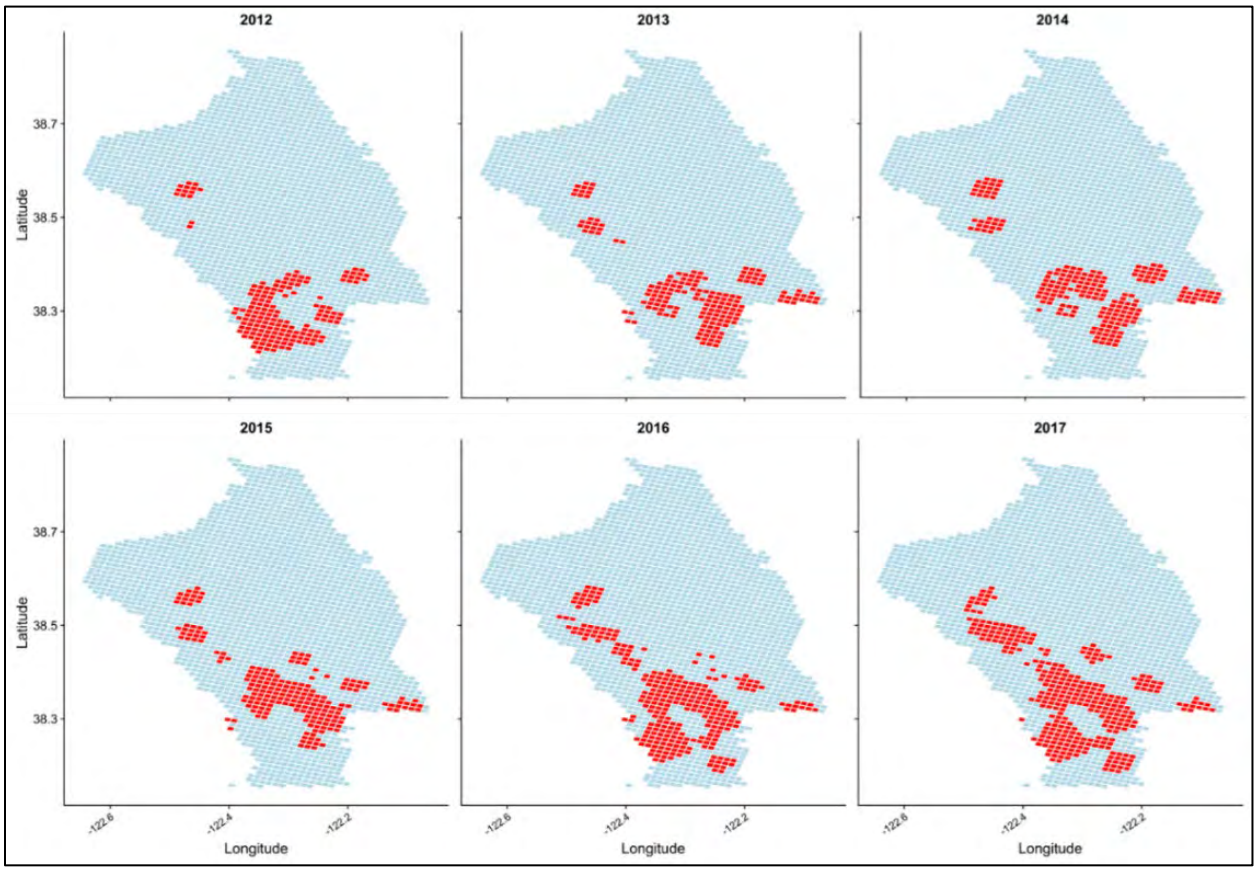

Figure 3. Hotspots (red) of VMB occurrence in Napa County, 2012-17.

Table 2. Mean estimates and standard errors (in meters) of yearly VMB spread generated via distance regression, square-root area regression, and boundary displacement methods.

	Distance		Square-root area		Boundary displacement	
Threshold	Mean	Error	Mean	Error	Mean	Error
Presence-only	365.9	31.0	779.8	261.2	832.6	41.0
10	310.1	39.9	572.8	230.1	848.1	41.0
100	299.5	87.8	94.9	257.3	890.3	62.6

Objective 2. Characterize the Landscape, Climatic, and Anthropogenic Factors Associated with Current VMB Occurrence to Predict Areas at Risk to Invasion

Analyses of the landscape, climatic, and anthropogenic factors associated with current VMB occurrence were also completed. All 2012-17 records of VMB presence and absence were compiled by considering unique traps that recorded VMB detections in at least one year (presence, n = 2,208) or were deployed in at least one year but never recorded a VMB detection (absence, n = 2,318). Variables of interest include 19 climate variables (WorldClim layers), elevation, percent impervious surface, and trap distance to nearest road and nearest winery. We also employed spatial eigenvector filtering to generate spatial predictors that reduce the signature of spatial autocorrelation among our presence-absence data. The final, Akaike Information Criterion (AIC)-informed model was used in conjunction with generalized linear models, boosted regression trees, and random forest algorithms to

assess the relative importance of each predictor variable, quantify the magnitude and directionality of each predictor-VMB relationship, and generate predictions of habitat suitability for VMB throughout Napa County.

All modeling and ensemble methods employed to predict habitat suitability for VMB in Napa County performed well (all Receiver Operating Characteristic (ROC) values > 0.8 and True Skill Statistic (TSS) values > 0.6). The boosted regression tree and random forest algorithm methods slightly outperformed the generalized linear modeling method. The grand ensemble method was the best-performing method employed (ROC = 0.953, TSS = 0.753).

The relative importance of our selected anthropogenic, climatic, and environmental predictor variables varied among the modeling and ensemble methods employed (**Figure 4**). In general, the amount of precipitation in the driest month, elevation, and trap distance to nearest winery were identified as the most important predictors of VMB occurrence. Precipitation in the driest month and trap distance to nearest winery were negatively associated with VMB occurrence whereas the probability of VMB occurrence increased slightly with increasing elevation (**Figure 5**). Conversely, trap distance to nearest road was the least important predictor across all modeling and ensemble methods and exerted little effect on the probability of VMB occurrence.

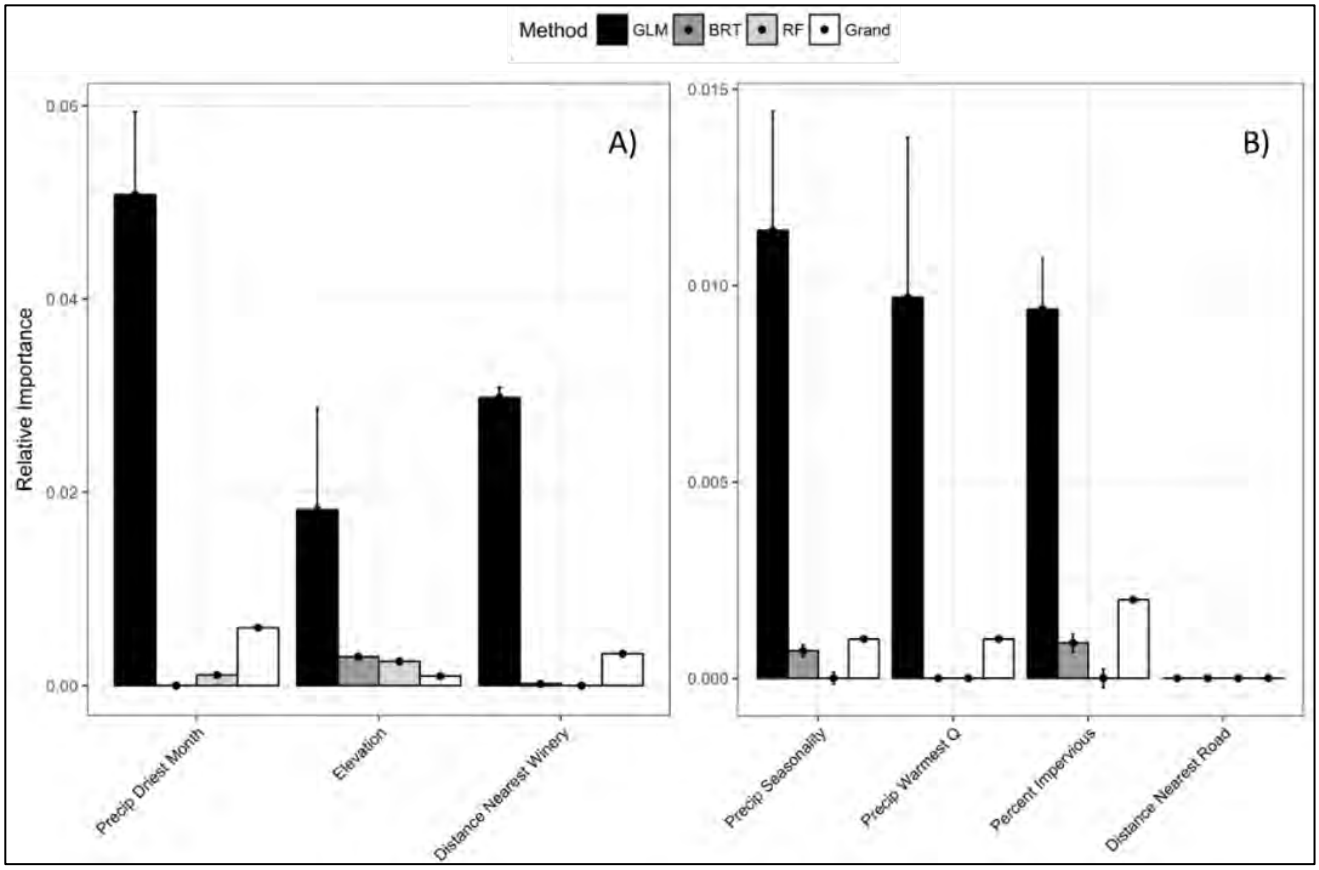

Figure 4. Relative importance of selected climatic, environmental, and anthropogenic variables in explaining VMB occurrence in Napa County. Note the different y-axis scale between panels.

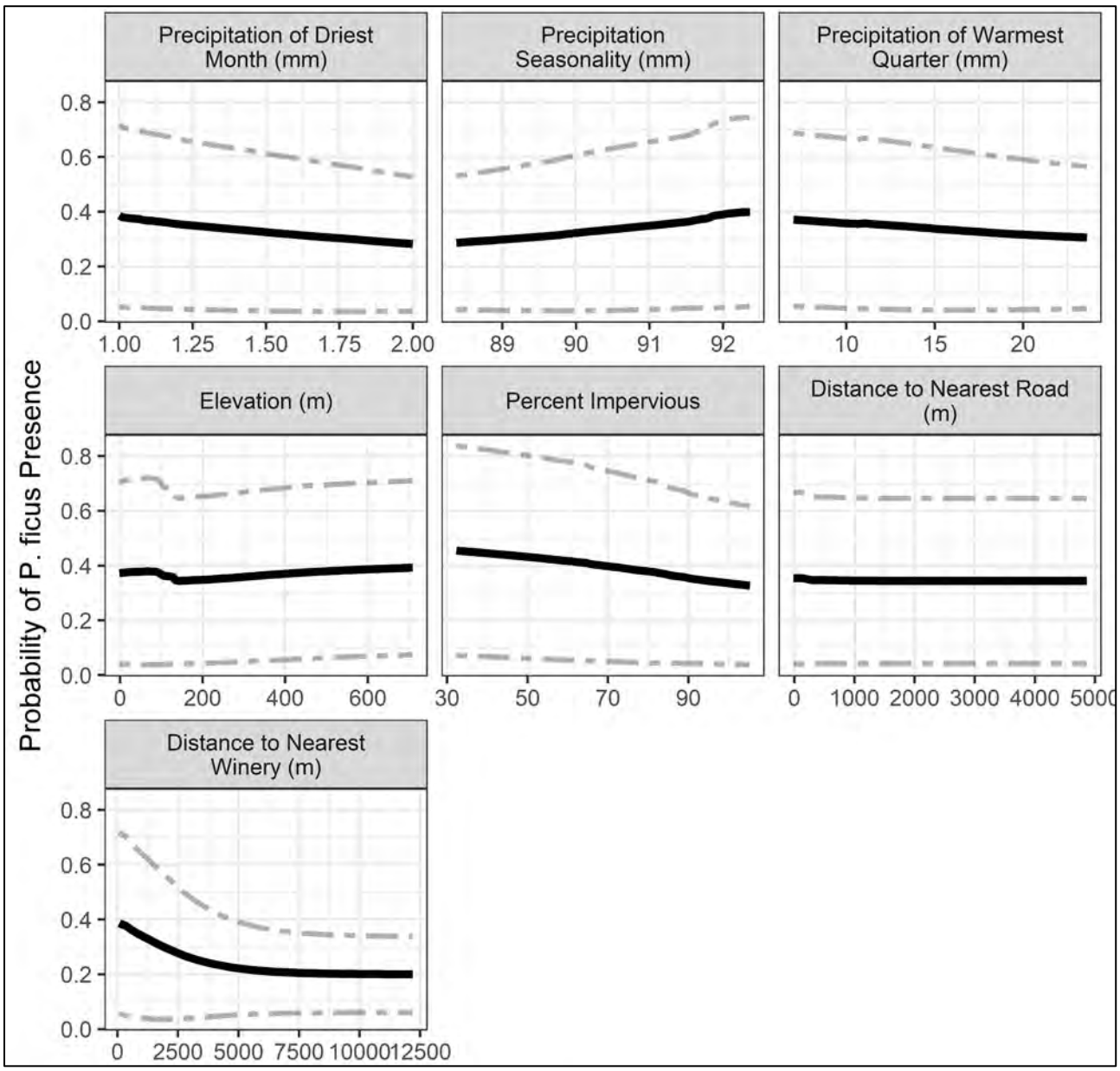

Figure 5. Mean fitted responses (solid black line) and 95% confidence interval (grey dashed line) of select landscape, climatic, and anthropogenic varaibles from grand ensemble predictions of habitat suitability.

Habitat suitability in the grand ensemble model was predicted to be greatest surrounding Napa and St. Helena, and the central-eastern portion of Napa County (**Figure 6**). Regions of Napa County where viticulture is largely absent, such as the northeastern portion of the county, are generally predicted to be of poor suitability.

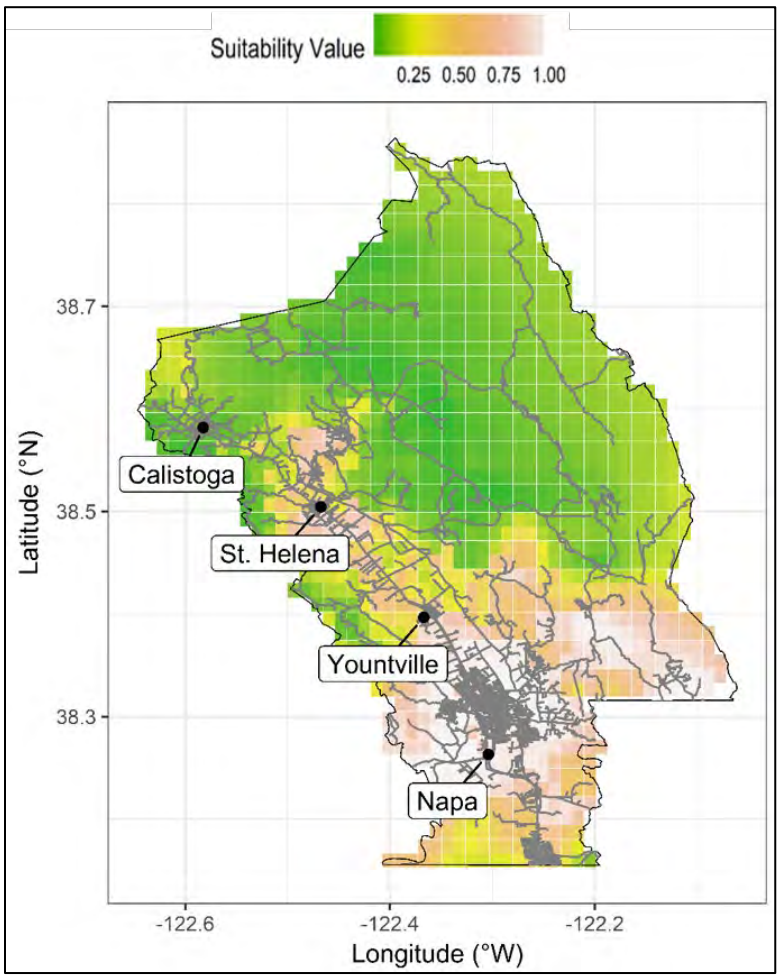

Figure 6. TSS-weighted grand ensemble prediction of habitat suitability for VMB in Napa County.

Objective 3. Validate and Update Predictions of VMB Risk Via In-Field Monitoring

Work on Objective 3 has commenced and will leverage the results of Objectives 1 and 2 to evaluate the accuracy of predictions of habitat suitability and risk of VMB infestation via in-field monitoring. Currently, we are working with grapegrowers and vineyard managers to identify vineyards that remain uninfested by VMB as well as vineyards where novel VMB infestations were recently identified. Within-vineyard data on pesticide application, use of mating disruption, and prevalence of infestation are being collected. These vineyards will be surveyed in Summer 2019, to evaluate the infestation status relative to predicted habitat suitability, VMB management, and the distance of each surveyed vineyard to the nearest prior VMB detection. This analysis will allow us to assses the explanatory power of VMB habitat suitability predictions and invasion kernels from prior detections, and to refine predictions for the areas most at risk to VMB infestation in the near future.

CONCLUSIONS

Our findings indicate that VMB invasion of Napa County is well beyond the initial invasion stages and is actively spreading throughout this region. Future VMB spread may continue to occur via natural and/or human-assisted pathways at rates upwards of 850 meters per year. We detected substantial heterogeneity in both the distribution of statistically significant hotspots of VMB detections and estimated habitat suitability for VMB over the study region. The amount of precipitation in the driest month, elevation, and trap distance to nearest winery were identified as the most important and strongly associated predictors of habitat suitability for VMB.

REFERENCES CITED

Almeida RPP, Daane KM, Bell VA, Blaisdell GK, Cooper ML, Herrback E, Pietersen G. 2013. Ecology and management of grapevine leafroll disease. *Frontiers in Microbiology* 4:94. doi:10.3389/fmicb.2013.00094.

Bradley BA, Wilcove DS, Oppenheimer M. 2010. Climate change increases risk of plant invasion in the Eastern United States. *Biological Invasions* 12:1855-1872.

Daane KM, Weber EA, Bentley WJ. 2004a. Vine mealybug – formidable pest spreading through California vineyards. *Practical Winery & Vineyard*. May/June: (www.practicalwinery.com).
Daane KM, Malakar-Kuenen R, Walton VM. 2004b. Temperature development of *Anagyrus pseudococci* (Hymenoptera: Encyrtidae) as a parasitoid of the vine mealybug, *Planococcus ficus* (Homoptera: Pseudococcidae). *Biological Control* 31:123-132.
Daane KM, Sime KR, Fallon J, Cooper ML. 2007. Impacts of Argentine ants on mealybugs and their natural enemies in California's coastal vineyards. *Ecological Entomology* 32:583-596.
Daane KM, Cooper ML, Triapitsyn SV, Walton VM, Yokota GY, Haviland DR, Bentley WJ, Godfrey K, Wunderlich LR. 2008. Vineyard managers and researchers seek sustainable solutions for mealybugs, a changing pest complex. *California Agriculture* 62:167-176.
Daane KM, Almeida RPP, Bell VA, Walker JTS, Botton M, Fallahzadeh M, Mani M, Miano JL, Sforza R, Walton VM, Zaviezo T. 2012. Biology and management of mealybugs in vineyards. In *Arthropod Management in Vineyards: Pests, Approaches and Future Directions*. Boustanian NJ, Vincent C, Isaacs R, eds. Springer, New York.
Gill R. 1994. Vine mealybug. *California Plant Pest and Disease Report*, January-June. California Department of Food and Agriculture, Sacramento, CA.
Godfrey KE, Daane KM, Bentley WJ, Gill RJ, Malakar-Kuenen R. 2002. Mealybugs in California vineyards. UC ANR Publ. 21612. Oakland, CA.
Gutierrez AP, Daane KM, Ponti L, Walton VM, Ellis CK. 2008. Prospective evaluation of the biological control of vine mealybug: refuge effects and climate. *Journal of Applied Ecology* 45:524-536.
Haviland DR, Bentley WJ, Daane KM. 2005. Hot water treatments to control *Planococcus ficus* (Hemiptera: Pseudococcidae) in grape nursery stock. *Journal of Economic Entomology* 98:1109-15.
Jiménez-Valverde A, Peterson AT, Soberón J, Overton JM, Aragón P, Lobo JM. 2011. Use of niche models in invasive species risk assessments. *Biological Invasions* 13:2785-2797.
Thuiller W, Richardson DM, Pyšek P, Midgley GF, Hughes GO, Rouget M. 2005. Niche-based modelling as a tool for predicting the risk of alien plant invasions at a global scale. *Global Change Biology* 11:2234-2250.
Tobin PC, Liebhold AM, Roberts EA, Blackburn LM. 2015. Estimating spread rates of non-native species: the gypsy moth as a case study. *Pest Risk Modelling and Mapping for Invasive Alien Species.* CABI International and USDA, Wallingford, pp.131-145.
Venette RC, Kriticos DJ, Magarey RD, Koch FH, Baker RH, Worner SP, Raboteaux NNG, McKenney DW, Dobesberger EJ, Yemshanov D, De Barro PJ. 2010. Pest risk maps for invasive alien species: a roadmap for improvement. *BioScience* 60:349-362.
Vicente JR, Alagador D, Guerra C, Alonso JM, Kueffer C, Vaz AS, Fernandes RF, Cabral JA, Araujo MB, and Honrado JP. 2016. Cost-effective monitoring of biological invasions under globalchange: a model-based framework. *Journal of Applied Ecology* 53:1317-132.

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RESISTANCE TO GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3 AND THE GRAPE MEALYBUG

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ABSTRACT

Leafroll is one of the most devastating and widespread viral diseases of grapevines. It causes economic losses by reducing yield, delaying fruit ripening, increasing titratable acidity, lowering sugar content in fruit juices, modifying aromatic profiles of wines, and shortening the productive lifespan of vineyards. Among the viruses associated with leafroll disease, grapevine leafroll-associated virus 3 (GLRaV-3) is dominant in vineyards. This virus is transmitted by several species of mealybugs, including the grape mealybug (*Pseudococcus maritimus*), which is its most abundant and widely-distributed vector, and a pest of grapes. Management of leafroll viruses and their mealybug vectors remains challenging due to a lack of recognized host resistance. Our research is exploring RNA interference (RNAi), a technology that has been successfully applied against viruses of fruit crops and phloem-feeding insects, to achieve resistance against GLRaV-3 and the grape mealybug. For RNAi against the grape mealybug, the osmoregulation genes *AQP1* and *SUC1* were characterized by reverse transcription polymerase chain reaction (RT-PCR) using total RNA from specimens from a colony maintained in the greenhouse, with overlapping degenerate primer pairs designed in conserved regions of the genes of interest based on alignments of similar sequences of other hemipterans. The cloned *AQP1* fragment is 490 base pairs (bp) in size and the cloned *SUC1* fragment is 394-bp in size. For RNAi efficacy, a nonspecific nuclease (*NUC*) was characterized from the grape mealybug to prevent the degradation of double-stranded RNA (dsRNA) constructs, using overlapping degenerate primer pairs designed in highly-conserved regions of similar sequences of other hemipterans, and used in RT-PCR with total RNA from the colony population. The cloned *NUC* fragment is 877-bp in size. Sequence analysis of the cloned PCR amplicons validated the nature of the *AQP1, SUC1,* and *NUC* products obtained. To evaluate the performance of dsRNA constructs against the grape mealybug, a transient assay based on detached Pixie grape leaves is developed. Preliminary results on dsRNA uptake using excised leaves are encouraging. In parallel, conserved nucleotide regions within the open reading frame coding for protein p19.7 (*p19.7*), a viral RNA silencing suppressor, the coat protein (*CP*), the RNA-dependent RNA polymerase (*RdRp*), and the heat shock 70 homolog (*HSP70h*) of GLRaV-3 were identified. Sets of overlapping primer pairs covering conserved regions of *p19.7*, *CP*, *RdRp*, and *HSP70* were designed and used in RT-PCR. Amplicons of the expected size were obtained, cloned, and validated by sequencing. One inverted-repeat *p19.7* construct was engineered and transferred into embryogenic calli of rootstock 110R via *Agrobacterium tumefaciens*-mediated transformation for the production of transgenic grapevines. It is anticipated that a pyramided approach for the simultaneous engineering of resistance against GLRaV-3 and the grape mealybug will protect grapevines against GLRaV-3 and the grape mealybug.

LAYPERSON SUMMARY

Leafroll disease affects yield, fruit ripening, and aromatic profiles of wines. Grapevine leafroll-associated virus 3 (GLRaV-3) is the predominant virus associated with leafroll disease in vineyards. This virus is transmitted by several species of mealybugs, including the grape mealybug (*Pseudococcus maritimus*), which is its most abundant and widely-distributed vector, as well as a pest of grapes. Management of leafroll viruses and their mealybug vectors is challenging due to a lack of recognized host resistance. We explore RNA interference (RNAi) technologies to achieve resistance against GLRaV-3 and the grape mealybug by simultaneously

interfering with the expression of key genes of the virus and its major vector. For RNAi against the grape mealybug, our targets are osmoregulatory genes that are expressed in the gut and required for water balance and survival. Two osmoregulation genes from the grape mealybug, as well as another gene that is essential for RNAi efficacy, were isolated and characterized. In parallel, a transient assay based on detached Pixie grape leaves was examined to evaluate the performance of double-stranded RNA (dsRNA) constructs against the grape mealybug. Preliminary results on dsRNA uptake using this assay are encouraging. For RNAi against the virus, conserved nucleotide sequence regions within four coding viral regions were identified and characterized. Among these four regions, an inverted-repeat p19.7 construct was engineered and used for the production of transgenic grapevines via *Agrobacterium tumefaciens*-mediated transformation. It is anticipated that an approach combining resistance against GLRaV-3 and the grape mealybug will protect grapevines against the major virus of leafroll disease and its widely distributed insect vector.

INTRODUCTION

Leafroll is one of the most devastating and widespread viral diseases of grapevines. It reduces yield, delays fruit ripening, increases titratable acidity, lowers sugar content in fruit juices, modifies aromatic profiles of wines, and shortens the productive lifespan of vineyards (Almeida et al., 2013; Naidu et al., 2014). 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 per acre in New York (Atallah et al., 2012).

Six major viruses named “grapevine leafroll-associated viruses” (GLRaVs), e.g., GLRaV-1, -2, -3, -4, -7, and -13, have been identified in diseased vines (Ito and Nakaune, 2016; Naidu et al., 2014; Naidu et al., 2015). Among these viruses, GLRaV-3 is the dominant leafroll virus in vineyards, including in California (Maree et al., 2013; Naidu et al., 2014; Naidu et al., 2015). This virus is phloem-limited and semi-persistently transmitted by several species of mealybugs, with acquisition and inoculation occurring within a one-hour access period of feeding by immature mealybug stages (Almeida et al., 2013). There is no significant effect of host plant tissue on transmission efficiency; nor is there specificity of transmission (Almeida et al., 2013; Naidu et al., 2014), indicating that many mealybug species may disseminate all transmissible strains of GLRaV-3.

Mealybugs are sap-sucking insects in the family Pseudococcidae. They are pests of grapes and many other important crops. At high densities, mealybugs can cause complete crop loss, rejection of fruit loads at wineries, and death of spurs, although small infestations may not inflict significant direct damage. In the feeding process, 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 immatures (crawlers) can be dispersed over long distances by wind and other means (Almeida et al., 2013).

In diseased vineyards, management strategies rely on the elimination of virus-infected vines and the reduction of mealybug populations through the application of systemic insecticides, primarily spirotetramat. However, managing leafroll viruses and their mealybug vectors remains challenging due to several factors, including a lack of recognized host resistance (Oliver and Fuchs, 2011). Resistance can be achieved by applying RNA interference (RNAi) technologies. The approach relies on the development of double stranded RNA (dsRNA) constructs targeting specific pathogen or insect genes and their use to specifically down-regulate their expression upon infection or feeding. The RNAi approach is highly specific, and is anticipated to reduce hazards of chemical pesticide applications. The fact that mealybugs transmit leafroll viruses offers an opportunity to explore a two-pronged approach to simultaneously target virus and vector (Fuchs, 2017).

The goal of our research is to develop a robust RNAi-based strategy against GLRaV-3 and the grape mealybug. The basis for our approach is three-fold. First, mealybug survival depends on two gene functions localized to the gut that prevent osmotic collapse and dehydration of the insect, as it feeds on its sugar-rich diet of plant phloem sap. These genes are the water channel aquaporin *AQP1* and the sucrase-transglucosidase *SUC1* (Jing et al., 2016), with evidence that insect mortality is enhanced by co-targeting these two genes with different molecular function but related physiological role (Tzin et al., 2015). Perturbing the expression of osmoregulatory genes required for water balance, specifically *AQP1* and *SUC1*, in the gut of phloem-feeding insects causes the insects to lose water from the body fluids and dehydrate, dying within two to three days (Karley et al., 2005; Shakesby et

al., 2009; Tzin et al., 2015). Second, the functions of *AQP1* and *SUC1* can be targeted by *in planta* RNAi, with evidence from related phloem-feeding insects that RNAi efficacy is enhanced by stacking these RNAi constructs with RNAi against the gut nuclease (*NUC1*) (Luo et al., 2017). Third, RNAi has been being successfully applied against viruses of fruit crops such as papaya (Gonsalves et al., 2008) and plum (Hily et al., 2004). The proposed research is to develop grapevines resistant to GLRaV-3 and the grape mealybug using RNAi by pyramiding dsRNA constructs against several 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.

OBJECTIVES

Our specific objectives are to:

1. Optimize RNAi constructs against the grape mealybug.
2. Develop a high throughput transient expression system to test the efficacy of RNAi constructs against the grape mealybug.
3. Characterize stably transformed RNAi grapevines.
4. Disseminate information to stakeholders through presentations at conventions and workshops.

RESULTS AND DISCUSSION

Objective 1. Optimize RNAi Constructs Against the Grape Mealybug

To optimize RNAi constructs against the grape mealybug, *AQP1* and *SUC1* have been characterized by reverse transcription polymerase chain reaction (RT-PCR) using total RNAs isolated from crawlers of a grape mealybug colony maintained on Pixie grapes in the greenhouse and overlapping primers. The cloned *AQP1* fragment is 490 base pairs (bp) in size, and the cloned *SUC1* fragment is 394-bp in size. The sequences of *AQP1* and *SUC1* were used to design dsRNA constructs which were cloned in a binary plasmid for expression *in planta*. Their expression is driven by the phloem-specific promoter s*ucrose-H+ symporter* (*SUC2)* to target RNAi expression to the preferred feeding sites of the grape mealybug.

To enhance the efficacy of RNAi against the grape mealybug, dsRNA constructs against the osmoregulation genes *AQP1* and *SUC1* were stacked. Additionally, we identified *NUC1,* a non-specific nuclease that is expressed in the gut and functions to degrade ingested dsRNA (Christiaens et al., 2014; Luo et al., 2013), by RT-PCR using overlapping primers and total RNA from crawlers. A dsRNA *NUC1* construct should protect dsRNA against degradation and dramatically increase insect mortality by stacking dsRNA against the osmoregulation genes with dsRNA against the nuclease, as recently documented (Luo et al., 2017). The *NUC1* dsRNA construct will be stacked with dsRNA constructs to *AQP1* and *SUC1*. The feasibility of this approach is assured by our previous research, in which up to five dsRNA constructs for *in planta* delivery were used with no effect on plant growth or development but with high mortality of psyllid and whitefly pests (Luo et al., 2017; Tzin et al., 2015).

For GLRaV-3, dsRNA0 constructs to the suppressor of RNA silencing *p19.7* and the coat protein (*CP*) open reading frame were engineered. Additional dsRNA constructs from conserved regions of the viral genome were developed by analysis of aligned virus nucleotide sequences available in GenBank and identification of short stretches of conserved regions. Emphasis was placed on the RNA-dependent RNA polymerase (*RdRp*) and the heat shock protein 70 homolog (*HSP70h*) open reading frames of GLRaV-3. Conserved regions were identified for *RdRp* and *HSP70h*. We retrieved full-length GRLaV-3 genome sequences available in GenBank and analyzed them to identify highly conserved nucleotide sequence regions. Search outputs revealed conserved nucleotide stretches of 100-300 nucleotides in size for *CP, RdRp,* and *HSP70h*. Individual conserved regions were amplified by RT-PCR using specific primers and total RNA from GLRaV 3-infected grapevines as template. The integrity of these constructs was verified by restriction digestions and sequencing. Each of these fragments was cloned into the plasmid pEPT8 - a plasmid derived from pUC19 that contains the cauliflower mosaic virus 35S promoter sequence and nopaline synthase terminator sequence - and subsequently into binary plasmid pGA482G for mobilization into *Agrobacterium tumefaciens* strain C58 for plant transformation. DsRNA constructs to GLRaV-3 *RdRp* and *HSP70h* will complete the *CP* and *p19.7* dsRNA constructs previously engineered.

Anticipating the engineering of stacked dsRNA constructs to the grape mealybug and GLRaV-3 for combined resistance to the virus and its most abundant vector, targeting the viral silencing suppressor *p19.7* (Gouveia et al., 2012) is not optimal. This is because RNAi should be fully effective and no silencing suppressor should be used

for maximal efficacy. Therefore, dsRNA constructs of GLRaV-3 *CP*, *RdRp*, and *HSP70h* will be stacked first, and these constructs will then be stacked with dsRNA constructs of *AQP1*, *SUC1*, and *NUC1* from the grape mealybug. The GLRaV-3 dsRNA construct pGA482G-LR3p19.7-4 (against the viral silencing suppressor *p19.7*) will continue to be used, but only for resistance against GLRaV-3.

Objective 2. Develop a High Throughput Transient Expression System to test the Efficacy of RNAi Constructs Against the Grape Mealybug

To develop a high throughput transient expression system to test the efficacy of RNAi constructs against the grape mealybug, optimizing the delivery of dsRNA constructs to grape tissue was a priority. This work is critical for the future development of RNAi transient bioassays to identify the most promising dsRNA constructs against the grape mealybug. Efforts included the monitoring of the behavior of the grape mealybug on tissue culture grown grape plantlets, anticipating that transient assays will be carried out on this type of plant material, perhaps via vacuum-assisted infiltration (Yepes et al., 2018). Crawlers were deposited on leaves and stems of tissue culture grown plantlets and observed over time (**Figure 1**). Unfortunately, this new habitat was not optimal for crawlers, as the majority of specimens did not survive the transfer from Pixie grapes onto stems or leaves of tissue culture grown grapevines, regardless of the nature of the plant material, i.e., *Vitis vinifera* cultivars or rootstock genotype, as shown by repeated counts within two to three weeks.

Figure 1. Grape mealybug crawlers on a stem of a tissue culture-grown *V. vinifera* cv. Syrah grape plantlet.

Since tissue culture grape material was shown to be suboptimal for transient assays with dsRNA constructs based on the behavior of the grape mealybug, the use of detached leaves of Pixie grapes was investigated. Pixie is a natural dwarf grapevine derived from the periclinal chimera of *V. vinifera* cv. Pinot Meunier. It has short internodes and is a preferred host of the grape mealybug. To test the feasibility of a detached leaf assay, we excised young Pixie leaves and placed them in microfuge tubes containing distilled water or a red food dye (10%). Red pigmentation was visible in the veins of Pixie leaves within one hour, and more pigment continued to disperse in subsequent hours (**Figure 2**). This initial work revealed that a food dye spreads from the stem of a detached Pixie grape petiole throughout the leaf, particularly to its very small veins. This result is very encouraging for the delivery of dsRNA constructs against the grape mealybug in transient assays.

Next, grape mealybugs from a colony maintained on potted Pixie vines in the greenhouse were deposited on detached Pixie leaves to evaluate their behavior on this new habitat. A high survival rate (more than 80%) of grape mealybugs was consistently obtained in replicated assays, even after two weeks of exposure (**Figure 3**).

Such conditions are anticipated to be well adapted to evaluate the effect of stacked *AQP1*, *SUC1*, and *NUC1* dsRNA constructs against the grape mealybug in a transient assay based on excised Pixie leaves. The next step was to determine if a dsRNA construct can be administered to an excised petiole of a Pixie leaf. We used a dsRNA construct to the green fluorescent protein (GFP) as a proxy for dsRNA constructs to the grape mealybug. First, we tested the stability of the GFP dsRNA construct in water over time. No degradation was observed for the

GFP dsRNA construct over the course of the experiment (0 to 24 hours), as shown by electrophoresis on an agarose gel (**Figure 4**).

Figure 2. Absorption of red food coloring by detached leaves of Pixie grape. Left panel: A subset of leaves are exposed to red food coloring (top) vs. distilled water (bottom). Middle and right panels: Close-up of primary, secondary, and tertiary veins of leaves exposed to water (left) versus red food coloring (right). Pictures were taken 18 hours after exposure.

Figure 3. Close-up of an excised Pixie leaf with its petiole immersed in water and mealybug adults feeding on secondary veins.

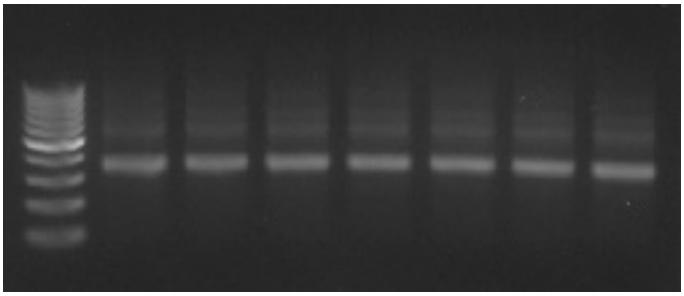

Figure 4. Analysis of the stability of a GFP dsRNA construct kept in water after 0 (lane 2), 0.5 (lane 3), 1 (lane 4), 2 (lane 5), 6 (lane 6), 12 (lane 7), and 24 (lane 8) hours by electrophoresis on an agarose gel.

Then, the GFP dsRNA construct (0.05 µg/µl in 200 µl solution) was added to the microfuge tubes containing excised Pixie leaves and its presence was tested by Northern blot hybridization in tissue collected from Pixie leaves at 24 hours post-soaking using a specific ^{32}P-labled probe (**Figure 5**). Analysis of the Northern blot image showed an uptake of the GFP dsRNA construct by excised Pixie leaves. Results were also consistent with the integrity of the GFP dsRNA detected in leaf tissue and some degradation possibly due to the plant RNAi machinery, since several DNA products of lower molecular mass than the 0.4kb full-length GFP dsRNA construct were detected.

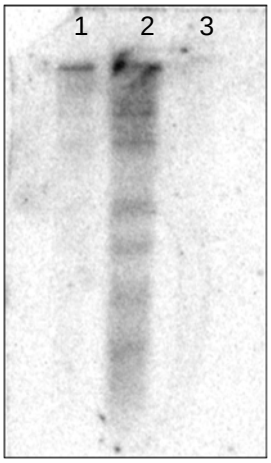

Figure 5. Northern blot hybridization of total RNA extracted from excised Pixie leaves for which the petiole was immersed into a GFP dsRNA solution for 24 hours (lane 2) or water (lane 3). Lane 1 is the GFP dsRNA construct in water as positive control.

Recently we initiated Northern blot hybridization experiments to determine whether the GFP dsRNA construct can be detected in grape mealybugs exposed to excised Pixie leaves soaked in a GFP dsRNA construct for 24-48 hours. This analysis is critical for determining whether the intact dsRNA construct is delivered to the insect and diced by the RNAi machinery of the insect to 21 nucleotide (nt) small interfering RNA (siRNA), with minimal nonspecific degradation. Optimizing such conditions is vital prior to running separate experiments with dsRNA constructs to *AQP1*, *SUC1*, and *NUC* and testing their effect on the survival of grape mealybugs.

Objective 3. Characterize Stably Transformed RNAi Grapevines

To characterize stably transformed RNAi grapevines, an inverted-repeat p19.7 construct was engineered and used for the production of transgenic grapevines via *A. tumefaciens*-mediated transformation. Embryogenic cultures of rootstock genotypes 110R and 101-14 were used for stable transformation experiments. Following transformation with *A. tumefaciens* elongation of embryogenic cultures was observed, with the highest efficacy obtained with 110R followed by 101-14. A few plants of the rootstock genotypes 110R that were subjected to transformation experiments were regenerated and micropropagated in tissue culture. Some putative transgenic plantlets were transferred to soil in the greenhouse by removing them from test tubes or polylethylene tissue culture bags using forceps, rinsing roots in water, and trimming roots to about one third in length to stimulate growth prior to transfer to Cornell mix in individual plastic pots. Plants were covered with plastic bags to avoid dehydration. Plastic bags were gradually opened following active growth in the greenhouse. In the near future, transgene insertion will be characterized by PCR and Southern blot hybridization using total plant DNA isolated from leaves of actively-growing putative transgenic plants. Additionally, RT-PCR and Northern blot hybridization will be carried out to confirm transgene expression and the accumulation of siRNA, respectively. Additional putative transgenic 110R and 101-14 rootstock plants will be transferred from tissue culture to the greenhouse for characterization of transgene insertion and expression. Efforts to transform *V. vinifera* cvs. Cabernet franc and eventually Pinot noir will be pursued.

Additional efforts to produce stable grapevine transformants with GLRaV-3 dsRNA constructs will focus on stacked dsRNA constructs of *CP*, *RdRp*, and *HSP70h*. These dsRNA constructs are vital for combining resistance

to the virus and the grape mealybug, as a dsRNA *p19.7* construct, which is coding for a silencing suppressor, would not be optimal for inclusion as one of the stacked constructs. Efforts to pyramid *CP*, *RdRp*, and *HSP70h* are under way. Pyramided GLRaV-3 dsRNA constructs will be stacked with dsRNA *AQP1, SUC1,* and *NUC* constructs as soon as the dsRNA constructs to the grape mealybug are validated in transient assays.

Objective 4. Disseminate Information to Stakeholders Through Presentations at Conventions and Workshops

To disseminate information to farm advisors and the industry, research results were communicated to farm advisors, extension educators, crop consultants, researchers, vineyard managers, and regulators at winter school meetings in California and New York. The targeted venues were (i) Sustainable Ag Expo in San Luis Obispo, California (550 participants), (ii) the Innovations and Insights in Plant Breeding Conference in Ithaca, New York (100 participants), and (iii) The Finger Lakes Forum in Geneva, New York on January 18, 2018 (60 participants).

CONCLUSIONS

Leafroll is one of the most devastating and widespread viral diseases of grapevines. GLRaV-3 is the dominant virus in leafroll diseased vineyards. This virus is transmitted by several species of mealybugs, including the grape mealybug, which is its most abundant and widely distributed vector in vineyards, and a pest of grapes. We are exploring RNAi to protect grapevines against GLRaV-3 and the grape mealybug. For RNAi to protect against GLRaV-3, conserved nucleotide sequence regions of *p19.7*, *CP*, *RdRp*, and *HSP70* were used to engineer dsRNA constructs. A few putative transgenic plants of the rootstock genotype 110R were obtained following *A. tumefaciens*-mediated transformation with a dsRNA *p19.7* construct, and established in the greenhouse. For the grape mealybug, key osmoregulatory genes *AQP1* and *SUC1* and the nonspecific nuclease *NUC* were obtained from crawlers of a grape mealybug colony established on Pixie grapes in the greenhouse. A bioassay using excised leaves of the Pixie grape is investigated to test the effect of dsRNA constructs on the survival of the grape mealybug. Ongoing efforts and preliminary results are encouraging. It is anticipated that pyramiding dsRNA constructs against GLRaV-3 and the grape mealybug will confer durable protection of grapevines.

REFERENCES CITED

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

Atallah S, Gomez M, Fuchs M, Martinson T. 2012. Economic impact of grapevine leafroll disease on *Vitis vinifera* cv. Cabernet franc in Finger Lakes vineyards of New York. *American Journal of Enology and Viticulture* 63:73-79.

Christiaens O, Swevers L, Smagghe G. 2014. DsRNA degradation in the pea aphid (*Acyrthosiphon pisum*) associated with lack of response in RNAi feeding and injection assay. *Peptides* 53:307-314.

Fuchs M. 2017. Pyramiding resistance-conferring gene sequences in crops. *Current Opinion in Virology* 26:36-42.

Gonsalves D, Ferreira S, Suzuki J, Tripathi S. 2008. Papaya. *Compendium of Transgenic Crop Plants*. Kole C, Hall TC (eds.). Oxford UK, Blackwell Publishing, pp. 131-162.

Gouveia P, Dandlen S, Costa S, Marques N, Nolasco G. 2012. Identification of an RNA silencing suppressor encoded by grapevine leafroll-associated virus 3. *European Journal of Plant Pathology* 133:237-245.

Hily J-M, Scorza R, Malinowski T, Zawadzka B, Ravelonandro M. 2004. Stability of gene silencing-based resistance to plum pox virus in transgenic plum (*Prunus domestica* L.) under field conditions. *Transgenic Research* 13:427-436.

Ito T, Nakaune R. 2016. Molecular characterization of a novel putative ampelovirus tentatively named grapevine leafroll-associated virus 13. *Archives of Virology* 161:2555-2559.

Jing XF, White TA, Luan J, Jiao C, Fei Z, Douglas AE. 2016. Evolutionary conservation of candidate osmoregulation genes in plant phloem-sap feeding insects. *Insect Molecular Biology* 25:251-258.

Karley AJ, Ashforda DA, Mintob LM, Pritchardb J, Douglas AE. 2005. The significance of gut sucrase activity for osmoregulation in the pea aphid, *Acyrthosiphon pisum*. *Journal of Insect Physiology* 51:1313-1319.

Luo Y, Wang X, Wang X, Yu D, Chen B, Kang L. 2013. Differential responses of migratory locusts to systemic RNA interference via double-stranded RNA injection and feeding. *Insect Molecular Biology* 22:574-583.

Luo Y, Chen Q, Luan J, Chung S-H, Van Eck J, Turgeon ER, Douglas AE. 2017. Towards an understanding of the molecular basis of effective RNAi against a global insect pest, the whitefly *Bemisia tabaci*. *Insect Biochemistry and Molecular Biology* 88: 21-29.

Maree HJ, Almeida RPP, Bester R, Chooi KM, Cohen D, Dolja VV, Fuchs MF, Golino DA, Jooste AEC, Martelli GP, Naidu RA, Rowhani A, Saldarelli P, Burger JT. 2013. Grapevine leafroll virus-associated 3. *Frontiers in Microbiology* 4:94.

Naidu RA, Rowhani A, Fuchs M, Golino DA, Martelli GP. 2014. Grapevine leafroll: A complex viral disease affecting a high-value fruit crop. *Plant Disease* 98:1172-1185.

Naidu RA, Maree HJ, Burger JT. 2015. Grapevine leafroll disease and associated viruses: A unique pathosystem. *Ann. Rev. Phytopathology* 53:613-634.

Oliver JE, Fuchs M. 2011. Tolerance and resistance to viruses and their vectors in *Vitis* sp.: A virologist's perspective of the literature. *American Journal of Enology and Viticulture* 62:438-451.

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

Shakesby AJ, Wallace IS, Isaacs HV, Pritchard J, Roberts DM, Douglas AE. 2009. A water-specific aquaporin involved in aphid osmoregulation. *Insect Biochemistry and Molecular Biology* 39:1-10.

Tzin V, Yang X, Jing X, Zhang K, Jander G, Douglas AE. 2015. RNA interference against gut osmoregulatory genes in phloem-feeding insects. *Journal of Insect Physiology* 79:105-112.

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

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ECOLOGY OF GRAPEVINE RED BLOTCH VIRUS

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ABSTRACT
Limited information is available on the ecology of grapevine red blotch disease, a new threat to the grape and wine industry. We characterized attributes of spread of grapevine red blotch virus (GRBV) in three distinct vineyards, a five-acre Cabernet franc vineyard in California with a 14% disease incidence 10 years post-planting, an adjacent four-acre Cabernet Sauvignon vineyard in California with a 2% disease incidence 10 years post-planting, and a two-acre Merlot vineyard in New York with a 60% disease incidence 10 years post-planting. Analysis of the spatiotemporal distribution of infected vines from 2014 to 2018 was consistent with a 2.5%, 0.5%, and 0% increase of infected vines annually in the Cabernet franc, Cabernet Sauvignon, and Merlot vineyards, respectively. An analysis of populations of the three-cornered alfalfa hopper (*Spissistilus festinus*; TCAH), the only known vector of GRBV of epidemiological importance so far, over two consecutive growing seasons indicated a 10-fold difference in abundance between the Cabernet franc and Cabernet Sauvignon vineyards, with 50 and five specimens caught on insect traps, respectively, including 25 of 50 (50%) and only one of five (20%), respectively, that had ingested GRBV, as shown by polymerase chain reaction. In contrast, no TCAH was found in the Merlot vineyard. Since legumes, not grapes, are preferred hosts of the TCAH and are often used in vineyard middle-row cover crops, we tested the potential of vineyard cover crops as hosts of GRBV and/or the TCAH. None of the cover crop samples collected in diseased vineyards in 2014 to 2018 tested positive for GRBV and no TCAH were observed in middle row cover crops, suggesting no major role of legumes in vineyard cover crops in disease epidemiology. Among artificially inoculated herbaceous plants with infectious GRBV clones in the laboratory, *Phaseolus vulgaris*, tomato (*Solanum lycopersicum*), and *Nicotiana benthamiana* were identified as local hosts, and TCAH was able to establish on *Phaseolus vulgaris.* To characterize the transmission mode of GRBV by TCAH, gut cleansing experiments revealed that the majority of TCAH that fed on infected grapevines tested positive for GRBV following a two-week feeding period on alfalfa, suggesting a circular transmission. This research is providing a strong foundation for disease management recommendations based on a careful selection of the planting material and reduction of the virus inoculum in vineyards through roguing and vineyard removal.

LAYPERSON SUMMARY
Red blotch is a recently recognized viral disease of grapevines that is widely distributed in vineyards in the United States. Limited information is available on the spread of grapevine red blotch virus (GRBV), its causal agent. Studying changes in disease incidence over time in selected vineyards in California and New York revealed an increase of virus infections in the two California vineyards, although at different rates (2.5% versus 0.5% annual increase of infected vines), but not in the New York vineyard. The differential dynamic of GRBV spread in two vineyards in California is associated with a 10-fold lower abundance of the three-cornered alfalfa hopper (*Spissistilus festinus*; TCAH), the only known vector of GRBV so far, in the vineyard where spread is limited compared to the vineyard where spread is readily occurring. This indicates that spread dynamics can be related to the TCAH population density. No TCAH was found in the New York study vineyard where GRBV spread is not occurring. Surveys of vineyard middle-row cover crops revealed that none of the plants tested in the spring of five consecutive years, especially legume species, were positive for GRBV, and no TCAH were found, suggesting no major role of vineyard cover crops in virus spread. Together, these findings stress the need to reduce the virus inoculum for effective red blotch disease management.

INTRODUCTION
Red blotch was described for the first time on Cabernet Sauvignon at the University of California Oakville Research Field Station in 2008 (Al Rwahnih et al., 2013; Calvi, 2011; Cieniewicz et al., 2017a; Sudarshana et al., 2015). Fruit ripening issues have been documented with diseased winegrapes. Reductions of 1-6°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., 2017a; Sudarshana et al., 2015). Poor fruit quality results from interference with the transcriptional and hormonal regulation of ripening (Blanco-Ulate et al., 2017). Based on the effect of the virus on fruit quality and ripening, numerous vineyard managers are culling infected vines and replacing them with clean vines derived from virus-tested stocks. The economic cost of the disease is estimated to range from $21,833 per acre (for a 5% initial infection in year three and a 25% price penalty for infected grapes) to $169,384 per acre (for a 60% initial infection in year three and a 100% price penalty for the proportion of infected grapes) 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.

Grapevine red blotch virus (GRBV) was documented in all major grape-growing U.S. States (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), in Canada (Poojari et al., 2017; Xiao et al., 2015) and in Mexico (Gasperin-Bulbarela and Licea-Navarro, 2018). The widespread occurrence of GRBV in the Americas and Mexico suggests that propagation material has played a significant role in its dissemination. The virus was also described in Switzerland (Reynard et al., 2018), South Korea (Lim et al., 2016) and India (GenBank accession number KU522121).

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 seven open reading frames (Cieniewicz et al., 2017a). We recently showed the causative role of GRBV in the etiology of red blotch disease using agroinoculation of tissue culture-grown grapevines with infectious clones of GRBV (Yepes et al., 2018).

The three-cornered alfalfa treehopper (*Spissistilus festinus*; TCAH) has been shown to transmit GRBV from infected to healthy vines under greenhouse conditions (Bahder et al., 2016a). The epidemiological significance of this finding was recently documented in a diseased vineyard in California (Cieniewicz et al., 2017b). Nonetheless, limited information is available on the ecology of red blotch disease, stressing the need to carry out studies in diseased vineyards. The overarching goal of our research is to advance our understanding of the ecology of red blotch disease with a major emphasis on attributes of GRBV spread and the potential epidemiological role of vineyard cover crops.

OBJECTIVES

Our specific objectives are to:

1. Characterize the spread of GRBV.
2. Investigate attributes of the spread of GRBV by TCAH.
3. Assess if vineyard cover crops can host GRBV and/or TCAH.
4. Determine the experimental host range of GRBV and TCAH.
5. Disseminate research results to farm advisors and to the grape and wine industry.

RESULTS AND DISCUSSION

Objective 1. Characterize the Spread of GRBV

To characterize the spread of GRBV, three distinct vineyards, a five-acre Cabernet franc vineyard in California, an adjacent four-acre Cabernet Sauvignon vineyard in California, and a two-acre Merlot vineyard in New York were selected. The three study vineyards were planted in 2008. Foliar symptoms were first noticed in 2012 in the Cabernet franc vineyard, in 2009 in the Cabernet Sauvignon vineyard, and in 2011 in the Merlot vineyard. The presence of GRBV was confirmed in the three study vineyards in 2013 and 2014, providing a foundation to investigate the spatiotemporal increase in incidence of GRBV.

In the Cabernet franc vineyard in California, an analysis of the number of symptomatic vines showed a disease incidence of 4% (305 of 7,691 vines) in 2014, 6% (461 of 7,691 vines) in 2015, 7% (547 of 7,691 vines) in 2016, 9% (696 of 7,691 vines) in 2017, and 14% (1,058 of 7,691 vines) in 2018 (**Figure 1**). These results were consistent with a 10% increase in disease incidence from 2014 to 2018 and a 2.5% annual increase in disease incidence over five consecutive years, likely as a result of TCAH-mediated transmission.

An investigation of the spatial distribution of symptomatic vines through an ordinary runs analysis, a statistical test for randomness of infected plants, revealed disease clustering in the majority of rows in the study area within the Cabernet franc vineyard (Cieniewicz et al., 2017b).

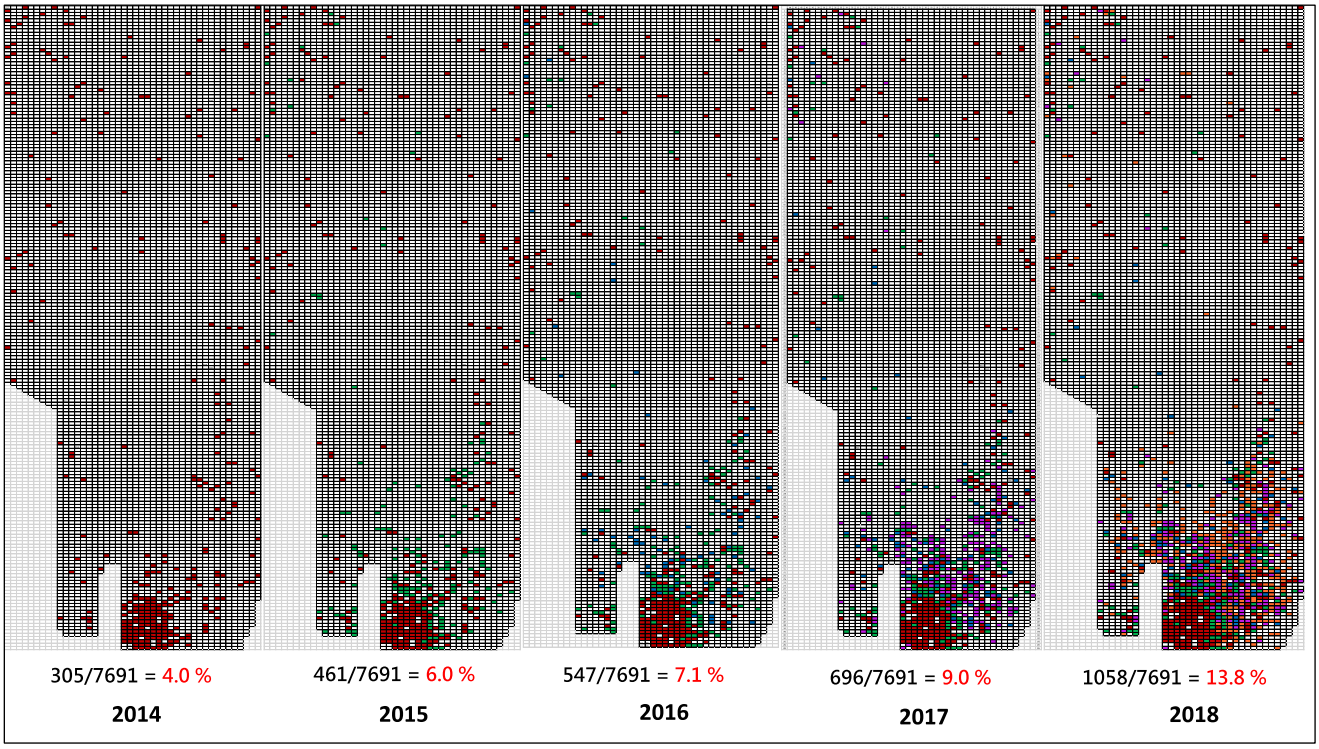

Figure 1. Red blotch disease progress in a five-acre Cabernet franc vineyard in California. Each cell represents a single vine that is asymptomatic (blank) or symptomatic (red in 2014, green in 2015, blue in 2016, purple in 2017, and peach in 2018).

The area of the Cabernet franc vineyard with highly aggregated GRBV-infected vines is proximal to a riparian area. An analysis of the distribution of diseased vines across rows illustrated a distinct dynamic of spread in the area of the Cabernet franc vineyard proximal to a riparian area versus the remainder of the vineyard (**Figure 2**). In the area proximal to the riparian area, disease incidence increased from 30% in 2014, 46% in 2015, 48% in 2016, 52% in 2016 and 71% in 2018. In contrast, in the remainder of the vineyard, disease incidence increased from 4% in 2014 to 6% in 2018. This represents a 10% and 0.5% annual increase of virus infected annually in the area of the vineyard close to the riparian area versus the remainder of the vineyard.

Probability-based modeling using the Markov Chain Monte Carlo algorithm, which integrates the spatial pattern and distance between newly infected vines to determine whether new infections are due to the influx of inoculum from within- or outside-vineyard sources of inoculum, suggested that spread in the Cabernet franc vineyard was primarily due to localized, within-vineyard sources (Cieniewicz et al., 2017b). Characterizing the genetic variability of GRBV isolates from infected vines in the aggregated area of the Cabernet franc vineyard by polymerase chain reaction (PCR) and sequencing indicated that most of them were nearly identical and grouped with phylogenetic clade 2 isolates (Krenz et al., 2014), validating within-vineyard spread (Cieniewicz et al., 2018a).

Close to 100 sentinel vines, i.e., healthy vines for which the mother stocks from which scion budwood and rootstock canes were tested and shown to be negative for GRBV, were planted in spring 2015 in the area of the Cabernet franc vineyard where infected vines are highly aggregated. These vines were used to gain direct evidence of insect-mediated GRBV spread. Sentinel vines replaced existing vines that were weak, regardless of their GRBV infection status. The presence of GRBV was tested in sentinel vines in 2015 to 2018 by PCR. None of the sentinel vines tested positive for GRBV in 2015 to 2017. However, a single asymptomatic vine tested

positive for GRBV in 2018. This suggested that three years were necessary for a sentinel vine to become infected, likely as a result of a TCAH-mediated transmission of GRBV, in an area of the vineyard where infected vines are highly aggregated and the annual increase of disease incidence is 10%.

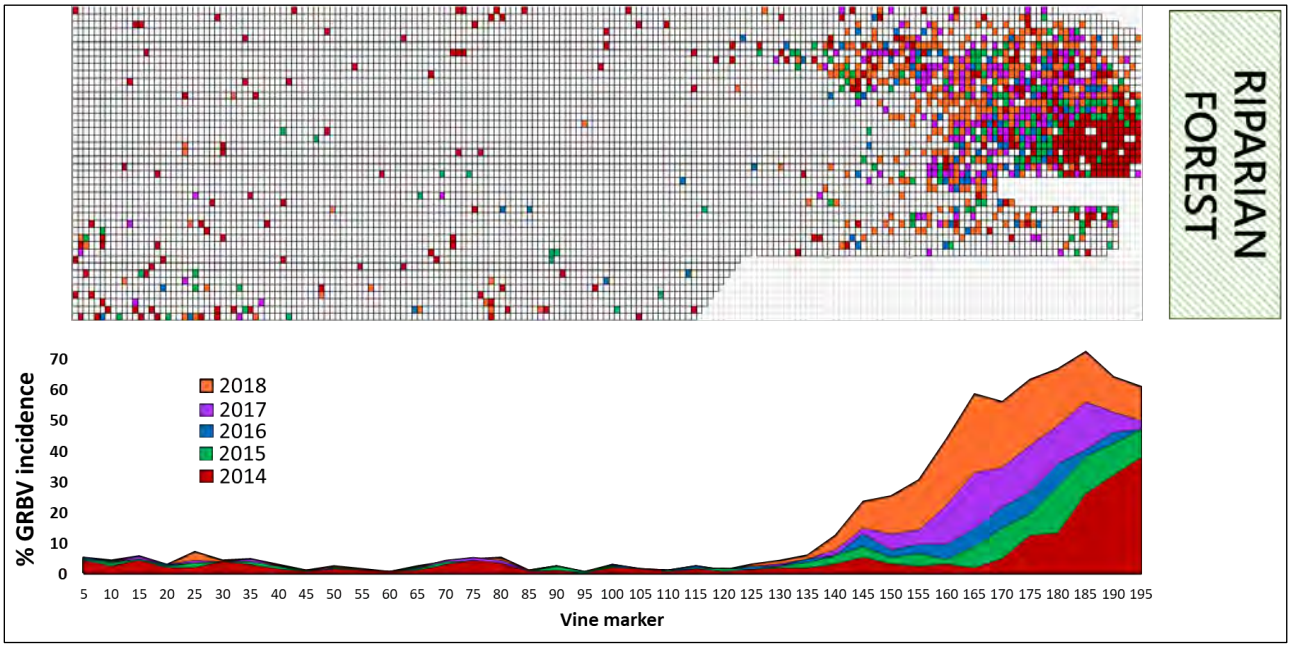

Figure 2. GRBV spread in a five-acre Cabernet franc vineyard in California over five years. The top graph shows the entire study vineyard with individual cells representing a single vine that is asymptomatic (blank) or symptomatic (colored). The bottom graph shows the distribution of diseased vines in five-vine panels across rows.

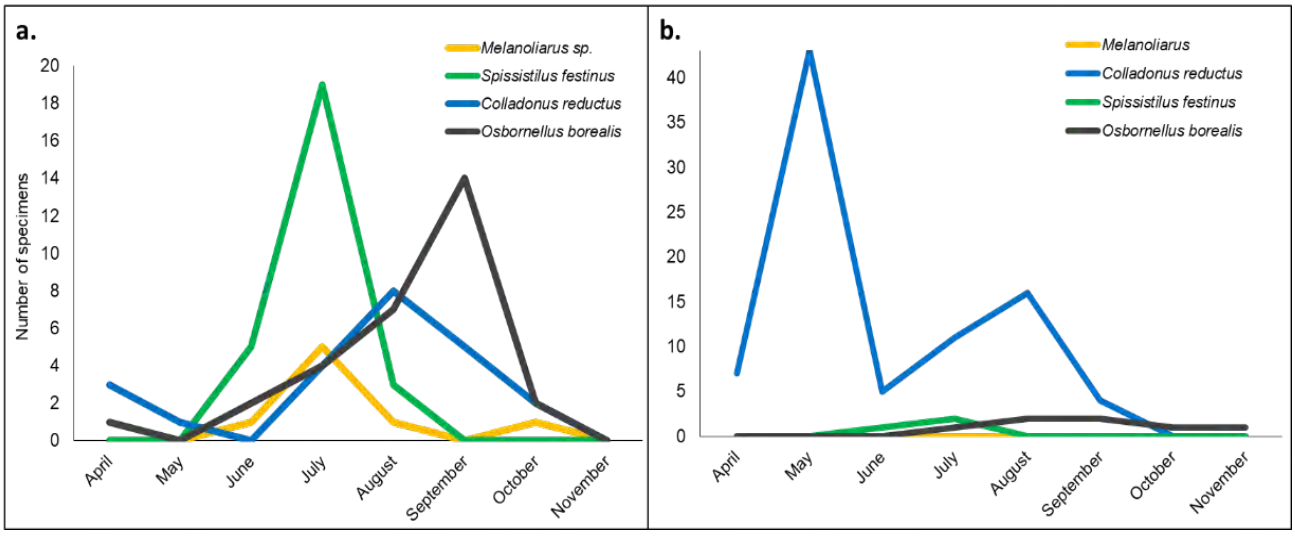

Figure 3. Populations of vector candidates in (a) a Cabernet franc vineyard where GRBV is readily spreading, and (b) an adjacent Cabernet Sauvignon vineyard where limited spread of GRBV is occurring.

The fact that extensive clustering of diseased vines occurred in one area of the selected Cabernet franc vineyard in California (**Figures 1 and 2**) provided an incentive to investigate the presence of potential vectors, particularly of TCAH. Insect sticky traps were placed in the area of the selected vineyard in California where extensive clustering of diseased vines is occurring. Traps were placed on diseased and healthy grapevines from early April to late November in 2014 and 2015 with the goal of catching insects visiting the vineyard (Cieniewicz et al., 2016a). Traps were rotated on a weekly basis. Each trap was analyzed for the presence of insects to establish a

census population and identify them at the species level, if possible, by using morphological parameters. Then, a subset of each insect family, genus, or species that was caught was removed from the traps and tested for the presence of GRBV by PCR. Results indicated that specimens of four species, among more than 40 species/taxa of Diptera, Apocrita, Coleoptera, Cicadellidae, Thysanoptera, Aphidae, Fulgoroideae, Phylloxera, Aleyrodidae, Membracidae, Blissidae/Lygaeidae, Psyloidea, Psocoptera, and Miridae that were caught on sticky traps, consistently carried GRBV (Cieniewicz et al., 2018a). The four species that consistently tested positive for GRBV were TCAH (currently the only known vector of GRBV), two leafhoppers (*Colladonus reductus* and *Osbornellus borealis*), and a planthopper (*Melanoliarus* spp.) (Cieniewicz et al., 2018a).

Populations of the four insect vector candidates caught on sticky traps were very low (~5-40 individuals per year) compared to populations of some typical grape pests, such as phylloxera, western grape leafhopper, variegated leafhopper and thrips (~500 to 1,500 individuals per year) (Cieniewicz et al., 2018a). The vector candidate populations peaked in July (TCAH and Cixiidae species) and September (*Colladonus reductus* and *Osbornellus* sp.) (**Figure 3a**). The four vector candidates are phloem-feeders, as would be expected for a GRBV transmitter. Of the four species that are able to acquire GRBV in the vineyard, none are considered a pest of grapevines.

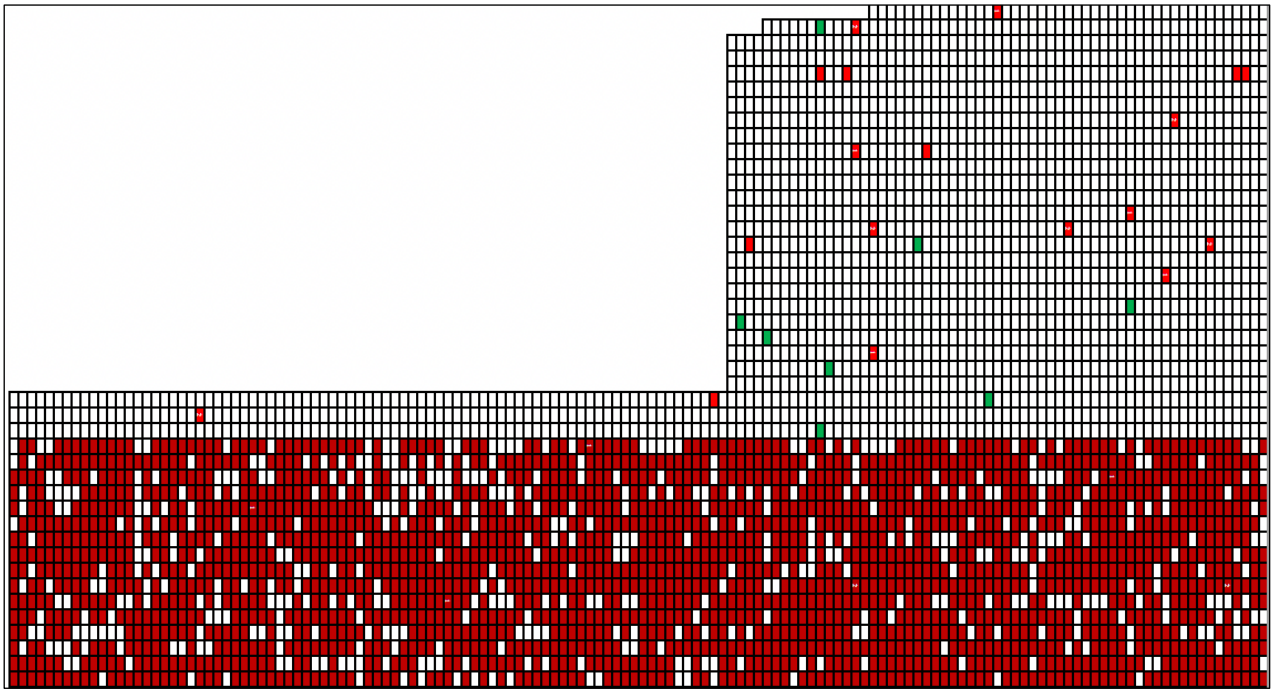

Figure 4. Map of GRBV incidence in the Cabernet Sauvignon vineyard in California. Almost all vines of clone 4 are symptomatic (bottom). Vines of clone 169 that became infected in 2017 and 2018 are shown in red and green, respectively (top).

The Cabernet Sauvignon vineyard selected for this study in California is adjacent to the Cabernet franc vineyard. The Cabernet Sauvignon vineyard is established with clones 4 and 169. Most vines of clone 4 were symptomatic following establishment, as observed by the vineyard manager and confirmed by mapping of diseased vines (**Figure 4**). This is clearly suggesting that the planting material was heavily infected with GRBV. In contrast, vines of clone 169 were clean when the vineyard was established and remained clean for several years, according to the vineyard manager. An analysis of the infection rate of GRBV in the section of the vineyard established with clone 169 showed a disease incidence of 1% (25 of 1,819 vines) in 2017 and 2% (36 of 1,819 vines) in 2018 (**Figure 4**). This is consistent with a 1% increase in disease incidence in the Cabernet Sauvignon vineyard from 2017 to 2018, likely as a result of TCAH-mediated transmission of GRBV.

The Merlot vineyard selected for this study in New York showed a high incidence (60% overall incidence) of red blotch disease following establishment, suggesting that the plant material was highly infected with GRBV. A spatiotemporal analysis of diseased vines in the Merlot vineyard in 2014 to 2018 did not provide any evidence of

an increased prevalence of GRBV over time. Over the five years of sampling and GRBV testing in this vineyard, negative vines consistently tested negative, with no vines that tested negative one year testing positive in a subsequent year. This indicated that, although GRBV is prevalent in this vineyard with a 60% overall incidence, no evidence of secondary spread was obtained. This suggested that a GRBV vector does not exist in the Merlot vineyard, or it eventually exists in the ecosystem at a very low population abundance, or it exists but does not visit the vineyard. Alternatively, the plant protection program used by the vineyard manager in New York is effective at reducing the vector population.

Research carried out in three distinct vineyards showed a difference in spread dynamic of GRBV. A relatively high rate of spread was documented in a Cabernet franc vineyard in California, a limited rate in the Cabernet Sauvignon vineyard in California, and no spread in the Merlot vineyard in New York. This prompted us to ask why there is a differential spread of GRBV in the study vineyards. In other words, why is GRBV readily spreading in the Cabernet franc vineyard but not much in Cabernet Sauvignon, in spite of the availability of a very low inoculum source (1%) in the former and a very high inoculum source (40%) in the latter following vineyard establishment? And, why is GRBV apparently not spreading in the Merlot vineyard in New York? Since GRBV shows equally striking symptoms in both Cabernet franc, Cabernet Sauvignon, and Merlot, we hypothesized that a difference in population or behavior of the TCAH vector (or other potential vectors) in these vineyards could result in the observed differential GRBV spread. To address this issue, a sticky card survey was conducted in 2017 and 2018 in the Cabernet Sauvignon vineyard (**Figure 5**) and the Merlot vineyard. The objective was to get a census of the TCAH populations that visited these two vineyards during the growing season and compare the population levels to those that visited the Cabernet franc vineyard.

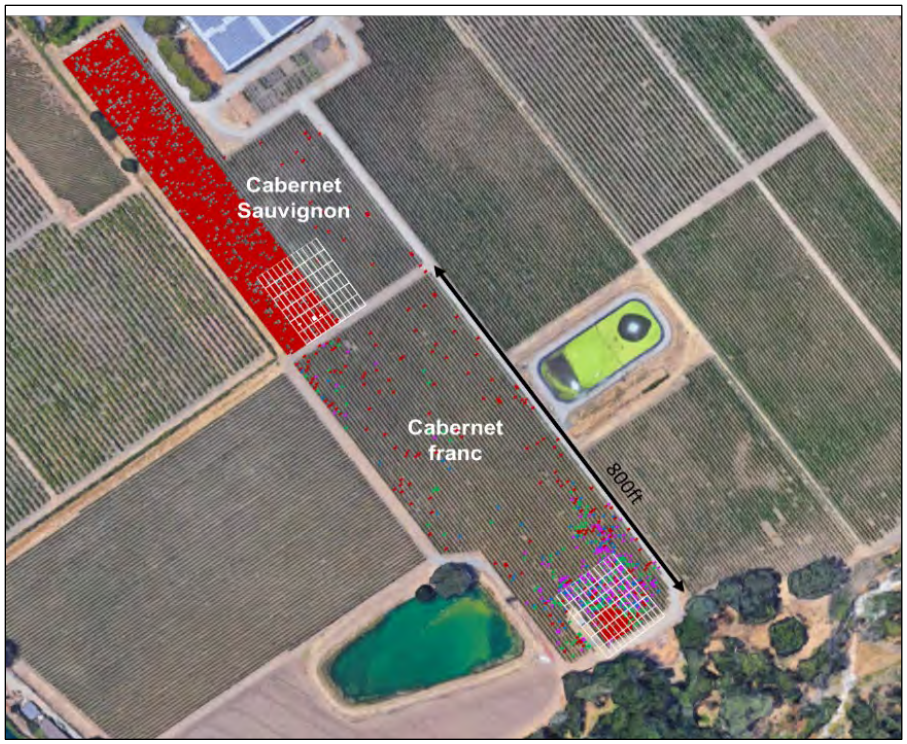

Figure 5. Landscape view of the Cabernet franc and Cabernet Sauvignon vineyards in California. White grids indicate areas of survey for insects in the Cabernet franc vineyard in 2015-16, and in the Cabernet Sauvignon vineyard in 2017-18.

Insect vector surveys showed that, although many of the same insects were present in the Cabernet franc and Cabernet Sauvignon vineyards in California, and the four insect species of interest peaked more or less at the same period during the growing season (**Figure 3**), the relative abundance of many of the species/taxa differed. For example, 25 TCAH were found in the Cabernet franc vineyard both in 2015 and 2016, but only 3 and 2 TCAH were found in the Cabernet Sauvignon vineyard in 2017 and 2018, respectively (**Figure 3b**). Similarly, there were fewer *Osbornellus borealis* and *Melanoliarus* spp. in the Cabernet Sauvignon vineyard compared to

the Cabernet franc vineyard, however, there was a greater abundance of *Colladonus reductus* in the Cabernet Sauvignon vineyard compared to the Cabernet franc. Additionally, 25 of 50 (50%) of the TCAH caught in the Cabernet franc vineyard (Cieniewicz et al., 2018a) and one of five (20%) of the TCAH caught in the Cabernet Sauvignon vineyard carried GRBV, as shown by PCR. A difference in insect vector community dynamics, particularly of the TCAH, including specimens carrying GRBV, could explain the differential spread of GRBV in the two study vineyards in California. Looking at the vineyard ecosystem, there is no major difference between the two study vineyards, except that the Cabernet franc vineyard is proximal to a riparian area and the Cabernet Sauvignon is about 800 feet from the riparian habitat (**Figure 5**). Could the degree of proximity to the riparian area explain a difference in the TCAH population that is visiting the two study vineyards? More work is needed to address this hypothesis.

Insect vector surveys in the Merlot vineyard in New York revealed several phloem-feeding leafhoppers and treehoppers, but not the TCAH, and none of them tested positive for GRBV. As expected, most species/taxa of leafhoppers and treehoppers in the New York vineyard were distinct from those in the California vineyards. This suggested that the absence of potential vectors of GRBV in this vineyard likely explains a lack of spread.

Objective 2. Investigate Attributes of the Spread of GRBV by TCAH

To characterize attributes of the TCAH-mediated spread of GRBV, TCAH were allowed to feed on GRBV-infected grapevines for five to eight days and were then transferred onto alfalfa, a non-host of GRBV. These gut cleansing experiments revealed that the majority of TCAH (18 of 28) tested positive for GRBV following a two-week feeding period on alfalfa, suggesting a persistent, circular transmission.

TCAH specimens were dissected under a stereoscope to isolate different organs (gut, salivary glands, and hemolymph) for testing by PCR following a one-week feeding period on GRBV-infected grapevines in the greenhouse (**Figure 6**). Results showed GRBV present in the salivary glands (4 of 4), hemolymph (7 of 8), and gut (8 of 8) of dissected TCAH. None of the TCAH that fed on healthy vines tested positive for GRBV in PCR. These observations support the hypothesis that GRBV is transmitted in a circulative mode. Additional experiments to confirm a circular transmission mode are under way.

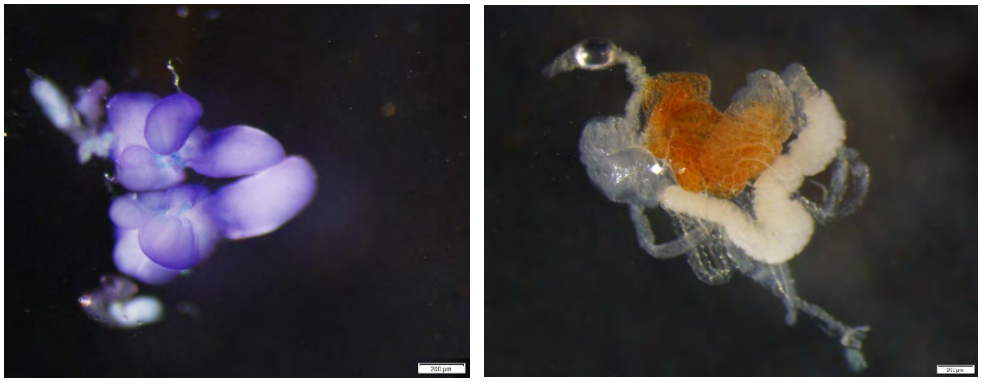

Figure 6. Dissected salivary glands (left) and gut (right) of TCAH, the only known vector of GRBV so far. The scale bar represents 200 μm.

Objective 3. Assess if Vineyard Cover Crops Can Host GRBV and/or TCAH

To determine if vineyard cover crops can host GRBV and/or TCAH, we surveyed vineyard middle row cover crop species for GRBV and TCAH in March of 2016 to 2018. The TCAH is a generalist feeder found throughout North America. While known to occur in vineyards, this insect is not considered a pest of grapevines. In addition, it does not complete its reproduction cycle on grape (Preto et al., 2018). However, the TCAH does infest legumes (Fabaceae) like alfalfa, peanut, and soybean. Legumes such as vetch, peas, bean, clover, and *Medicago* sp. are often sown in vineyard row middles as cover crops. Determining the capacity of legumes in and around vineyards to serve as sources of GRBV inoculum is critical for optimal disease management, including cover cropping strategies and weed management. Our 2017 and 2018 surveys of legume cover crop species within and adjacent to red blotch diseased vineyards showed that none of the 518 cover crop samples collected in diseased vineyards of

Sauvignon blanc, Cabernet Sauvignon, Cabernet franc, and Merlot in California tested positive for GRBV. Similarly, no TCAH were caught by extensive sweep netting vineyard middle-row cover crops in 2017 and 2018. Together with similar work carried out in 2014 to 2016, these results indicate that vineyard cover crops do not have a major role, if any, as reservoirs of GRBV and hosts of the TCAH; thus, cover crops, particularly legumes, are not involved in red blotch disease epidemics.

Objective 4. Determine the Experimental Host Range of GRBV and TCAH

To determine the experimental host range of GRBV and TCAH, and complement surveys of vineyard cover crop species, we agroinoculated a few legume species such as clover, vetch, bean, and peas with infectious clones of GRBV (Yepes et al., 2018) in the greenhouse. Plants were assayed for GRBV two weeks post-agroinoculation via pricking or vacuum-assisted infiltration by PCR and reverse transcription (RT)-PCR (Yepes et al., 2018). The RT-PCR assay is critical to detect the accumulation of spliced transcripts, as a proxy for GRBV replication. Our results showed that bean (*Phaseolus vulgaris*), tomato (*Solanum lycopersicum*), and *Nicotiana benthamiana* sustain the replication of GRBV in inoculated leaves. Similar work with other legumes is inconsistent, although preliminary data suggested that clover, vetch, and pea do not act as alternate hosts of GRBV. If confirmed, these results will provide compelling evidence that legume species used in vineyard cover crop mixes are unlikely involved in red blotch disease epidemiology. Additionally, TCAH can be maintained on beans.

Objective 5. Disseminate Research Results to Farm Advisors and to the Grape and Wine Industry

To disseminate information to farm advisors and the industry, research results were communicated to farm advisors, extension educators, crop consultants, researchers, vineyard managers, and regulators at winter school meetings in California, New York, and Missouri. The targeted venues were (i) Sustainable Ag Expo in San Luis Obispo, California (500 participants), (ii) the Show Me Grape and Wine Conference in Columbia, Missouri (52 participants), (iii) Cornell Recent Advances in Viticulture and Enology Conference in Ithaca, New York (60 participants), and (iv) the Summer Grape Conference and Field Day in Dunkirk, New York (75 participants).

CONCLUSIONS

Characterizing the spatiotemporal distribution of infected vines in two vineyards in California and one vineyard in New York documented distinct spread patterns, ranging from a relatively high rate of spread (an average of 10% increase in infected vines annually) to no spread. TCAH was documented as a vector of epidemiological importance. Populations of TCAH peaked in July in the two study vineyards in California, but their abundance was relatively low. Higher populations of TCAH were found at the edge of a California vineyard proximal to a riparian area, where spread is readily occurring compared to more within the vineyard, highlighting the likely importance of riparian areas as habitat of the TCAH. In addition, an association was found between the rate of GRBV spread in the two California vineyards and the abundance of TCAH populations, with high rates of spread correlated to high TCAH populations. No TCAH were found in the New York vineyard, where spread is not occurring. Preliminary work suggests that the transmission mode of GRBV by the TCAH is circulative. Surveys of vineyard cover crops, particularly of legumes, for GRBV in spring from 2016 to 2018 revealed that none of the species tested were infected with the virus. Similarly, no TCAH was found in vineyard cover crops by sweep netting, suggesting that cover crops, including legumes, have limited, if any, role in disease epidemiology. Research findings were communicated to the wine and grape industry during winter grower conferences.

REFERENCES CITED

Al Rwahnih M, Dave A, Anderson M, Rowhani A, Uyemoto JK, Sudarshana MR. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology* 10:1069-1076.

Al Rwahnih M, Rowhani A, Golino DA, Islas CM, Preece JE, Sudarshana MR. 2015a. Detection and genetic diversity of grapevine red blotch-associated virus isolates in table grape accessions in the National Clonal Germplasm Repository in California. *Canadian Journal of Plant Pathology* 37:130-135.

Al Rwahnih M, Rowhani A, Golino D. 2015b. First report of grapevine red blotch-associated virus in archival grapevine material from Sonoma County, California. *Plant Disease* 99:895.

Bahder BW, Zalom FG, Sudarshana MR. 2016a. An evaluation of the flora adjacent to wine grape vineyards for the presence of alternative host plants of grapevine red blotch-associated virus. *Plant Disease* 100:1571-1574.

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

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

Calvi B. 2011. Effects of red-leaf disease on Cabernet Sauvignon at the Oakville Experimental Vineyard and mitigation by harvest delay and crop adjustment. MS thesis. University of California, Davis.

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

Cieniewicz E, Pethybridge S, Gorny A, Madden L, Perry KL, McLane H, Fuchs M. 2017b. Spatiotemporal spread of grapevine red blotch-associated virus in a California vineyard. *Virus Research* 230:59-62.

Cieniewicz E, Pethybridge SJ, Loeb GM, Perry KL, Fuchs M. 2018a. Insights into the ecology of grapevine red blotch virus in a diseased vineyard. *Phytopathology* 108:94-102.

Cieniewicz E, Thompson JR, McLane H, Perry KL, Dangl GS, Corbett Q, Martinson T, Wise A, Wallis A, O'Connell J, Dunst R, Cox K, Fuchs M. 2018b. Prevalence and diversity of grabloviruses in free-living *Vitis* spp. *Plant Disease*, https://apsjournals.apsnet.org/doi/pdf/10.1094/pdis-03-18-0496-re.

Gasperin-Bulbarela J, Licea-Navarro AF. 2018. First report of grapevine red blotch virus in Mexico. *Plant Disease*, https://apsjournals.apsnet.org/doi/pdf/10.1094/PDIS-07-18-1227-PDN.

Krenz B, Thompson J, Fuchs M, Perry P. 2012. Complete genome sequence of a new circular DNA virus from grapevine. *Journal of Virology* 86:7715.

Krenz B, Thompson JR, McLane HL, Fuchs M, Perry KL. 2014. Grapevine red blotch-associated virus is widespread in the United States. *Phytopathology* 104:1232-1240.

Li R, Fuchs MF, Perry KL, Mekuria T, Zhang S. 2017. Development of a fast AmplifyRP Acceler8 diagnostic assay for grapevine red blotch-associated virus. *Journal of Plant Pathology* 99:657-662.

Lim S, Igori D, Zhao F, Moon JS. 2016. First report of grapevine red blotch-associated virus on grapevine in Korea. *Plant Disease* 100:1957.

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

Perry KL, McLane H, Thompson JR, Fuchs M. 2018. A novel grablovirus from non-cultivated grapevine (*Vitis* sp.) in North America. *Archives of Virology* 163:259-262.

Poojari, S, Alabi OJ, Fofanov V, Naidu RA. 2013. A leafhopper-transmissible DNA virus with novel evolutionary lineage in the family *Geminiviridae* implicated in grapevine redleaf disease by next-generation sequencing. *PLoS One* 6:e64194.

Poojari S, Lowery T, Schmidt A-M, Rott M, McFadden-Smith W, Stobbs L, Urbez-Torres JR. 2016. Red blotch and the virus in Canada. *Webinar on Red Blotch Disease*, February 26, 2016. http://www.ipmcenters.org/index. cfm/center-products/ipm-eacademy/upcoming-events/red-blotch-speakers/.

Poojari S, Lowery DT, Rott M, Schmidt AM, Úrbez-Torres JR. 2017. Incidence, distribution, and genetic diversity of grapevine red blotch virus in British Columbia. *Canadian Journal of Plant Pathology* 39:201-211.

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

Reynard JS, Brodard J, Dubuis N, Zufferey V, Schumpp O, Schaerer S, Gugerli P. 2018. Grapevine red blotch virus: Absence in Swiss vineyards and analysis of potential detrimental effect on viticultural performance. *Plant Disease*, https://doi.org/10/1094/PDIS-07-17-1069-RE.

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

Seguin J, Rajeswaran R, Malpica-Lopez N, Martin RR, Kasschau K, Dolja VV, Otten P, Farinelli L, Pooggin MM. 2014. *De novo* reconstruction of consensus master genomes of plant RNA and DNA viruses from siRNAs. *PLoS One* 9: e88513.

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

Varsani A, Roumagnac P, Fuchs M, Navas-Castillo J, Moriones E, Idris I, Briddon RW, Rivera-Bustamante R, Murilo Zerbini F, Martin DP. 2017. *Capulavirus* and *Grablovirus*: Two new genera in the family *Geminiviridae. Archives of Virology* 162:1819-1831.

Xiao H, Kim WS, Meng B. 2015. Comparison and improvement of methodologies for isolation of quality RNA from diverse woody plant species and utilization in detection of viral pathogens. *Virology Journal* 12:171.

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

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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 to December 1, 2018.

ABSTRACT

The goal of this project is to determine when grapevine red blotch virus (GRBV) is spreading in the vineyard. Knowing when the virus is spreading will provide important information on effective management of GRBV and help focus the efforts to identify additional vectors. This information will also help target control measures to times of the season when the virus is being transmitted in the field. Three vineyards where GRBV has been spreading are being used in this study. One vineyard is adjacent to a riparian zone, with most virus spread occurring near that edge of the vineyard nearest the riparian zone. In this case the trap plants are placed in a grassy area between the riparian zone and the vineyard. The second vineyard is adjacent to an alfalfa field, and since the one vector reported to transmit the virus is the three-cornered alfalfa hopper (*Spissistilus festinus*), the plants were placed perpendicular to the alfalfa field, and within vineyard rows. 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 and going through September 15. After one month in the field the plants are returned to Corvallis, treated with a systemic insecticide, and maintained in a screenhouse. All 300 plants from the 2016 field trials were tested for GRBV in late October 2016 and in October 2017, and will be retested in the fall of 2018. Given the lack of positive results in the 2016 trials, 25% of the 400 plants from the 2017 field trials were tested in early November 2017. These plants will be tested again in the fall of 2018 and 2019.

LAYPERSON SUMMARY

The goal of this project is to determine when grapevine red blotch virus (GRBV) is spreading in the vineyard. Knowing when the virus is spreading will provide important information on effective management of GRBV and help focus the efforts to identify additional vectors. This information will also help target control measures to times of the season when the virus is being transmitted in the field. Three vineyards where GRBV has been spreading were used in 2016 and four vineyards are being used in 2017. One vineyard is adjacent to a riparian zone, with most virus spread occurring near that edge of the vineyard nearest the riparian zone. In this case the trap plants are placed in a grassy area between the riparian zone and the vineyard. The second vineyard is adjacent to an adjacent alfalfa field, and since the one vector reported to transmit the virus is the three-cornered alfalfa hopper (*Spissistilus festinus*), the plants were placed perpendicular to the alfalfa field, and within vineyard rows. This vineyard was removed after the 2016 season, and another nearby vineyard with GRBV was substituted for the 2017 field trials. The third vineyard 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 and going through September 15 in 2016, and starting May 2 in 2017 and continuing until October. 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 in the 2016 growing season were tested by PCR in May 2017. Again, all plants were negative for GRBV. The entire set of 300 plants was tested in October 2017 and will be tested again in September 2018. Twenty-five percent of the plants from the 2017 trial were tested in November 2017 and were negative for GRBV. All 400 of the test plants from the 2017 field trial will be tested in the fall of 2018 and 2019.

INTRODUCTION

In 2012, a new virus was identified in Cabernet franc in New York's Finger Lakes region and also in Cabernet Sauvignon plants in the Napa Valley. These plants exhibited leafroll-like symptoms but tested negative for leafroll viruses. At a meeting of the International Committee on the Study of Viruses and Virus-like Diseases of Grapevine in October 2012, the name "grapevine red blotch-associated virus" (GRBaV) was agreed upon for this new virus (later changed to "grapevine red blotch virus"; GRBV).

This research aims to determine when GRBV is spreading in the field. So far, the three-cornered alfalfa hopper (*Spissistilus festinus*; TCAH) 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 of TCAH as a vector early in 2016. Regardless if this is the only vector or one of multiple vectors, the timing of transmission will be important information in developing a vector management plan.

If we know when the virus moves, efforts at vector control can be targeted to a specific time frame rather than throughout the growing season. Also, knowing when the virus is moving in the vineyards will help focus on transient insects, which may be present in vineyards for only a short period of time, or insects that feed on grapevines but have other preferred hosts. In either case these vectors could escape detection and identification in standard insect surveys. If transmission is more efficient in 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 using in-house (USDA-ARS) funds to ensure we could get the first year of field work done in 2016. Funding from the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board became available July 1, 2016 and is being used for the remainder of the project. Three hundred grapevines (Merlot on 3309 rootstock) were obtained from (donated by) Duarte Nursery, repotted into three-gallon pots, and held in a screenhouse until being used in the field, or held in a canyard near Corvallis isolated from any vineyards. Plants were tested for GRBV prior to use in the field experiment and all plants tested negative for GRBV in polymerase chain reaction (PCR) assays using two sets of primers. Beginning in April 2016, 15 plants were placed in each of three vineyards for a one-month period (45 plants each month total). Then, in mid-May, these plants were returned to Corvallis, treated with a systemic insecticide, and stored in a screenhouse. The second set of plants was taken to the vineyards in mid-May, and the process was repeated each month through September. The last set of plants was returned to the greenhouse in Corvallis in mid-October. There are 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. In 2017, four vineyards were used in the study, two in southern Oregon and two in the Willamette Valley, again with 15 plants per vineyard per month. After the last set of plants was collected, all 300 plants were tested for GRBV in November 2016. A subset of the plants was tested in May 2017 and all were tested in October 2017 and October 2018. A subset (25%) of the trap plants from the 2017 study were tested in October 2017, and all 400 will be tested in the fall of 2018 and 2019.

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 place between plants in a single row of the vineyard near the edge of where symptoms were observed. A fourth vineyard was added in 2017, another vineyard in the Willamette Valley, with spread of GRBV based on discussions with the grower.

Each plant was numbered (1-300 in 2016, and 1-400 in 2017) and the location of each plant and the month it was in the vineyard 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 was 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 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 fall of 2018 and 2019. The plants are being treated with systemic insecticides and treated for powdery mildew. The plants are not being pruned, since there is not good information on how quickly the virus moves systemically throughout the plants. Next testing of the 700 plants will be in October 2018.

In 2018, insects were collected in a vineyard with a high incidence of GRBV and sorted into groups by J. Lee, entomologist. Each group of insects was then placed on infected vines in separate cages for a six-day acquisition access feeding period. Then, four healthy plants were added to each cage and allowed a six-day inoculation access period. After the inoculation access period the insects were fumigated and the healthy plants were removed, treated with a systemic insecticide, and held for observation. This was repeated every two weeks from early June through mid-October. The plants used in the 2018 trials will also be tested in late October.

The experimental setup went according to plan and plant rotation went smoothly. We had feeding damage similar to that observed with TCAH on 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 TCAHs. 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 the membracid insects. The entomologists have been working on insect monitoring in vineyards in Oregon in 2016 and 2017. Based on recent information from M. 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 (V. Walton and R. Hilton) did catch several species of membracids in Oregon vineyards in 2016 and 2017, and the 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 V. Walton's group at Oregon State University and, as of meetings we had in January 2018, they had not obtained any positive transmissions in the greenhouse using these two membracids.

The plants from the 2016, 2017, and 2018 trials are being tested in late October 2018 and the results will be reported at the annual conference in December.

REFERENCES CITED

Al Rwahnih M, Dave A, Anderson MM, Rowhani A, Uyemoto JK, Sudarshana MR. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology* 103:1069-76. doi:10.1094/PHYTO-10-12-0253-R.

Bahder BW, Zalom FG, Sudarshana MR. 2016. An evaluation of the flora adjacent to wine grape vineyards for the presence of alternative host plants of grapevine red blotch-associated virus. *Plant Dis*. 100:1571-1574. http://dx.doi.org/10.1094/PDIS-02-16-0153-RE.

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

Krenz B, Thompson JR, Fuchs M, Perry KL. 2012. Complete genome sequence of a new circular DNA virus from grapevine. *J. Virol*. 86:7715. doi:10.1128/JVI.00943-12.

Krenz B, Thompson JR, McLane HL, Fuchs M, Perry KL. 2014. Grapevine red blotch-associated virus is widespread in the United States. *Phytopathology* 104:1232-40. doi: 10.1094/PHYTO-02-14-0053-R. PubMed PMID: 24805072.

Perry KL, McLane H, Hyder MZ, Dangl GS, Thompson JR, Fuchs MF. 2016. Grapevine red blotch-associated virus is present in free-living *Vitis* spp. proximal to cultivated grapevines. *Phytopathology* 106:663-70. doi:10.1094/PHYTO-01-16-0035-R.

Sudarshana MR, Perry KL, Fuchs MF. 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Phytopathology* 105:1026-32. doi: 10.1094/PHYTO-12-14-0369-FI.

Varsani A, Roumagnac P, Fuchs MF, Navas-Castillo J, Moriones E, Idris I, Briddon RW, Rivera-Bustamante R, Murilo Zerbini F, Martin DP. 2017. Capulavirus and Grablovirus: Two new genera in the family Geminiviridae. *Arch. Virol*. 162:1819-1831.

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EDUCATION AND OUTREACH FOR THE GRAPEVINE REGISTRATION AND CERTIFICATION PROGRAM, AND AN ASSESSMENT OF RECENTLY ESTABLISHED PRODUCTION VINES FROM INCREASE BLOCKS

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ABSTRACT / LAYPERSON SUMMARY

Disease-inducing viruses of grapevine are believed to have been around since the crop's earliest cultivation, following the spread of the plant around the world (Reynolds, 2017). In California, most growers did not perceive virus diseases of grapevine as very important until several decades ago, likely because rootstocks that were previously widespread, particularly AXR1 and St. George, reduced the expression of certain virus symptoms (Golino, 1993; Golino et al., 2008). The shift to diverse new rootstocks in the 1980s, accelerated following the failure of AXR1 due to new pest pressure, brought new focus on grapevine viruses, such as those causing leafroll disease and fanleaf degeneration, that were present but asymptomatic or of little impact in older vineyards (Golino et al., 2008; Reynolds, 2017). Grafting of infected budwood collected from existing vineyards onto new rootstock varieties with varying levels of virus tolerance often resulted in more significant disease with impacts on yield, quality, and vineyard longevity. In this context, the value of certified stock that has been screened and produced with protections against virus infection has gained greater appreciation. However, the certification program in California has certain limitations and is confronted by the fact that certain viruses, in particular, grapevine leafroll-associated virus 3, are readily spread by mealybugs from vine to vine and from vineyard to vineyard, often spreading disease to vineyards only recently planted with certified stock or even to vineyard blocks used for propagation of certified material. This has led to the impression with some growers that the certification process is not adequate or reliable. The spread of leafroll viruses by mealybugs, however, depends on the presence of infected vines, and therefore a coordinated effort to reduce the incidence of the viruses on the landscape locally or regionally can significantly reduce the chances of newly-established vineyards becoming subsequently infected. Over time, less effort is required to protect new plantings. The success of this kind of coordinated effort was first exemplified by a workgroup in Napa County. The aim of this project is to facilitate other winegrape growing regions to pursue similar coordinated management through outreach about California's Grapevine Registration and Certification Program, the nature of grapevine virus diseases, how they spread, management options, and the benefits of coordinating efforts. Additionally, a field survey has been conducted to evaluate baseline incidence and migration of grapevine viruses into blocks recently established using certified stock.

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 (GLRaV-3) is spreading in California, in addition to the discovery of grapevine red blotch virus (GRBV) (Al Rwahnih et al., 2013; Golino et al., 2008), grape producers question the quality of certified vines. 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 by vectors, 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, and there is 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. To develop a grower information pack and slide presentation to summarize the Grapevine Registration and Certification Program.
2. To hold grower meetings in key grape-growing regions of California to explain the functioning, efficacy, and limitations of the certification program.
3. To quantify the impact of education and outreach by issuing pre-test and post-test surveys at grower meetings.
4. To assess the level of potential contamination or reinfection in newly-established vineyard blocks when material is sourced from increase blocks.
5. To assess the level of reinfection of GLRaV-3 and GRBV in increase blocks between certification sampling bouts.

RESULTS AND DISCUSSION

Grapevine virus diseases represent a complex challenge that require the consideration of many factors for effective planning and management. While most grape growers will not have the opportunity to become experts, informed discussions with nursery and diagnostic laboratory representatives, pest control advisors, and farm advisors can lead them to more realistic expectations and practical strategies. Over the last year meetings with nursery and laboratory representatives, tours of facilities and operations, participation with grower groups focused on virus disease management, and discussions with farm advisors were used to gather perspectives and to refine and focus outreach and educational materials. Growers in Monterey County and Lodi have initiated organized efforts to manage the spread of GLRaVs and there is widespread interest in learning more about disease-tested and certified planting stock. The role of the project has been to support these efforts while making new connections to develop interest in other regions.

Objective 1. To Develop a Grower Information Pack and Slide Presentation to Summarize the Grapevine Registration and Certification Program

Outreach material has been focused on facilitating and enhancing informed discussions between growers and viticulturists with nurseries, diagnostic labs, and other supporting industries. Meetings with such representative stakeholders, as well as with other industry and extension professionals including farm advisors, have informed further development and refining of educational materials to provide accessible education to growers about the value of the California Grapevine Registration and Certification Program, its technical structure, and limits. Outreach about grapevine virus disease management has been focused primarily on leafroll disease, which poses the greatest risk for spread in production and propagation vineyards. This information is structured around major concepts in the integrated management of grapevine leafroll disease, describing key tools and practices in detection and monitoring, removal of infected vines (roguing), management of mealybugs, and the importance of coordinated regional management. Additional outreach material addresses the latest science on GRBV and its management. This structure has been adapted to existing outreach material and used in presentations to the Monterey County Vintners and Growers Association (MCVGA) and Temecula Small Growers Association. A written format of the material is also under development.

Objective 2. To Hold Grower Meetings in Key Grape-Growing Regions of California to Explain the Functioning, Efficacy, and Limitations of the Certification Program

Regular participation in meetings of various regional management groups have included the Lodi Winegrape Commission Virus Research Focus Group, coordinated by Stephanie Bolton, and the MCVGA, coordinated by Kim Stemler and Greg Gonzalez. A working relationship with Craig MacMillan of the Vineyard Team

organization continues to explore possibilities in the Central Coast, and includes participation in the Sustainable Agriculture Expo in November 2018. An initial presentation to the MCVGA in July anticipated a proposed series of presentations in the winter of 2018/2019 on the California Grapevine Registration and Certification Program and integrated management of leafroll disease and other grapevine virus diseases. A presentation to the Temecula Small Growers Association was coordinated by farm advisor Carmen Gispert, and Greg Pennyroyal.

Objective 3. To Quantify the Impact of Education and Outreach by Issuing Pre-Test and Post-Test Surveys at Grower Meetings

Following previous leads in the development of surveys, informative and practical surveys to assess both the needs and the interest of stakeholders with respect to grapevine virus diseases and to evaluate the impact of outreach are under development.

Objective 4. To Assess the Level of Potential Contamination or Reinfection in Newly-Established Vineyard Blocks When Material Is Sourced from Increase Blocks

Samples were collected in the fall of 2017 from 18 vineyards established with certified planting stock in the previous one to three years in diverse viticultural areas in the state. These included vineyards in Napa, Sonoma, Mendocino, San Joaquin, Kern, Fresno, San Luis Obispo, Santa Barbara, El Dorado, and Placer counties. Twenty vines in groups of four (two by two facing in adjacent rows) were sampled from each block, and the vines tagged for repeat sampling in the following year. The samples were analyzed for 11 graft-transmissible grapevine viruses by total nucleic acid extraction and quantitative polymerase chain reaction. In addition to testing for the agents of grapevine virus diseases of greatest concern in California (GLRaV-3 and GRBV), samples were also tested for GLRaVs -1, -2, and -4, and grapevine vitiviruses, grapevine viruses A and B, nepoviruses, grapevine fanleaf virus, grapevine fleck virus, and grapevine Pinot Gris virus. The unregulated grapevine rupestris stem-pitting-associated virus (GRSPaV) was also included to establish the baseline presence of this virus in new vineyards planted with certified stock.

Ten of the 18 vineyards had no detections of regulated grapevine viruses. Two had a single incidence of GLRaV-3, and two others of GRBV. In all cases the same virus species was present in neighboring blocks. GRSPaV was frequent in eight of the 18 blocks. Overall the results suggest that, in general, vineyards planted with certified stock are free from important viruses in the first three years, with occasional occurrence of vectored viruses such as GLRaV-3 and GRBV, where the contexts suggest they may have spread to the new block from the neighboring infected vines.

Most sites sampled in 2017 were sampled again in 2018, with the addition of a few new blocks in the Temecula Valley, San Diego County, and in Sonoma County. Analysis of the samples is in process.

Objective 5. To 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 (CDFA) has been contacted in order to access the diagnostic information when it becomes available.

REFERENCES CITED

Al Rwahnih M, Dave A, Anderson MM, Rowhani A, Uyemoto JK, Sudarshana MR. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology* 103(10):1069-1076.

Golino DA. 1993. Potential interactions between rootstocks and grapevine latent viruses. *American Journal of Enology and Viticulture* 44(2):148-152.

Golino DA, Weber E, Sim S, Rowhani A. 2008. Leafroll disease is spreading rapidly in a Napa Valley vineyard. *California Agriculture* 62(4):156-60.

Reynolds AG. 2017. The Grapevine, Viticulture, and Winemaking: A Brief Introduction. In *Grapevine Viruses: Molecular Biology, Diagnostics, and Management*. Springer, pp. 3-29.

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IDENTIFICATION OF GRAPE CULTIVARS AND ROOTSTOCKS WITH RESISTANCE TO VINE MEALYBUG

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ABSTRACT
Vine mealybug (*Planococcus ficus*) is the primary mealybug pest on grapes in California. This pest costs the industry an estimated $126 - $500/acre in pesticides and replant costs annually. In addition, this insect can transmit the economically important grapevine leafroll-associated virus, which costs growers $12,106 to $91,623 per acre annually in California. Mealybug resistant cultivars are not available, but will be integral for long-term management of this pest. Ten grape cultivars, breeding lines, and species were evaluated for mealybug resistance in two potted plant field trials. Grape species and rootstocks had lower mealybug incidence than the Cabernet Sauvignon when evaluated in 2017 and 2018. In the second study, fewer mealybugs were observed on inter-specific hybrid rootstocks RS-3 and IAC 572 than on the *Vitis vinifera* cultivars evaluated. Overall, grape rootstocks IAC 572, RS-3, and 10-17A had fewer mealybugs on average than any of the grape cultivars evaluated and may serve as potential sources of resistance or tolerance.

LAYPERSON SUMMARY
Vine mealybug (*Planococcus ficus*) is a major pest to the California grape industry. Insecticide sprays provide inconsistent control due to problems associated with timing and poor contact with the insect. As concerns about the development of insecticide resistance increase, alternate systems for controlling mealybug are essential. Resistant grape cultivars are not currently available and could take more than a decade to breed. In the interim, resistant rootstocks could provide sufficient control either alone or in combination with insecticides. Six grape cultivars were evaluated bi-weekly for susceptibility to vine mealybug including potentially resistant rootstocks 10-17A and IAC 572. Plants were evaluated for the total number of visible mealybugs and egg sacs. Greater numbers of mealybugs and egg sacs were observed on the grape cultivar Cabernet Sauvignon compared to each of the other species evaluated. Potential sources of resistance, IAC 572 and 10-17A, had few mealybugs present on most, but not all, of the plants evaluated in 2017 and 2018 compared to Cabernet Sauvignon. In a separate outdoor cage study, rootstocks IAC 572 and RS-3 had few to no mealybugs compared to the four scion cultivars evaluated. From our results, RS-3, IAC 572, and 10-17A are all good potential candidates for breeding mealybug-tolerant cultivars. These materials are currently available to nurseries, researchers, and grape breeders through Foundation Plant Services at the University of California, Davis.

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). Mealybug control costs are estimated at $50 per acre, in vineyards with small mealybug populations and many natural predators, up to $500 per acre for vineyards with moderate populations and few parasitoids (Ricketts et al., 2015). Vine mealybug (*Planococcus ficus*) is one of six mealybug species that threaten the California grape industry. This introduced (ca. 1994) pest can rapidly reproduce and spread, outcompeting other mealybug species and making it the most important mealybug pest of grape in California (Daane et al., 2012).

Insecticides are the main form of mealybug control. Mating disruption and parasitoids have been implemented with success in vineyards, however these forms of control are more expensive or can be impeded by Argentine ant populations which “tend” the mealybugs (Daane et al., 2007; Mansour et al., 2011; UC IPM Pest Management Guidelines: Grape). Resistant grapes, and specifically resistant rootstocks, could directly reduce mealybug populations developing or overwintering under the bark and on roots in the vineyard.

In Brazil, one study identified a single rootstock with lab-based resistance to citrus mealybug (Filho et al., 2008). This resistance was described as a reduction in the number of viable offspring produced per female compared to susceptible cultivars, Cabernet Sauvignon and Isabel (Filho et al., 2008). This was later confirmed in a similar lab experiment performed by a different lab group (Bertin et al., 2013). These results, while promising, are based on mealybug species (*Dysmicoccus brevipes* and *Planococcus citri*) of minor importance to California. The only other report of mealybug resistance in grape comes from field observations by Michael McKenry and David Ramming (unpublished), suggesting that rootstock RS-3 may have resistance to an unknown species of mealybug in addition to nematode resistance.

OBJECTIVES

The objective of this project is to develop a novel control strategy for vine mealybug using host resistance as part of an integrated management program. Identified grape material with resistance to vine mealybug will be further evaluated for use as rootstocks and 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. Multi-season sustainability of resistance to vine mealybug in identified grape rootstocks and cultivars.

RESULTS AND DISCUSSION

Objective 1. Develop a Method to Evaluate Mealybug Host Resistance and Identify Grape Material with Leaf Resistance to Vine Mealybug

A vine mealybug colony was established in the lab on butternut squash as per K. Daane's recommendations, and clip cages were constructed to complete Objective 1. Grape plants were propagated for Flame Seedless, Autumn King, IAC 572, Tampa, and Cabernet Sauvignon in the greenhouse. Three first or second stage mealybug crawlers were placed into a clip cage (**Figure 1**) on a single leaf from each cultivar. Three leaves per cultivar were evaluated. Surviving mealybugs and life stage were evaluated after three and six weeks. High crawler mortality was observed for each cultivar, making statistical comparisons impractical.

Figure 1. Insect clip cages on grapes.

Detached leaves from each of the listed cultivars were placed into petri dishes in the lab and ten first or second stage mealybug crawlers were placed on each leaf. Five leaves were evaluated for each cultivar. Similar to clip cages, high mortality rates among crawlers were observed.

Objective 2. Evaluate Grape Materials with Identified Resistance to Vine Mealybug

Rooted cuttings of grape cultivars Flame Seedless, Cabernet Sauvignon, IAC 572, Autumn King, Valley Pearl, and Chardonnay were grown in pots in outdoor cage studies. One hundred stage one and two crawlers were placed onto each plant, with a second set inoculated onto the plant a week later. Five replicate plants were used for each cultivar. Plants were evaluated bi-weekly for mealybug colony growth. Greatest mealybug numbers were observed on cultivars Chardonnay and Cabernet Sauvignon. Rootstocks IAC 572 and RS-3 had the lowest number of mealybugs.

Table 1. Cultivars and species evaluated for mealybug resistance.

Cultivar	Species	Features
Flame Seedless	*V. vinifera*	Table grape control
Cabernet Sauvignon	*V. vinifera*	Wine grape control
IAC 572	*Interspecific hybrid*	Citrus mealybug resistance
RS-3	*Interspecific hybrid*	Mealybug resistance (anecdotal)
Autumn King	*V. vinifera*	Table grape
Chardonnay	*V. vinifera*	Wine grape
Valley Pearl	*V. vinifera*	Table grape

Objective 3. Multi-Season Sustainability of Resistance to Vine Mealybug in Identified Grape Rootstocks and Cultivars

Six *Vitis* genotypes were evaluated for susceptibility to vine mealybug (**Table 2**). Two mealybug ovisacs (average of 10-20 crawlers per ovisac) were placed onto each plant to promote colonization by the insect. Visible mealybugs, ovisacs, predators, and ants were counted every two weeks (July - Sept.) on each plant. During the winter, plants were pruned and visible mealybugs removed from above ground tissues. Mealybug evaluations began in June and continued through September in year two.

Table 2. Cultivars and species currently being evaluated for mealybug colonization and overwintering.

Cultivar	Species
USDA 1-1	*V. champinii*
PCO-349-11	*Interspecific hybrid*
IAC 572	*V. caribbea*
10-17A	*Interspecific hybrid*
USDA 1-2	*V. australis*
USDA 1-3	*V. candicans*
Cabernet Sauvignon	*V. vinifera*

In year one, highest numbers of mealybugs were observed in mid-August, with visible mealybug numbers decreasing into September. Initial results suggest that mealybug colonization was higher on Cabernet Sauvignon than the other species evaluated (**Figure 2**). High variability was observed among replicate plants, with most plants having few to no visible mealybugs. Cabernet Sauvignon was the exception, with moderate to high levels (10-50) of mealybugs visible on most replicates. Cultivars IAC 572, USDA 1-1, and 10-17A had low numbers of mealybugs detected throughout the season. In year two, mealybug numbers steadily increased across all cultivars compared to year one. Numbers peaked in August, and steadily decreased throughout September.

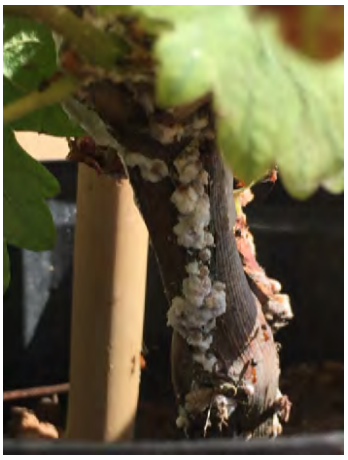

Figure 2. Adult vine mealybugs on Cabernet Sauvignon grape.

CONCLUSIONS

Clip cages were a better method for evaluating mealybug survival and growth than detached leaf assays for grape, however, whole plant assay was the best method. Six grape cultivars were evaluated biweekly for susceptibility to vine mealybug including rootstocks 10-17A and IAC 572 in 2017 and 2018. Plants were evaluated for the total number of visible mealybugs, egg sacs, and ants. High variability in the number of mealybugs was observed between plants, but differences among cultivars was evident. Greater numbers of mealybugs, ants, and mealybug egg sacs were observed on the grape cultivar Cabernet Sauvignon compared to each of the other species evaluated. This was also consistent between the first and second years of evaluation. In a separate outdoor cage study, rootstocks IAC 572 and RS-3 had few to no mealybugs compared to grape cultivars Chardonnay, Cabernet Sauvignon, Flame Seedless, Valley Pearl, and Autumn King. Based on these data, rootstocks RS-3, IAC 572, and 10-17A have greater tolerance to vine mealybug than scion cultivars and may be useful within a breeding program to incorporate insect tolerance.

REFERENCES CITED

Bertin A, Bortoli LC, Botton M, Parra JRP. 2013. Host plant effects on the development, survival, and reproduction of *Dysmicoccus brevipes* (Hemiptera: Pseudococcidae) on grapevines. *Annal Ent Soc Amer* 106:604-609.

Daane KM, Almeida RPP, Bell VA, Walker JTS, Botton M, Fallahzadeh M, Mani M, et al. 2012. Chapter 12: Biology and Management of Mealybugs in Vineyards. *Arthropod Management in Vineyards: Pests, Approaches, and Future Directions*. Bostanian NJ, Vincent C, Isaacs R. (eds.). Springer, Dordrecht, pp. 271-307.

Daane KM, Sime KR, Fallon J, Cooper ML. 2007. Impacts of Argentine ants on mealybugs and their natural enemies in California's coastal vineyards. *Ecol Ent* 32:583-596.

Filho M, Grutzmacher AD, Botton M, Bertin A. 2008. Biology and fertility life table of *Planococcus citri* in different vegetative structures of grape cultivars. *Pesq. Agropec. Brasileira* 43:941-947.

Mansour R, Suma P, Mazzeo G, Lebedi KG, Russo A. 2011. Evaluating side effects of newer insecticides on the vine mealybug parasitoid *Anagyrus* sp. near *Pseudococci*, with implications for integrated pest management in vineyards. *Phytoparasitica* 39:369 doi:10.1007/s12600-011-0170-8.

Ricketts KD, Gomez MI, Atallah SS, Fuchs MF, Martinson TE, Battany MC, Bettiga LJ, Cooper ML, Verdegaal PS, Smith RJ. 2015. Reducing the economic impact of grapevine leafroll disease in California: Identifying optimal disease management strategies. *Am J Enol Vitic* 66:2 pp 138-146.

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INVESTIGATION OF THE IMPACT OF GRAPEVINE RED BLOTCH VIRUS ON GRAPE RIPENING AND METABOLISM

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ABSTRACT
Red blotch is a recently identified disease caused by grapevine red blotch virus (GRBV). Since its discovery in 2011, its widespread presence has been confirmed in 14 states in the U.S. as well as in Canada, and it has been found in white and red winegrape varieties, table and raisin grapes, interspecific hybrids, and rootstocks. Prior to our research little was known about the impact of red blotch disease (RBD) on grape and wine composition. After four years of study across multiple varieties and sites we have good baseline data about the range of impact. Results indicate mostly a substantial impact on berry ripening in all varieties studied (Oberholster, 2015, 2016), along with variable impacts on primary and secondary metabolites depending on site and season which had a larger impact than variety (Oberholster 2015, 2016; Eridon 2016). However, the impact of RBD on metabolic pathways remains to be explored in depth. Limited previous research indicated transcriptional suppression of primary and secondary metabolic pathways by GRBV when studied in Zinfandel for one season. The current project aims to expand this research to other varieties and sites over multiple seasons to determine any potential varietal, as well as environmental, impact on RBD expression. Only after virus functioning is well understood can tools be developed to mitigate the impact thereof.

LAYPERSON SUMMARY
Prior to the Oberholster lab research over the past four years, little was known about the impacts of grapevine red blotch virus on grape composition and the resulting wine quality. Through our research, it was found that there are variable impacts on primary and secondary metabolite levels, depending on the variety, season, and rootstock. In addition, research performed by Blanco-Ulate et al. in 2017, observed changes in transcriptional factors and regulatory networks relating to an inhibition of berry ripening in infected fruit. The current project aims to further this research across varieties, seasons, sites, and rootstocks to understand the potential variable impacts the disease has on berry ripening. By doing so, a deeper knowledge of the virus functioning will be gained, and possible mitigation strategies can be achieved.

INTRODUCTION
Grapevine red blotch virus (GRBV), a causative agent for red blotch disease (RBD), is a recently discovered virus that has been identified in vineyards in 14 states across the U.S., as well as in Canada. Symptoms of GRBV include red blotches on leaves as well as reddening of primary and secondary veins for red varieties and chlorotic regions within leaf blades and marginal burning similar to potassium deficiency on white varieties (Sudarshana, Perry et al., 2015). Over the past four years, the Oberholster group has researched the impacts of GRBV on grape development and composition and the resulting impact on wine quality across varieties, sites, seasons, and rootstocks. Results indicate mostly a substantial impact on berry ripening in all varieties studied (Oberholster 2015, 2016), along with variable impacts on primary and secondary metabolites depending on site and season (Oberholster 2015, 2016; Eridon 2016). Through transcriptomics and metabolomics, the present study aims to investigate the impact GRBV has on transcriptional factors and regulatory networks. Previous research investigated the impact of GRBV on Zinfandel infected fruit for one season, and found that there was an inhibition of the phenylpropanoid metabolic pathway along with other regulatory networks responsible for berry ripening (Blanco-Ulate, Hopfer et al., 2017). This research needs to be expanded across varieties, sites, seasons, and rootstocks to determine any potential varietal, as well as environmental, impact on GRBV and RBD expression. Only once understanding of virus functionality is obtained, can tools be developed to mitigate the impact of GRBV other than the removal of infected vines.

OBJECTIVES

The main objectives of this project are the following:

1. To determine the impact of GRBV on grape metabolism during ripening.
2. To determine the impact of GRBV on hormone abundances and enzymatic activity.
3. To determine the potential impact of variety, rootstock, site, and season on GRBV functioning.

The first step is to understand GRBV and grapevine interaction. How does GRBV infection influence grape metabolism and thus ripening? What potential synergy exists between environmental stresses and RBD expression? Answers to these questions are the first step in developing an RBD management strategy. Outcomes from this study will add much needed information to understand the influence of GRBV on grape metabolism and development. This can be used to develop a measurement tool to determine disease impact as well as vineyard management recommendations to mitigate potential impact on grape quality and guide judicious removal of grapevines.

RESULTS AND DISCUSSION

We establish a protocol for RNA extraction and quality assurance in consultation with cooperator Mysore Sudarshana and the Expression Analysis Core Facility at the UC Davis Genome Center. Test samples have been utilized to determine the optimal sample treatment and RNA sequencing method. The next few months will be spent using the Qiagen RNeasy Plant MiniKit to extract RNA from grape tissue in conjunction with the Qiagen RNase-Free DNase Set. Finally, the isolated RNA will be purified using the RNeasy Kit. Once the RNA is isolated, we will test the integrity and purity of the RNA using a 2100 Bioanalyzer and NanoDrop 2000c Spectrophotometer, respectively. Subsequently, the samples will be sent to the Expression Analysis Core Facility for library preparation and sequencing using 3'-Tag RNA sequencing. Sample preparation for targeted metabolomics will commence with completion of RNA extraction.

CONCLUSIONS

The first portion of this project was method validation. Based on sample throughput and cost, we decided to perform RNA extraction, DNA clean up, and RNA purification using Qiagen RNeasy Plant MiniKit. This will extract total RNA instead of mRNA for the Expression Analysis Core to use for building the library. In addition, a quality assurance method was decided upon, ensuring the purity and integrity of the total RNA sent to the Expression Analysis Core. Next, RNA isolation will commence on the 2015-2016 and 2016-2017 samples. Results will help us determine the impact of GRBV on grape berry ripening across varieties, seasons, sites, and rootstocks. By doing so, a deeper knowledge of virus functioning will be gained, and possible mitigation strategies can be achieved.

REFERENCES CITED

Andreasson A, Kiss NB, Juhlin CC, Höög A. 2013. Long-term storage of endocrine tissues at -80°C does not adversely affect RNA quality or overall histomorphology. *Biopreservation and Biobanking* 11(6):366- 370.

Blanco-Ulate B, Hopfer H, Figueroa-Balderas R, Ye Z, Rivero RM, Albacete A, Perez-Alocea F, Koyama R, Anderson MM, Smith RJ, Ebeler SE, Cantu D. 2017. Red blotch disease alters grape berry development and metabolism by interfering with the transcriptional and hormonal regulation of ripening. *Journal of Experimental Botany*: 10.1093/jxb/erw506.

Eridon SS. 2016. Assessing the effect of different percentages of red blotch affected fruit on wine composition for Cabernet Sauvignon. MSc, University of California, Davis.

Hendrickson DA, Lerno LA, Hjelmeland AK, Ebeler SE, Heymann H, Hopfer H, Block KL, Brenneman C, Oberholster A. 2016. Impact of mechanical harvesting and optical berry sorting on grape and wine composition. *American Journal of Enology and Viticulture* 67(4):385-397.

Hjemeland AK, King ES, Ebeler SE, Heymann H. 2013. Characterizing the chemical and sensory profiles of United States Cabernet Sauvignon wines and blends. *American Journal of Viticulture and Enology* 64(2):169-179.

Oberholster A. 2015. Investigation of the impact of grapevine red blotch-associated virus on grape and wine composition and quality. American Vineyard Foundation.

Oberholster, A. 2016. Investigation of the impact of grapevine red blotch-associated virus (GRBaV) on grapevine health and subsequent grapes and wine composition and style. American Vineyard Foundation.

Sudarshana M, Perry K, Fuchs M. 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Plant Disease* 105:1026-1032.
Theodoridis G, Gika H, Franceschi P, Caputi L, Arapitsas P, Scholz M, Masuero D, Wehrens R, Vrhovsek U, Mattivi F. 2012. LC-MS based global metabolite profiling of grapes: Solvent extraction protocol optimisation. *Metabolomics* 8:175-185.
Toffali K, Zamboni A, Anesi A, Stocchero M, Pezzotti M, Levi M, Gusso F. 2011. Novel aspects of grape berry ripening and ost-harvest withering revealed by untargeted LC-ESI-MS metabolomics analysis. *Metabolomics* 7:424-436.

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STRUCTURE-FUNCTION STUDIES ON GRAPEVINE RED BLOTCH VIRUS TO ELUCIDATE DISEASE ETIOLOGY

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ABSTRACT
Grapevine red blotch virus (GRBV) is a serious threat to North American vineyards that the Pierce's Disease and Glassy-winged Sharpshooter Board is addressing by investing in applied research focused on vectors, epidemiology, ecology, and field transmission. An understanding of the molecular mechanisms evolved by GRBV to mount successful infection is essential to develop resistance strategies against the virus. Towards this end, to date five GRBV open reading frames were polymerase chain reaction amplified from field-infected samples from Temecula and Cloverdale, California, and Jacksonville, Oregon, and cloned under the *35S* cauliflower mosaic virus promoter. The cassettes will be cloned in a binary vector for identifying the viral suppressor protein(s) by transient assays in *Nicotiana benthamiana* transgenic line expressing green fluorescent protein.

LAYPERSON SUMMARY
Understanding how grapevine red blotch virus (GRBV) causes disease can provide cogent strategies for combating this threat to a multibillion-dollar industry. The etiology of GRBV effects on the host plant is completely unknown but is hypothesized to involve derangement of host small RNAs (sRNAs), which function as negative regulators of growth and development. It is likely that microRNAs (miRNAs) and trans-acting small-interfering RNAs (tasi-RNAs) operate systemically by moving through vasculature, raising prospects of genetic engineering of grapevine rootstocks for GRBV resistance in non-genetically modified organism (GMO) scions[1-4] using combinatorial RNA interference strategies[5]. The Pierce's Disease and Glassy-winged Sharpshooter Board has suggested investment in research on genetic modification of genes involved in diffusible signals (here, applies to viral sRNA suppressor proteins) and host chemical specificity for disease etiology (here applies to host target sRNAs). The Principal Investigator has characterized in many dicot species including grape a sugar-, inorganic phosphate (P_i)-, and stress hormone (abscisic acid, ABA) regulatory network controlling expression of microRNA828 (miR828), its targets MYeloBlastosis viral oncogene-like (*MYB)* transcription factors (a class of regulatory gene found in all animals and plants) and *Trans-Acting-Small RNA locus4* (*TAS4*) that down-regulate anthocyanin biosynthesis by targeting related *MYB* genes for post-transcriptional gene silencing (PTGS). This regulon is hypothesized as the mechanism by which GRBV and grapevine leafroll viruses cause symptoms by virtue of encoding sRNA silencing suppressor proteins. The novel miR828/*TAS4* target MYB transcription factors (VvMYBA6/A7 in grape) are known effectors of anthocyanin accumulation and hypothesized to be the specific targets of GRBV novel silencing suppressor genes of unknown function encoded in the virus. We are directly testing this hypothesis by expressing cloned GRBV genes in a facile transient PTGS assay system.

INTRODUCTION

Geminiviruses are single-stranded (ss) DNA viruses that cause major losses to a number of economically important crops throughout the world[6-8] and encode suppressors of post-transcriptional gene silencing (PTGS)[9], which is the grounds for the Principal Investigator's claim that red blotch host symptoms are the direct consequence of grapevine red blotch virus (GRBV) suppression of microRNA828 (miR828) biogenesis and/or action. *Geminiviridae* constitutes the second largest family of plant viruses. Geminiviruses are characterized by small, circular, ssDNA genomes encapsidated in twinned (hence, the name Gemini) icosahedral particles[10-12]. They are vector-transmissible and infect both monocotyledonous and dicotyledonous plants[13]. The genomes are either monopartite or bipartite with circular DNA molecules of 2.5 to 3 x 10^3 nucleotides. Geminiviruses possess a highly conserved common region (CR) of ~200 nucleotides. An inverted repeat within this region forms a hairpin loop and within the loop is the invariant 9-nt sequence 5'-TAATATT$^{\downarrow}$AC-3'. The viral gene products are required for its replication and transmission. Successful commercialization of engineered viral resistance of crops to date[14-17] (examples are papaya, squash, tomato, and potato) includes strategies for blocking virus replication.

Koch's postulates have been established for GRBV as the cause of red blotch disease in grapevine[18], which was first observed in California in 2008[19] and has been reported extant in numerous *Vitis* species and germplasms[19b,c], including the European Agroscope grapevine virus collection[19d]. GRBV is a single-stranded DNA virus of genome size 3.2 -3.6 kb. It infects grapevines and has a high resemblance to monopartite geminiviruses[20-25].

Recent work by Bahder et al.[26] identified the three-cornered alfalfa hopper (*Spissistilus festinus*) as a vector of GRBV under laboratory conditions. *Tortistilus* spp. treehoppers are also proposed as vectors because infestation of virus-infected grapevines with *T. albidosparsus* and *T. wickhami* is correlated with acquisition and persistence of virus in the insect, associated with leaf petiole girdling (characteristic damage by insect feeding) in the field. However, the ability of *Tortistilus* spp. to transmit the virus to non-infected plants has yet to be demonstrated[26b,c]. Additionally, preliminary results indicate GRBV is persistent for at least five weeks after acquisition in greenhouse-infected treehoppers[26c], and genomic analysis over successive years confirms GRBV spread in Oregon vineyards[26c,d]. Notwithstanding, it is likely that propagation materials have played a significant role in GRBV dissemination. Disease symptoms of viral infection initiates as red patches in the middle of the grapevine leaf, veins, and petiole which then coalesce at the end of the season resulting in a red leaf[25]. GRBV infection results in delayed and uneven berry ripening and higher titratable acid, reduced sugar, and reduced anthocyanin content in the berry[27]. The reduced fruit quality adversely affects both the table grape and wine industries[28].

Consistent with geminiviruses, GRBV possesses the conserved nonanucletide sequence, and open reading frame (ORF) predictions confirm transcription is bidirectional[25]. GRBV encodes three ORFs in the virion strand (*V1*, *V2*, and *V3*) and three in the complementary strand (*C1*, *C2*, and *C3*; **Figure 1**). Similar to mastrevirus (a monopartite geminivirus), GRBV complementary-sense ORF *C1* encodes RepA, the replication protein. Another spliced transcript encompassing the *C1* and *C2* ORFs encodes Rep, the replication protein[22, 25, 29, 30]. GRBV virion-sense strand ORFs *V2* and *V3* are predicted to encode movement proteins whereas *V1* ORF encodes coat protein.

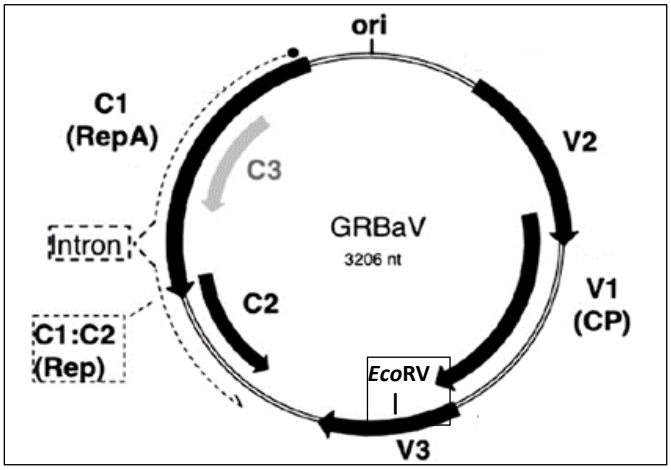

Figure 1. Genome organization of GRBV.

The functions of the predicted GRBV ORFs are yet to be elucidated experimentally. Understanding the molecular mechanisms by which the virus mounts a successful infection is fundamental and essential to developing cogent engineered resistance strategies. The geminivirus genome encodes a small number of proteins which act in an orchestrated manner to infect the host. However, the practical issue is that the few proteins encoded by the virus are multifunctional and modulate several host regulatory genes, a mechanism uniquely evolved by the viruses to balance the genome size-constraint emplaced by the capsid. A comprehensive analysis of host transcriptome profiles during berry development and select metabolite and enzyme quantitation for GRBV-infected berries from two different vineyards suggest several host regulatory pathways are modulated by the virus[31]. The induction of pathways associated with early berry development and repression of ripening and phenylpropanoid pathways was documented for GRBV-infected post-veraison berries. GRBV infection results in deranged expression of post-transcriptional machinery, transcription factors, and several hormone biosynthesis and response pathways. PTGS processes involving miRNAs and small interfering RNAs (siRNAs) are known to regulate immune responses to viruses and microbes, as well as normal plant development and hormonal signaling[32]. Hence, we postulate GRBV manifests disease by specifically targeting the host PTGS machinery, thereby driving the observed reprogramming of multiple host regulatory and metabolic pathways for its successful replication and transmission.

PTGS has evolved as a major host defense mechanism against invasive pathogens, including viruses. miRNAs and siRNAs are the specificity "guide" for nucleases of the ARGONAUTE (AGO) class which cleave or otherwise repress protein-coding transcripts in a nucleotide sequence-specific manner[33, 34]. The presence of a robust viral counter defense mechanism is underscored by the ubiquitous presence of one or more silencing suppressor proteins in the genomes of many plant viruses. The arms race between host silencing of pathogen transcripts and silencing suppression by pathogen gene products results in resistance or susceptibility to the pathogen. Geminiviruses encode silencing suppressor proteins that target PTGS, transcriptional gene silencing (TGS), and/or cellular regulatory genes (**Figure 2**). Mungbean yellow mosaic virus (MYMV) AC2 suppress PTGS by inducing the expression of host suppressor protein *WEL1*, a homologue of Werner-like exonuclease (*WEX*)[35] (**Figure 2a**). AC2 of tomato golden mosaic virus (TGMV) and L2 of beet curly top virus (BCTV) suppress PTGS by inactivating adenosine kinase[36, 37] (**Figure 2b**). Beet severe curly top virus (BSCTV) C2 suppresses PTGS by stabilizing S-adenosyl methionine decarboxylase1 (SAMDC1)[38] (**Figure 2c**). TGMV and *cabbage leaf curl virus* (CaLCuV) AC2 and BCTV L2 suppress TGS by inactivating adenosine kinase and stabilizing SAMDC1[39] (**Figure 2b and c**) and also by inhibiting the histone methyltransferase SUVH4/KYP[40], which can bind to viral chromatin and control its methylation to combat virus infection (**Figure 2g**). AC2 of TGMV and L2 of BCTV have been shown to interact and inactivate a serine-threonine kinase. SNF1-related kinase (SnRK1) is a key regulator of cellular stress responses and a component of innate antiviral defense[41] (**Figure 2d**). Suppression is mediated by elevation of cellular cytokinin levels by TGMV AC2 and C2 of spinach curly top virus (SCTV)[42] (**Figure 2e**). Tomato yellow leaf curl virus (TYLCV) C2 interacts with host CSN5 and interferes with the cellular ubiquitination machinery and inhibits jasmonate signaling[43] (**Figure 2f**). AC4 of African cassava mosaic virus (ACMV) binds ss-miRNAs[44] and Rep of wheat dwarf virus (WDV) binds ss-and duplexed 21 and 24 nt siRNAs[45] (**Figure 2h**) and suppress PTGS.

Previous work on the model plant *Arabidopsis* in the Principal Investigator's (PI's) lab showed altered source-sink distributions of sucrose and the stress hormone abscisic acid (ABA)[46] interact to regulate anthocyanin accumulation via miR828, *Trans-Acting Small-interfering locus4 (TAS4),* and their target MYeloBlastosis viral oncogene-like (v-MYB) transcription factors, viz. Vvi-MYBA6/7 and close homologues targeted by miR828 in grapevine[47, 48]. The transcriptome profiling study of GRBV host berries identified significant repression of ABA biosynthesis loci *NCED2* and *NCED3* (first described by the PI[49]) in infected berries[31].

Our working model (**Figure 2i**) is that GRBV infection interferes with the normal PTGS pathways of the host by the activity of viral-encoded suppressor proteins. miRNAs/tasi-RNAs/phasi-RNAs (phased siRNAs) regulate a large array of host gene expression at the post-transcriptional level and transcriptional level. Viruses utilize plant miRNAs to facilitate pathogenesis, and plants have co-opted miRNAs for plant innate immunity[50-55]. Under P_i starvation, reduced ABA and sugar regulate the expression of miR399/827 and miR156 and facilitate anthocyanin biosynthesis by MYB-bHLH-WD40 complexes. Increases in MYB-bHLH-WD40 transcription factors result in up-regulation of miR828 via the conserved auto-regulatory loop[46] involving miR828/*TAS4* to regulate *MYBA6/A7* levels and thereby anthocyanin levels (**Figure 2i**). We hypothesize the red blotch phenotype observed in GRBV-

infected grape leaves is a consequence of viral suppressor proteins targeting the miR828/TAS4/MYBA6/A7 autoregulatory loop which keep a check on the anthocyanin levels.

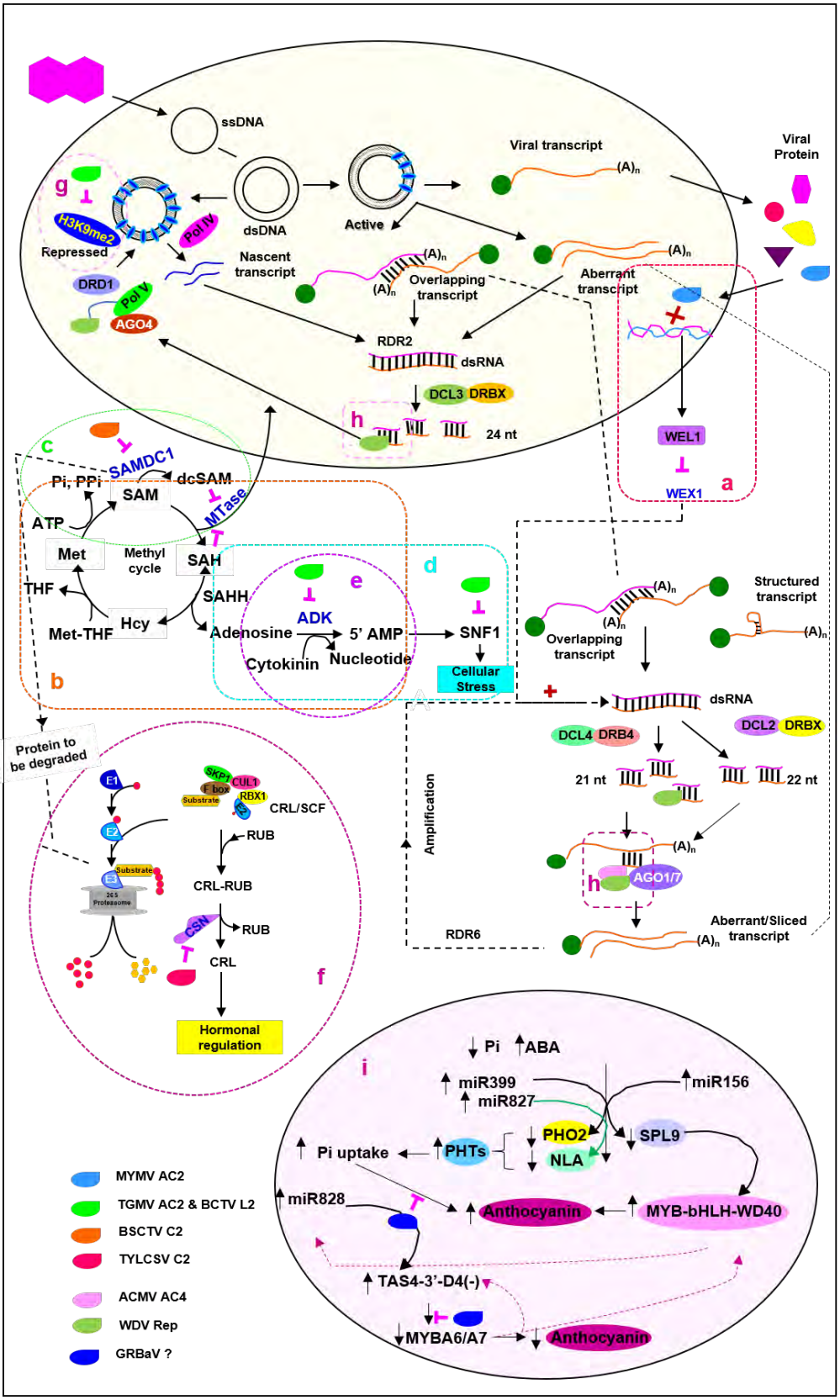

Figure 2. Model of GRBV mechanisms of action in grapevine to derange anthocyanin and hormone regulatory pathways.

We hypothesize the mis-regulation of anthocyanin in GRBV-infected plants might be a visual cue for the three-cornered alfalfa hopper, which has been shown to carry and is able to transmit GRBV in a lab setting[26]. Short distance dispersal within vineyards suggestive of spread by an insect vector has been observed in some areas[56], and while several other insects carry GRBV it is not yet clear whether one of those insects, a nematode, or some other method of transmission exists[57, 58]. A report from researchers at Washington State University suggests that the Virginia creeper leafhopper (*Erythroneura ziczac*) can be a vector for GRBV under greenhouse conditions[23], but this claim has yet to be independently verified[59]. In the future, beyond the scope of one year, transgenic GRBV resistance plants will be developed using hairpin suppressors or *Agrobacterium vir*E2 as transgene. We will test the transgenic plants for GRBV resistance by agroinoculation. In the future there is scope for collaboration and best practices sharing to develop vector transmission and feeding preference assays once the vector(s) is/are validated in the field (potentially in collaboration with Rodrigo Almeida). We hypothesize green leaves may not be a preferred diet by vectors compared to red-leafed GRBV-infected plants. This would provide critical evidence for the role of anthocyanins in disease transmission and potentially suggest strategies (e.g., leaf-specific transgenic suppression of anthocyanin biosynthetic pathways) for combating spread.

Prior work has reported GRBV effects on berry development[31]. **Table 1** provides preliminary evidence drawn from this publicly available berry transcriptome data supporting our model. As per our hypothesis, we observe a near-statistically significant downregulation of Vvi-*TAS*4c at veraison and post-veraison, indicating the miR828-*TAS4*-MYB pathway is a specific target of GRBV. This is supported by the strong up-regulation of MYBA6 at harvest, the target of a deeply conserved TAS4c tasi-RNA 3'D4(-), and several other MYBs known to function in the phenylpropanoid/flavonol pathway targeted by miR828. Interestingly, we observe up-regulation of AGO, DCL, and SGS3 proteins, all major proteins of the post-transcriptional machinery and themselves subject to PTGS and spawning of amplified phasi-RNAs. It will be very interesting to determine if transitivity of these loci is deranged by GRBV infection; we hypothesize a repression of silencing machinery upon virus infection but the evidence is the host is compensating by overexpressing PTGS effector pathways, setting the stage for discovery of a novel host homeostatic mechanism for PTGS effectors in response to infection, and/or differential effects of hypothesized virus silencing suppressors. This unexpected observation could be because the data we analyzed is from different developmental stages of berry ripening. Berry ripening is also under post-transcriptional regulation and hence an interaction between the virus infection and berry ripening could result in up-regulation of components of silencing machinery. These preliminary results underscore the need to perform transcriptome and small RNA analysis from different parts of the infected grapevine including girdled petioles at the sites of vector feeding to decipher the targets of GRBV.

Table I. Analysis of publicly available transcriptome data^ for GRBaV-infected berries across development

developmental stage:			pre-veraison		veraison		post-veraison		harvest	
target; sRNA effector	gene ID	Phase Score	beta ~LFC	pval	beta~LFC	pval	beta~LFC	pval	beta~LFC	pval
GRBaV genome	JQ901105.2	n.d.	6.26	1.91E-15	NA	NA	NA	NA	6.76	3.47E-32
Vvi-TAS4c; miR828	chr1:2961251:2961747	3375	NA	NA	-1.01	0.13	-1.01	0.13	0.38	0.53
AGO1a; miR168/530	VIT_17s0053g00680	n.d.	0.06	0.55	0.17	0.04	0.17	0.04	0.16	0.05
AGO1b; miR168/530	VIT_19s0014g01840	n.d.	0.26	0.47	0.43	0.04	0.43	0.04	0.08	0.75
MYBA6, TAS4	VIT_14s0006g01290	22.2	NA	NA	NA	NA	NA	NA	1.25	0.09
MYB^{PAL1}; miR828	VIT_00s0341g00050	476	0.52	0.01	0.12	0.39	0.12	0.39	0.13	0.31
MYB; miR828	VIT_17s0000g08480	1330	0.62	0.09	0.33	0.35	0.33	0.35	NA	NA
MYB; miR828	VIT_04s0079g00410	24.6	0.39	0.01	0.17	0.04	0.17	0.04	-0.06	0.46
AGO2a; miR403	VIT_10s0042g01180	50	0.61	0.02	0.36	0.07	0.36	0.07	0.82	0.02
AGO2b; miR403	VIT_10s0042g01200	n.d.	0.04	0.81	0.03	0.81	0.03	0.81	-0.16	0.29
DCL2; unknown	VIT_04s0023g00920	33.8	0.39	0.25	0.47	0.03	0.47	0.03	0.11	0.57
SGS3; unknown	VIT_07s0130g00190	177.4	0.04	0.69	0.23	0.01	0.23	0.01	0.16	0.06
DCL1; miR162	VIT_15s0048g02380	n.d.	-0.05	0.62	0.05	0.54	0.05	0.54	-0.21	0.15

^ Oakville vineyard dataset (ref. 31) analysed by kallisto/sleuth.

OBJECTIVES

1. Characterize hypothesized silencing suppressor protein(s) encoded by GRBV to establish the molecular mechanism by which GRBV (and grapevine leafroll-associated virus, by inference) cause disease by derangement of host miRNAs, tasi-RNAs, and phased-tasi-RNAs (phasi-RNAs).
2. Identify the host grapevine targets of GRBV suppressor proteins.
3. Create model system transgenics for future characterization of the host targets of GRBV suppressor proteins.

RESULTS AND DISCUSSION

Objective 1. Characterize Hypothesized Silencing Suppressor Protein(s) Encoded by GRBV to Establish the Molecular Mechanism by Which GRBV (and Grapevine Leafroll-Associated Virus, by Inference) Cause Disease by Derangement of Host miRNAs, tasi-RNAs, and Phased-tasi-RNAs (phasi-RNAs)

Earlier work by S. Sukumaran has established that geminivirus mungbean yellow mosaic virus (MYMV) AC2 and AC4 function as suppressors of silencing[35, 60]. Hence, MYMV AC2 and AC4 will be used as positive controls in the ongoing experiments. To prove which of the GRBV protein(s) act as a silencing suppressor and gain further insights into the molecular mechanisms of GRBV interactions with the host, a facile transient expression assay in *Nicotiana benthamiana* line 16c[61] is being employed. In this system, RNA silencing of the stably integrated jellyfish green fluorescent protein (GFP*)* transgene is induced by transient co-expression of another GFP(trigger)-expressing vector. When a silencing suppressor protein construct is co-infiltrated with GFP(trigger), the infiltrated zone recovers fluorescence as an indication of suppression of silencing mediated by the test construct (i.e., GRBV protein-coding sequences). Five days post infiltration, local GFP silencing of infiltrated leaf will be observed under long wave ultraviolet light as mild red (chlorophyll, no GFP) fluorescence. RNA blot analysis of agroinfiltrated leaf tissues will be performed using a *gfp* probe. Red fluorescence, absence of GFP transcript, and presence of GFP siRNAs will validate PTGS. Green fluorescence, presence of GFP transcript, and absence of GFP siRNAs will validate suppression of PTGS by the candidate GRBV gene product(s).

The GRBV ORF genes V3, C1, and C3 have been successfully polymerase chain reaction (PCR)-amplified from genomic DNA extracted from GRBV-infected grape leaf tissue (**Figure 3**). Field-collected samples from 2016 and 2018 were tested for viral presence using V2 primers, verifying prior results (**Figure 3a**).

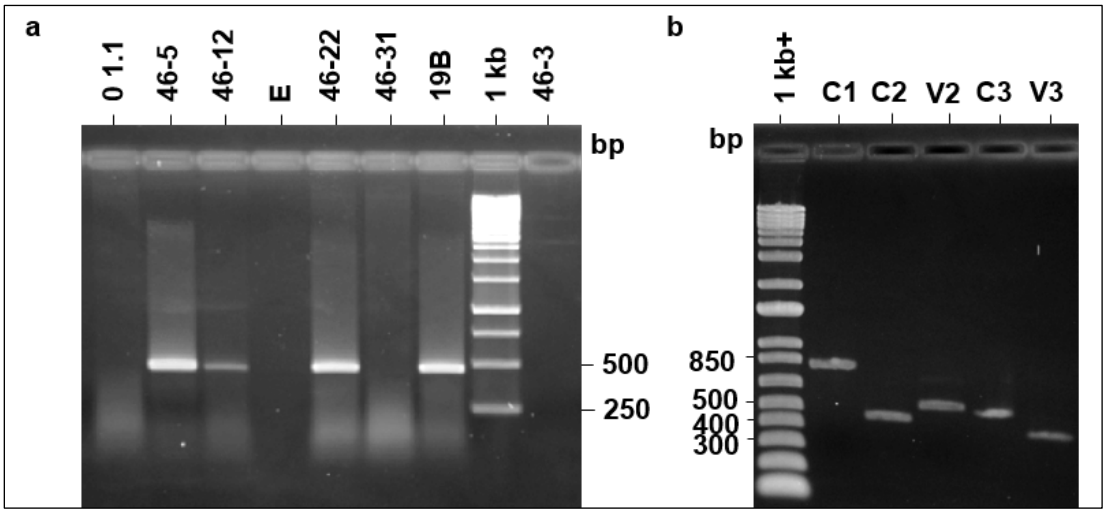

Figure 3. PCR amplification of 516 bp viral ORF V2 from validated GRBV-infected vines collected in July 2018 from counties of Sonoma and Riverside, CA and Jackson, OR. (**a)** Genomic DNA was extracted from various field leaf samples from 2016 and 2018 collections, which were validated by sRNA library sequencing or PCR results and tested for viral presence using V2 primers. (**b)** PCR amplification of C1, C3, and V3 ORF with *Hin*dIII/*Sac*I restriction sites and C2 and V2 with *Hin*dIII/*Eco*RI restriction sites from validated genomic DNA from Temecula, CA Merlot 2016 field sample "19B." (Lane legend for (a): 0 1.1: Cabernet Franc, Cloverdale, CA; 46-5: Pinot Noir, Jacksonville, OR; 46-12: Pinot Noir, Jacksonville, OR; E: empty lane; 46-22: Pinot Noir, Jacksonville, OR; 46-31: Pinot Noir, Jacksonville, OR; 19B: Merlot, Temecula, CA; 46-3: negative control, Pinot Noir, Jacksonville, OR.)

Viral ORFs C1 (795 bp), C3 (483 bp), and V3 (372 bp) were amplified by introducing *Hin*dIII/*Sac*I restriction sites in the primers and C2 (435 bp) and V2 (516 bp) were amplified by introducing *Hin*dIII/*Eco*RI restriction sites in the primers and cloned in the corresponding site of pJIC-35S vector[62]. The clones were initially screened by PCR using primers flanking the *35S* promoter and 3' polyA signal sequence (**Figure 4**).

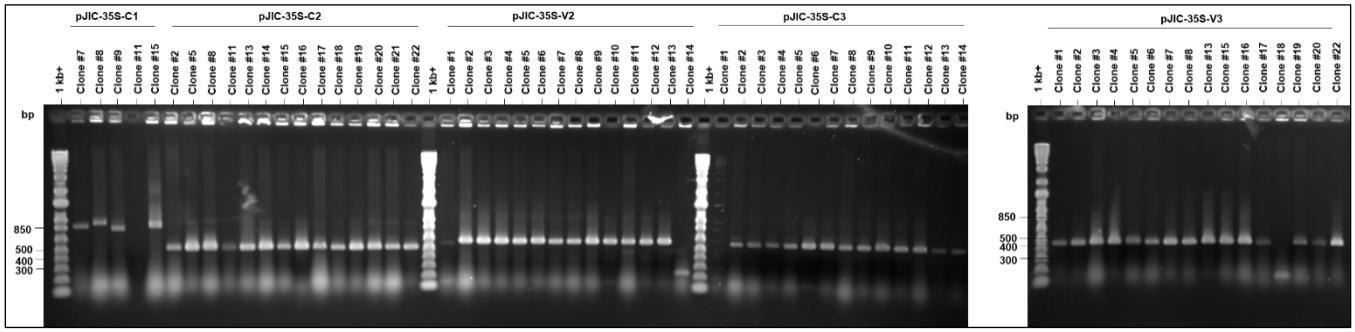

Figure 4. Colony PCR assay for screening the clones for presence of inserts using primers flanking the *35S* promoter and polyA signal.

Four positive clones from each transformation were subjected to diagnostic restriction digestion analysis of engineered sites flanking the ORF inserts and digested with *Eco*RV which has sites flanking the 35S: polyA signal cassette and an internal site at nt 96 of ORF V3, to verify the recombinant chimaeras (**Figure 5**).

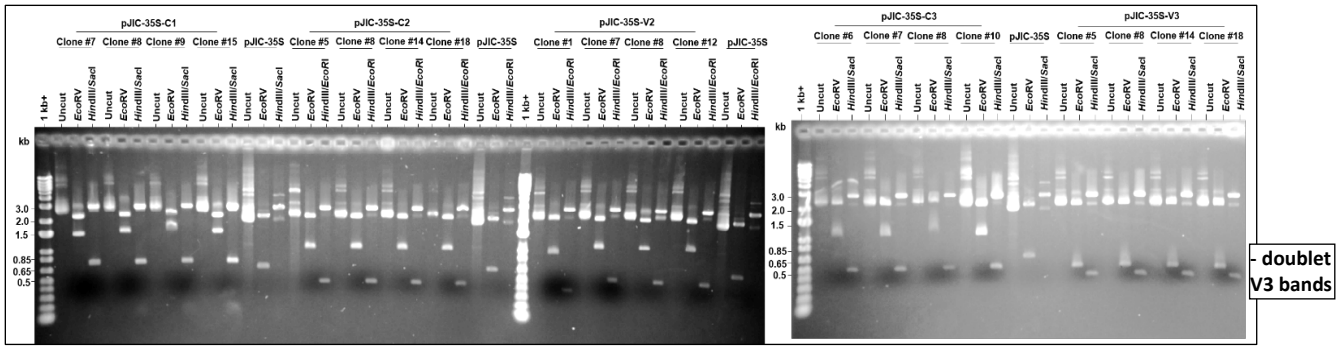

Figure 5. Restriction fragment digestion patterns of independent candidate pJIC-35S:C1, pJIC-35S:C2, pJIC-35S:V2, pJIC-35S:C3, and pJIC-35S:V3 constructs mapped with multiple restriction enzymes.

Going forward, the 35S cassette including the 35S promoter-viral ORF-35S polyA transcription termination sequence will be excised as an *Eco*RV fragment and cloned into the *Sma*I site of T-DNA binary vector pCAMBIA2300 and propagated in *Escherichia coli*. The constructs will be electroporated into *Agrobacterium tumefaciens* strain EHA105 obtained under duly-issued USDA APHIS permit P526-180523-008. To identify the suppressors of silencing, the 16c plants will be agroinfiltrated with P35S-*gfp* alone as positive control for non-silencing and in parallel a 1:1 test mixture of the *A. tumefaciens* strains harboring P35S-V1/ P35S-V2/P35S-V3/ P35S-C1/ P35S-C2/ or P35S-C3, respectively, pairwise with P35S-*gfp* silencing trigger.

Objective 2. 2. Identify the Host Grapevine Targets of GRBV Suppressor Proteins

We hypothesize GRBV suppressor proteins target host miRNA/siRNAs and alter the expression of their target genes. Prior work reported GRBV effects on the host berry transcriptome[31]. Deep sequencing of GRBV-infected and control healthy grapevine leaf sRNA libraries and mRNA transcriptome libraries can reveal the specific host genes in vegetative tissues deranged by the pathogen and provide leads for understanding the underlying mechanisms, e.g., specific miRNA effectors of host gene regulatory networks controlling plant immunity. We have in hand GRBV-infected and control samples from the 'Calle Contento' vineyard (cv. Merlot) in Temecula, California collected in 2016 and July 2018, the former validated by sequencing data. We also have GRBV-validated Pinot Noir leaf samples (A. KC, pers. comm.) from the DeBoer vineyard in Jacksonville, Oregon

collected in July 2018, and GRBV-validated[26] (R. Smith, pers. comm.) Cabernet Franc leaf samples (**Figure 6, left**) collected in Cloverdale, California.

Figure 6. Left: July 18, 2018 Cabernet Franc leaf sample from Cloverdale, CA vineyard showing GRBV symptoms. Right: July 25, 2018 Merlot leaf samples from Temecula, CA 'Calle Contento' vineyard showing GRBV symptoms.

Table 2 shows the results of anthocyanin quantitation of Temecula and Cloverdale 2018 samples, supporting the disease state of vines previously validated for GRBV infection. Small RNA libraries will be prepared using purified small RNA as input (50 ng) according to the instructions provided by TruSeq Small RNA Sample Preparation Kit (Illumina®). To validate the targets of differentially expressed sRNAs, a degradome and RNA sequencing analysis of the corresponding GRBV-infected and control samples will be done in the coming months.

Table 2. Anthocyanin quantitation of GRBV-infected leaves from Cloverdale and Temecula, CA, July 2018.

Location/Genotype	Condition	µmoles cyanidin-O-glucoside equiv/mg fresh weight	s.e.m.	pval†
Cloverdale/Cab Franc	control healthy	2.2	0.2	
	GRBV infected*	14.2	1.2	0.008
Temecula/Merlot	control healthy	8.5	0.9	
	GRBV infected§	19.2	1.1	0.002

* based on PCR assay results coordinated by Cooperator Rhonda Smith.
† significantly different than control; two-sided Student's t-test, unequal variance assumed (n = 3).
§ provisional until sequencing confirms; 2016 sample '19B' confirmed GRBV positive (**Figure 3a**).

Objective 3. 3. Create Model System Transgenics for Future Characterization of the Host Targets of GRBV Suppressor Proteins

We have yet to initiate experiments for Objective 3 and describe below the future planned work to validate if the differential expression of putative targets is caused by suppressor protein(s) identified in Objective 1. Towards this end, the suppressor protein(s) will be over-expressed in *Arabidopsis* and tobacco. The constructs shown to be functional in transient expression assays will be in hand, and thus will be used for stable transformation and regeneration of transgenics for future characterization, beyond the scope of one year of support. The high degree of evolutionary conservation of miRNAs and targets[44] would allow future characterizations of small RNA and RNA sequencing transcriptome profiles in suppressor protein-overexpressing *Arabidopsis* and tobacco transgenics. For example, sRNA blots and total RNA blots of the putative targets in the over-expressing lines. The hypothesized concordance in expression profiles of putative targets in over-expression lines with that of sequencing data (Objective 2) could independently validate the sRNA targets of GRBV suppressor proteins. Also

beyond the scope of one year of funding is to test if the mechanism of silencing suppression is by binding miRNA/siRNA. Labelling of probe and *in vitro* binding assay would be performed as described by Chellappan et al.[44] to discover host proteins that bind physically to GRBV suppressor proteins.

In the future, we will identify host proteins that physically interact with suppressor proteins by yeast-two-hybrid cloning. We plan to make transgenic grapevine rootstocks expressing short hairpin cassettes directed against GRBV suppressor protein transcripts, or that overexpress VirE2, and test transgenic grapevine plants for GRBV resistance via GRBV agroinoculation, and to test the plants for GRBV resistance by vector transmission assay in collaboration with Rodrigo Almeida at the University of California, Berkeley.

CONCLUSIONS

The comprehensive approaches involving structure-function studies on GRBV proteins (Objective 1), and sRNA, mRNA, and degradome host sequencing (Objective 2) will provide candidate viral effector and host sRNA targets of hypothesized GRBV suppressor proteins. The molecular approaches proposed here can have significant impacts on viticulture by: (i) applying deep knowledge from model plant species and other viral diseases to grapes; (ii) facilitating optimal selection of parents for breeding and immediate selection of elite progeny with multiple desirable traits, e.g., specific *MIR828/TAS4/MYB* haplotypes; (iii) accessing abundant genetic variation[63] (grape varieties currently face severe pathogen pressures, and the long-term sustainability of the industry relies on the exploitation of natural genetic diversity); (iv) understanding other viral pathogen etiologies like grapevine leafroll-associated virus; and (v) advancing genetically modified organism (GMO) technologies for mobile sRNAs engineered to confer viral resistance in the scion without the presence of transgenes. This approach to controlling a plant virus can reduce use of sprays to control arthropod vectors while not altering how the crop is grown.

REFERENCES CITED

1. Palauqui JC, Elmayan T, Pollien JM, Vaucheret H. 1997. Systemic acquired silencing: Transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. *EMBO J* 16:4738-4745.
2. Pant BD, Buhtz A, Kehr J, Scheible W-R. 2008. MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. *Plant J* 53:731-738.
3. Molnar A, Melnyk CW, Bassett A, Hardcastle TJ, Dunn R, Baulcombe DC. 2010. Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. *Science* 328:872-875.
4. Bai S, Kasai A, Yamada K, Li T, Harada T. 2011. A mobile signal transported over a long distance induces systemic transcriptional gene silencing in a grafted partner. *J Exp Bot* 62:4561-4570.
5. Lambeth LS, Van Hateren NJ, Wilson SA, Nair V. 2010. A direct comparison of strategies for combinatorial RNA interference. *BMC Mol Biol* 11:77.
6. Boulton MI. 2003. Geminiviruses: Major threats to world agriculture. *Ann Appl Biol* 142:143-143
7. Moffat AS. 1999. Geminiviruses emerge as serious crop threat. *Science* 286:1835-1835.
8. Rojas MR, Hagen C, Lucas WJ, Gilbertson RL. 2005. Exploiting chinks in the plant's armor: Evolution and emergence of geminiviruses. *Annu Rev Phytopathol* 43:361-394.
9. Vanitharani R, Chellappan P, Pita JS, Fauquet CM. 2004. Differential roles of AC2 and AC4 of cassava geminiviruses in mediating synergism and suppression of posttranscriptional gene silencing. *J Virol* 78:9487-9498.
10. Hanley-Bowdoin L, Settlage SB, Orozco BM, Nagar S, Robertson D. 1999. Geminiviruses: Models for plant DNA replication, transcription, and cell cycle regulation. *Crit Rev Plant Sci* 18:71-106.
11. Lazarowitz SG, Shepherd RJ. 1992. Geminiviruses: Genome structure and gene function. *Crit Rev Plant Sci* 11:327-349.
12. Palmer KE, Rybicki EP. 1998. The molecular biology of mastreviruses. *Advances in Virus Research.* Maramorosch K, Murphy FA, Shatkin AJ. Academic Press (eds.). Vol. 50:183-234.
13. Report: Family - Geminiviridae. 9th International Committee on Taxonomy of Viruses (San Diego). 2012. *Virus Taxonomy* (Ninth Edition). King AMQ, Lefkowitz E, Adams MJ, Carstens EB (eds.). Elsevier, pp. 351-373.
14. Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL, Sanford JC. 1992. Virus resistant papaya plants derived from tissues bombarded with the coat protein gene of papaya ringspot virus. *Bio/Technology* 10:1466-1472.

15. Ferreira SA, Pitz KY, Manshardt R, Zee F, Fitch M, Gonsalves D. 2002. Virus coat protein transgenic papaya provides practical control of Papaya ringspot virus in Hawaii. *Plant Disease* 86:101-105.
16. Jongedijk E, Huisman MJ, Cornelissen BJC. 1993. Argonic performance and field resistance of genetically modified, virus-resistant potato plants. *Sem Virol* 4:407-416.
17. Hu JS, Pang SZ, Nagpala PG, Siemieniak DR, Slightom JL, Gonsalves D. 1993. The coat protein genes of squash mosaic virus: Cloning, sequence analysis, and expression in tobacco protoplasts. *Arch Virol* 130:17-31.
18. Yepes LM, Cieniewicz E, Krenz B, McLane H, Thompson JR, Perry KL, Fuchs M. 2018. Causative role of grapevine red blotch virus in red blotch disease. *Phytopathology* 108:902-909.
19. Calvi BL. 2011. Effects of red-leaf disease on Cabernet Sauvignon at the Oakville experimental vineyard and mitigation by harvest delay and crop adjustment. *MSc thesis University of California, Davis*.
19b. Thompson T, Petersen S, Londo J, Qui W. 2018. First report of grapevine red blotch virus in seven *Vitis* species in a U.S. *Vitis* germplasm repository. *Plant Disease* 102:828.
19c. Yao X-L, Han J, Domier LL, Qu F, Lewis Ivey ML. 2018. First report of grapevine red blotch virus in Ohio vineyards. *Plant Disease* 102:463.
19d. Reynard JS, Brodard J, Dubuis N, Zufferey V, Schumpp O, Schaerer S, Gugerli P. 2018. Grapevine red blotch virus: Absence in Swiss vineyards and analysis of potential detrimental effect on viticultural performance. *Plant Disease* 102:651-655.
20. Seguin J, Rajeswaran R, Malpica-López N, Martin RR, Kasschau K, Dolja VV, Otten P, Farinelli L, Pooggin MM. 2014. *De novo* reconstruction of consensus master genomes of plant RNA and DNA viruses from siRNAs. *PLoS ONE* 9:e88513.
21. Krenz B, Thompson JR, Fuchs M, Perry KL. 2012. Complete genome sequence of a new circular DNA virus from grapevine. *J Virol* 86:7715.
22. Krenz B, Thompson JR, McLane HL, Fuchs M, Perry KL. 2014. Grapevine red blotch-associated virus is widespread in the United States. *Phytopathology* 104:1232-1240.
23. Poojari S, Alabi OJ, Fofanov VY, Naidu RA. 2013. A leafhopper-transmissible DNA virus with novel evolutionary lineage in the family *Geminiviridae* implicated in grapevine redleaf disease by next-generation sequencing. *PLoS ONE* 8:e64194.
24. Rwahnih MA, Dave A, Anderson MM, Rowhani A, Uyemoto JK, Sudarshana MR. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology* 103:1069-1076.
25. Sudarshana MR, Perry KL, Fuchs MF. 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Phytopathology* 105:1026-1032.
26. Bahder BW, Zalom FG, Jayanth M, Sudarshana MR. 2016. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* as a vector of grapevine red blotch-associated virus. *Phytopathology* 106:1223-1230.
26b. Daane K, Wilson H, Cooper M, Almeida R, Golino D, Anderson J, Ooi K-L, Yazdani A. 2018. Searching for potential vectors of grapevine red blotch-associated virus. *CDFA-PD/GWSS Final Report 15-0428-SA.* Available at http://www.piercesdisease.org/reports.
26c. Walton V, Daane K, Dalton DT, Hilton R, Achala NKC, Kaiser C, Sudarshana M, Zalom F 2018. Integrative studies of vector-related field epidemiology for grapevine red blotch associated virus. *CDFA-PD/GWSS Interim Report 17-0418-000-SA.* Available at http://www.piercesdisease.org/reports.
26d. Dalton DT, Hilton RJ, Kaiser C, Daane KM, Sudarshana MR, Vo J, Zalom FG, Buser JZ, Walton VM. 2018. Grapevine red blotch virus spread and associative mapping of grapevine leafroll associated virus-3 in Oregon vineyards. *Plant Disease* (in press, per https://ourenvironment.berkeley.edu/people/kent-m-daane).
27. Oberhoster A, Girardello RC, Lerno LA, Eridon S, Cooper MLY, Smith RH, Brenneman CA, Heymann H, Sokolowsky M. 2016. Impact of red blotch disease on grape and wine composition. *Workshop on Recent Advances in Viticulture and Enology, UC Davis, Dec 9, 2016*: available at http://ucanr.edu/repository/fileaccess.cfm?article=162938&p=YQCEFO.
28. Rwahnih MA, Rowhani A, Golino DA, Islas CM, Preece JE, Sudarshana MR. 2015. Detection and genetic diversity of grapevine red blotch-associated virus isolates in table grape accessions in the National Clonal Germplasm Repository in California. *Canadian J Plant Pathol* 37:130-135.
29. Dekker EL, Woolston CJ, Xue YB, Cox B, Mullineaux PM. 1991. Transcript mapping reveals different expression strategies for the bicistronic RNAs of the geminivirus wheat dwarf virus. *Nucl Acids Res* 19:4075-4081.

30. Wright EA, Heckel T, Groenendijk J, Davies JW, Boulton MI. 1997. Splicing features in maize streak virus virion- and complementary-sense gene expression. *Plant J* 12:1285-1297.
31. Blanco-Ulate B, Hopfer H, Figueroa-Balderas R, Ye Z, Rivero RM, Albacete A, Pérez-Alfocea F, Koyama R, Anderson MM, Smith RJ, Ebeler SE, Cantu D. 2017. Red blotch disease alters grape berry development and metabolism by interfering with the transcriptional and hormonal regulation of ripening. *J Exp Bot* 68:1225-1238.
32. Vaucheret H. 2006. Post-transcriptional small RNA pathways in plants: Mechanisms and regulations. *Genes & Development* 20:759-771.
33. Carbonell A, Fahlgren N, Garcia-Ruiz H, Gilbert KB, Montgomery TA, Nguyen T, Cuperus JT, Carrington JC. 2012. Functional analysis of three *Arabidopsis* ARGONAUTES using Slicer-defective mutants. *Plant Cell* 24:3613-3629.
34. Wang X-B, Jovel J, Udomporn P, Wang Y, Wu Q, Li W-X, Gasciolli V, Vaucheret H, Ding S-W. 2011. The 21-nucleotide, but not 22-nucleotide, viral secondary small interfering RNAs direct potent antiviral defense by two cooperative Argonautes in *Arabidopsis thaliana*. *Plant Cell* 23:1625-1638.
35. Trinks D, Rajeswaran R, Shivaprasad PV, Akbergenov R, Oakeley EJ, Veluthambi K, Hohn T, Pooggin MM. 2005. Suppression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes. *J Virol* 79:2517-2527.
36. Bisaro DM. 2006. Silencing suppression by geminivirus proteins. *Virology* 344:158-168.
37. Wang H, Hao L, Shung C-Y, Sunter G, Bisaro DM. 2003. Adenosine kinase is inactivated by geminivirus AL2 and L2 proteins. *Plant Cell* 15:3020-3032.
38. Zhang Z, Chen H, Huang X, Xia R, Zhao Q, Lai J, Teng K, Li Y, Liang L, Du Q, Zhou X, Guo H, Xie Q. 2011. BSCTV C2 attenuates the degradation of SAMDC1 to suppress DNA methylation-mediated gene silencing in *Arabidopsis*. *Plant Cell* 23:273-288.
39. Buchmann RC, Asad S, Wolf JN, Mohannath G, Bisaro DM. 2009. Geminivirus AL2 and L2 proteins suppress transcriptional gene silencing and cause genome-wide reductions in cytosine methylation. *J Virol* 83:5005-5013.
40. Castillo-González C, Liu X, Huang C, Zhao C, Ma Z, Hu T, Sun F, Zhou Y, Zhou X, Wang X-J, Zhang X. 2015. Geminivirus-encoded TrAP suppressor inhibits the histone methyltransferase SUVH4/KYP to counter host defense. *eLife* 4:e06671.
41. Hao L, Wang H, Sunter G, Bisaro DM. 2003. Geminivirus AL2 and L2 proteins interact with and inactivate SNF1 kinase. *Plant Cell* 15:1034-1048.
42. Baliji S, Lacatus G, Sunter G. 2010. The interaction between geminivirus pathogenicity proteins and adenosine kinase leads to increased expression of primary cytokinin responsive genes. *Virology* 402:238-247.
43. Lozano-Durán R, Rosas-Díaz T, Gusmaroli G, Luna AP, Taconnat L, Deng XW, Bejarano ER. 2011. Geminiviruses subvert ubiquitination by altering CSN-mediated derubylation of SCF E3 ligase complexes and inhibit jasmonate signaling in *Arabidopsis thaliana*. *Plant Cell* 23:1014-1032.
44. Chellappan P, Vanitharani R, Fauquet CM. 2005. MicroRNA-binding viral protein interferes with *Arabidopsis* development. *Proc Natl Acad Sci USA* 102:10381-10386.
45. Wang Y, Dang M, Hou H, Mei Y, Qian Y, Zhou X. 2014. Identification of an RNA silencing suppressor encoded by a mastrevirus. *J Gen Virol* 95:2082-2088.
46. Luo Q-J, Mittal A, Jia F, Rock CD. 2012. An autoregulatory feedback loop involving *PAP1* and *TAS4* in response to sugars in *Arabidopsis*. *Plant Mol Biol* 80:117-129.
47. Rock CD. 2013. *Trans-acting small interfering RNA4*: Key to nutraceutical synthesis in grape development? *Trends Plant Sci* 18:601-610.
48. Pantaleo V, Szittya G, Moxon S, Miozzi L, Moulton V, Dalmay T, Burgyan J. 2010. Identification of grapevine microRNAs and their targets using high-throughput sequencing and degradome analysis. *Plant J* 62:960-976.
49. Rock CD, Zeevaart JA. 1991. The *aba* mutant of *Arabidopsis thaliana* is impaired in epoxy-carotenoid biosynthesis. *Proc Natl Acad Sci USA* 88:7496-7499.
50. Fahlgren N, Howell MD, Kasschau KD, Chapman EJ, Sullivan CM, Carrington JC. 2007. High-throughput sequencing of *Arabidopsis* microRNAs: Evidence for frequent birth and death of *MIRNA* genes. *PLoS ONE* 2:e219.
51. Li Y, Zhang QQ, Zhang JG, Wu L, Qi YJ, Zhou JM. 2010. Identification of microRNAs involved in pathogen-associated molecular pattern-triggered plant innate immunity. *Plant Physiol* 152:2222-2231.

52. Padmanabhan C, Zhang XM, Jin HL. 2009. Host small RNAs are big contributors to plant innate immunity. *Curr Opin Plant Biol* 12:465-472.
53. Zhang W, Gao S, Zhou X, Chellappan P, Chen Z, Zhou X, Zhang X, Fromuth N, Coutino G, Coffey M, Jin H. 2011. Bacteria-responsive microRNAs regulate plant innate immunity by modulating plant hormone networks. *Plant Mol Biol* 75:93-105.
54. Zhang XM, Zhao HW, Gao S, Wang WC, Katiyar-Agarwal S, Huang HD, Raikhel N, Jin HL. 2011. *Arabidopsis* Argonaute 2 regulates innate immunity via miRNA393*-mediated silencing of a Golgi-localized SNARE gene, MEMB12. *Mol Cell* 42:356-366.
55. Navarro L, Jay F, Nomura K, He SY, Voinnet O. 2008. Suppression of the microRNA pathway by bacterial effector proteins. *Science* 321:964-967.
56. Cieniewicz EJ, Pethybridge SJ, Gorny A, Madden LV, McLane H, Perry KL, Fuchs M. 2017. Spatiotemporal spread of grapevine red blotch-associated virus in a California vineyard. *Virus Res* 241:156-162.
57. Daane K, Almeida R, Cooper M, Golino D, Wilson H, Anderson J, Ooi K-L. 2017. Searching for potential vectors of grapevine red blotch-associated virus. *Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes*. December 2017. California Department of Food and Agriculture, Sacramento, CA, pp. 202-214.
58. Cieniewicz EJ, Pethybridge SJ, Loeb G, Perry K, Fuchs M. 2017. Insights into the ecology of grapevine red blotch virus in a diseased vineyard. *Phytopathology* 108:94-102.
59. Bahder BW, Zalom FG, Sudarshana MR. 2016. An evaluation of the flora adjacent to wine grape vineyards for the presence of alternative host plants of grapevine red blotch-associated virus. *Plant Disease* 100:1571-1574.
60. Sunitha S, Shanmugapriya G, Balamani V, Veluthambi K. 2013. *Mungbean yellow mosaic virus* (MYMV) AC4 suppresses post-transcriptional gene silencing and an AC4 hairpin RNA gene reduces MYMV DNA accumulation in transgenic tobacco. *Virus Genes* 46:496-504.
61. Ruiz MT, Voinnet O, Baulcombe DC. 1998. Initiation and maintenance of virus-induced gene silencing. *Plant Cell* 10:937-946.
62. Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM. 2000. pGreen: A versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Mol Biol* 42:819-832.
63. Myles S, Boyko AR, Owens CL, Brown PJ, Grassi F, Aradhya MK, Prins B, Reynolds A, Chia J-M, Ware D, Bustamante CD, Buckler ES. 2011. Genetic structure and domestication history of the grape. *Proc Natl Acad Sci USA* 108:3530-3535.

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UNDERSTANDING SYMPTOMOLOGY AND PHYSIOLOGICAL EFFECTS OF RED BLOTCH DISEASE IN VINEYARDS IN OREGON'S WILLAMETTE VALLEY

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ABSTRACT
Grapevine red blotch disease is an important virus of grapevine that has become of major concern for the U.S. wine industry, in part because of the concerns about reduced fruit and wine quality that results with infected vines. Much of the information about the virus has been shared from virus biology and insect vector work that has been conducted in recent years, but information is lacking on how the virus impacts grapevine growth, productivity, and fruit composition. We designed a two-year study to evaluate the impacts of grapevine red blotch associated virus on grapevines in the Willamette Valley of Oregon. This study determines the impacts of the virus on vine growth, photoassimilation, water status, vine nutrient status, and fruit composition. To date we have found limited impact on vine water status or photoassimilation based on virus status. There was limited impact on vine nutrient status or vine growth. Further work in this project will help determine if the disease can be managed, and if so, suggest potential vineyard management practices to be evaluated in the future.

LAYPERSON SUMMARY
Grapevine red blotch disease is a newly identified virus of grapevines that is causing substantial concern for commercial grape producers, as it is thought to reduce fruit and wine quality. Many producers fear that infected vineyards will require removal and replacement which comes at a substantial cost and may not be feasible economically. This research is being conducted to better understand how the virus impacts vine growth and fruit composition. This is an important first step towards understanding how to manage the virus and whether it can be managed. Results will help determine ways for producers to manage vines in infected vineyard to remain profitable and avoid having to rogue and replant entire vineyard blocks.

INTRODUCTION
Grapevine red blotch disease (RBD) has recently become a major concern for winegrape producers in Oregon and other areas of the U.S. The causal agent of the disease, grapevine red blotch-associated virus (GRBaV), was first identified by researchers in California and New York (Al Rwahnih et al., 2013; Krenz et al., 2014). The disease has been at the forefront of industry concern during a time of significant industry expansion (vineyard planting) since spread has primarily been through infected nursery stock (NCPN, 2017).

Anecdotal evidence from industry indicates that fruit stops ripening in the most severe cases. Studies indicate that sugar levels can lag by 1 to 2.7 °Brix (Shudarshana et al., 2015), and that fruit lack normal ripening as a result of altered secondary metabolite production that is important for wine quality (Blanco-Ulate et al., 2017). The lack of fruit ripening is a major concern for premium winegrape producers in cool climate regions such as the Willamette Valley, where ripening is a challenge in typical years due to the limited season length and heat units.

There is significant research underway to understand the virus biology and to identify insect vectors of the virus. While researchers in virology and entomology have made great strides in a matter of a few years to understand the virus-insect complex (Bahder et al., 2016), there is little definitive evidence of the impacts of the virus on vine physiology, and few research projects are focused on understanding the growth effects on grapevines.

As we seek to provide management options for growers, we need information about how the virus may be influencing vine growth and fruit ripening. We have observations from GRBaV-infected vineyards in Oregon that range from having little to no impact while others are claiming that their vineyards are no longer economically viable. The best advice to date is to remove vines that are infected and replant with "clean" plant material, but the cost of removal and replacement may not be economically feasible (Ricketts et al., 2017).

OBJECTIVES

1. Determine vine growth and physiology effects related to RBD in vineyards in Oregon's Willamette Valley.
2. Determine the effects of RBD on fruit ripening for vineyards in Oregon's Willamette Valley.

RESULTS AND DISCUSSION

Two vineyards were monitored during summer 2018 for symptoms, and vine physiological measures were taken to understand the impacts of the virus on grapevine growth and productivity. Vineyard 1 is located in the Eola-Amity Hills American Viticultural Area (AVA) near Amity, Oregon and is planted (in 2007) to Pinot Noir clone 828 grafted to Riparia Gloire. Vineyard 2 is located in the Dundee Hills AVA near Lafayette, Oregon and is planted (in 2002) to Pinot Noir clone 777 grafted to 101-14. This report will contain information from Vineyard 1, as the virus status was pre-determined in a preliminary trial during 2017 so that all 2018 data were collected earlier in the reporting period based on virus and symptomology status. Vineyard 2 was new for 2018 and virus detection results lagged and vineyard data were collected based on symptoms only until the virus testing results were received (Sep. 28, 2018) and data are still undergoing statistical analysis.

Objective 1. Vine Growth and Physiological Effects

Leaf single-photon avalanche diode (SPAD) data (an indicator of chlorophyll) was monitored in Vineyard 1 from July 17, 2018 through August 22, 2018 and first began to show lower SPAD in RBD+ compared to RBD- vines by August 7, 2018 (berry touch stage) in basal leaves only and was consistent through the following sample date (August 22, 2018). Leaves in the mid- to upper- canopy did not differ in SPAD readings, indicating a similar level of leaf greenness throughout the summer. In general, SPAD readings were high, averaging ~41, and the minimum value reported (of basal leaves) was 26. The vines were vigorous and healthy with sufficient canopy greenness throughout the summer. Vine leaf blade nitrogen was high (2.4-2.5%N) for both RBD+ and RBD- vines, and there were no differences by virus status. Leaf blade potassium differed by symptom status but not virus status, with asymptomatic vines having higher K than symptomatic vines at 0.99 and 0.79 % K, respectively. There were no other nutrient differences for macro- or micronutrients for leaf blades analyzed at veraison.

The first virus-associated symptoms in Vineyard 1 were observed in leaves at veraison (late August 2018), starting with interveinal reddening of the most basal leaves. There was little to no leaf chlorosis during the pre-veraison or post-veraison time period. A slight chlorosis of leaves was visible by harvest (September 28, 2018), but only on some of the basal leaves of RBD+ vines. By harvest, the symptoms were visible primarily in basal leaves with some occasional mid- and upper-canopy leaves having interveinal reddening (**Figure 1**).

Leaf photoassimilation and stomatal conductance was measured on 20 individual vines on seven dates from July 5, 2018 to September 6, 2018 to detect any potential differences based on virus or symptom status. Leaves in two zones were measured on each vine, including basal leaves and mid-upper canopy leaves. Photoassimiilation and stomatal conductance gradually declined as the season advanced, as expected with increasing soil moisture deficit and vine water stress. There was rarely a difference in photoassimilation or stomatal conductance of the mid-upper canopy leaves. However, vines without virus symptoms had higher basal leaf photoassimlation and stomatal conductance than those that were asymptomatic for two of the seven dates that this was measured (**Figure 2**). There was no difference in photoassimilation or stomatal conductance based on virus status for any of the dates measured (**Figure 3**), suggesting that vines differentially express virus symptoms and the symptoms may influence physiology more than the virus status alone.

Figure 1. A Pinot noir vine in Vineyard 1 that is positive for GRBaV. This vine shows symptoms on the day of harvest (September 28, 2018). The entire canopy remained green with primarily basal leaves having interveinal reddening (somewhat purplish in color). Some mid- and upper-canopy leaves also show symptoms but at lower incidence.

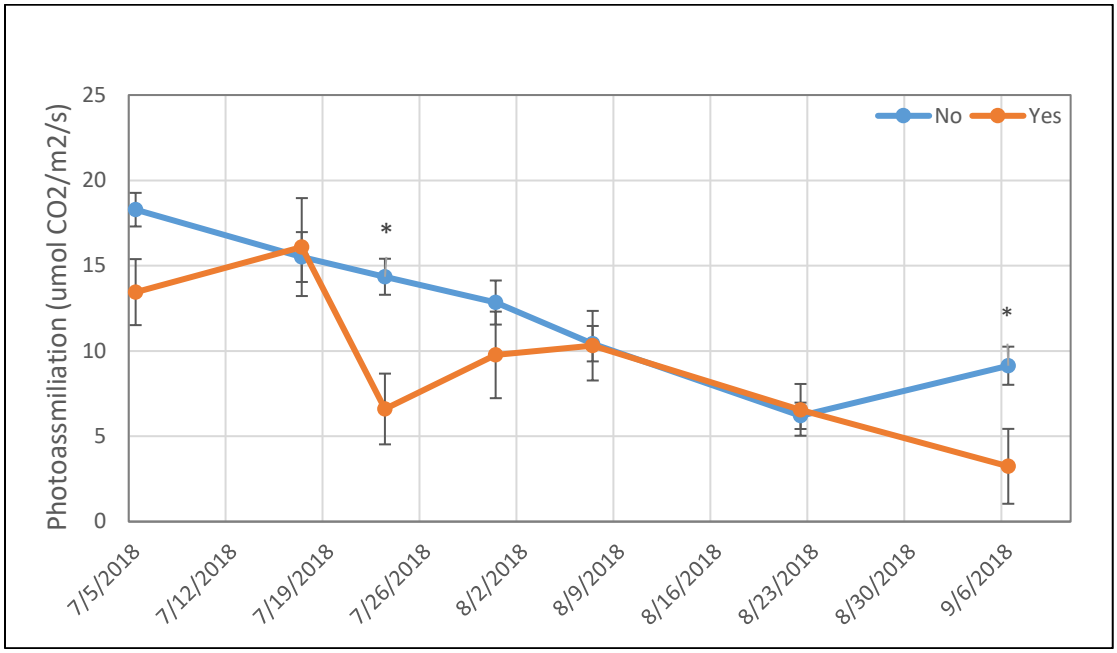

Figure 2. Single leaf photoassimilation (mean ± SE) measured on basal leaves of vines that had RBD symptoms (yes = symptomatic, no = asymptomatic). *indicates a difference in means at $p<0.05$.

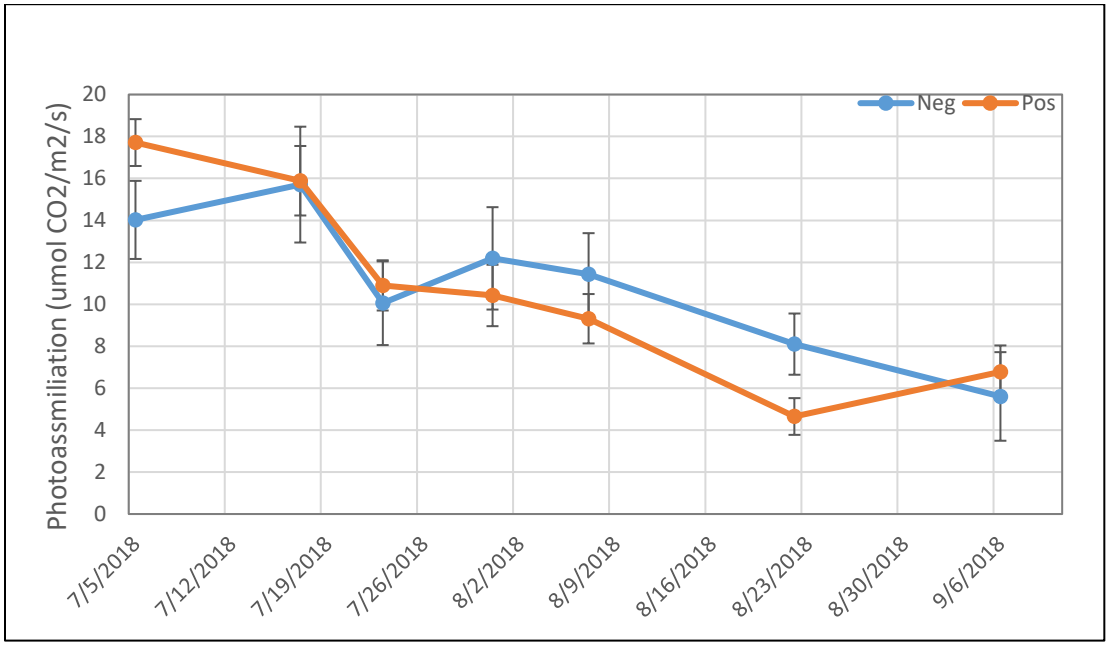

Figure 3. Single leaf photoassimilation (mean ± SE) measured on basal leaves of vines that tested positive (Pos) or negative (Neg) for GRBaV. There were no statistical differences in the means shown above for any date.

Leaf and stem water potential were also measured on vines during three dates in August (pre-veraison and at veraison). There were no differences in leaf or stem water potential based on virus or symptom status. This is an irrigated vineyard and drip irrigation was applied judiciously only when vines experienced stress late season. Across the three dates measured, mean leaf water potential was -0.74, -1.3, and -0.96 on August 1, August 8, and August 20, respectively.

With sufficient canopy growth, vine nutrient status, and leaf greenness and the lack of differences in vine water status, leaf photoassimilation and stomatal conductance, suggest that vines should have sufficient capacity to ripen fruit. Fruit ripeness has recently been assessed. Although the data have not yet been fully analyzed, there appear to be no differences in total soluble solids, pH, or titratable acidity at harvest.

Objective 2. Fruit Ripening and Composition

Fruit composition analysis of total phenolics, including total anthocyanins, total phenolics, and total tannins will be analyzed during the final quarter of 2018. Fruit analysis is currently underway after harvest, as the research blocks were recently harvested on September 28, 2018 and October 1, 2018.

CONCLUSIONS

At this stage of the research it is too early to draw clear conclusions. However, when we combine the current 2018 data from Vineyard 1 with our 2017 preliminary data, we find that it is possible for infected vines to have limited visual symptoms and minimal or no impact on fruit quality at harvest. Vine water status and photoassimilation were not reduced based on virus status. Given that there were few, if any, differences in nutrient status, suggests that the virus may not be ameliorated by a specific nutrient fertilization program. Further analysis of fruit from 2018 and the second vineyard site will help clarify differences in the symptom expression and physiology of the virus within another vineyard that may have greater environmental stress.

REFERENCES CITED

Al Rwahnih M, Dave A, Anderson M, Rowhani A, Uyemoto JK, Sudarshana MR. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology* 103:1069-1076.

Bahder BW, Zalom FG, Jayanth M, Sudarshana MR. 2016. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* as a vector of grapevine red blotch-associated virus. *Phytopathology* 106:1223–30.

Blanco-Ulate B, Hopfer H, Figueroa-Balderas R, Ye Z, Rivero RM, Albacete A, Perez-Alfocea F, et al. 2017. Red blotch disease alters grape berry development and metabolism by interfering with the transcriptional and hormonal regulation of ripening. *J. Exp. Bot.* 14.

Krenz B, Thompson JR, McLane HL, Fuchs M, Perry KL. 2014. Grapevine red blotch-associated virus is widespread in the United States. *Phytopathology* 104:1232-1240.

NCPN- Fact Sheet: *Grapevine Red Blotch Disease*. National Clean Plant Network.

Ricketts KD, Gómez MI, Fuchs MF, Martinson TE, Smith RJ, Cooper ML, Moyer MM, Wise A. 2017. Mitigating the economic impact of grapevine red blotch: Optimizing disease management strategies in U.S. vineyards. *Am. J. Enol. Vitic.* 68:127–35.

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

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INTEGRATIVE STUDIES OF VECTOR-RELATED FIELD EPIDEMIOLOGY FOR GRAPEVINE RED BLOTCH VIRUS

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ABSTRACT

Distribution, non-crop host plants, and seasonal phenology of candidate vector insects were confirmed during 2017 and 2018. Treehoppers primarily oviposit in perennial suitable host plants surrounding vineyards. These plants include oak, apple, and pear. Hatching nymphs move to vetch and wild carrot, to develop to adults. Adults move to perennial green plant tissues as soon as annual plant tissues dry out during the latter portion of the growing season. Adults feed on host plants, including grapevines, potentially spreading red blotch virus. These trends are confirmed by D-Vac sampling, sweep netting, sticky traps, and feeding symptoms on vines. Controlled transmission trials showed persistence of virus in the candidate insect vector species up to five weeks after acquisition. Transmission biology experiments were conducted during both 2017 and 2018. Virus testing of plants receiving virus-infected vector insects is needed in order to confirm transmission with these insects.

Regionally, we found candidate vector insect species in all production regions. Earlier work showed spread of virus over successive years using genomic analysis. Red blotch virus can be found in all of the winegrape production regions, indicating the significant magnitude of this problem. Several extension outreach activities were conducted during both 2017 and 2018.

Growers can use this information because we successfully identified host plants and the lifecycle of candidate insect species. This information will help growers identify the risk of their vineyard surroundings hosting potential vector insects. The seasonal vector distribution and presence were highlighted and described. This information will help growers to determine of insects are present in high risk vineyards, simply by being able to look in specific vineyard locations, and by looking at areas on the vineyard edges, close to surrounding vegetation. We additionally demonstrated that the virus is persistent in the candidate insects for up to five weeks, strongly pointing towards these insects being vectors of the virus.

LAYPERSON SUMMARY

Distribution, non-crop host plants, and seasonal phenology of candidate vector insects of grapevine red blotch virus (GRBV) were confirmed during 2017 and 2018. Adult treehopper insects (Hemiptera: Membracidae) feed and lay eggs in the fall on suitable perennial host plants, including grapevines. These insects overwinter as eggs in oak, apple, and pear. Nymphs hatch from eggs, move to annually growing vetch and wild carrot plants as soon as temperatures become suitable for development in the spring, and develop to adults. Late instar nymphs move to perennial host plants as soon as annual plants dry out in mid-late summer. This life cycle was observed in 2017

and 2018 by a combination of collection techniques including vacuum sampling, sweep netting, sticky trap monitoring, and observing feeding symptoms on vines.

Feeding damage and distribution of treehoppers is concentrated on vineyard edges in close proximity to suitable wild habitat. Feeding on grapevines typically can be found on green canes with a diameter of up to 0.08 inches. This information will help growers identify potential host plants, assess whether the vineyard landscape is favorable to candidate vector insects, and determine whether such vectors are present.

The candidate insect vector treehopper species *Spissistilus festinus* (three-cornered alfalfa hopper), *Tortistilus albidosparsus*, and *T. wickhami* showed persistence of GRBV for at least five weeks after acquisition in greenhouse transmission trials. Additional transmission biology experiments were conducted in the greenhouse in 2017 and 2018. Additional virus testing of the greenhouse plants is currently being conducted in order to confirm if these insects are indeed vectors of the virus.

Regionally, we found treehoppers in southern Oregon, the Willamette Valley, and Columbia Gorge. Earlier work showed spread of virus over successive years of genomic analysis (Dalton et al., submitted). GRBV can be found in all winegrape production regions of the Pacific coast, indicating the significant magnitude of this problem.

Several extension outreach activities were conducted during both 2017 and 2018.

INTRODUCTION

Grapevine virus diseases are of serious concern for vineyard managers and winemakers in all western production regions. Grapevine red blotch virus (GRBV) infection impacts grape berry quality, resulting in berries with lower °Brix at harvest (Al Rwahnih et al., 2013: Sudarshana et al., 2015) and necessitating the removal of symptomatic vines from vineyards. GRBV is spreading in many Oregon vineyards; ecological mapping of GRBV-positive vines, as verified by quantitative polymerase chain reaction (qPCR) during 2013-2016, showed a significant trend of virus increase over time in two of three areas studied in Oregon (Dalton et al., submitted). The role of an insect vector has not been confirmed in the field, and the available information from greenhouse studies implicates treehopper insects as the most likely vectors.

OBJECTIVES

1. Follow insect vector distribution and disease progression in relation to management.
2. Conduct controlled transmission biology experiments.
3. Obtain baseline information on the current levels and extent of red blotch.
4. Extension of information on grapevine red blotch virus and insect vectors.

RESULTS AND DISCUSSION

Objective 1. Follow Insect Vector Distribution and Disease Progression in Relation to Management

Follow Insect Vector Distribution and Incidence. In 2017, vineyards in seven locations were surveyed for the presence of treehoppers (Hemiptera: Membracidae). Additional sites where treehoppers were trapped included Southern Oregon University Sustainability Farm (SOU) and Southern Oregon Research and Extension Center (SOREC). In 2018, five sites were surveyed in the Willamette Valley, and nine sites were surveyed in southern Oregon. Site WV1 was a natural area removed from agricultural production and did not contain *Vitis* plants. Site WV3 was a research vineyard managed by Oregon State University (OSU). All other sites were in commercial vineyards. Nymphs from Willamette Valley sites that survived to the adult stage were tentatively identified to species. Adults of three treehopper species (*Spissistilus festinus*, *Tortistilus albidosparsus*, and *T. wickhami*) were found in OSU surveys. The taxonomic identification of the two *Tortistilus* species is tentative. For the purposes of this report, all adult *Tortistilus* treehoppers without pronotal horns are considered to be *T. wickhami* and all horned individuals are *T. albidosparsus*.

Several surveying techniques were used based on the time of season and host plant in order to improve collection efficiency (**Table 1**). The most effective methods during early season collection were microscopic examination of dormant tissues allowing determination of the presence of younger life stages (eggs and first instar nymphs), caging (young instars), visual surveys coupled with hand collection (second and third instar nymphs, adults),

vacuum sampling (fourth and fifth instar nymphs), sweep netting (adults), and deploying sticky cards (adults). Beat sheeting was ineffective and only yielded three insects across all sites during 2017.

Table 1. Treehopper collection method and species from AV (southern Oregon) during 2017 and from nine southern Oregon sites in 2018.

	2017			2018		
Sampling Method	***S. festinus***	***T. albidosparsus***	***T. wickhami***	***S. festinus***	***T. albidosparsus***	***T. wickhami***
Hand	0	20	3	1	74	340
Sweep	0	51	3	33	53	28
Vacuum	2	1	0			
Beating	0	0	2			
Sticky cards	0	52	2	0	0	76
Total	**2**	**124**	**10**	**34**	**127**	**444**

Seasonal Observations of Treehoppers in the Willamette Valley, Oregon. YV is a commercial vineyard in the Willamette Valley and was surveyed in 2017 every 14 days from spring through fall and was surveyed in 2018 every 14-21 days. Several vineyard blocks are at YV, ranging in age and size. The primary study area was a block of Pinot Noir grapevines and the adjacent surrounding habitat. To the west of the vineyard block was a mix of riparian habitat at the bottom of steep, heavily vegetated slopes. Riparian habitat was dominated by Oregon ash (*Fraxinus latifolia*) and wild blackberry (*Rubus armeniacus*). Dominant woody species above the riparian areas included seedling apple (*Malus domestica*), Oregon white oak (*Quercus garryana*), wild plum (*Prunus domestica*), wild blackberry, bigleaf maple (*Acer macrophyllum*) and hawthorn (*Crataegus* spp.). Minor species included wild rose (*Rosa* spp.), poison oak (*Toxicodendron diversilobum*), and wild hazelnut (*Corylus cornuta*). Herbaceous species in the adjacent habitat included wild carrot (*Daucus carota*), vetch (*Vicia* spp.), Canada thistle (*Cirsium arvense*) and unidentified grasses. Alleys between grapevine rows were maintained as wild-growing grass with occasional seedling blackberry and oak plants. Infrequent mowing and herbicide applications were used to control weeds in 2017, but routine mowing in 2018 effectively minimized interrow vegetation. No irrigation or insecticide sprays were applied to the vineyard study block. CRV is an experimental vineyard managed by OSU and was surveyed in October 2017 and April 2018. Habitat adjacent to a young planting of winegrapes (planted in 2015) was primarily grass, and 10-15 yards to the east and southeast of the block was a stand of oak trees (*Quercus* spp.) that contained rose and blackberry. Heirloom apple, cherry (*Prunus avium*), and plum trees were to the northeast of the study block.

T. albidosparsus lays its eggs behind the bud scales of woody hosts (Yothers, 1934). Collection of woody materials from study vineyards and surrounding habitat 2017 and 2018 provided a reading of the percent of buds infested with treehopper eggs. Treehopper eggs collected from the Willamette Valley field sites were found only behind the bud scales of deciduous trees. In 2017 eggs from greenhouse-infested plants were found either behind bud scales (insects of Willamette Valley origin, putatively *T. albidosparsus*) or in slits along mature wood (insects of southern Oregon origin, *T. wickhami*).

At CRV, treehopper eggs were found only on samples of the two respective oak species in 2017. Oak, heritage apple, cherry, and plum trees were recorded growing 30 yards away from the vineyard edge and 5-50 yards from woody surrounding habitat. Rose, Oregon white oak, and red oak were the dominant species immediately adjacent to the vineyard block. Nymphs were observed in April 2018, and only eggs were found at CRV during October 2017. Nymphs surviving to the adult stage from CRV resembled *T. albidosparsus* found at YV, and all eggs were laid under bud scales. At CRV, eggs were found on red oak, Oregon white oak, and mock orange (*Philadelphus lewisii*) in spring of 2018. Across both years, eggs from YV were found from the highest to lowest proportion of infested buds on oak, apple, hawthorn, and plum. Overall, buds that did contain eggs tended to host a single egg.

The YV site was surveyed repeatedly from spring through fall of 2017 and 2018 in order to track the phenology of treehoppers. A clear seasonal progression of the *T. albidosparsus* lifecycle was recorded (**Figure 1**). Collected nymphs of all instar stages developed into adult *T. albidosparsus* in the laboratory. First instar treehopper nymphs emerged from apple wood cuttings and rose cuttings held in a walk-in cold room (44 ºF) in 2017. First instar

nymphs were found in the field in 2018 and also emerged from woody cuttings collected in late April. Second instar nymphs appeared around the same time as the first instar nymphs, indicating that egg hatch likely occurred over a period of several weeks. Significant overlap of insect instar stages was observed in 2018 from mid-June to early July. By July 10, the first adult *T. albidosparsus* was collected at YV and all nymphs were in advanced instar stages. The first adult field collection of *T. albidosparsus* in 2017 was two weeks later in the season. Wild carrot hosted the majority of the fourth and fifth instar nymphs. The above observations can be summarized as follows to describe the seasonal lifecycle of *T. albidosparsus* treehoppers at YV (**Figure 2**). Insects overwinter in woody vegetation as eggs and start to emerge in early May. Immature nymphs molt five times (juvenile instar stages 1-5), eventually giving rise to winged adults. The early instar stages may remain on the woody host for a period of time but will eventually drop to the understory vegetation. Juvenile insects feed on lush green tissue such as vetch until the host plant dries out in early summer. Later instar nymphs will migrate to drought-hardy or evergreen perennial plants, including grapevines, which can provide a nutritional or water resource. Adults mate toward the latter portion of the season and females lay eggs on suitable perennial woody host plants. Treehopper feeding produces characteristic girdling damage on affected leaves and stems. In addition to surveying for phenology of *T. albidosparsus*, feeding damage was documented at YV on the edge rows in summer and fall.

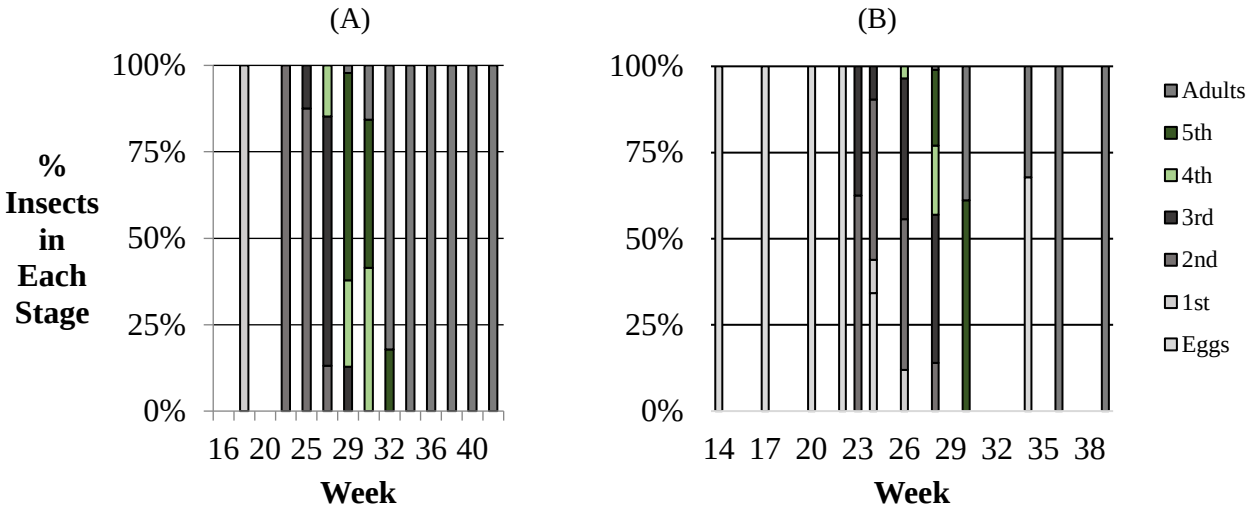

Figure 1. Proportion of *T. albidosparsus* life stages observed throughout the growing season at YV during 2017 (A) and during 2018 (B).

In 2017 the two outer-most rows of the study block were surveyed six times, and the incidence of girdling was noted. In the lab, the caliper of the damaged tissue was measured above the girdling point (**Figure 3**). In 2018, the same rows were surveyed two times. The average number of girdles per vine is depicted across seasons. Most vines (91.2%) in Row 1 nearest the field edge had at least one girdle, whereas 69.0% of the vines in Row 2 were affected. Up to 10 girdles were found on individual grapevines over the course of the season in 2017, whereas in 2018 the highest number of girdles on a vine was five. Girdling consistent with treehopper feeding damage was also observed on other woody and herbaceous hosts, including hawthorn, apple, wild carrot, and vetch.

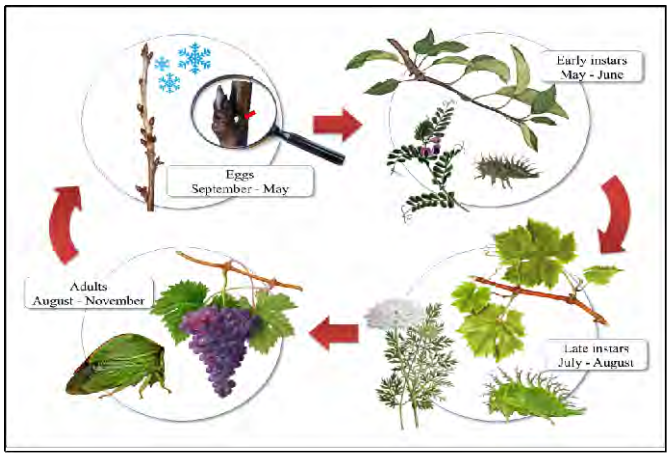

Figure 2. The seasonal lifecycle of *Tortistilus albidosparsus* at YV (Willamette Valley). The insect overwinters in the egg stage hidden in dormant bud scales of suitable perennial host plants. In spring, the early instar emerges and drops onto suitable green herbaceous plant hosts. In mid-summer, the later instar migrates to drought-hardy herbaceous or woody host vegetation. In late summer, the adult emerges and mates, and eggs are laid into the buds of suitable perennial host plants in the fall.

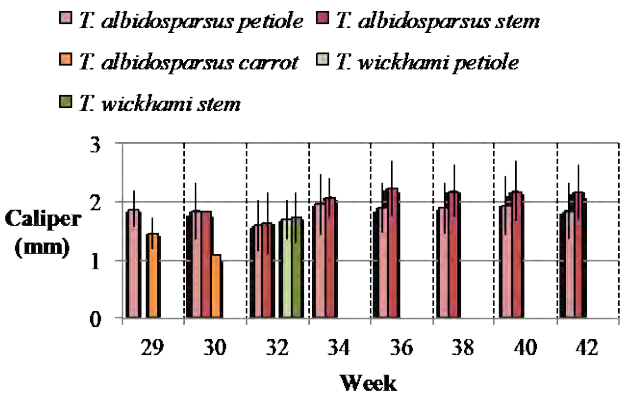

Figure 3. Caliper of treehopper-damaged leaf and stem tissue damaged by *Tortistilus albidosparsus* from YV (Willamette Valley) and *T. wickhami* from CJV (Southern Oregon), as measured above the girdling point. Data from 2017 field season.

Surveys occurred in fall 2016 and were repeated in 2018. A survey to document symptomatic vines was conducted in a separate block at YV in 2016 and in 2018. Between the two seasons, visual surveys produced consistent findings in 777/958 vines (81.1%). In total, 37.5% of the surveyed vines appeared to have symptoms of GRBV, whereas 43.3% of surveyed vines appeared to be asymptomatic. Several vines showed questionable symptoms. In 2016 a subset of asymptomatic vines was sampled for GRBV analysis using qPCR. Collections were made from the same vines in 2018, but the samples have not been analyzed to date.

Seasonal Observations of Treehoppers in Southern Oregon. In 2017, a total of 19 *S. festinus* (three-cornered alfalfa hopper; TCAH) adults were found in southern Oregon and were either associated with vineyards or from sampling in alfalfa fields. Most of the TCAH were collected by sweeping the groundcover vegetation. Sampling in alfalfa fields resulted in TCAH collected on a single date in two adjacent alfalfa fields. In 2018, TCAH was, with a sole exception, found in sweep net samples in one vineyard and comprised just over 5% of the total treehoppers found (see **Table 1**). All but one of the 125 *T. albidosparsus* collected in southern Oregon in 2017 were from AV and a mixed orchard adjacent to the vineyard. Both the orchard and vineyard were farmed organically. In 2018 *T. albidosparsus* was detected in five of the nine vineyards and made up 21% of the sampled treehoppers. This species was found primarily in visual searches (see **Table 2**). The one location where it was found in sweep net sampling was the organic vineyard/orchard where it was often found in the orchard floor vegetation. The most abundant treehopper collected (n = 804) in southern Oregon 2017 and 2018 was *T. wickhami* (**Table 1**). Most specimens were collected at CJV, and collections indicated a strong edge effect of treehopper distribution. Most of the *T. wickhami* were found by visual searching; however, in 2018, 17% of the total *T. wickhami* were trapped in sticky cards and about 6% with the sweep net. *T. wickhami* was the only treehopper trapped in the yellow sticky cards. The visual searching and sweep netting were not done in a systematic fashion so those results should be considered qualitative. However, in 2018 the sticky traps were deployed and checked on a fairly uniform and regular basis beginning at the end of June and extending through September. At the end of the season each trap location was examined, and the degree of treehopper girdling activity was assessed on the vine where the sticky trap was placed, along with the two neighboring vines. Girdling was observed both on leaf petioles and on shoots. In very rare instances girdling was observed on the fruit rachis. The results of the 2018 girdle assessment and the number of *T. wickhami* per sticky card are shown in **Table 2**.

Table 2. Treehopper activity in nine southern Oregon vineyards as evidenced by trap catch and associated girdling activity.

Vineyard	Mean Number Per Vine			Mean Number of *T. wickhami* Per Yellow Sticky Card
	Petiole Girdles	Shoot Girdles	Total Girdles	
SO1	0.24	0.43	0.67	0
SO2	0.46	1.26	1.72	0.31
SO3	0	0	0	0
SO4	0.64	2.64	3.28	0
SO5	1.61	3.06	4.67	0.75
SO6	0.15	0.08	0.23	0
SO7	0.38	0.63	1.01	0.24
SO8	1.06	1.24	2.30	1.62
SO9	0.28	1.14	1.42	0

Two trials were conducted to test the effects of imidacloprid systemic insecticide application and organic deterrent sprays on treehopper distribution within a vineyard. On August 1, 2018, a grower made a foliar treatment of imidacloprid. TCAH were collected and caged on the treated foliage the day after treatment and a comparable number of individuals were placed on untreated grapevines located at the SOREC research station. *T. albidosparsus* were collected the following day from the same alfalfa field primarily from one edge that was bordered by a hedgerow, and again comparable numbers of *T. albidosparsus* were caged on treated and untreated foliage. Four sleeve cages were used for TCAH and three cages for *T. albidosparsus* in each of the treated and untreated areas with four to six treehoppers being placed in all of the cages. An initial assessment of mortality was made in the field on August 6, 2018. The cages were removed on August 10, and a final determination of mortality was made in the lab. Mortality of TCAH was 94.7% on the initial evaluation date but decreased to 84.2% after the insects had been exposed to the treated foliage for eight days. The degree of intoxication was variable, and observation of some moribund individuals complicated the final evaluation. The mortality of *T. albidosparsus* was 30.8% on the initial sample date but increased to 100% after seven days exposure to the treated foliage. Mortality in untreated vines was appreciably higher for TCAH than for *T. albidosparsus*. In this small-scale study, exposure to grapevines freshly treated with imidacloprid resulted in treehopper mortality but the effect on TCAH was not as clear-cut as the effect on *T. albidosparsus* (**Table 3**).

Table 3. Results of caging TCAH and *T. albidosparsus* on vineyard foliage treated with imidacloprid on 08/01/2018 in comparison to caging on untreated vines.

Date Evaluated	% Treehopper Mortality			
	TCAH caged on 8/2		*T. albidosparsus* caged on 8/3	
	Treated vines (n = 19)	Untreated vines (n = 21)	Treated vines (n = 13)	Untreated vines (n = 14)
8/6	94.7	19	30.8	0
8/10	84.2	28.6	100	6.7

The organic crop protectant Surround®, a sprayable formulation of kaolin clay, was applied to vines in a certified organic vineyard where considerable treehopper activity and girdling damage to vines had been observed in 2017. Treatments were applied to the outside row of the vineyard where the girdling damage had been most evident. The product was applied according to label instructions and was applied at either a two-week interval (sprays applied on 6/27, 7/11, 7/26, and 8/10), or a four-week interval (sprays applied on 6/27 and 7/26), and both treatments were compared to an untreated control. The treatments were replicated five times and each replicate consisted of three vines with the middle vine being evaluated for treehopper activity. The vines were inspected on two dates, 8/16 and 9/27. The results of the vine inspections (**Table 4**) yielded fairly consistent evidence of treehopper activity in the untreated vines while there was no treehopper activity in the vines that had been treated with Surround® every two weeks. Just two leaf petioles with girdling were observed on the first evaluation date in the treatment where the vines had been treated twice with kaolin, but no girdling damage was seen on the subsequent evaluation. Most of the girdling damage in the untreated vines was on the shoots, while no shoot

girdling was seen in any of the treated vines. One treehopper was observed on an untreated vine during the second evaluation. The data were analyzed using a randomized complete block analysis of variance (ANOVA), and the difference in degree of girdling among treatments was not statistically significant on the first evaluation date but was on the second date, with the untreated vines having a significantly higher level of damage than either of the Surround treatments. This small-scale study showed that treehoppers were effectively deterred by repeated applications of Surround®, which is Organic Materials Review Institute (OMRI) certified for use in certified organic production. This trial was essentially a choice test, and follow-up testing on a large scale is needed to determine if the repellent effect of the compound would still occur when entire blocks are treated.

Table 4. Girdles and treehopper capture following treatment of vines in an organic vineyard with Surround® sprayable kaolin clay.

Treatment	Mean Number Per Vine							
	Petiole Girdles		Shoot Girdles		Total Girdles		Treehoppers	
	on 8/16	on 9/27	on 8/16	on 9/27	on 8/16	on 9/27	on 8/16	on 9/27
Untreated check	0.2	0.6	2.8	2.2	3.0	2.8	0	0.2
14-day interval	0.6	0	0	0	0	0	0	0
28-day interval	0	0	0	0	0	0	0	0

Objective 2. Conduct Controlled Transmission Biology Experiments

Greenhouse GRBV Transmission Bioassays. We initiated controlled greenhouse trials in 2016 to determine whether GRBV could be successfully transmitted by *T. wickhami* or *T. albidosparsus*. The initial virus status of all plant material was verified using qPCR. Field-collected live insects were placed on known GRBV-infected plant material for a 48-hour acquisition access feeding period (AAFP) and single insects were then transferred to GRBV-free plants for a 48-hour inoculation access feeding period (IAFP). All surviving insects were subsequently transferred individually at one-week intervals to new plants with no virus infection. This process was repeated weekly until all insects had died. In total, 113 initially GRBV-free grapevines were infested with *T. wickhami* in 2016, and 90 vines were infested with *T. albidosparsus*. Control vines (n = 120) that were not infested with treehoppers were also maintained. Plants were tested periodically with qPCR in 2017, and no plants from the 2016 greenhouse transmission bioassay had tested positive for GRBV by that time. Leaf collections from the same vines were collected in fall 2018 and qPCR analysis of the latest tissue collection is ongoing at the present time.

The greenhouse bioassay was repeated in 2017 with modified methodology. In August 2017, rooted cuttings of GRBV-infected plants were infested with *T. wickhami* and *T. albidosparsus* adults. Following a six-day AAFP, cohorts of five insects of the same species were put onto disease-free vines. The IAFP was seven days, after which all insects of each cohort were placed onto previously uninfested GRBV-negative vines. At the end of each IAFP, one cohort of each species was collected directly from a randomly selected infested vine and stored in 95% ethanol at -9 ºF until genetic analysis. Grapevines were infested with *T. albidosparsus* (n = 62 vines) and with *T. wickhami* (n = 53 vines) over a six-week period. In addition, control plants (n = 60 vines) were never infested with treehoppers. All vines used in the 2017 greenhouse bioassay were tested in the fall of 2018 for presence of GRBV.

Cohorts of insects used in the 2017 greenhouse bioassay were tested for the presence of GRBV using qPCR to evaluate whether virus particles can be taken up by the treehopper species under examination, as well as to determine the persistence of the virus in the insect body. Laboratory-reared insects of TCAH, a confirmed treehopper vector of GRBV (Bahder et al., 2016), served as a positive control and were placed onto GRBV-positive material for a six-day AAFP, at which point they were collected and frozen at -112 ºF until genetic analysis. Insects were prepared for genetic analysis as per Bahder et al. (2015) and then tested by qPCR for the presence of GRBV using primers F1580 and 1693R to amplify virus DNA. The results showed that treehoppers provided an AAFP of six days will uptake GRBV particles. The virus persisted within the tested treehopper species for the entire five-week period in both *T. albidosparsus* and *T. wickhami*.

Because greenhouse infestation trials in 2016 and 2017 used insects in the adult stage, a study took place in summer 2018 to test transmission of GRBV by immature treehoppers. *T. albidosparsus* nymphs were collected

from the field on June 26 and were placed onto GRBV-positive rooted grapevine cuttings in the greenhouse on June 28. Following a six-day AAFP, the instar stages of all nymphs were estimated, and cohorts of five nymphs were transferred onto GRBV-negative grapevines to provide a seven-day IAFP. Nymphs that had died were collected into 70% ethanol for later genetic analysis. Surviving nymphs were removed from grapevines on July 11, and their instar stages were estimated. Fifth-instar nymphs were then placed onto cover crop species maintained in small containers in a growth chamber.

Cover Crop Trials. Surviving fifth instar nymphs from the greenhouse bioassay were selected for placement on cover crops. Cover crops included frosty berseem clover (*Trifolium alexandrinum*), 'GO-MOB' red clover (*Trifolium pratense*), 'Oregon Trail' snap pea (*Pisum sativum*), 'Lonestar' annual ryegrass (*Lolium multiflorum)*, and 'Carlinda' turnip rape (*Brassica rapa*). Three replicates of each cover crop were used. Five nymphs were placed on the vegetation in each pot on July 11. Each pot was secured within an organza mesh bag that was tied closed at the top to prevent escape. Every two days the cover crops were examined for surviving insects. The majority of nymphs had died on turnip rape by July 17, and all specimens on this cover crop species had died by July 29. At the end of the observation period, most nymphs had emerged as adults in all other cover crop species (**Figure 4**). On August 6 only adults were remaining, and mating pairs were established on previously uninfested potted grapevines. When possible, females were paired with males that had fed on the same cover crop species. The remaining females were paired with males that had fed on a different cover crop species. Emergence of treehoppers will be tracked in spring 2019 to test the effect of late-instar feeding source on reproduction.

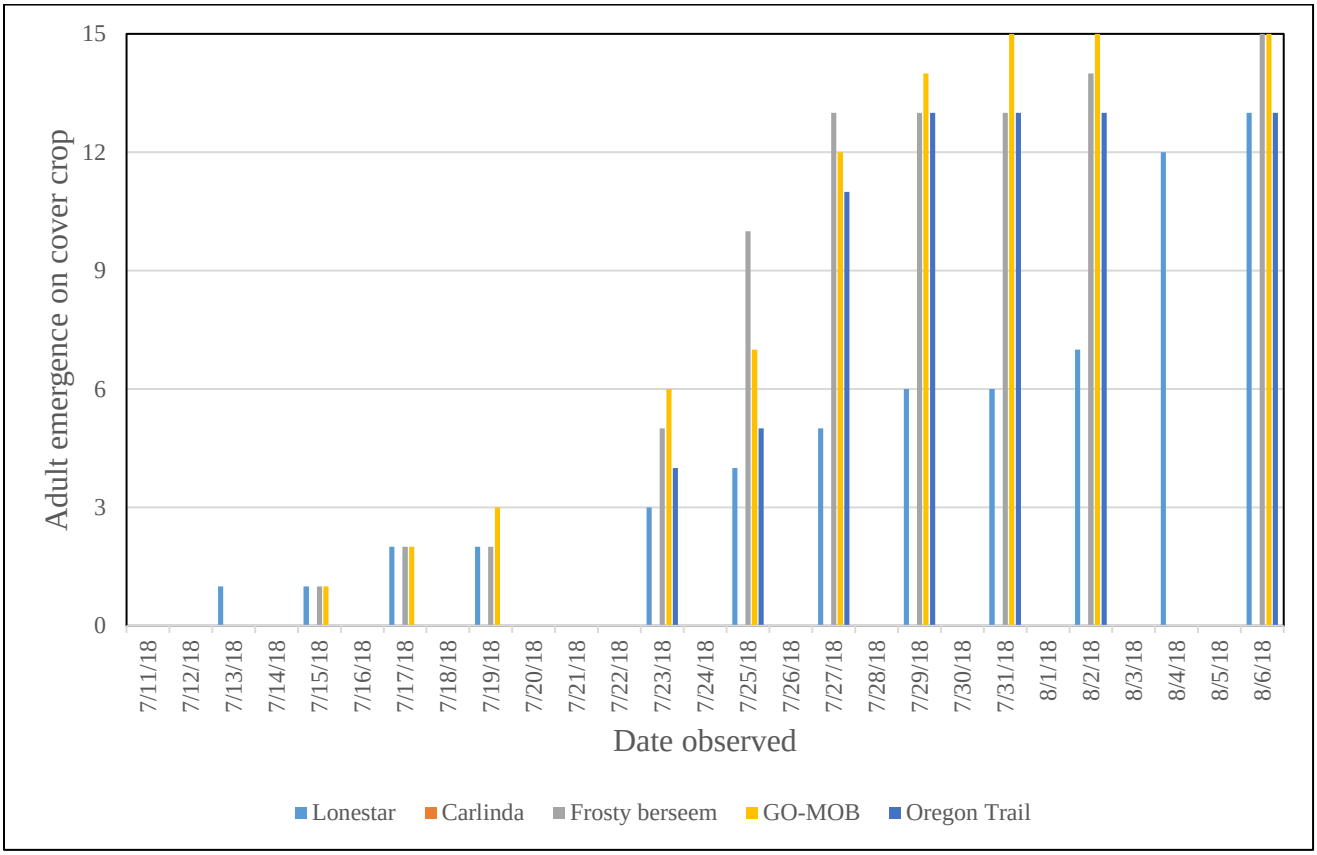

Figure 4. Emergence of adult *T. albidosparsus* following placement on cover crop species *Lolium multiflorum* 'Lonestar,' *Brassica rapa* 'Carlinda,' *Trifolium pratense* 'GO-MOB,' *Trifolium alexandrinum* 'Frosty berseem,' and *Pisum sativum* 'Oregon Trail.'

Virus Movement Within Vine. A trial testing migration of virus particles within the grapevine was conducted beginning in September 2018. Adults of *T. albidosparsus* were placed onto field-grown GRBV-positive grapevines in small clip cages to provide a six-day AAFP. Insects were then individually caged onto petioles of GRBV-negative grapevines for a 24-hour IAFP or a 72-hour IAFP. Representative insects were collected directly into 70% ethanol for further genetic analysis for presence of GRBV. The area of petiole tissue that was directly exposed to the insect within a clip cage was collected, and five additional samples from different areas of the infested grapevine were also collected. The procedure was repeated in October 2018 using TCAH. These samples will be assessed for presence of GRBV particles to determine the dynamics of within-plant movement of GRBV.

Results to date have not shown evidence of GRBV infection mediated by *Tortistilus* species in the tested grapevines. Genetic analyses of insect samples and previously infested grapevine materials are currently in process. However, persistence of GRBV within the insects provides indirect support that they may be vectors of the virus. Our trials in 2018 will help to confirm the ability of TCAH to transmit GRBV to greenhouse-grown grapevines and will help to elucidate the potential role of other treehopper species to vector GRBV.

Objective 3. Obtain Baseline Information on Current Levels and Extent of GRBV
In 2018 we collected grapevine samples from plants at YV that had previously tested negative for GRBV in 2016. While visual symptoms were largely unchanged from the 2016 field survey, a small number of grapevines had become symptomatic by 2018. The samples originated from a separate block at the same vineyard than had been previously assessed for spread of GRBV (Dalton et al., submitted).

Limited samples were received in 2017 from a grower in eastern Oregon. These samples tested negative for GRBV. Additional samples were received in October 2018 from eastern Oregon. These samples have not been analyzed to date for presence of GRBV.

Objective 4. Extension of Information on Grapevine Red Blotch Virus, and Insect Vectors
In 2017, results were presented a total of 13 times in-person to growers, grape industry representatives, and OSU Cooperative Extension personnel through grower reports, seminars, and national webinars. We organized a regional vineyard workshop on vectors and vineyard disease transmission for growers and industry in 2017. In 2018 outreach activities continued, primarily targeting local grower groups. Vaughn Walton, 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. A recently submitted manuscript documents the spread of virus over successive years of genomic analysis. Several extension outreach activities were conducted during 2017. Additional results will be published in popular and scientific journals. 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. Several presentations have been given at scientific meetings and public research expositions (see report for the list of applicable publications).

CONCLUSIONS
Objective 1. Follow Insect Vector Distribution and Disease Progression in Relation to Management
Distribution, non-crop host plants, and seasonal phenology of candidate vector insects was determined in 2017 and confirmed in 2018. The complete treehopper lifecycle was identified. The insects overwinter as eggs in perennial host plants. Nymphs hatch from eggs, move to plants including vetch and wild carrot, and develop to adults. Adults use woody hosts such as oak, apple, and grapevine for reproduction in the fall. Insects were captured in 2017 by a combination of collection techniques including vacuum sampling, sweep netting, sticky trap monitoring, and observing feeding symptoms on vines. In 2018, collections were best carried out by visual searches for nymphs and adults.

Objective 2. Conduct Controlled Transmission Biology Experiments
Greenhouse transmission trials showed persistence of GRBV in the candidate insect vector species for at least five weeks after acquisition. Additional transmission biology experiments were conducted in the greenhouse in 2017 and in 2018. Testing of greenhouse materials is ongoing. It is anticipated that grapevines infested in 2018 with virus-containing treehoppers will be tested in summer 2019 for the presence of GRBV. To date, successful transmission of GRBV by *Tortistilus* treehoppers remains elusive.

Objective 3. Obtain Baseline Information on the Current Levels and Extent of Red Blotch
Vineyards in southern Oregon were surveyed for symptoms of GRBV and GLRaV. Genetic testing of symptomatic vines is ongoing to determine degree of co-infection of the two viruses. Plant samples from eastern Oregon tested negative for GRBV. Field materials from southern Oregon and the Willamette Valley are currently being retested.

Objective 4. Extension of Information on Grapevine Red Blotch Virus and Insect Vectors.
A manuscript was submitted in 2017 documenting the spread of GRBV over successive years of genomic analysis. Several extension outreach activities were conducted during 2017 and 2018. Several presentations have been given at scientific meetings and public research expositions.

REFERENCES CITED
Al Rwahnih M, Ashita D, Anderson MM, Rowhani A, Uyemoto JK, Sudarshana MR. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology* 103:1069-1076.

Bahder BW, Bollinger ML, Sudarshana MR, Zalom FG. 2015. Preparation of mealybugs (Hemiptera: Pseudococcidae) for genetic characterization and morphological examination. *J Insect Sci* 15(1):104.

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

Dalton DT, Hilton RJ, Kaiser C, Daane KM, Sudarshana MR, Vo J, Zalom F, Buser JZ, Walton VM. [submitted]. Spatial associations of vines infected with grapevine red blotch virus in Oregon vineyards. *Plant Disease*.

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

Yothers MA. 1934. Biology and control of tree hoppers injurious to fruit trees in the Pacific Northwest. *USDA Technical Bulletin No. 402*. 55 pp.

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BIOLOGY AND ROLE OF TREEHOPPERS IN GRAPEVINE RED BLOTCH DISEASE

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Reporting Period: The results reported here are from work conducted July 2017 to June 2018.

ABSTRACT
Funding for this project from the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board was approved effective July 1, 2017, but work on the objectives had been initiated earlier by the Zalom and Sudarshana labs at UC Davis, and by the Daane lab at UC Berkeley. Results presented summarized our monitoring of the population dynamics of the three-cornered alfalfa hopper (*Spissistilus festinus*; 3CAH) in vineyards and surrounding landscapes from the time when these studies began during the 2016 season and continued over the 2017 season and into spring 2018 in vineyards and along transects from vineyards to natural areas. Results of field transmission studies, 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, and the status of grape as a reproductive host of 3CAH are also reported.

LAYPERSON SUMMARY
The results of this project are intended 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. Studies to determine possible transmission by other treehoppers found in vineyards where GRBV is spreading were initiated. 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
A grapevine disease with symptoms that resembled those of grapevine leafroll was found in Napa County vineyards in 2007 (Calvi, 2011). The disease was named grapevine 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). The virus was also found independently in grapevines in New York, Oregon, and Washington (Krenz, et al., 2012; Poojari et al., 2013; Seguin et al., 2014), and it is now known to be widely distributed in the U.S. Consultants and researchers working in California vineyards infected with the virus, especially those planted to red varieties, report substantial impact to grape quality, substantially reducing their value.

Red blotch disease epidemiology is 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 in a pattern that was consistent with a motile insect vector. The virus has been isolated from wild grapevines, mainly open-pollinated *Vitis californica* (Bahder et al., 2016; Perry et al., 2016), even at a considerable distance from commercial vineyards. After surveying many of the hemipteran insect species found in commercial vineyards where there was evidence that red blotch disease was spreading, the three-cornered alfalfa hopper (*Spissistilus festinus*; 3CAH) (Hemiptera: Membracidae) 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 where red blotch disease was believed to be spreading in California, southern Oregon, and the Willamette Valley (Zalom and Sudarshana, unpublished; Dalton and Walton, unpublished), but the status of *Tortistilus* treehoppers as GRBV vectors has yet to be confirmed. Although some aspects of 3CAH biology is mentioned in the scientific literature, the majority of this information comes from legume cropping systems such as soybean, peanut, and alfalfa where it is considered to be a pest (Wildermuth, 1915; Beyer et al., 2017). The biology of 3CAH and more especially the other treehoppers that are found 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 and between vineyards. The research objectives addressed through this Pierce's Disease and Glassy-winged Sharpshooter (PD/GWSS) Board sponsored research began in 2014 with funding from the CDFA Specialty Crops Block Grant Program (to Sudarshana and Zalom), USDA-ARS National Program funds (to Sudarshana), and the American Vineyard Foundation (to Daane). Funding for this PD/GWSS Board grant was finally received on October 10, 2017, due to complications in contracting between UC and CDFA, however, we continued the proposed research prior to formally receiving funding in order to avoid temporal gaps in the ongoing research during critical parts of the growing season.

OBJECTIVES

The long-term objectives of this study address improved 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 abundance of treehoppers, focusing on the 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 3CAHs 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 for two years starting March 2016 through March 2018. The vineyard block consists of 53 rows. All odd-numbered rows were tilled late March of both years 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. Captured adults were sexed. The vineyard was removed due to increasing red blotch disease incidence following the last sampling date in March 2018. Similar late winter capture patterns were observed each year (2016-2018). We now believe that this marks the initiation of activity of the overwintering generation into the vineyard. Bud break occurred in early April of both 2016 and 2017, about seven weeks after the first 3CAH adult was found in the vineyard. The first nymphs were collected on May 16 and May 23 of those years, coinciding with an increase in adult 3CAH captures (**Figure 1**) and the grapevine phenological marker of bloom. Increase in captures of fourth and fifth 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. Vineyard weeds, which constitute the ground cover sampled at Oakville, started to noticeably dry in early August of both years, corresponding with a drop in adult 3CAH. This also corresponded to an increase in the number of girdles on vines (**Figure 2**). Girdles were only sampled in 2017, but we are

counting girdles weekly again in 2018 in three vineyards to see if the pattern remains similar and across locations. Data from the 2018 girdle counts will be summarized at the conclusion of the 2018 season.

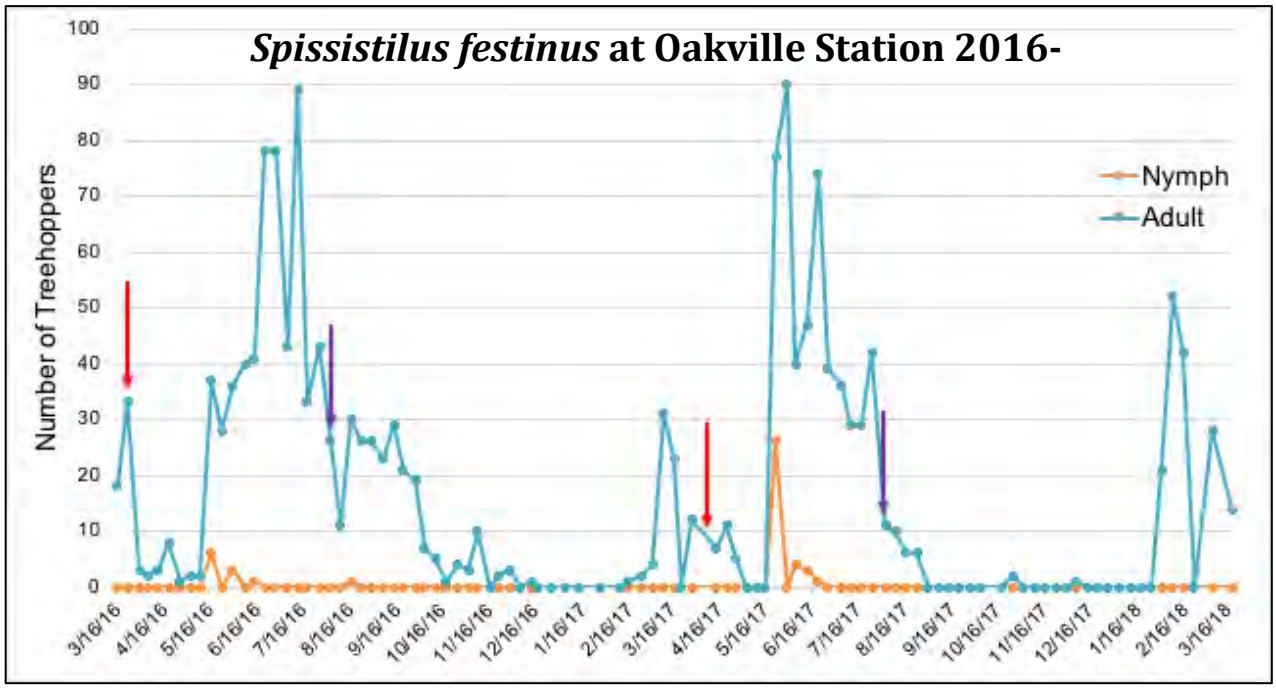

Figure 1. Weekly sweep net sampling of vineyard ground cover for 3CAH at Oakville, 2016-2018. Red arrows indicate bud break and purple arrows indicate time when ground cover was completely dried.

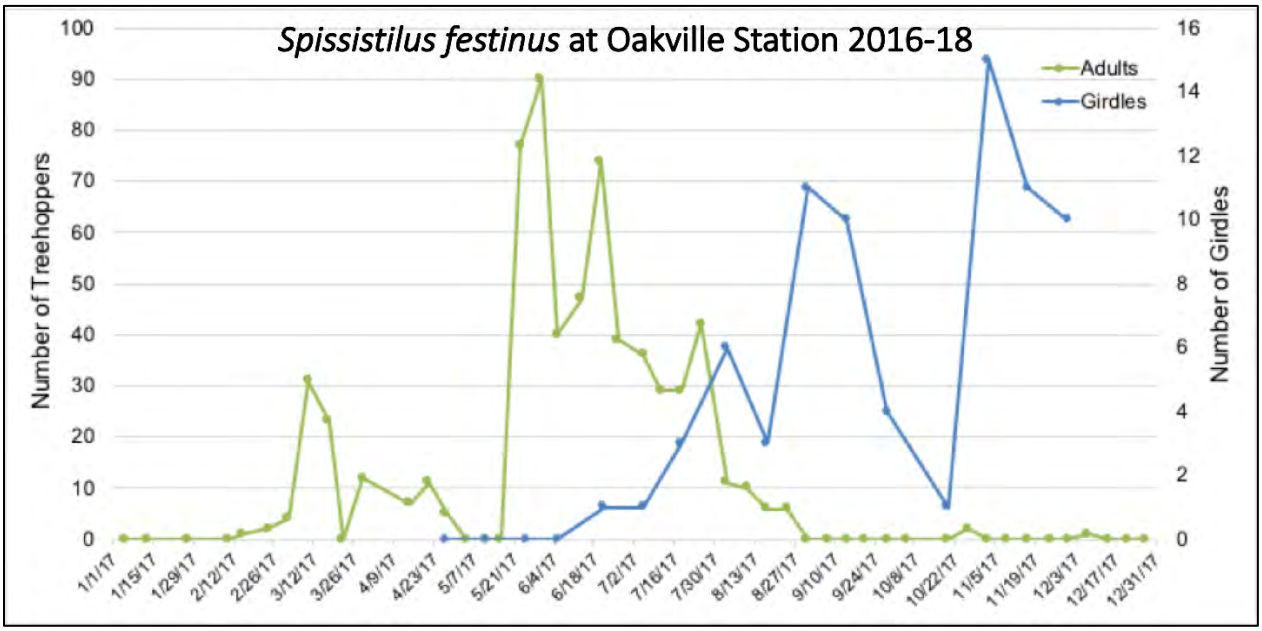

Figure 2. Weekly sweep net sampling of vineyard ground cover for 3CAH and weekly number of girdles on 30 study vines at Oakville in 2017.

Salivary glands were extracted from the 3CAH collected at the Oakville vineyard to test for the presence of GRBV biweekly beginning March 3, 2017, just prior to bud break, and throughout the season. A total of 96 usable samples were collected. Salivary glands from 3CAH reared from eggs were dissected on each collection date, and these served as negative controls. The salivary glands were removed, placed in 180 uL ATL and 20 uL proteinase K, incubated four hours at 56ºC, and stored in a -80ºC freezer until they were analyzed by quantitative polymerase

chain reaction (qPCR) for GRBV detection in February 2018. None of the salivary glands tested positive for GRBV.

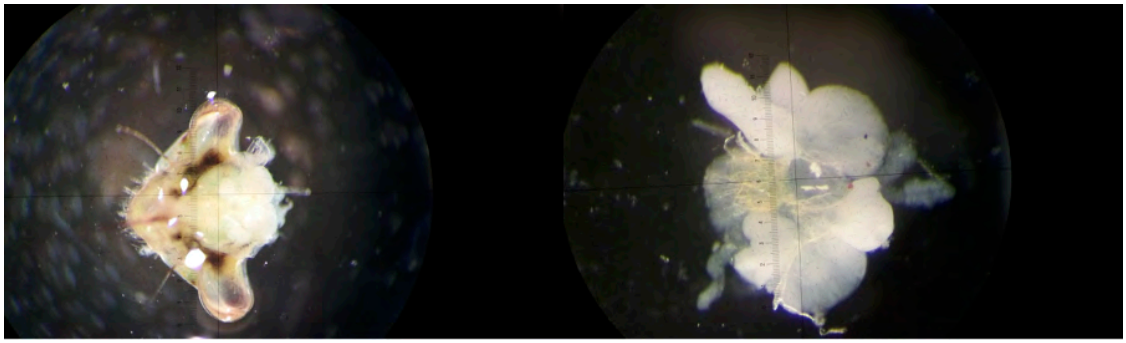

Figure 3. 3CAH salivary gland dissections showing salivary glands within head capsule (left) and removed from head capsule (right).

In a related study conducted by Houston Wilson of the Daane lab, 3CAH populations and crop damage were sampled along transects that extend out from natural habitats into vineyards. At each sampled point along the transect, 3CAH densities were measured on both ground covers and in the crop canopy along with petiole girdling. Densities were evaluated at approximately two-week intervals beginning in March 2017 using a combination of yellow sticky-traps, sweep-nets and beat-sheet sampling. Field sites consisted of five vineyard blocks greater than two acres in size adjacent to riparian and/or oak woodland habitat located in Napa and Sonoma counties. 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. 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). The edge of the vineyard and natural habitat are typically separated by a roadway or path that is about five meters wide. 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 approximately 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^2 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. Findings to date (March 2017 – May 2018) indicate that 3CAH activity showed a strong temporal trend, with densities generally increased between June and August along with some activity observed in March and again in October and November (**Figures 4A-D**). While there was no clear gradient of 3CAH activity across the transect points, densities on the yellow sticky traps and in sweep net samples were slightly elevated in natural habitats in early June just prior to increases observed in the vine canopy at both the vineyard edge and interior in the following round of sampling (**Figures 4C and 4D**). 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 5A**), some 3CAH could still be found on the little bit of ground

cover that remained later in the season (**Figure 5B**). Surprisingly, these late season 3CAH adults were most frequently encountered on ground covers in the vineyard interior (**Figure 4B**). Finally, petiole girdling became apparent in August, with a higher proportion of girdles located at the vineyard interior (**Figure 6**). This increase in girdling in August follows increased 3CAH densities observed in the vine canopy between June and August.

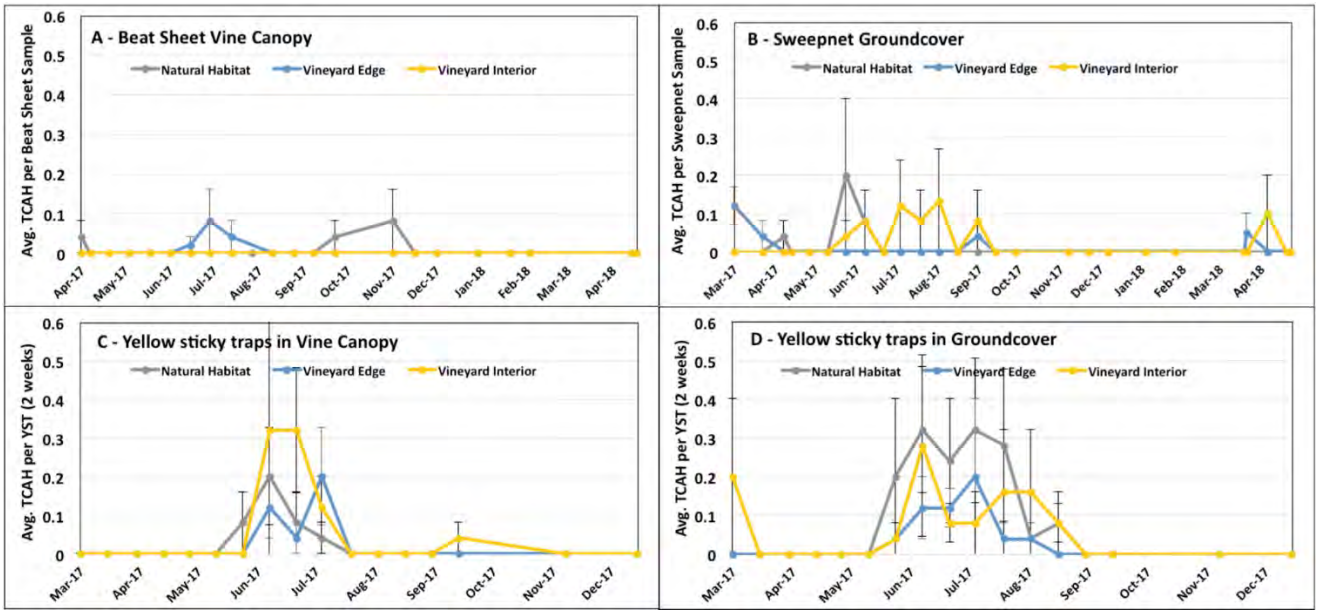

Figure 4. 3CAH densities sampled along the transect using (A) beat sheet in the vine canopy or perennial vegetation canopy; (B) sweep-net on ground covers; (C) yellow sticky traps in the vine canopy or at vine canopy height; and (D) yellow sticky traps at ground cover height (~ 0.3 meters).

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.

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 despite considerable effort, 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, and we continue to test the grape plants for transmission using 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. Subsequent qPCR analysis of these plants has failed to detect presence of GRBV. We also attempted to conduct a transmission assay in a more natural environment using 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 (AAP) of six days then transferred to qPCR confirmed healthy Cabernet Sauvignon recipient vines and allowed an inoculation access period (IAP) of six days. Testing of these plants began in July 2018, but preliminary results are not presented in this report.

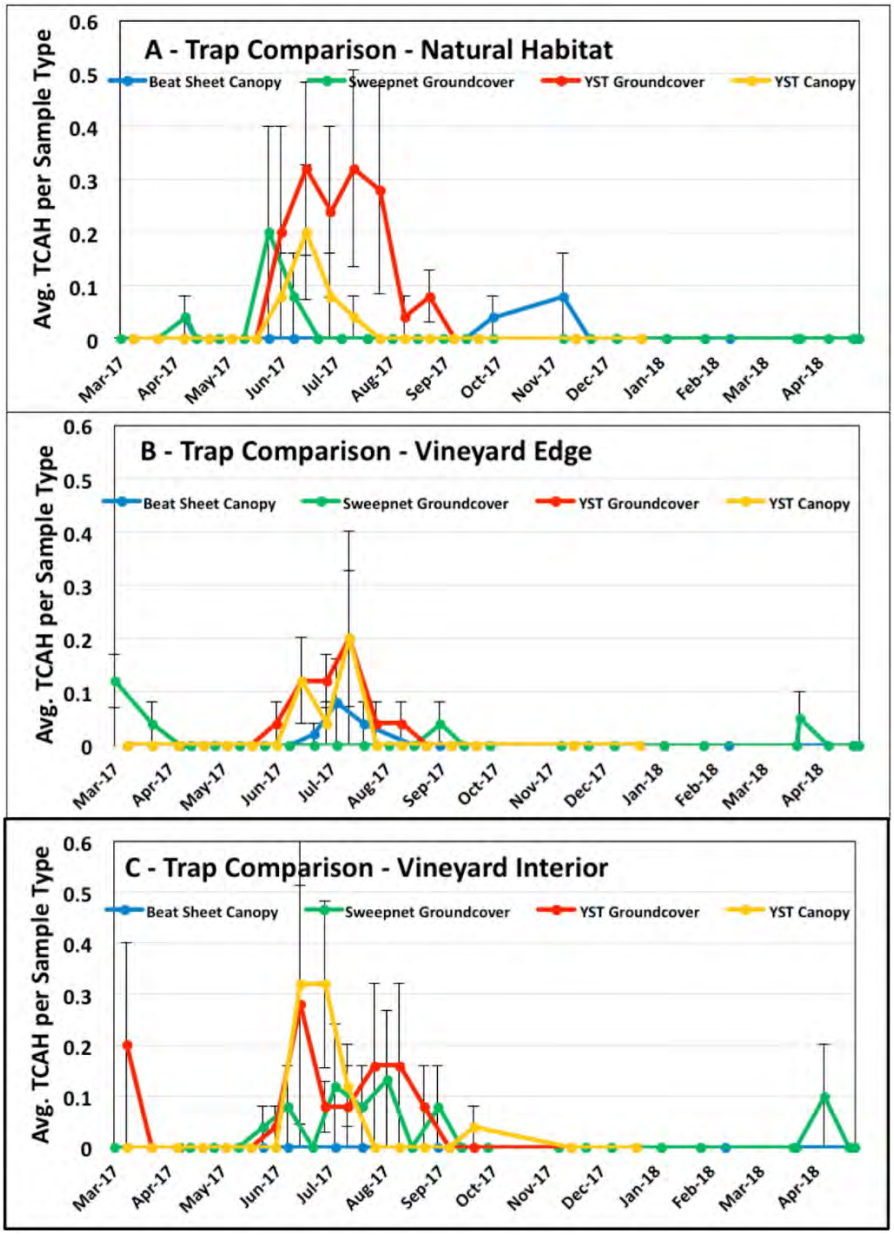

Figure 5. 3CAH densities varied according to sampling technique across the (A) natural habitat; (B) vineyard edge; and (C) vineyard interior. Generally, the yellow sticky traps picked up more 3CAH than sweep-nets or beat sheets.

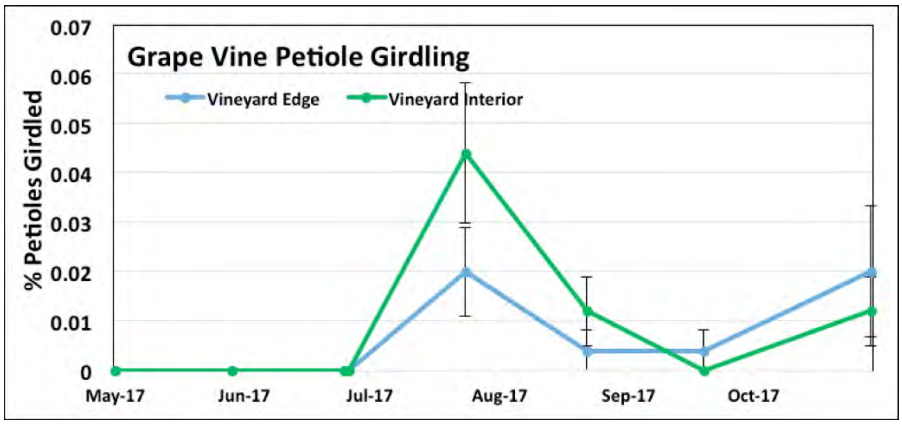

Figure 6. Petiole girdling became apparent in late July and early August 2017, with a higher proportion of girdles located at the vineyard interior.

All *Tortistilus* removed from the grapevines post-inoculation were placed inside of 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. In addition, 15 salivary glands removed from *Tortistilus* collected from test positive wild grapevine have been tested with only one of the 15 testing positive.

In winter 2018, we collected cuttings from Zinfandel grapevines in an Amador County vineyard that tested positive for GRBV and have rooted and potted the cuttings for transmission studies that will begin in summer 2018. We have been working in the Amador County vineyard for the past three years and have documented GRBV spread. It is particularly interesting since no known grape viruses other than GRBV have been found in the vineyard. One concern that we have is that perhaps the GRBV in the source plants that we had been using are no longer capable of being transmitted via a vector. We have initiated transmission tests using these new source plants in August 2018 with both 3CAH and *Tortistilus* treehoppers to test that hypothesis.

The Sudarshana lab planted a Cabernet Sauvignon vineyard planted on Freedom rootstock using nursery plants that were determined to be free of GRBV by qPCR at the UC Davis Plant Pathology Field Station (Armstrong Tract) in 2015 for the purpose of documenting transmission and spread (**Figure 7**, left photo). 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 grapevines. A three-meter-wide alfalfa strip was planted on the edge of the vineyard nearest the 'infected' vines in summer 2016, and 3CAH were found in the alfalfa planting by mid-summer. Testing of the recipient vines for GRBV presence during 2017 though June 2018 has not documented GRBV presence in any of the vines, but testing will continue through 2018. In July 2018, we planted 15 source vines produced from cuttings from the aforementioned Amador County Zinfandel grapevines within one of the vine rows of our Armstrong Tract vineyard near the alfalfa strip that has since become infested naturally by 3CAH in order to determine if spread of this GRBV genotype is occurring at that site.

A survey of the vineyard for 3CAH girdles conducted during 2017 indicated the presence of girdles in the Armstrong Tract block from August through fall (**Figure 8**). Sampling is continuing during 2018. If transmission was successful from the caged inoculation attempts in fall 2016, we anticipate that this site will provide a controlled model for studying details of GRBV spread by both clades. The introduction of the new Amador County source plants into the block in 2018 is intended to provide similar information, but using the different GRBV source material.

Figure 7. 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.

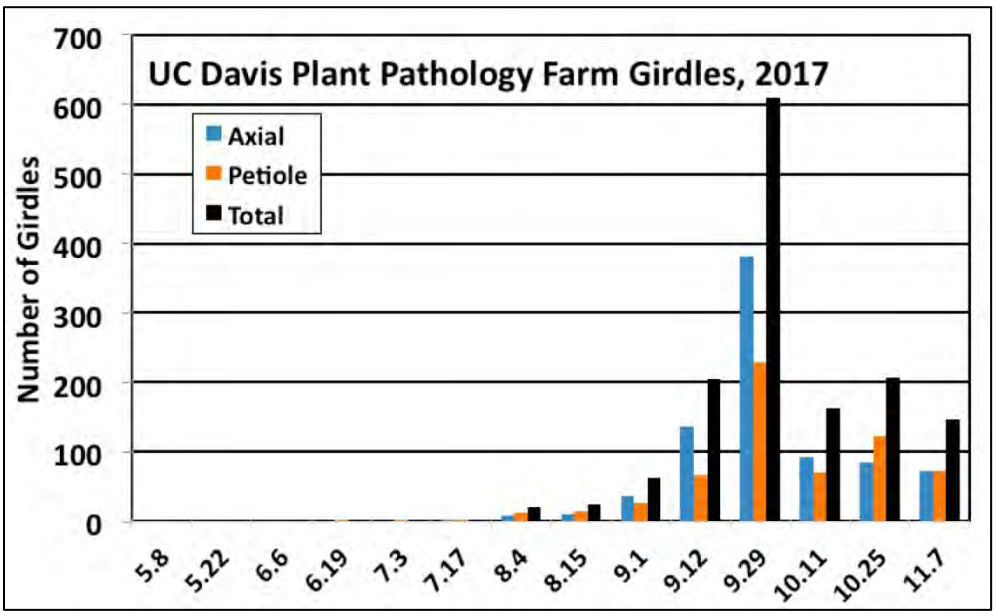

Figure 8. Weekly 3CAH girdle counts in a Cabernet Sauvignon vineyard at the UC Davis Plant Pathology Field Station (Armstrong Tract) in 2017.

Objective 3. Determine the Transmission Efficiency of 3CAH to Identify Virus Acquisition Periods and Persistence in the Insect

Studies related to this objective that were proposed to be conducted by the Daane lab have been initiated, but no results are available as yet in part because the length of time required to initiate a study and then obtain results is beyond the timeframe of this project.

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, and sampled by sweep net for presence of treehoppers. Unfortunately, most of the cover crop species we planted at the two commercial sites in Napa and Yolo counties only produced a very sparse stand that was largely indistinguishable from resident vegetation. At the third site at UC Davis we were able to successfully establish cover crops (**Figure 9**), but we did not capture any 3CAH adults or nymphs in our weekly sweep sampling of the ground cover from January through April 2017. Given that experience, for 2017-18 we decided to concentrate the study at UC Davis where we had the option of establishing the cover crops with irrigation and maintaining them better, and intended to cage adult 3CAH on twelve individual plants in each plot three times during the winter to assess overwintering success and reproduction. On October 24, 2017, we planted five cover crops (bell beans, magnus peas, blando brome, California red oats, and mustard) in a randomized block design with four replicates in a Syrah vineyard at UC Davis. A resident vegetation plot within each replicate served as a control. However, the source of the 3CAH for the study, a colony that we had established the previous summer, crashed, so we did not have a source of insects for the study.

Objective 5. Determine the Status of Common Weed and Cover Crops as Feeding and Reproductive Hosts for 3CAH

Feeding and reproductive common vineyard weed and cover crop hosts of 3CAH were determined in the greenhouse in a series of no-choice experiments. This study represented 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 9**). The cages were opened weekly for four weeks to determine adult survival (defined as percent survival on caged plants for two weeks), 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 weeds and cover crops evaluated as feeding and reproductive hosts of 3CAH are presented in **Tables 1 and 2**. This study identified plant species in the families Asteraceae, Convolvulaceae, Fabaceae, and Poaceae that are capable of serving as feeding and reproductive hosts. Plants in the family Fabaceae were previously reported as their preferred hosts in the southern U.S. (Wildermuth, 1915;

Mueller and Dumas, 1987). Spanish clover, dandelion, birdsfoot trefoil, common groundsel, field bindweed, magnus peas, bell beans, blando brome, purple vetch, black medick, subterranean clover, crimson clover, and woollypod vetch were all found to be reproductive hosts in our study. Our results also indicate that buckhorn plantain, Kentucky bluegrass, wild carrot, mustard, oats, and Bermuda grass are poor feeding hosts, not reproductive hosts, and likely would not be of significance for maintaining 3CAH populations in vineyards where more suitable hosts are present.

Figure 9. Weeds and cover crops caged with 3CAH in a greenhouse study at UC Davis.

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 (four known reproductive hosts from the completed no-choice experiment) were randomly arranged in a large dome-shaped cage in the greenhouse and replicated three times (**Figure 10**). Purple vetch was included in each evaluation as a standard. Ten male and ten female 3CAH were released into each cage and allowed to freely feed and oviposit. All adults were removed from the cages after one week. Nymphs were counted and collected from individual plants on weeks two and three. Destructive sampling of all plants and collection of nymphs were conducted at week four. The plant species that exhibited the greatest nymph emergence in each of the three groups tested were all in the family Fabaceae (**Figure 11**). Interestingly, while the five plant species tested in cover crop group 1 were all in the family Fabaceae there were differences among them, suggesting that even within the Fabaceae reproductive preference exists, with the two vetch species tested being preferred over the two clover species (**Figure 11A**). Cover crop group 2 consisted of four plants in the Fabaceae family and one in the Poaceae family. Two of those Fabaceae had significantly greater nymph emergence than did the plant species in the family Poaceae (**Figure 11B**). Group 3, which consisted of vineyard weeds, included three species in the family Fabaceae, one in the family Asteraceae, and one in the family Convolvulaceae. The three Fabaceae plant species yielded significantly more nymphs than did the plants in the other two families (**Figure 11C**). These results further support a hypothesis that plants of the family Fabaceae are preferred hosts of 3CAH. The results of this study have been published (Preto et al., 2018).

Table 1. Weed species tested as feeding and reproductive hosts for 3CAH.

Scientific Name	Common Name	Family	Girdles	Nymphs	% Survival[a]
Acmispon americanus	Spanish clover	Fabaceae	Yes	Yes	92
Taraxacum officinale	Dandelion	Asteraceae	Yes	Yes	71
Lotus corniculatus	Birdsfoot trefoil	Fabaceae	No	Yes	58
Poa pratensis	Kentucky bluegrass	Poaceae	Yes	No	25
Senecio vulgaris	Common groundsel	Asteraceae	Yes	Yes	21
Plantago lanceolata	Buckhorn plantain	Plantaginaceae	No	No	8
Daucus carota	Wild carrot	Apiaceae	Yes	No	4
Convolvulus arvensis	Field bindweed	Convolvulaceae	Yes	Yes	4
Kickxia elatine	Sharppoint fluvellin	Plantaginaceae	No	No	0
Cynodon dactylon	Bermuda grass	Poaceae	No	No	0

[a] Survival of adults for first two weeks on plants.

Table 2: Cover crop species tested as feeding and reproductive hosts for *Spissistilus festinus.*

Scientific Name	Common Name	Family	Girdles	Nymphs	% Survival[a]
Pisum sativum	Magnus Peas	Fabaceae	Yes	Yes	92
Vicia faba	Bell beans	Fabaceae	No	Yes	83
Bromus hordeaceus	Blando brome	Poaceae	Yes	Yes	33
Vicia benghalensis	Purple vetch	Fabaceae	Yes	Yes	30
Medicago lupulina	Black medick	Fabaceae	Yes	Yes	25
Trifolium subterraneum	Subterranean clover	Fabaceae	Yes	Yes	17
Trifolium incarnatum	Crimson clover	Fabaceae	Yes	Yes	13
Vicia villosa ssp. varia	Woollypod vetch	Fabaceae	Yes	Yes	13
Brassica sp.	Mustard	Brassicaceae	No	No	0
Avena sativa	California red oats	Poaceae	No	No	0

[a] Survival of adults for first two weeks on plants.

Figure 10. Four reproductive hosts plus purple vetch as a standard caged with 3CAH in a greenhouse preference study at UC Davis.

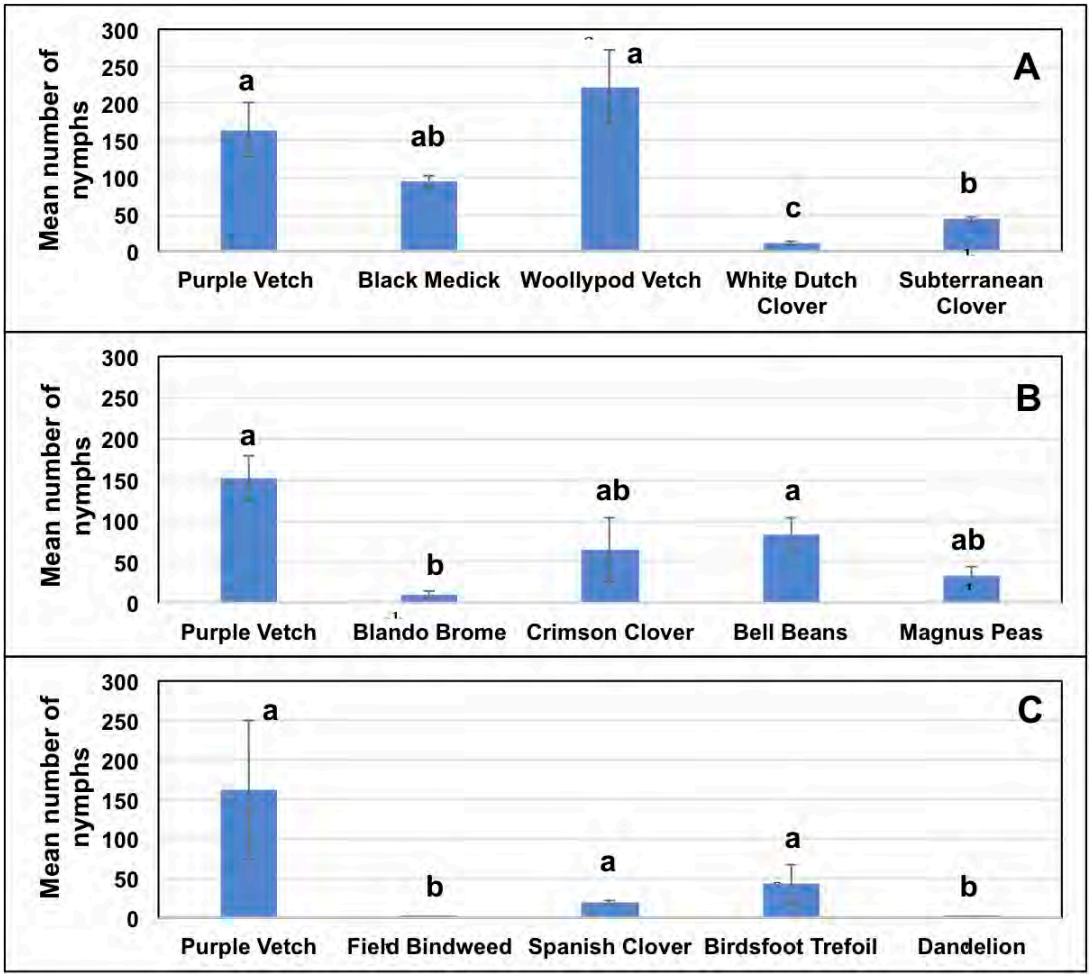

Figure 11. Results of post-hoc pairwise comparisons of mean number (±SEM) of 3CAH nymphs emerging from (A) cover crop - group 1 (purple vetch, black medick, woollypod vetch, white Dutch clover, subterranean clover), (B) cover crop - group 2 (purple vetch, blando brome, crimson clover, bell beans, magnus peas), and (C) weeds (purple vetch, field bindweed, Spanish clover, birdsfoot trefoil, dandelion). Means followed by the same letter are not significantly different (Tukey HSD test, $P \leq 0.05$).

CONCLUSIONS

The studies of the seasonal population dynamics of 3CAH in vineyards and surrounding landscapes presented in this report represents the first extensive study of timing of vineyard colonization, movement between ground covers and the crop canopy, and seasonal occurrence of girdling. Sampling data are presented both from an intensive sampling of a vineyard from 2016-2018, and from transects that extend out from vineyards into natural habitats. The transect sampling also allowed for comparison of different sampling methods that could prove useful in establishing guidelines for 3CAH monitoring by consultants and growers in the future. We have also initiated studies to identify the role, if any, of *Tortistilus* treehoppers that occur in vineyards where GRBV spread is confirmed and 3CAH are not found or occur at very low densities. Studies using *T. albidosparsus* were initiated in 2017, but have not yet indicated successful GRBV transmission. However, we do not know the timeframe necessary to first detect the presence of new infections in the field. Our results on the association of 3CAH in relation to cover crops and resident vegetation expands the confirmed list of feeding and reproductive hosts, and represents the first study evaluating common plant species used as cover crops or present as weeds in California vineyards. An associated preference study based on results of this no-choice test confirmed that plants of the family Fabaceae are preferred hosts of 3CAH. Knowledge of plant species present in vineyards that serve as alternative hosts for 3CAH is an additional contribution in understanding the relationship of GRBV and its presumed vector 3CAH. Our studies will provide a needed and sound foundation for developing management strategies for 3CAH to mitigate GRBV spread.

REFERENCES CITED

Al Rwahnih M, Dave A, Anderson M, Rowhani A, Uyemoto, JK, Sudarshana MR. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology* 10:1069-1076.

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

Bahder BW, Zalom FG, Sudarshana MR. 2016. An evaluation of the flora adjacent to wine grape vineyards for the presence of alternative host plants of grapevine red blotch-associated virus. *Plant Dis*. 100:1571-1574.

Beyer BA, Srinivasan R, Roberts PM, Abney MR. 2017. Biology and management of the three-cornered alfalfa hopper (Hemiptera: Membracidae) in alfalfa, soybean, and peanut. *J. Integr. Pest Manag*. 8(1):1-10.

Calvi B. 2011. Effects of red-leaf disease on Cabernet Sauvignon at the Oakville Experimental Vineyard and mitigation by harvest delay and crop adjustment. MS thesis. University of California, Davis, CA.

Krenz B, Thompson J, Fuchs M Perry P. 2012. Complete genome sequence of a new circular DNA virus from grapevine. *J. Virology* 86:7715.

Mueller AJ, Dumas BA. 1987. Host plants of the three-cornered alfalfa hopper (Hemiptera: Homoptera: Membracidae). *Environ. Entomol*. 16:513-518.

Perry KL, McLane H, Hyder MZ, Dangl GS, Thompson JR, Fuchs MF. 2016. Grapevine red blotch-associated virus is present in free-living *Vitis* spp. proximal to cultivated grapevines. *Phytopathology* 106:663-70.

Preto CR, Sudarshana MR, Zalom FG. 2018. Feeding and reproductive hosts of *Spissistilus festinus* (Hemiptera: Membracidae) found in Californian vineyards. *J. Econ. Entomol*. https://doi.org/10.1093/jee/toy236.

Seguin, J, Rajeswaran R, Malpica-López N, Martin RR, Kasschau K, Dolja VV, Otten P, Farinelli L, Pooggin MM. 2014. *De novo* reconstruction of consensus master genomes of plant RNA and DNA viruses from siRNAs. *PLoS One* 9:e88513.

Poojari S, Alabi OJ, Fofanov VY, Naidu RA. 2013. A leafhopper-transmissible DNA virus with novel evolutionary lineage in the family geminiviridae implicated in grapevine redleaf disease by next-generation sequencing. *PLoS One* 2013 8:e64194.

Sudarshana MR, Perry KL, Fuchs MF. 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Phytopathology* 105:1026-32.

Wildermuth VL. 2015. Three-cornered alfalfa hopper. *J. Agric. Res*. 3:343-364.

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EVALUATION OF SPRAYABLE PHEROMONE FOR MATING DISRUPTION OF VINE MEALYBUG, 2017

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Reporting Period: The results reported here are from the 2017-18 funding cycle.

ABSTRACT

Mating disruption (MD) is a relatively new technique with the potential to improve integrated pest management programs for vine mealybug (*Planococcus ficus*; VMB). Mating disruption works by inundating the vineyard with artificially-produced VMB pheromone. This inhibits the ability of males to find females. If females do not mate they are unable to produce offspring. Likewise, delays in mating can result in decreased offspring.

A new method of applying pheromone to disrupt mating of VMB became available in 2016. The new product is called CheckMate VMB-F (Suterra). This new formulation contains pheromone that is contained within tiny capsules (microencapsulated) contained within a liquid. The product is poured into a standard spray tank with water and applied to the vines. This results in millions of tiny capsules on the leaves that slowly release pheromone into the vineyard.

Research in 2016 and 2017 using 10-acre plots compared to no-MD controls showed that an application of CheckMate VMB-F at a rate of five gallons of active ingredient per acre can inhibit the ability of male VMB to find pheromone traps for approximately 30 days. This time period is similar to the length of one generation of VMB during summer months. When multiple applications were made at monthly intervals, trials showed reductions in male captures of 93 to 95% from June through the end of October. At one research site, plots treated aggressively with insecticides plus mating disruption every 30 days had no mealybug-infested clusters compared to 0.08% infested clusters in plots receiving only the insecticides. At a second research site plots treated aggressively with insecticides plus mating disruption every 30 days had 0.58 and 0.67% infested clusters compared to 1.20% in clusters in plots receiving only the insecticides. At a third site there were significant reductions in male mealybug captures in traps, but no mealybugs in the clusters at harvest in any plots.

During 2018 trials are underway to evaluate sprayable pheromone compared to two new commercial MD systems that use passive dispensers that are hung on the vine at the beginning of the year. Mealybug populations were monitored throughout the spring and summer and harvest evaluations were completed in September 2018. Evaluation of data is underway and should be available for presentation in December 2018.

FUNDING AGENCIES

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

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We thank Chelsea Gordon, Eryn McKinney, Laurren Heppner, and Minerva Gonzalez for assistance with data collection, and Kern County table grape growers and their pest control advisors for hosting this research within their vineyards.

COMPARISON OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3 AND GRAPEVINE RED BLOTCH VIRUS EFFECTS ON FOLIAR AND STEM PHENOLIC COMPOUND LEVELS

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Reporting Period: The results reported here are from work conducted September 2017 to September 2018.

ABSTRACT
Grapevine leafroll-associated virus 3 (GLRaV-3) and grapevine red blotch virus (GRBV) are of emerging concern to vineyard production in California and elsewhere. Key to management is early detection. One potential early detection strategy involves monitoring changes in host physiology that occurs in viral infected grapevines, either by assessing key compound levels in the field with portable technologies or looking for indirect signatures via remote sensing. One compound class associated with viral infection is phenolics. Although foliar phenolics were observed to increase after symptom development in GRBV-infected grapevines, little is known about how levels change in leaves and stems throughout the growing season. Likewise, it is unknown whether GLRaV-3 affects phenolic levels. Therefore, research was untaken to monitor phenolic levels in leaves and stems of GLRaV-3 or GRBV Cabernet franc or Cabernet Sauvignon grapevines on either 101-14MG or St. George rootstocks in May, July, and September. Results from May, July, and September of 2018 are pending completion of extractions. However, results from an initial trial in September of 2017 determined that GLRaV-3 had significantly greater foliar phenolic levels than controls and plants infected by GRBV ($F = 5.835$; $P = 0.011$). Rootstocks did not have significant effects on foliar phenolic levels. Stem phenolic levels were unaffected by viral infection, but the 101-14MG rootstock was observed to have greater phenolic levels than St. George. Results from 2018 are pending, and the experiment will be repeated in full in 2019. Conclusions should reveal the specificity of using phenolics to identify infections by either GLRaV-3 or GRBV via changes in plant physiology, with implications for remote sensing techniques.

FUNDING AGENCIES
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ACKNOWLEDGEMENTS
We thank Foundation Plant Services at the University of California, Davis for the kind use of their experimental vineyards to accomplish this project.

ABBREVIATIONS

3CAH	three-cornered alfalfa hopper
AAFP	acquisition access feeding period
AAP	acquisition access period
ABA	abscisic acid
ACMV	African cassava mosaic virus
AIC	Akaike information criterion
ANOVA	analysis of variance
APHIS	Animal and Plant Health Inspection Service
ARS	Agricultural Research Service
AUDPC	area under the disease progress curve
AVA	American Viticultural Area
BAC	bacterial artificial chromosome
BAP	benzylaminopurine
BC	backcross
BCTV	beet curly top virus
BGSS	blue-green sharpshooter
bp	base pair
BPI	bacterial permeability increasing
BSA	bovine serum albumin
BSCTV	beet severe curly top virus
CAP	chimeric antimicrobial protein
CAT	computed tomography
CDFA	California Department of Food and Agriculture
cDNA	complementary DNA
cfu	colony forming unit
Chr	chromosome
cM	centimorgan
CMI	cane maturation index
CR	common region
CRISPR	clustered regularly interspaced short palindromic repeats
CSWA	California Sustainable Winegrowing Alliance
Ct	cycle threshold
CTAB	cetyl trimethylammonium bromide
CWDE	cell wall degrading enzyme
DAMP	damage-associated molecular pattern
DE	differentially expressed
DEG	differentially expressed gene
DNA	deoxyribonucleic acid
DSF	diffusible signal factor
dsRNA	double-stranded RNA

ELISA	enzyme-linked immunosorbent assay
EM	electron microscope
EPG	electropenetrography
EPS	extracellular polymeric substances
FL	fiducial limit
FPS	Foundation Plant Services
GBR	General Beale Road
GFP	green fluorescent protein
GIS	geographic information system
GLMM	generalized linear mixed-effects model
GLRaV	grapevine leafroll-associated virus
GMO	genetically modified organism
GPS	global positioning system
GRBV	grapevine red blotch virus
GRSPaV	grapevine rupestris stem-pitting-associated virus
GWSS	glassy-winged sharpshooter
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HTH	helix-turn-helix
HWY	highway
IAFP	inoculation access feeding period
IAP	inoculation access period
JA	jasmonic acid
Kb	kilobase
LAMP	loop mediated isothermal amplification
LBP	lipid binding protein
LC	liquid chromatography
LPS	lipopolysaccharide
LRR	leucine-rich repeat receptors
MAMP	microbe-associated molecular pattern
MAPK	mitogen-activated protein kinase
MAS	marker-assisted selection
MCVGA	Monterey County Vintners and Growers Association
MD	mating disruption
microCT	micro-computed tomography
miRNA	microRNA
MLST	multi-locus sequence typing
mRNA	messenger RNA
MS	mass spectrometry
MYMV	mungbean yellow mosaic virus
NGS	next generation sequencing
NOS	nopaline synthase
nt	nucleotide

OG	oligogalacturonide
OMRI	Organic Materials Review Institute
OP	organophosphate
ORF	open reading frame
OSU	Oregon State University
P	phosphorus
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PCA	principal component analysis -or- pest control advisor
PCD	programmed cell death
PCR	polymerase chain reaction
PD	Pierce's disease
PEG	polyethylene glycol
PG	polygalacturonase
PGIP	polygalacturonase inhibitory protein
Phi	phosphite
PI	principal investigator
PM	pit membrane -or- powdery mildew
PPR	pentatrico-peptide repeat
PR	pathogenesis-related
PTGS	post-transcriptional gene silencing
PTI	PAMP-triggered immunity
qPCR	quantitative PCR
QTL	quantitative trait locus
RAP	ray and axial parenchyma
RBD	red blotch disease
RGA	resistance gene analog
RNA	ribonucleic acid
RNAi	RNA interference
RNA-seq	RNA sequencing
ROC	receiver operating characteristic
ROS	reactive oxygen species
RT-PCR	reverse transcription PCR -or- real-time PCR
SAC	spatial autocorrelation
SE	standard error
SEM	scanning electron microscopy
SEUS	southeastern United States
SIP	Sustainability in Practice
siRNA	small interfering RNA
SJVASC	San Joaquin Valley Agricultural Sciences Center
snoRNA	small nucleolar RNA
SNP	single-nucleotide polymorphism

SOREC	Southern Oregon Research and Extension Center
SOU	Southern Oregon University
SPAD	single-photon avalanche diode
sRNA	small RNA
ss	single-stranded
SSR	simple sequence repeat
STL	Stags' Leap
T2SS	type II secretion system
tasi-RNA	trans-acting small-interfering RNA
TCAH	three-cornered alfalfa hopper
T-DNA	transfer DNA
TDZ	thidiazuron
TE	transposable element
TGMV	tomato golden mosaic virus
TGS	transcriptional gene silencing
TSS	total soluble solids -or- true skill statistic
TYLCV	tomato yellow leaf curl virus
UC	University of California
U.S.	United States
USDA	United States Department of Agriculture
VMB	vine mealybug
WDV	wheat dwarf virus
WPM	woody plant medium
Xf	*Xylella fastidiosa*

AUTHOR INDEX