Interim Progress Report for CDFA Agreement Number 15-0428-SA

"Searching for Potential Vectors of Grapevine Red Blotch-Associated Virus"

Principal Investigator:
Kent Daane
Dept. Environmental Science, Policy and Management
University of California
Berkeley, CA 94720-3114
kdaane@ucanr.edu

Co-Principal Investigator:
Rodrigo Almeida
Dept. Environmental Science, Policy and Management
University of California
Berkeley, CA 94720-3114
rodrigoalmeida@berkeley.edu

Co-Principal Investigator:
Monica Cooper
UC Cooperative Extension
1710 Soscol Ave, Suite 4
Napa, CA 94559
mlycooper@ucanr.edu

Co-Principal Investigator:
Deborah Golino, Director,
Foundation Plant Services
One Shields Ave
University of California
Davis, CA 95616
dagolino@ucdavis.edu

Post Doctoral Researcher:
Houston Wilson
Dept. Environmental Science, Policy and Management
University of California
Berkeley, CA 94720-3114
houston@berkeley.edu

Post Doctoral Researcher:
Jeremy Anderson
Dept. Environmental Science, Policy and Management
University of California
Berkeley, CA 94720-3114
jandersen@berkeley.edu

Laboratory Technician:
Kei-Lin Ooi
Dept. Environmental Science, Policy and Management
University of California
Berkeley, CA 94720-3114
keilinooi@berkeley.edu

REPORTING PERIOD: The results reported here are from work conducted July 2015 – February 2017.

INTRODUCTION

In 2006 an increase in grapevine leafroll disease (GLD) and vines with "red leaf" symptoms was observed by growers in vineyards located within Napa Valley, CA. Symptoms were also observed at the Oakville Experimental Vineyard (OEV) by Jim Wolpert (UC Davis Viticulture Extension Specialist), Ed Weber (former UCCE Viticulture Farm Advisor), and Mike Anderson (UC Davis Staff Research Associate). Tissue samples were collected from symptomatic vines and tested by commercial laboratories and UC Davis Foundation Plant Service. Test results were most often negative for known grapevine leafroll-associated viruses (GLRaVs).

The increasing awareness of blocks containing vines with grapevine leafroll disease symptoms, primarily in Napa and Sonoma Counties, but testing negative for grapevine leafroll-associated viruses resulted in a renewed focus on virus species and strains causing GLD. New GLRaV-3 strains have been discovered (e.g., Sharma et al. 2011); however, this did not fully explain all of the observed symptomatic vines. In 2010, next generation sequencing analyses identified a new pathogen (Al Rwahnih et al. 2013). Soon after a circular DNA virus, similar to members of the family Geminiviridae, was isolated (Krenz et al. 2012) and, concurrently, PCR primers were developed (Al Rwahnih et al. 2013) for this pathogen now known as Grapevine Red Blotch-associated Virus (GRBaV). GRBaV has since been isolated from vines throughout North America and in Switzerland (Krenz et al. 2014), highlighting either a rapid dissemination or, more likely, its long hidden presence (e.g., misidentified as GLD).

This project focuses on possible vectors of GRBaV. Multiple viruses in the Geminiviridae are insect transmissible (Ghanim et al. 2007, Chen and Gilbertson 2009, Cilia et al. 2012), and there has been some initial evidence that leafhoppers may transmit GRBaV (Poojari et al. 2013) and better evidence that a membracid may transmit the pathogen (Bahder et al. 2016). However, there has been mixed evidence of GRBaV field spread in association with leafhoppers. Concern for the spread of GRBaV led to an off-cycle project in summer 2013, funded by the "Napa County Winegrape Pest and Disease Control District" to initiate appropriate scientific studies of possible insect vectors of GRBaV. The work was continued in 2014 with American Vineyard Foundation (AVF) and Napa County funds.

Table 1. Arthropods targeted for GRBaV tests

Common name	Scientific Name	Common Distribution
Western grape leafhopper	*Erythroneura elegantula*	North Coast (north of Tehachapi Mtns.)
Variegated leafhopper	*Erythroneura variabilis*	Central Valley (San Joaquin Co. to So. Cal.)
Virginia creeper leafhopper	*Erythroneura ziczac*	Northern CA
Potato leafhopper	*Empoasca* sp.	Sporadic vineyard populations
Vine mealybug	*Planococcus ficus*	California vineyards
Grape mealybug	*Pseudococcus maritimus*	North Coast and San Joaquin Valley
Obscure mealybug	*Pseudococcus viburni*	Central and North Coast
Blue-green sharpshooter	*Graphocephala atropunctata*	Northern CA
European fruit lecanium scale	*Parthenolecanium corni*	North Coast
Grape phylloxera	*Daktulosphaira vitifoliae*	North Coast, Sacramento Delta, Foothills
Grape whitefly	*Trialeurodes vittatas*	California
Mites	*Tetranychus spp.*	California

Our goal is to test potential vectors to provide concrete evidence that organisms can or cannot move GRBaV among vines. Determining field epidemiology of GRBaV is critical in the development of a control program – whether the pathogen is moved via infected nursery material, mechanically or, as with the focus of this study, by a vector. There are ample California vineyard sites where the pathogen is present but does not appear to have moved from infected vines over a period of many years, but in some vineyards, vine to vine movement has been recorded. This difference – whether there is no vector movement and disease presence is exclusively from infected nursery material or there is a vector – completely changes the needed control programs.

Our proposed work will screen all common vineyard arthropods, as well as the "long shots" that are potential GRBaV vectors, thereby providing the proper target for control. Table 1 provides a partial list of the common vineyard insect species that should be screened as potential vectors of GRBaV, based on their incidence and distribution in California vineyards.

Once tested organisms are either identified as vectors or our work shows that they are either not vectors or that they are so inefficient that spray programs are not needed, this information will be disseminated to farmers, PCAs and extension personnel, thereby having a practical, direct and immediate impact on control decisions to "spray or not to spray".

OBJECTIVES

To screen potential vectors for their ability to acquire and transmit Grapevine Red Blotch-associated Virus (GRBaV) and, if a vector is discovered, to determine vector efficiency. Objectives for this research program are as follows:

1. Screen common vineyard insects and mites as potential vectors for GRBaV.
2. Screen uncommon organisms that feed on vines as potential vectors for GRBaV.
3. Follow disease progression in established vineyard plots to collect preliminary data on field epidemiology.

Objective 1. Screen common vineyard insects and mites as potential vectors of GRBaV.

2013-2014 – Initial Transmission Trials with Potted Vines

In 2013 and 2014, we prioritized the screening of leafhoppers (*E. elegantula* and *E. ziczac*), grape whitefly (*Trialeurodes vittatas*), mealybugs (*Planococcus ficus* and *Pseudococcus maritimus*), and blue-green sharpshooter (*Graphocephala atropunctata*) because of the published work by Poojari et al. (2013), their prevalence in California vineyards, and/or their phloem feeding (this category of viruses [Geminiviridae] are phloem-limited, although the biology and ecology of GRBaV is not fully understood).

In both years, canes were collected from Cabernet Sauvignon (clone 6) and Cabernet Franc (clone 04) vines in vineyard blocks where vines are known to have tested positive for GRBaV, and negative for all known GLRaVs and other known grapevine viruses. PCR test results for these vines were made and canes negative for all viruses except GRBaV and RSP (UC Berkeley and FPS test results) were transferred to UC Berkeley Oxford Tract Greenhouse and established in pots on a mist bench. Vines were maintained in the greenhouse, strictly treated to be insect and mite-free, and isolated from other vines that may have harbored viral pathogens. As indicators for these studies, we used Cabernet Sauvignon vines propagated from material provided by FPS and maintained under similar conditions.

Initial tests were conducted using the most mobile stages of key species, including adults of the *Erythroneura* (leafhopper) species and the grape whitefly, and crawlers of the vine mealybug crawlers and grape phylloxera. We employed standard transmission protocols to evaluate the potential of these insects to transmit GRBaV, as has recently been done for GLRaVs (Tsai et al. 2008, Tsai et al. 2011) and Pierce's Disease (Almeida and Purcell 2003a, b). We used a standard Acquisition Access Period (AAP) and Inoculation Access Period (IAP) of 120 hours (5 d) each for all tested insect species except the more delicate grape whitefly, which could feed on plants for an AAP and IAP of 48 hours (2 d) each. In the "controlled trials", known infected source plants or uninfected control plants in pots (1-liter size) were inoculated with 30-50 insects for the AAP, and surviving insects were then transferred to uninfected plants for the IAP. Field-collected leafhopper adults and blue-green sharpshooter adults were taken from an insectary colony and released on plants that were placed singly in 61 x 61 x 61 cm BugDorm cages. Grape whitefly adults reared from pupae were collected in Napa County vineyards and then released into nylon bags enclosing 5 leaves on potted grape plants. Mealybug crawlers were moved onto individual grape leaves (3 leaves per plant) using a brush, and grape leaves were then enclosed with white paper bags. Following the IAP, all vines were treated with a contact insecticide to kill any remaining insect species. All insects were collected and tested for GRBaV within 48 hours after the AAP period. Every four months thereafter, three petioles were collected from each host plant and assayed for GRBaV infection. A total of 20 test vines were inoculated for each of the above insect species in the 2014 trials.

Results from the 2013/2014 trials have not indicated that any of these insects (i.e. leafhoppers [*E. elegantula* and *E. ziczac*], grape whitefly [*Trialeurodes vittatas*], mealybugs [*Planococcus ficus* and *Pseudococcus maritimus*], and blue-green sharpshooter [*Graphocephala atropunctata*]) are capable of transmitting GRBaV to uninfected grape vines. Inoculated vines from these trials are being held for a two-year period, during which petioles are tested for GRBaV every four months and vines are visually evaluated for symptoms every fall. All insects that fed on infected plant material in these trials have tested negative as well. That said, we have recently begun to redesign our insect testing procedures to improve the sensitivity and accuracy of these

laboratory tests. Insects from the 2013/2014 trials are being re-tested using new protocols that have been developed and verified.

2015 – Improved "Bouquet" Transmission Trials

In 2015 and 2016, protocols for these transmission experiments were modified due to concerns about (a) potentially low virus titer levels in the potted vines grown from cuttings of GRBaV-positive vines at vineyard field sites and (b) small number of insects per trial. Our concern is that candidate vector ability to transmit GRBaV is confounded by low titer levels in the GRBaV-positive vines used in previous trials and/or inadequate insect sample size.

The new approach involves using "bouquets" of mature grape leaves collected from GRBaV-positive vines at vineyard field sites that were not sprayed with insecticides. Each bouquet consists of ten mature grape leaves held in a 16 oz. plastic container that contains moist perlite. Ten leaves were collected from each of ten GRBaV-positive vines (nodes 1-5) in an established vineyard in Napa County (100 leaves total). Each bouquet consisted of one leaf from each of the ten vines, totaling ten leaves per bouquet and ten total bouquets (i.e. one bouquet per replicate). Bouquet degradation was initially evaluated by testing petioles for GRBaV 6-48 hours after collection. Results indicated no degradation of the petioles. Finally, each trial now contains at least 100 insects/replicate (when possible) and 10 replicates per treatment.

Since July 2015, we have completed trials using the bouquets with Virginia Creeper leafhopper adults (*Erythroneura ziczac*), vine mealybug crawlers (*Planococcus ficus*), and foliar form grape phylloxera crawlers (*Daktulosphaira vitifoliae*). Due to concerns about bouquet degradation, these experiments used an AAP of 48 hours (2 day) and an IAP of 72 hours (3 d). Clip-cages (7 cm diameter x 2 cm height) were used to confine 10 insects/leaf to each bouquet (100 insects/bouquet). Bouquets with insects were placed in a 61 x 61 x 61 cm BugDorm cage and there was a total of 10 replicates per treatment. After the 48 hour AAP, clean potted vines were introduced into the cages. The clip cages were then removed, thus allowing the insects to move onto the clean vine. Bouquets were also removed at this time, after ensuring that they were free of the candidate vectors. Petioles from the bouquets were then collected for GRBaV testing as well as a sub-sample of the candidate vectors (10-50 insects per replicate). After the 72 hour IAP, another subsample of the candidate vectors was collected for testing (10-50 insects per replicate) and the potted vines were then treated with a contact insecticide to kill any remaining insects. Three petioles were sampled from each vine (nodes 1-5) for immediate testing. Vines are now being maintained for a two-year period and petioles tested for GRBaV every four months.

Bouquet experiments with grape phylloxera were initially unsuccessful due to their rejection of the bouquet material. Following the 48 hour AAP it was observed that none of the phylloxera crawlers had settled on the leaves and instead were mostly desiccated inside the cages. As such, we reverted to the previous experimental approach utilizing potted vines that were confirmed to be GRBaV positive. This time, two-year-old GRBaV-positive vines were used in these trials to possibly provide vines having elevated virus titer levels. Negative control source vines were one year old. Vines were placed in 61 x 61 x 61 cm BugDorm cages and inoculated by pinning ten leaf discs containing a large number of galls (>15) on each vine. The galls on these discs had been cut open with a razor to encourage movement of the crawlers onto the vine. After 25 days all of the potted vines exhibited >50 galls (i.e. 25 day AAP). At this point, clean vines were introduced into the cages and sub-samples of grape phylloxera adults, eggs and crawlers were collected for testing. Acquisition and inoculation vines remained together in the cages until the inoculation vines had >50 galls/vine, which resulted in a 38 day IAP. At this point vines were treated with both a contact and systemic insecticide. As before, vines will be held for a two-year period and tested every four months. So far, our 2015 and 2016 "bouquet" trials have shown no transmission of GRBaV by either the Virginia Creeper leafhopper or vine mealybug. Similarly, the trial with foliar form grape phylloxera on two-year-old GRBaV-positive vines did not show any transmission.

Testing Plant Material for GRBaV

To test for the presence of GRBaV in grapevine petioles potentially infected with red blotch disease (Sharma et al. 2011), whole genomic DNA was extracted from three randomly selected petioles (nodes 1-5) from each target grapevine using the ISOLATE II Plant DNA Extraction Kit (Bioline Corp.). Briefly, 0.1 g of each petiole tissue was homogenized in Mo-Bio 2.0 ml tough tube containing a Boca chrome steel ball-bearing using a Precellys 24 Tissue Homogenizer set for two 10-second cycles at 6,500 Hz for with a 30-second intermission between cycles. DNA was then extracted following the manufacturers protocols. The presence of GRBaV clade 1 and/or clade 2 viruses were then determined using quantitative polymerase chain reaction (qPCR). Duplicate qPCR reactions were run for each petiole with both either primers specific to clade 1 or clade 2 (in total four qPCR reactions were run for each sample). Reactions were conducted on an Applied Biosystems 7500 Fast Real-Time PCR System with SDS Software used for analysis with the following reaction conditions: 12.5 µl Promega GoTaq master mix, 2.5 µl of 10 µM primers (either GVGF1 and GVGR1 to test for the presence of clade 1 or GVGF2 and GVGR2 to test for the presence of clade 2), 0.25 µl CXR reference dye, 8ul water, and 2 µl of each target sample (Al Rwahnih et al. 2013). Thermocycling conditions included one cycle of 95°C for 2 minutes; forty cycles of 95°C for 15 seconds, 58°C for 1 minute; and one cycle of 72°C for 10 minutes, followed by a final dissociation cycle. Results were then analyzed by the 7500 Fast System SDS Software, accounting for the Ct values, melting temperatures, and component curves, with infected samples scored as those with positive amplification curves prior to 30 cycles (See Fig. 1 for an example). All reactions were run with positive and negative controls.

Testing Insects for GRBaV

After field collection, insects were frozen at -80°C prior to testing for the presence of GRBaV. Whole genomic DNA was extracted from individual insects using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Corp.) based on the manufacturers protocol. Prior to extraction, insects were homogenized using the same method as above. Recently, (Bahder et al. 2016) found that that digital PCR (dPCR) may be an effective tool for identifying the presence of GRBaV virus in insect vectors. The development of digital droplet PCR (ddPCR), however, now allows us to build upon the increased sensitivity of the dPCR system, with the added benefit of being able to directly quantify gene copy numbers (i.e. virus infection loads) for each insect. Therefore, we developed two directly labeled primer-probe sets that can be used to simultaneously determine whether collected insects are infected with both GRBaV clades 1 and/or clades to GRBaV and to quantify the infection. ddPCR reactions were conducted on a Bio-RAD QX200 ddPCR system with 12.5 µl of BioRad ddPCR 2x MasterMix (BioRad, Inc.), 1.25 µl of each primer-probe pair, and 10 µl of extracted DNA, with the following thermocycler conditions: one cycle of 95°C for 1 minutes; forty cycles of 94°C for 30 seconds, 56°C for 1 minute; followed by a final hold at 12°C prior to quantification with the Bio-RAD QuantaSoft™ software. All reactions were run in duplicate, with an example of the results for infected and uninfected insects presented in Fig. 2.

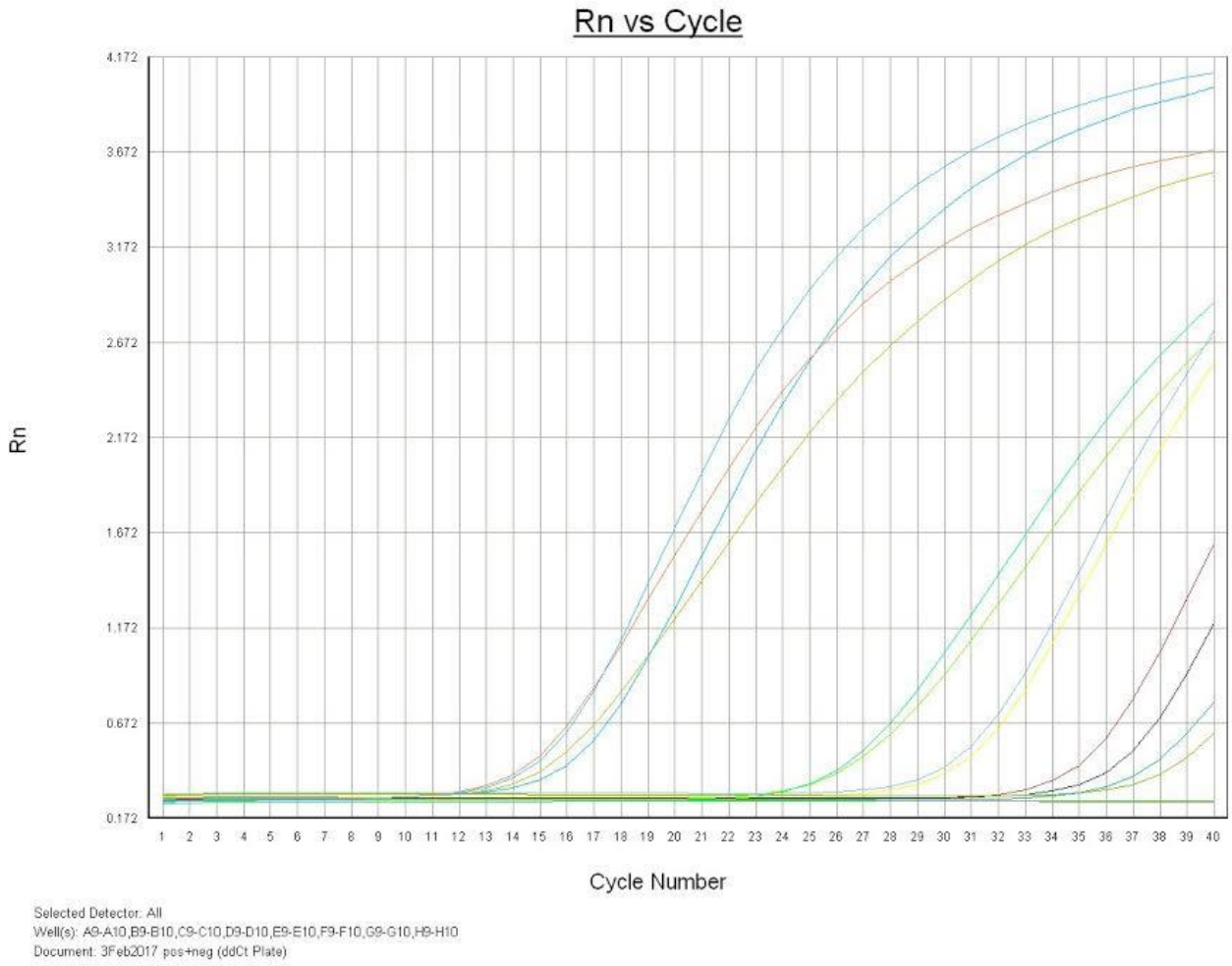

Fig. 1. Example results from GRBaV plant petiole testing. Samples with amplification curves present prior to 30 cycles (x-axis) are scored as infected (first eight curves) and those with amplification after 30 cycles are scored as uninfected (final four curves).

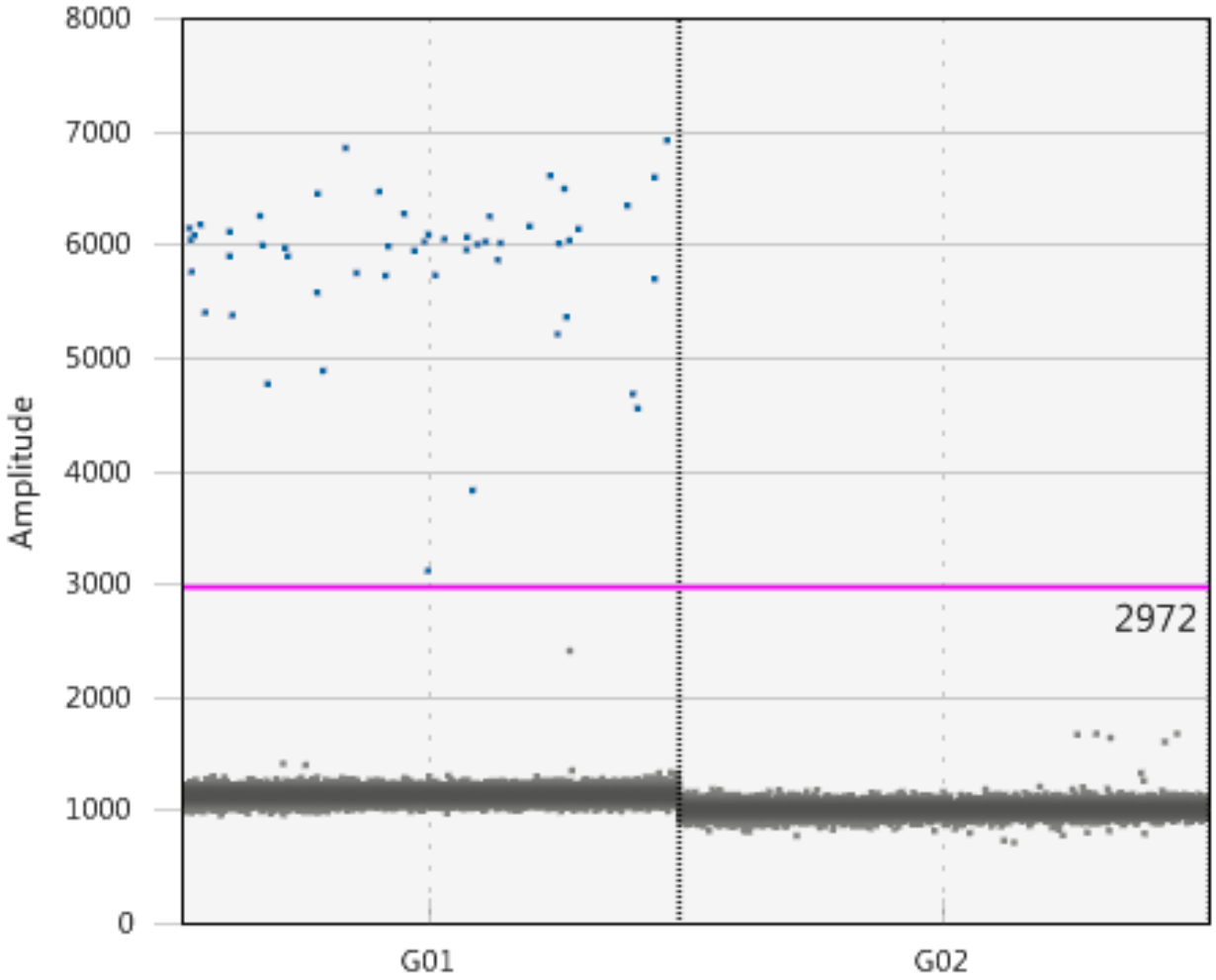

Fig. 2. Results for ddPCR analysis of infected (left) and uninfected (right) insects. Each blue dot represents a copy of GRBaV, and the total infection level for each insect is calculated as the ratio of droplets containing amplified GRBaV and the number of droplets without amplified GRBaV. The infected sample has a GRBaV concentration of 88 copies per 20 μl of sample, and the uninfected sample has a GRBaV concentration of 0 copies per 20 μl of sample.

Conclusion –No Transmission Observed to Date

We have evaluated a total of 7 vector candidates, which includes grape leafhopper (*Erythroneura elegantula*), Virginia Creeper leafhopper (*E. ziczac*), grape whitefly (*Trialeurodes vittatas*), mealybugs (*Planococcus ficus* and *Pseudococcus maritimus*), blue-green sharpshooter (*Graphocephala atropunctata*) and foliar form grape phylloxera (*Daktulosphaira vitifoliae).* In 2015 and 2016, we modified experimental protocols that were designed to overcome perceived limitations in previous transmission experiments from 2013-2014. This led to the re-evaluation of 2 candidates, Virginia Creeper leafhopper and vine mealybug, as well as evaluation of a new candidate, foliar form grape phylloxera.

To date, none of the candidate vectors have tested positive for GRBaV and no transmission has been observed, although testing of insect and plant material from these experiments is on-going. Transmission vines from these experiments were most recently tested in October 2016.

Objective 2. Screen uncommon organisms that feed on vines as potential vectors for GRBaV.

Vineyard Insect Survey

We used the same methodologies described for Objective 1 to screen lessor known vineyard organisms or unlikely vectors. Insects were collected 1x/month from 5 established vineyards where movement of GRBaV has been observed or reported (assumed to have happened). Samples were collected from grape vines, ground covers and non-crop vegetation in the surrounding landscape using a combination of sweep-nets (on ground covers, 5 samples per site, 30 sweeps per sample) and a D-Vac type suction sampling machine (on grape vines and non-crop vegetation), which consisted of a 25cc gas blower/vacuum (Craftsman) fitted with a 5-gallon (18.9 liter) bucket on the vacuum tube to create a 1 ft^2 (0.093 m^2) sampling cone. Each D-Vac sample consisted of five thrusts with the D-Vac running at full speed (5 samples of grape vine per site, 5-10 samples of non-crop vegetation). All samples were held in a cooler and brought to the laboratory for immediate processing. Specimens were incapacitated using CO_2 gas, sorted and identified to species or genus, and then stored in 95% EtOH and stored at -80^o C until testing. So far we have collected leafhoppers in the genera *Aceratagallia* sp., *Acinopterus* sp., *Alconeura* sp., *Colladonus* sp., *Empoasca* spp., *Macrosteles* sp., *Osbornellus* sp., *Scaphytopius* spp., as well as the species *Deltocephalus fuscinervosus, Dikrella californica*, and *Euscelidius schenki*. Other organisms include members of the families Acanaloniidae, Cixidae, Membracidae, Miridae, Lygaeidae, Psyllidae, and Tingidae.

Many novel insects have been collected from vineyard sites where movement of GRBaV is suspected, but to date none have tested positive for GRBaV, although many specimens are still in the process of being tested, and as mentioned above, we are still in the process of refining our laboratory techniques to improve sensitivity of detection for insect material.

Non-crop Plant Survey

As a complement to the insect collection and testing, plant material was also collected from non-crop vegetation and tested for GRBaV in order to identify plant species that serve as reservoirs of GRBaV outside of the vineyard. Plant material was sampled from maple (*Acer* sp.), California buckeye *(Aesculus californica*), alder (*Alnus rhombifolia*), madrone (*Arbutus menziesii*), manzanita (*Arctostaphylos* sp.), coyotebrush (*Baccharis pilularis*), Oregon ash (*Fraxinus latifolia*), English ivy (*Hedera helix*), toyon (*Heteromeles arbutifolia*), California walnut (*Juglans californica*), wild cucumber (*Marah macrocarpa*), olive (*Olea europaea*), plum (*Prunus* sp.), coast oak (*Quercus agrifolia*), blue oak (*Q. douglasii*), valley oak (*Q. lobata*), wild rose (*Rosa californica*), blackberry (*Rubus* spp.), willow (*Salix* sp.), elderberry (*Sambucus* sp.), California bay (*Umbellularia californica*), periwinkle (*Vinca major*), wild grape (*Vitis californica*) as well as various vineyard ground covers and weedy vegetation (*Artemisia douglasiana, Avena fatua, A. sativa, Brassica* spp., *Calendula officinalis, Conium maculatum, Convolvulus arvensis, Foeniculum vulgare, Malva parviflora, Raphanus sativa, Taraxacum officinale, Vicia fava*, and *Vigna* sp.). To date, most of this plant material has tested negative for GRBaV, except for wild grape which has tested positive fairly consistently across multiple sites. It should be noted that "wild grape" at these sites may be a hybrid form *Vitis californica* x *vinifera* due to its proximity to commercial vineyards.

Vineyard Insect and Plant Survey – Preliminary Findings

The insect and non-crop plant survey concluded in May 2016, marking one full year of monthly insect and plant sampling in five vineyards with suspected spread of GRBaV. As mentioned, testing of plant and insect material is on-going, but here we present some preliminary summaries of the data based on findings to date. In our surveys, the only non-crop plant species to test positive for GRBaV has been wild grape (*V. californica* x *V. vinifera*), indicating a potential role of this plant in the spread of GRBaV into commercial vineyards. Here we present a summary of the insect community found on wild grapes in our survey (Table 2). Diptera (flies) and *E. elegantula* (Western grape leafhopper) make up >50% of the insects found on wild grape and >90% of organisms are represented when we include the parasitic Aprocita (parasitoid wasps), spiders,

Formicidae (ants), *Empoasca* spp., Coleoptera (beetles), *Chrysoperla* sp. (green lacewings), *E. variabilis* (Variegated leafhopper), *Osbornellus* sp., Psocoptera (book lice), Trichoptera (caddisflies), aphids and Miridae. From this group, only *E. elegantula*, *Empoasca* spp., *E. variabilis*, *Osbornellus* sp., aphids and the Miridae are likely to feed directly on wild grape tissue and only *E. elegantula* and *E. variabilis* are known to successfully reproduce on it.

Evaluating insect community overlap between wild and wine grape could help identify novel insect vectors of GRBaV. Organisms that were found on both wild and wine grape include aphids, Berytidae, *Chrysoperla* sp., Coleoptera, *Deltocephalus fuscinervosus*, Diptera, *Empoasca* spp., *E. elegantula*, *E. variabilis*, Formicidae, Galerucinae, parasitic Aprocrita, Lepidoptera, Lygaeidae, *Spissistilus festinus* (three-cornered alfalfa hopper), Miridae, *Orius* sp., Psocoptera, Psyllidae, *Scaphytopius* spp., spiders, Thysanoptera, Trichoptera and a small number of unknown Ciccadellids. Of these organisms that co-occur on both wild and wine grape, *Deltocephalus fuscinervosus*, *Empoasca* spp., *E. elegantula*, *E. variabilis*, Lygaeidae, Miridae, Psyllidae, *Scaphytopius* spp., *Spissistilus festinus*, Thysanoptera, and the unknown Ciccadellids will likely feed directly on grape plant tissue and only *E. elegantula* and *E. variabilis* are known to reproduce on these species. The most commonly encountered organism on cultivated wine grape was *E. elegantula* (35%), followed by *E. variabilis* (11%), Thysanoptera (5%), aphids (2%) and Lygaeidae (1%). All other organisms represented <1% of the community found on wine grapes. From this group of likely feeders that occur on both wild and wine grape, we have conducted GRBaV transmission experiments with *E. elegantula* and *E. variabilis*, which represent some of the commonly encountered organisms on both wild and wine grape. Results from these trials have not indicated any ability of these insects to transmit the virus.

Table 2. Arthropod Community on Wild Grapes and Cultivated Wine Grapes. Data shows mean annual abundance per sample ±SEM and percentage of total arthropods found on the plant.

Order	Family	Genus/Species	Wild Grape		Wine Grape	
			Abundance	%	Abundance	%
Araneae			0.39 ±0.12	6%	0.02 ±0.02	2%
Coleoptera	Galerucinae		0.02 ±0.02	<1%	0.01 ±0.01	<1%
	Cantharidae		-	-	<0.01	<1%
	Other		0.18 ±0.09	3%	0.08 ±0.02	2%
Dermaptera			0.04 ±0.03	1%	-	-
Diptera	Syrphidae		-	-	<0.01	<1%
	Other		2.80 ±0.68	41%	1.24 ±0.14	28%
Hemiptera	Acanaloniidae		0.02 ±0.02	<1%	-	-
	Alydidae		-	-	<0.01	<1%
	Anthocoridae	*Orius* sp.	0.04 ±0.04	1%	0.03 ±0.01	<1%
	Aphididae		0.08 ±0.05	1%	0.09 ±0.02	2%
	Berytidae		0.04 ±0.03	1%	<0.01	<1%
	Ciccadellidae	*Acinopterus angulatus*	-	-	0.01 ±0.01	<1%
		Deltocephalus fuscinervosus	0.02 ±0.02	<1%	0.02 ±0.01	<1%
		Dikraneura rufula	-	-	<0.01	<1%
		Dikrella sp.	0.02 ±0.02	<1%	-	-
		Empoasca spp.	0.22 ±0.13	3%	<0.01	<1%
		Erythroneura elegantula	0.80 ±0.43	12%	1.51 ±0.44	35%
		Erythroneura variabilis	0.14 ±0.07	2%	0.47 ±0.19	11%

		Graphocephala atropunctata	-	-	<0.01	<1%
		Macrosteles quadrilineatus	-	-	<0.01	<1%
		Osbornellus sp.	0.12 ±0.10	2%	-	-
		Scaphytopius spp.	0.02 ±0.02	<1%	0.02 ±0.01	<1%
		Sophonia sp.	-	-	<0.01	<1%
		Unknown	0.04 ±0.03	1%	0.01 ±0.01	<1%
	Geocoridae	*Geocoris* sp.	-	-	<0.01	<1%
	Lygaeidae		0.06 ±0.05	1%	0.06 ±0.04	1%
	Membracidae	*Spissistilus festinus*	0.02 ±0.02	<1%	0.02 ±0.01	<1%
	Miridae		0.08 ±0.05	1%	<0.01	<1%
	Psyllidae		0.02 ±0.02	<1%	0.02 ±0.01	<1%
	Rhopalidae		0.02 ±0.02	<1%	-	-
	Tingidae		-	-	0.01 ±0.01	<1%
Hymenoptera	Apoidea (non-*Apis*)		-	-	0.02 ±0.01	<1%
	Aprocrita (parasitic)		0.57 ±0.17	9%	0.17 ±0.03	4%
	Formicidae		0.37 ±0.12	6%	0.01 ±0.01	<1%
	Vespidae		0.02 ±0.02	<1%	-	-
Ixodida	Ixodidae		0.04 ±0.04	1%	-	-
Lepidoptera			0.04 ±0.04	1%	<0.01	<1%
Neuroptera	Chrysopidae	*Chrysoperla* sp.	0.14 ±0.12	2%	0.01 ±0.01	<1%
Orthoptera			0.02 ±0.02	<1%	-	-
Psocoptera			0.08 ±0.05	1%	0.07 ±0.02	2%
Thysanoptera			0.04 ±0.03	1%	0.22 ±0.08	5%
Trichoptera			0.08 ±0.05	1%	<0.01	<1%

While it is notable that *S. festinus*, a known vector of GRBaV (Bahder et al. 2016), was found on both wild and wine grapes, on both plant species they represented <1% of total organisms. Regardless of the overall low populations encountered in vineyards, data on host plant associations of *S. festinus* (Fig. 3) provides new information on population dynamics in vineyards. This species was primarily found in the late spring on groundcovers in and around the vineyard, which included various weedy grasses as well as overwintering grass/legume cover crops. As groundcovers died down, *S. festinus* was intermittently found in low abundance on wild grape, wine grape, toyon (*Heteromeles arbutifolia*) and coast oak (*Quercus agrifolia*) throughout the growing season. These are not necessarily reproductive hosts for this species and further work is needed to better understand the life cycle of *S. festinus* on the non-crop habitats in and around vineyards.

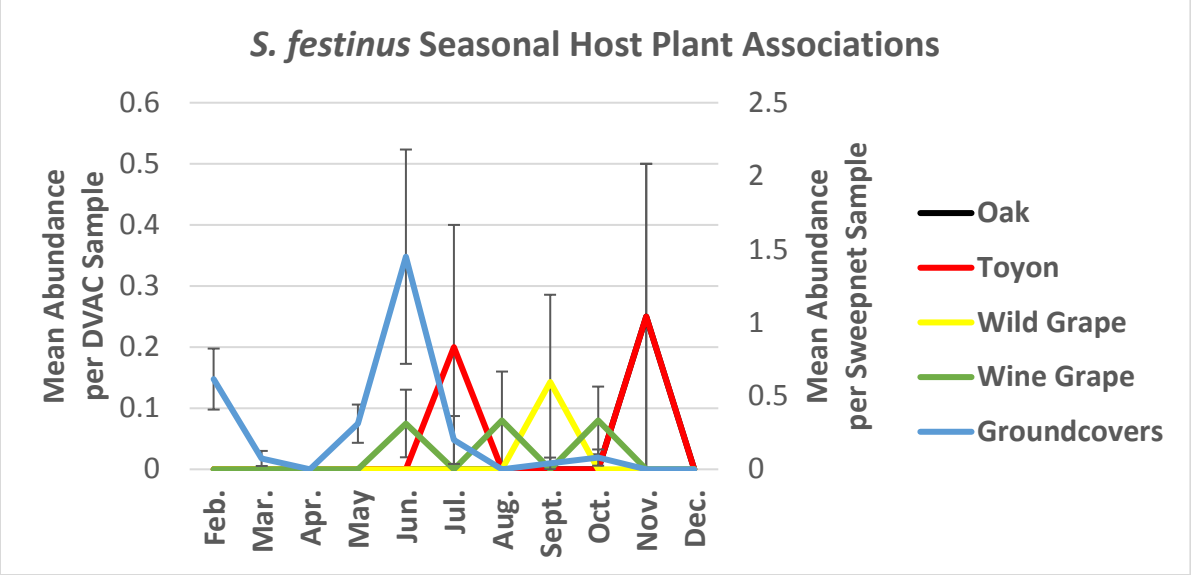

Fig. 3. Seasonal host plant associations of *S. festinus* in North Coast vineyards. High densities of *S. festinus* were found on groundcovers in the late spring and then intermittently on wild grape, wine grape, coast oak and toyon. Plant species shown are not necessarily reproductive hosts. Right Y-axis denotes abundance on groundcovers, left Y-axis denotes abundance on all other plants.

Establishing Colonies of Novel Vectors (2015-present)

Due to the low abundance of novel candidate vectors (e.g. *Empoasca* spp., *S. festinus*, *D. fuscinervosus*), we have been working to establish colonies of these insects at the UC Berkeley greenhouse facilities in order to rear a large enough population suitable for GRBaV transmission experiments, which typically require >200 individuals per trial. Data is scant for many of these species and information on reproductive hosts is limited. As such, this spring we collected candidate species from vineyards and introduced them into cages containing various potential host plants. So far, we have seen successful reproduction of *Aceratagallia* sp. and *Euscelidius schenki* on select host plants. We also collected large populations of *S. festinus* from alfalfa fields and are now seeing reproduction in our colonies.

Transmission Experiment with *S. festinus* (2016)

A GRBaV transmission experiment was conducted with field collected *S. festinus* in July 2016. Individuals were collected from an organic alfalfa field and introduced into cages with GRBaV positive or negative vines. Each cage contained a single potted vine (11 cages each with a single GRBaV-positive vine and 9 cages each with a single GRBaV-negative vine) and received 20 *S. festinus* adults. Adults could feed for 48 hours (AAP), after which the GRBaV-positive/negative vine was removed and a GRBaV-negative vine was introduced into each cage. The adults could feed on the negative vine for 48 hours (IAP) and were then removed from the vine. As with previous transmission experiments, the vines are now being held for a 2-year period and will be tested for GRBaV every 4 months. While it has been demonstrated that *S. festinus* can vector GRBaV (Bahder et al. 2016), our goal is to first confirm these findings and then begin evaluating transmission efficiency of this species under laboratory and field conditions.

Evaluating *S. festinus* Overwintering Habitat and Seasonal Activity in Vineyards (March – October 2017)

With the confirmation of *S. festinus* as a known vector of GRBaV, new information is needed on the seasonal ecology of this organism in vineyards.

Overwintering Habitat

Ground covers and other non-crop plants in natural habitats adjacent to vineyards will be sampled in March to identify *S. festinus* overwintering habitat use. Sampling will take place in the natural habitats adjacent to Napa and Sonoma County vineyards. There will be at least 4 sites sampled each month. Natural habitat will

consist of patches of riparian and/or oak woodland habitat > 400 m^2. Sweep-nets will be used to sample ground covers and perennial plant species in the natural habitats and at the periphery of adjacent vineyards. At each site, 10 sets of 30-sweeps will be collected from groundcovers using a 30.5 cm diameter sweep-net (BioQuip Products, Rancho Dominguez, CA). Ground cover species composition will be recorded. Sweep-nets will also be used to sample the canopy of at least 10 non-crop plant species at each site. For each sample, the sweep-net is held beneath the canopy while vigorously shaking the plant for 30 seconds to dislodge insects into the net.

Seasonal Activity

In February 2017, we established a study in five Napa and Sonoma County vineyards to evaluate the activity of *S. festinus* populations along transects that extend out from large patches of natural habitat into vineyards. At each site, insects will be sampled along five parallel transects (positioned 20 m apart) that extended out from the riparian or oak woodland habitat (i.e. "natural habitat") into the vineyard. Each transect will be 160 m long – 10 m into the natural habitat and 150 m into the vineyard (Fig. 1). Along each transect samples will be taken at the edge (10 m) of the natural habitat as well as at the edge (10 m) and interior (150 m) of the vineyard.

Densities of *S. festinus*, *Erythroneura* leafhoppers and other hemipterans will be monitored along the transects approximately every 2 weeks using a combination of yellow sticky-traps, sweep-nets and beat-sheet sampling. Two yellow sticky-traps (16 x 10 cm, Seabright Laboratories, Emeryville, CA) will be placed at each transect point. In the vineyard, one trap will be placed in the vine canopy (approximately 3.5 feet above the ground surface) and another trap will be hung from irrigation lines (approximately 1.5 feet above the ground surface). In the natural habitat, two sticky-traps will be hung from a pole at each transect point at a height equal to those in the vineyard (i.e. one trap 3.5 feet and the other 1.5 feet above the ground surface). Traps will be replaced approximately every 2 weeks from March to October. Sweep-nets will be used to sample ground covers. At each transect point, a set of 30-sweeps will be collected from the groundcovers using a 30.5 cm diameter sweep-net (BioQuip Products, Rancho Dominguez, CA). Ground cover species composition and percentage cover will be recorded. A modified beat-sheet will be used at each transect point to sample the canopy of grape vines (in the vineyard) and non-crop species (in the natural habitat). The beat-sheet consists of a 1 m^2 nylon funnel that feeds into a detachable 1 gallon plastic bag. For each sample, the funnel is held beneath the canopy while vigorously shaking the plant (or vine) for 30 seconds to dislodge insects into the funnel and plastic collection bag. Each month, vines along each vineyard transect point will be evaluated for signs of TCAH feeding damage (i.e. girdling of leaf petioles). At each vineyard transect point, 5 randomly selected vines will be visually inspected each month for leaf girdling. Total leaf girdles per vine will be recorded.

Objective 3. Follow disease progression in established vineyard plots to collect preliminary data on field epidemiology.

<u>Large Block Mapping (1 site, 2009-2015)</u>

We have been studying grapevine leafroll disease (GLD) movement at one particular site in Napa Valley, beginning in 2009. The block is a 20 ha newly planted (in 2008) block of Cabernet Sauvignon. Each year in September, incidence of GLD and more general "red leaf" symptoms were mapped at this site and location recorded with GPS. As early as 2009, many of the vines displayed "red leaf" symptoms but tested negative for grapevine leafroll-associated virus (GLRaV). In our subsequent surveys these symptoms appeared to spread through the vineyard, although most these "red leaf" symptom vines continued to test negative for GLRaV over this period. We began testing vines for both GLRaV and grapevine red blotch-associated virus (GRBaV) in 2014 and found that 136 vines tested positive for red blotch, 9 tested positive for leafroll and 11 tested positive for both red blotch and leafroll. Plant material from the 2015 survey is still in the process of being tested, but we recorded about 250 "red leaf" symptomatic vines, all of which had tested negative for GLRaV in 2014. With the development of new and more complete primers for both leafroll and red blotch,

we are now in the process of re-testing plant material from the 2009-2013 survey to verify whether or not GRBaV is present in the "red leaf" symptom vines that previously tested negative for GLRaV.

In 2016, the "large block mapping" program was replaced with a "small block mapping" program (see below). Monitoring spread of GRBaV in small plots at multiple sites will allow for the comparison of spread patterns across multiple locations, each with their own unique set of features (variety-rootstock combination, environmental factors, insect communities, relation to natural habitats etc.). This type of multi-site comparison could potentially provide novel insights into the spatial and temporal dimensions of GRBaV spread. Smaller blocks does not necessarily mean less data, as the overall number of vines being monitored for GRBaV under this new "small blocks" program is actually greater than in the "large blocks" program.

Small Block Mapping (8 sites, 2015-present)

In September 2015, we began to map and test for GRBaV (using the protocols described previously) at the same 5 established vineyards mentioned in Objective 2. At each site, an area consisting of 6 rows by 20 vines per row (120 vines/site total) was visually evaluated for GRBaV and petiole samples collected from each vine (3 petioles/vine) for diagnostic testing. At some sites canes were sampled instead of petioles because samples were collected after vines had dropped their leaves. Cane samples consisted of a composite sample of three canes per vine. Each piece of cane material was taken from between nodes 1-5.

The idea is to return to these same blocks in September 2016 and 2017 to repeat this detailed mapping in order to evaluate if the virus appears to be spreading from vine to vine. In October 2015 we learned that one of these established vineyard sites (Napa – Yountville) was going to be removed due to intolerable levels of GRBaV incidence. In December 2015, we located an alternate site (Napa – Oakville 2) to replace the lost site and conducted the same detailed mapping protocol. Unfortunately, this site was also subsequently replanted at the end of 2016, as was the Napa – Oakville 1 site. A new site has been located to replace these lost sites (Napa – Mt. Veeder). In fall 2016, additional sites in the Sierra Foothills were added to the mapping effort. See Table 3 for a summary of the sites sampled over the past 2 years. Sampling in 2016 was expanded to include separate samples of 3 and 6 petioles from each vine to evaluate the sensitivity of virus detection. Visual evaluations were eliminated in 2016 as well, since it is now well-known that symptom expression does not correlate with GRBaV infection.

Table 3. Sites sampled in the small block mapping program.

Site (County – Area)	Year Mapped		
	2015	2016	2017
Napa – Carneros	3 petioles	3 + 6 petioles	
Napa – Mt. Veeder		3 + 6 petioles	
Napa – Oakville 1	3 petioles	3 + 6 petioles	Replant
Napa – Oakville 2	3 canes	Replant	
Napa – St. Helena	3 petioles	3 + 6 petioles	
Napa – St. Helena	3 petioles	3 + 6 petioles	
Napa – Yountville	3 petioles	Replant	
Amador – Sutter Creek		3 canes	
El Dorado – Placerville		3 canes	

Red Blotch Titers Survey

Concerns about the possibility of low GRBaV titer levels in potted vines used in the transmission trials (see Objective 1) led us to initiate a broader survey to quantify GRBaV titer levels throughout grapevines over the course of the year. Between April 2015 – May 2016, plant material was collected each month from various parts (roots, trunk, canes etc.) of at least 10 GRBaV positive vines at each of 3 vineyard sites in Napa Valley. The goal is understanding whether the virus localizes in certain regions of the grapevine during the year. If

this is the case, it could improve the focus of our search for novel vectors (i.e. vectors that preferentially feed on parts of the vine with high GRBaV titer levels).

PUBLICATIONS PRODUCED AND PENDING, AND PRESENTATIONS MADE THAT RELATE TO THE FUNDED PROJECT – July 2016 – February 2017.

"Searching for potential vectors of grapevine red blotch-associated virus." 2016 Pierce's Disease Research Symposium. San Diego, CA, Dec. 2016 (Poster) ***Dr. Kent Daane and Dr. Houston Wilson***

"Mealybug pests in California vineyards – their role in the transmission of plant pathogens and their controls." Unified Wine and Fruit Outreach Day. Walla Walla, WA, Jan. 2017. ***Dr. Kent Daane***

RESEARCH RELEVANCE STATEMENT

Findings from this research help improve our understanding of GRBaV transmission and field epidemiology in order to develop better recommendations and control programs for commercial growers. Greenhouse trials to evaluate GRBaV transmission by both suspected and novel insects aim to clarify which, if any, insects can transmit this virus and, if so, how efficiently they do so. Similarly, screening insects from field sites with suspected spread of GRBaV allows us to identify additional novel vectors for subsequent evaluation in greenhouse trials. Testing plant material from non-crop species in the natural habitats surrounding vineyards provides new information on potential reservoirs of GRBaV outside of the vineyard. Closer evaluation of the insects associated with non-crop reservoirs of GRBaV will further reinforce efforts to identify novel vectors. We are now also conducting closer evaluations of the lone insect vector that has been identified to date, the three-cornered alfalfa hopper. This includes studies of transmission efficiency and seasonal population trends in vineyards, which will improve our understanding of how rapidly this insect could potentially spread GRBaV under field conditions. Detailed mapping of GRBaV at multiple sites where spread of this virus has been suspected will allow us to confirm if this is the case as well as evaluate spatial trends of infected vines relative to pertinent landscape features, such as riparian habitats or adjacent vineyard blocks with high levels of GRBaV infection. Finally, quantifying GRBaV titer levels throughout the vine will aid in the search for novel vectors that may feed on specific areas of the vine where the virus is concentrated.

LAYPERSON SUMMARY

Grapevine Red Blotch-associated Virus (GRBaV) is a newly identified vineyard pathogen causing vine damage similar to other Grape Leafroll Diseases (GLD). There has been some initial laboratory evidence that leafhoppers are a potential vector of GRBaV; however, there have been mixed reports of possible vector-borne movement in vineyards and recent work at UC Davis identified an insect called a 'tree hopper' as a likely vector. Our goal is to identify and test potential vectors to provide concrete evidence that organisms can or cannot move GRBaV among vines. This work must be completed to develop a control program for "Red Blotch" and develop accurate information on the epidemiology of this newly reported pathogen. To date, we have tested many leafhoppers (which are common in vineyards), grape whitefly, mealybugs (which are also commonly found in vineyards), blue-green sharpshooter, and foliar form grape phylloxera. None of these insects have moved the pathogen from an infected plant or plant material to a clean plant in laboratory studies. We have begun transmission experiments evaluating a treehopper (three-cornered alfalfa hopper) to determine its efficiency. Our field studies have surveyed insects and potential non-crop reservoirs in vineyards with suspected movement of red blotch. None of the herbivores in this survey have tested positive for the virus responsible for red blotch, although many samples are still being tested in the laboratory. We have recently initiated another field study to evaluate seasonal population trends of the three-cornered alfalfa hopper in vineyards adjacent to large patches of natural habitat, where it is thought to overwinter and then migrate into vineyards. We have also conducted detailed mapping of red blotch in vineyards where movement of the virus is suspected to evaluate spatial trends related to virus spread. Similarly, we are also mapping

GRBaV titers levels within the vine itself to help with the identification of novel vectors which may preferentially feed on regions of the vine where the virus is localized.

STATUS OF FUNDS

As of the February 2017 UC Berkeley invoice (from KM Daane's "PI Portfolio" of funds spent), there was $69,811 remaining of the total $242,044 awarded for this project. This is behind schedule for Years 1 and 2 spending because of a late start in getting a fund number from the UC Berkeley SPO office, and because our primary technician working on virus identification left for graduate school in May 2016. We have now hired a new molecular biology specialist (Kei-Lin Ooi) and she is in the process of evaluating all of the 2016 and some of the 2015 plant and insect samples. We plan to request an extension for the use of these funds, which places the end-date for this project on June 30, 2018.

SUMMARY AND STATUS OF INTELLECTUAL PROPERTY ASSOCIATED WITH THE PROJECT

There is no intellectual property associated with this project.

REFERENCES CITED

Almeida, R. P. P., and A. H. Purcell. 2003a. *Homalodisca coagulata* (Hemiptera, Cicadellidae) transmission of *Xylella fastidiosa* to almond. Plant Disease 87: 1255-1259.

Almeida, R. P. P., and A. H. Purcell. 2003b. Transmission of *Xylella fastidiosa* to grapevines by *Homalodisca coagulata* (Hemiptera : Cicadellidae). Journal of Economic Entomology 96: 264-271.

Al Rwahnih, M. A., A. Dave, M. M. Anderson, A. Rowhani, J. K. Uyemoto, and M. R. Sudarshana. 2013. Association of a DNA Virus with grapevines affected by red Blotch disease in California. Phytopathology 103:1069–1076.

Bahder B.W., Zalom, F.G., Jayanth, M., and Sudarshana M.R. (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., J. R. Thompson, M. Fuchs, and K. L. Perry. 2012. Complete genome sequence of a new circular DNA virus from grapevine. Journal of Virology 86:7715–7715.

Krenz, B., J. R. Thompson, H. McLane, M. Fuchs, and K. L. Perry. 2014. Grapevine red blotch-associated virus is widespread in the United States. Phytopathology.

Poojari, S., O. J. Alabi, V. Y. Fofanov, and R. A. Naidu. 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.

Sharma, A. M., J. B. Wang, S. Duffy, S. M. Zhang, M. K. Wong, A. Rashed, M. L. Cooper, K. M. Daane, and R. P. P. Almeida. 2011. Occurrence of grapevine leafroll-associated virus complex in Napa Valley. PLoS ONE 6.

Tsai, C. W., J. Chau, L. Fernandez, D. Bosco, K. M. Daane, and R. P. P. Almeida. 2008. Transmission of grapevine leafroll-associated virus 3 by the vine mealybug (*Planococcus ficus*). Phytopathology 98: 1093-1098.

Tsai, C. W., A. Rowhani, D. A. Golino, K. M. Daane, and R. P. P. Almeida. 2010. Mealybug transmission of grapevine leafroll viruses: an analysis of virus-vector specificity. Phytopathology 100:830–834.