Title of Report: Final report for CDFA Agreement Number 15-0428-SA

"SEARCHING FOR POTENTIAL VECTORS OF GRAPEVINE RED BLOTCH-ASSOCIATED VIRUS"

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REPORTING PERIOD: The results reported here are from work conducted July 2015 – June 2018.

ABSTRACT

Grapevine Red Blotch-associated Virus (GRBaV) is a newly identified vineyard pathogen causing vine damage similar to other Grape Leafroll Diseases (GLD). Initial laboratory evidence implicated a common leafhopper as a potential vector of GRBaV. However, a recent study has shown that a membracid, the three-cornered alfalfa hopper (*Spissistilus festinus*) (TCAH) can indeed transmit GRBaV. Our goals were to (a) test potential vectors commonly found in vineyards (leafhoppers, mealybugs etc.), (b) identify additional, novel candidate insect vectors, (c) test non-crop plants outside of vineyards as potential reservoirs of the virus, (d) document spread of GRBaV in commercial vineyards and (e) quantify virus titre levels throughout the grape vine over the course of the growing season. This work was undertaken to develop a GRBaV control program and provide accurate information on the epidemiology of this newly reported pathogen, its insect vectors and non-crop hosts. Our field studies have identified wild grape (*Vitis* spp.) as the sole reservoir of GRBaV outside of vineyards. Insects that have tested positive for the virus include *E. elegantula*, *Colladonus coquillet*, *Acinopterus angustatus*, *Scaphytopius* spp. and of course TCAH. We conducted laboratory transmission experiments with leafhoppers (*Erythroneura elegantula*, *E. variabilis*, *E. ziczac*), grape whitefly (*Trialeurodes vittatas*), mealybugs (*Planococcus ficus* and *Pseudococcus maritimus*), blue-green sharpshooter (*Graphocephala atropunctata*), and foliar form grape phylloxera (*Daktulosphaira vitifoliae*) – none of which moved the pathogen from an infected plant or plant material to a clean plant. The key findings from this project to date are that none of the common, abundant insects tested have been shown to transmit this virus, and the virus appears to be limited to plants in the genus *Vitis*. As such, rapid spread of this virus by an insect in commercial vineyards seems unlikely, and current movement is dependent on pathogen and TCAH abundance in or near vineyards. Additional results are yet to come, as many samples still need to be tested in the laboratory.

LAYPERSON SUMMARY

Grapevine Red Blotch-associated Virus (GRBaV) is a newly identified vineyard pathogen that leads to negative impacts on fruit quality similar to other Grape Leafroll Diseases (GLD). The goals of this project were to

(a) conduct transmission experiments with insects commonly found in vineyards (leafhoppers, mealybugs etc.), (b) identify which vineyard insects acquire GRBaV, (c) test non-crop plants outside of vineyards for GRBaV, (d) document spread of GRBaV and (e) quantify infection levels throughout the grape vine over the course of the growing season. To date, the tested leafhoppers (*E. elegantula, E. variabilis, E. ziczac*), grape whitefly (*Trialeurodes vittatas*), mealybugs (*Planococcus ficus* and *Pseudococcus maritimus*), blue-green sharpshooter (*Graphocephala atropunctata*), and foliar form grape phylloxera (*Daktulosphaira vitifoliae*) did not transmit the virus under our laboratory conditions, although many of tested leafhoppers 'acquired' the pathogen after feeding on plants. The sole reservoir of GRBaV outside of vineyards is wild grape (*Vitis* spp.) and insects that tested positive for the virus include *Erythroneura elegantula, Colladonus coquillet, Acinopterus angustatus, Scaphytopius* spp. and of course TCAH, the only known vector identified to date. The key findings from this project are that none of the pest insects abundantly found in vineyards have been shown to transmit this virus, and the virus appears to be limited to plants in the genus *Vitis*. As such, rapid spread of this virus by an insect in commercial vineyards seems unlikely and would be dependent on abundances of the pathogen and the known vector (TCAH) in or near the vineyard.

INTRODUCTION

In 2006 an increase in grapevine leafroll disease (GLD), or vines with "red leaf" symptoms, was observed by growers in vineyards located within Napa Valley, CA. Tissue samples were collected from symptomatic vines and tested by commercial laboratories and UC Davis Foundation Plant Service, with test results 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), described as a circular DNA virus, similar to members of the family Geminiviridae (Krenz et al. 2012). PCR primers were developed (Al Rwahnih et al. 2013) for this pathogen now known as Grapevine Red Blotch-associated Virus (GRBaV), which 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 focused on identifying 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. Our goal is to test potential vectors to provide concrete evidence that different common vineyard insects 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' – changes the needed control programs. We screened common vineyard arthropods, as well as the "long shots" that are potential GRBaV vectors. This information has been disseminated to farmers, PCAs and extension personnel, thereby having a practical, direct and immediate impact on control decisions to "spray or not to spray" and is currently being prepared for journal publication.

OBJECTIVES

Our goal was to screen potential vectors for their ability to acquire and transmit GRBaV and, if a vector is discovered, to determine vector efficiency. Objectives for this research program were 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 data on field epidemiology.

RESULTS AND DISCUSSION

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

Initial Transmission Trials with Potted Vines. 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).

Canes were collected from Cabernet Sauvignon (clone 6) and Cabernet Franc (clone 04) vines in vineyard blocks where vines were 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 showed none of the tested 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*]) transmitted GRBaV to uninfected grape vines. Inoculated vines from these trials were 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 (note that in other trials leafhoppers have tested positive).

<u>"Bouquet" Transmission Trials</u>. Because of the poor acquisition of the GRBaV pathogen by insects, the experiment protocols 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. The new approach utilized "bouquets" of mature grape leaves from GRBaV-positive vines at field sites that were not sprayed with insecticides. Each bouquet consisted of ten mature grape leaves held in a 16 oz. plastic container that contained moist perlite. All leaves were collected from GRBaV-positive vines (nodes 1-5) in an established vineyard in Napa County. Bouquet degradation was initially evaluated by testing petioles for GRBaV 6-48 hours after collection (no degradation of the petioles during the test period was found). We also increased the number of insects tested to a minimum of 100 insects per replicate (when possible) and 10 replicates per treatment.

We 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 days) and an IAP of 72 hours (3 days). Clip-cages (7 cm diameter x 2 cm height) were used to confine 10 insects/leaf to each bouquet (100 insects/bouquet). Bouquets with insects were placed in a 61 x 61 x 61 cm BugDorm cage and there was a total of 10 replicates per treatment. After the 48-hour AAP, clean potted vines were introduced into the cages. The clip cages were then removed, thus allowing the insects to move onto the clean vine. Bouquets were also removed at this time, after ensuring that they were free of the candidate vectors. Petioles from the bouquets were then collected for GRBaV testing as well as a 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 >15 galls per 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 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.

Results from the "bouquet" trials showed 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. Leafhopper from these trials have tested positive for the GRBaV virus, as has field collected material, but insect acquisition does not imply that the insect can aid in the movement of the pathogen among plants.

Conclusion – No Transmission Observed to Date. We have evaluated a total of 7 vector candidates (all commonly found in vineyards), 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).* To date, none of the candidate vectors have transmitted the GRBaV from an infected plant to a clean plant. We were concerned that the initial trials had either plants with too low a virus titre, or the trials used too few insects; however, adjustments were made for the 'bouquet' trials to offsets these concerns and the results (no transmission) ere the same.

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

Vineyard Insect and Non-Crop Plant Survey. We used the same methodologies described for Objective 1 to screen lessor known vineyard organisms or unlikely vectors. Insects were collected 1x/month (May 2015 – May 2016) 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.

As a complement to the insect collection and testing, plant material was also collected from non-crop vegetation and tested for GRBaV 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.).

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. So far, most of the plant material has tested negative for GRBaV. One exception to this is wild grape, which has tested positive across multiple sites, indicating a potential role of this plant in the spread of GRBaV into commercial vineyards.

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. With regards to the insects, many novel families, genera and/or species have been collected, especially many different types of leafhoppers (Cicadellidae), including the genera *Aceratagallia* sp., *Acinopterus* sp., *Alconeura* sp., *Colladonus* spp., *Empoasca* spp., *Macrosteles* sp., *Osbornellus* sp., *Scaphytopius* spp., as well as the species *Deltocephalus fuscinervosus, Dikrella californica*, and *Euscelidius schenki*. Other organisms include specimens from the families Acanaloniidae, Cixidae, Membracidae, Miridae, Lygaeidae, Psyllidae, and Tingidae. While many of these samples are still in the process of being tested, some individuals of *Colladonus coquillet, Acinopterus angustatus, Scaphytopius* spp. and of course three-cornered alfalfa hopper (*Spissistilus festinus*) (TCAH), a known vector of GRBaV, have tested positive for GRBaV. It is critical to note here that an insect's ability to pick up this virus does not necessarily mean that it is able to transmit the virus to a grape vine, and further testing is absolutely needed to verify whether any of these organisms (aside from TCAH, a known vector) can successfully transmit GRBaV.

Reconciling the findings from this plant and insect survey, we summarized the insect community found on wild grapes in our survey (Table 1). 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 Apocrita (parasitoid wasps), spiders, Formicidae (ants), *Empoasca* spp., Coleoptera (beetles), *Chrysoperla* sp. (green lacewings), *E. variabilis* (Variegated leafhopper), *Osbornellus* sp., Psocoptera (book lice), Trichoptera (caddisflies), aphids and Miridae. From this group, only leafhoppers, 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 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 Apocrita, Lepidoptera, Lygaeidae, *Spissistilus festinus* (TCAH), 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* (TCAH), 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 plants. 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, we have conducted GRBaV transmission experiments with *E. elegantula* and *E. variabilis*, which represent two of the most 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. That said, both TCAH and *Scaphytopius* spp. tested positive for GRBaV and are found on both wild and cultivated grapes, albeit in very low densities. Studies have now demonstrated TCAH ability to vector GRBaV between grape vines, and the additional data now presented in this report indicates that it would be worthwhile to evaluate Scaphytopius spp. as a vector, along with the other novel candidate vectors *Colladonus coquillet* and *Acinopterus angustatus.*

While it is notable that TCAH, 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 densities encountered in vineyards, data on TCAH host plant associations (Fig. 3) provides new information on population dynamics in vineyards. TCAH was primarily found in the late spring on groundcovers in and around the vineyard, which included various weedy grasses and overwintering grass/legume cover crops. As groundcovers died down, TCAH was intermittently found in low abundance on wild grape, wine grape, toyon (*Heteromeles arbutifolia*) and coast oak (*Quercus agrifolia*). These are not necessarily reproductive hosts and further work is needed to better understand the role of non-crop habitats nears vineyards in the TCAH life cycle.

Table 1. 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* (TCAH)	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%
	Apocrita (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%

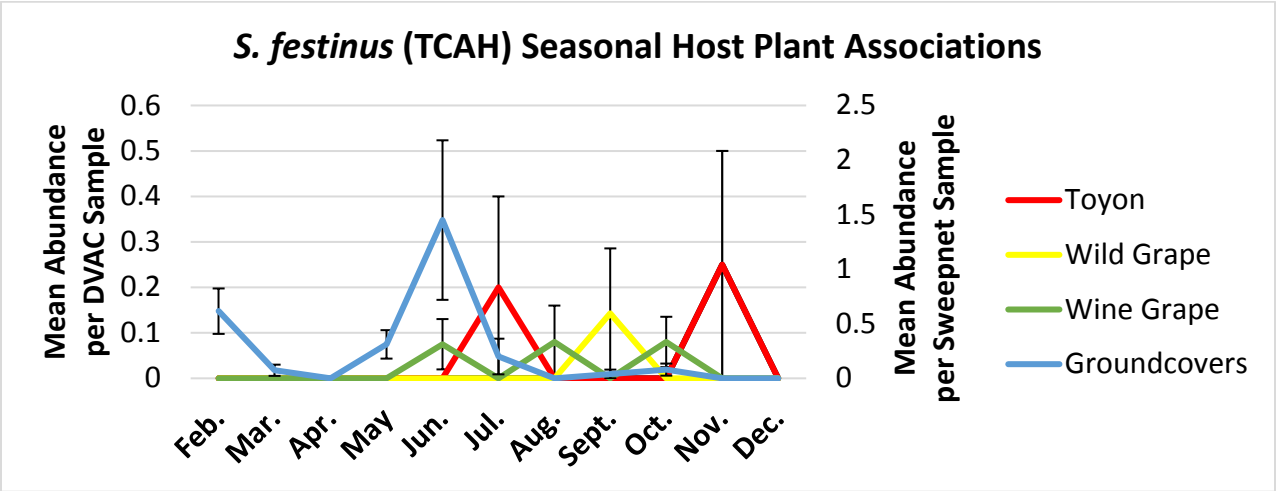

Fig. 3. Seasonal host plant associations of *S. festinus* (TCAH) in North Coast vineyards. High densities of TCAH were found on groundcovers in the late spring and then intermittently on wild grape, wine grape, coast oak and toyon. Plant species shown are not necessarily reproductive hosts. Right Y-axis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

Establishing Colonies of Novel Vectors. Due to the low abundance of novel candidate vectors (e.g. *Aceratagallia* spp., TCAH, *D. fuscinervosus*), we have been working to establish colonies of these insects at the UC Berkeley greenhouse facilities to rear large populations suitable for GRBaV transmission experiments, which typically require >200 individuals per trial. Data on some of these species are scant, including reproductive hosts. As such, in spring 2016 and 2017 we collected candidate species from vineyards and began to evaluate host plant preferences. So far, we have seen successful reproduction of *Aceratagallia* sp. and *Euscelidius schenki* on some hosts. We also collected large populations of TCAH from alfalfa fields and now have a reproductive population in our colonies, which is being used for ongoing projects.

Transmission Experiment with TCAH. GRBaV transmission experiment were conducted with field collected TCAH, and this work is continuing. 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 TCAH 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. The initial results have shown TCAH adults tested positive for the virus after the acquisition period. The vines are now being held for a 2-year period and are being tested for GRBaV every 4 months. While it has been demonstrated that TCAH can vector GRBaV (Bahder et al. 2016), our goal is to first confirm these findings and then evaluate transmission efficiency under laboratory and field conditions. This project is continuing with new funding.

Evaluating TCAH Overwintering Habitat and Seasonal Activity in Vineyards. With the confirmation of TCAH as a known vector of GRBaV, new information is needed on the seasonal ecology of this organism in vineyards. As such, we have used other funds to establish a study to evaluate overwintering habitat and seasonal population trends of TCAH in North Coast vineyards. This study was established in winter 2017 and will be continue into spring 2019 (new funding was obtained). While this work is not part of the stated objectives for the CDFA project (15-0428-SA), we present the preliminary data here because to its relevance to the project objectives.

Overwintering Habitat of TCAH. Ground covers and other non-crop plants in natural habitats adjacent to vineyards were sampled in March 2018 to identify TCAH overwintering habitat use. Sampling is being conducted in the natural habitats adjacent to Napa and Sonoma County vineyards, using 4 sites sampled each month. Natural habitat consists of patches of riparian and/or oak woodland habitat > 400 m^2. Sweep-nets are being 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 are 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 are also

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 of TCAH in Vineyards. In February 2017, we established a study in Napa 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 5 total study sites. All vineyard blocks are red varietals that are at least 5 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 m apart) that extend out from the riparian or oak woodland habitat (i.e. "natural habitat") into the vineyard. Each transect is 160 m long – going 10 m into the natural habitat and 150 m into the vineyard. Along each transect samples are taken at the interior of the natural habitat (10 m into the habitat) as well as at the edge and interior of the vineyard (10 and 150 m into the vineyard, respectively). The edge of the vineyard and natural habitat are typically separated by a roadway or path that is about 5 m wide. Densities of TCAH, *Erythroneura* leafhoppers and other hemipterans are being 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) are placed at each transect point. In the vineyard, one trap is placed in the vine canopy (approximately 4 feet above the ground surface) and another trap is hung from irrigation lines (approximately 1 foot the above 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 4 feet and the other 1 foot above the ground surface). Traps are replaced approximately every 2 weeks between March 2017 and March 2019. Sweep-nets are used to sample ground covers. At each transect point, a set of 30 unidirectional sweeps are collected from the groundcovers using a 30.5 cm diameter sweep-net (BioQuip Products, Rancho Dominguez, CA). Proportion of ground cover to bare soil is recorded along with species composition and ground cover status (i.e. proportion of cover that was still green/healthy). A modified beat-sheet is used at each transect point to sample the canopy of 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 are evaluated for signs of TCAH feeding damage (i.e. girdling of leaf petioles). At each vineyard transect point, 1 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 – 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 (YST), followed by sweep-nets and then beat sheets. While there was no clear gradient of TCAH activity across the transect points, densities on the YSTs 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 – August.

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

Large Block Mapping. We have been studying grapevine leafroll disease (GLD) movement for the past seven years at a 20 ha Cabernet Sauvignon block in Napa County, planted in 2008. 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 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 do 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). 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 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. Sampling in 2017 was somewhat reduced in the North Coast due to several sites being replanted. In total we have 2 years of mapping data from 4 sites (2 North Coast, 2 Sierra Foothills) and 3 years of mapping data from 2 sites (both North Coast).

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

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

Red Blotch Titers Survey. Concerns about the possibility of low GRBaV titer levels in potted vines used in the transmission trials (see Objective 1) led us to initiate a broader survey to quantify GRBaV titer levels throughout grapevines over the course of the year. Between April 2015 – May 2016, plant material was collected each month from various parts (roots, trunk, canes etc.) of at least 10 GRBaV positive vines at each of 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). This plant material is stored and will be tested later, when we get through the back log of insect samples collected.

CONCLUSION

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

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FUNDING AGENCIES

Funding for this project was provided by the “Napa County Winegrape Pest and Disease Control District” in 2013, the work was continued in 2014 with American Vineyard Foundation (AVF) and Napa County funds and was funded by this grant (CDFA Agreement Number 17-0460-800-SA) from July 2015 – June 2018.