Title of Project: Ecology of grapevine red blotch virus

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Time period covered by the report
July 1, 2018 to March 15, 2019

List of objectives
- Characterize attributes of the spread of grapevine red blotch virus by *Spissistilus festinus*
- Complete a study on the potential role of vineyard cover crops in disease epidemiology
- Complete an investigation on the experimental host range of grapevine red blotch virus and *S. festinus*
- Disseminate research results to the grape and wine industry, and to farm advisors

Description of activities conducted to accomplish each objective, and summary of accomplishments and results of each objective

To address objective #1 and characterize attributes of the spread of grapevine red blotch virus (GRBV) by Spissistilus festinus, we expanded on earlier studies to investigated the transmission mode of GRBV by *S. festinus*. By analogy with other members of the family *Geminiviridae*, we hypothesized that GRBV does not replicate within *S. festinus*. In order to test this hypothesis, a time course experiment was conducted with 32 adult specimens that were caged on GRBV-infected grapevines for two weeks. Specimens were then transferred to alfalfa to remove access to GRBV for up to 12 days. The day of transfer was time 0 (T_0). At 3-day time intervals (T_1=3 days, T_2=6 days, T_3=9 days, T_4=12 days), cohorts of six adults which acquired GRBV were removed and immediately frozen at -80°C. DNA was extracted from individual specimens and tested for GRBV using qPCR (Setiono *et al.* 2018). Fifty ng of DNA was added to each reaction, and assays were run in triplicate. An external standard was included on each qPCR plate using a dilution series containing 1×10^9 to 1×10^2 copies of the GRBV monomer, in order to

obtain a standard curve of GRBV copy number. GRBV was quantified using the comparative Cq method and standard curve method described by Setiono *et al.* (2018). Variance among groups was estimated by ANOVA in R studio. Both quantification methods showed a decreasing GRBV titer over time in *S. festinus* after GRBV-exposure is removed (Figure 1). These results revealed a non-propagative transmission mode of GRBV by *S. festinus.*

In parallel, we investigated the distribution of GRBV-infected vines in a 4-acre Cabernet Sauvignon vineyard in Napa Valley, CA and a 3-acre Cabernet franc vineyard on Long Island, NY. The two study vineyards were planted in 2008. The Cabernet Sauvignon vineyard was established with clones 4 and 169. Vines of the two Cabernet Sauvignon clones were purchased from two different nurseries. Almost every vine of clone 4 exhibited typical red blotch symptoms soon after planting whilst vines of clone 169 remained asymptomatic. The California vineyard was visually surveyed for disease symptoms in October of 2017 and 2018. Additionally, leaf and petiole samples (6-8 leaves per vine, 3-4 from each side of the trunk) were collected on selected Cabernet Sauvignon vines for GRBV testing by multiplex polymerase chain reaction (PCR) (Krenz *et al.* 2014).

Fig. 1. Reduction in GRBV DNA in *S. festinus* over time after exposure to GRBV source was removed. Two methods of qPCR analysis were used: (Top) GRBV copy number compared to external reference and (Bottom) Comparative Cq method of Rep GRBV target and 18S *S. festinus* target). Following feeding on GRBV-infected grapevine for two weeks, specimens were transferred to alfalfa and tested immediately (T0), and at three (T1), six (T2), nine (T3) and 12 (T4) days post-transfer. Letters above standard error bars indicate significance groups, as determined by ANOVA in R.

Based on visual assessment in the Cabernet Sauvignon in California, red blotch symptoms were apparent in very few (<15) vines throughou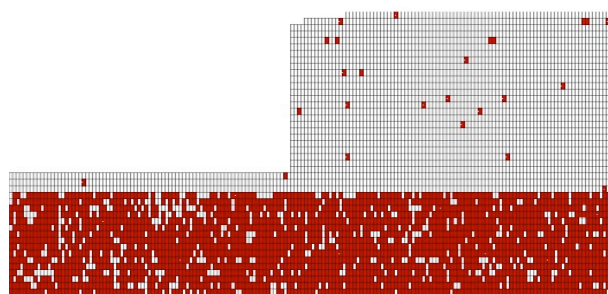t clone 169 in the years between planting in 2008 and our survey in 2017 (Figure 2). In the vineyard section established with clone 169, disease incidence increased from 0.86% (24 of 2799 vines infected) in 2017 to 1.2% (33 of 2799 vines infected) in 2018. This result was consistent with limited spread of GRBV in vines of clone 169 despite the availability of a large initial source of inoculum (estimated 48% at planting), confined primarily to adjacent vines of clone 4 (calculated 81% of clone 4 vines or 48% of the entire Cabernet Sauvignon vineyard in 2017) (Figure 2).

Fig. 2. Distribution of diseased vines in a Cabernet Sauvignon vineyard with clone 4 (bottom) and clone 169 (top).

In the 3-acre Merlot vineyard in New York vineyard, red blotch disease symptoms were observed in 2009, the year after planting. Disease incidence could not be reliably assessed visually in this vineyard because foliar symptomatology was confounded by extensive leaf
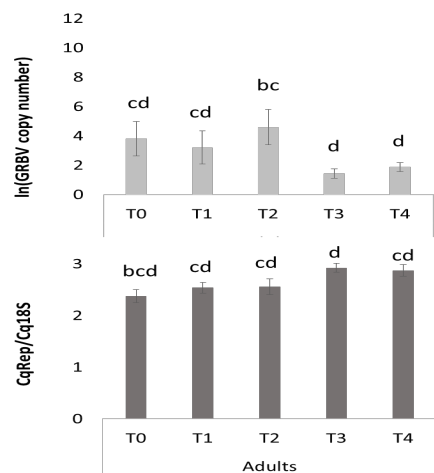
removal in the fruiting zone by the vineyard manager at a post-véraison development stage, thereby removing the oldest leaves where foliar red blotch symptoms are pronounced. Nonetheless, a cursory visual assessment suggested an overall 40% disease incidence at planting. Leaf and petiole samples were collected from a subset of 65 vines in late August or September of each year from 2014 to 2018. In 2017-2018, samples were collected from only vines which tested negative for GRBV in the previous years. All petiole samples were tested for GRBV using multiplex PCR (Krenz *et al.* 2014). No indication of spread in the Merlot vineyard was obtained as all vines that tested negative in 2014 continued to test negative for GRBV through the 2018 season.

To investigate an eventual association between the population of *S. festinus* and rate of GRBV spread, yellow sticky cards were placed at the east end in the Cabernet Sauvignon vineyard in California (closest to a 5-acre Cabernet franc vineyard) from April thru November in 2017-2018 on diseased and asymptomatic vines of clones 169 and 4, across six rows of each clone (Figure 3, white grid). Sticky cards were rotated on a weekly basis. Each trap was analyzed for the presence of insects to establish a census population and identify them at the species level by using morphological parameters. Then, a sub-set of each insect family, genus or species that was caught was removed from the traps and tested for the presence of GRBV by multiplex PCR. Emphasis was on the four species that were identified as vector (*S. festinus*) or as vector candidates (*Colladonus reductus*, *Osbornellus borealis*, and *Melanoliarus* sp.).
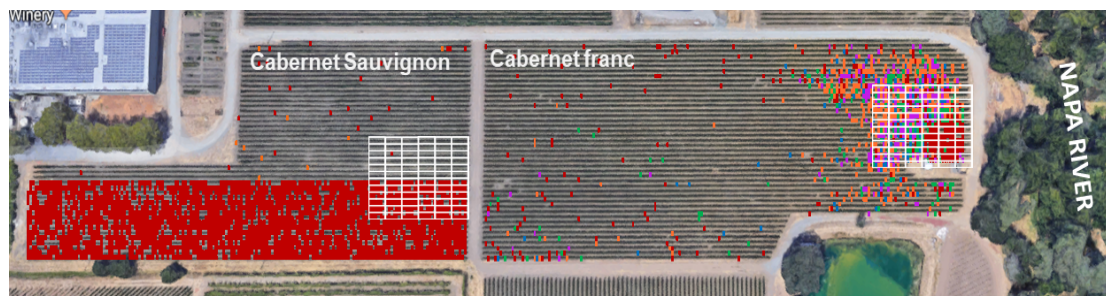

Fig. 3. Map of GRBV incidence in a 4-acre hectare Cabernet Sauvignon and a 5-acre Cabernet franc vineyard within ten years-post planting. In the Cabernet Sauvignon vineyard, red overlay indicates diseased vines in 2017 at the onset of the study and orange indicates new GRBV infections in 2018. In the Cabernet franc vineyard, red overlay indicates initial disease incidence at the onset of the study in 2014. Green, blue, purple, and orange indicate new infections in 2015, 2016, 2017, and 2018 respectively. White grids indicate areas where insect survey was conducted in 2015-2016 in the Cabernet franc (Cieniewicz *et al.* 2018) and in 2017-2018 in the Cabernet Sauvignon.

Results indicated a substantially lower population of *S. festinus* in the Cabernet Sauvignon (N = 3) vineyard (this study) compared to the Cabernet franc vineyard (N = 25) (Cieniewicz et al., 2018a). For Cicadellidae, more *C. reductus* (N = 63) but less *O. borealis* (N = 6) were found in the Cabernet Sauvignon vineyard (this study) compared to the Cabernet franc vineyard (N = 23 and 31) (Cieniewicz et al., 2018a). For the *Melanoliarus* sp., none was caught in the Cabernet Sauvignon vineyard (this study) while eight was found in Cabernet franc vineyard (Cieniewicz et al., 2018a). Additionally, only one out of three (33%) *S. festinus,* 19 out of 63 (30%) *C. reductus* and four out of six (67%) *O. borealis* tested positive for GRBV in PCR. These results revealed a lower population of *S. festinus*, *O. borealis* and *Melanoliarus* sp. that ingested GRBV in the Cabernet Sauvignon vineyard (this study) compared to the Cabernet franc vineyard (Cieniewicz et al., 2018a). Similar work with insect traps in the Merlot vineyard in New York in 2017-2018 revealed no *S. festinus*; neither were any *C. reductus*, *O. borealis*, or *Melanoliarus* sp. caught on sticky traps. Altogether, these results validated our hypothesis about an

association between GRBV spread and the dynamics of *S. festinus* populations in a vineyard ecosystem.

To address objective #2 and complete a study on the potential role of vineyard cover crops in disease epidemiology, we surveyed cover crop species, particularly legume species, in 13

vineyards of Sauvignon blanc, Cabernet franc, Merlot and Cabernet Sauvignon in California (Figure 4). These vineyards were selected for this study because they are infected with GRBV or proximal to vineyards infected with GRBV. A total of over 500 legume samples including fava beans (*Vicia faba*)*,* purple vetch *(Vicia americana),* red and white clover (*Trifolium spp.*), and field peas (*Pisum sativum* subsp. *Arvense)* and other non-legumenous species (barley, oats, rye and grasses) from vineyard middle-row cover crops were collected in early March in 2017 and 2018. These samples were tested for GRBV by multiplex PCR (Krenz et al., 2014).

Fig. 4. Vetch, birdsfoot trefoil and other cover crop species in vineyard middle rows in spring 2017.

Results showed that none of the samples tested was positive for GRBV in PCR. These findings are consistent with similar work that did not yield any positive for GRBV in 2014-2016. This work suggested that legumes or other cover crop species have limited, or any, role as reservoirs of GRBV and likely do not contribute to the epidemiology of red blotch disease in vineyards.

The same 13 diseased California vineyards were surveyed for *S. festinus* and the other three vector candidates, i.e. *C. reductus, O. borealis,* and *Melanoliarus* sp. (Cieniewicz *et al.,* 2018a) by sweep netting. Time spent sweep-netting ranged from 15 to 50 minutes in duration for each vineyard. Sweep-netting surveys were essentially done in the morning. In spite of extensive sweep netting efforts for a ntotal of more than seven hours, no specimen of *S. festinus* or any of the other vector candidates was caught. This result suggested that the vector and vector candidates of GRBV are likely not abundant in vineyard middle row cover crops in spring. It will be interesting to see if similar findings are confirmed in other vineyard ecosystems.

To address objective #3 and complete an investigation on the experimental host range of GRBV and S. festinus, we used our GRBV infectious clones to agroinoculate seedlings of clover, vetch, bean, Medicago and peas by needle pricking. *Nicotiana benthamiana* and *Solanum lycopersicum* 'Florida Lanai' were also included in this study. This is because *N. benthamiana* is a common herbaceous host used in plant virology studies (Goodin *et al.* 2008), and *S. lycopersicum* 'Florida Lanai' has been described as an optimal model host for studying geminiviruses of tomato (Rajabu *et al.* 2018).

Seedlings (4-5 leaf stage) agroinoculated as previously described (Yepes *et al.,* 2018). Negative controls included a mock inoculated (sterile needle) and non-inoculated plant. At seven days post-inoculation (dpi) the inoculated leaves were collected and tested by RT-PCR. At 14 and 21 dpi, leaves were collected from apical (non-inoculated) leaves to test for systemic movement of GRBV. RT-PCR was carried out using primers designed to detect the accumulation of spliced transcripts and the 18S of *S. festinus*. The RT-PCR is critical to determine virus replication in

agroinoculated plants and distinguish virus infection from the GRBV genetic information in *Agrobacterium tumefaciens* carrying the infectious clone (Yepes *et al.,* 2018). Results indicated the accumulation of GRBV spliced transcripts in inoculated leaves of bean, *N. benthamiana* and *S. lycopersicum* by 7 dpi. However, throughout the duration of these experiments, GRBV was not detected in apical (non-inoculated) leaves. This suggests that GRBV is replicating locally in inoculated leaves of these three plant species, but not moving systemically. None of the other herbaceous hosts tested sustained the replication of GRBV.

To examine the reproductive potential of *S. festinus* on different host plants, groups of 10-20 female and male *S. festinus* adults were placed on *Vitis vinifera*, alfalfa and bean in cages in the greenhouse. Treehoppers were evaluated for feeding behavior, oviposition and reproduction, particularly for the emergence of eggs and nymphs. Results showed that *S. festinus* reproduced on the three plant species. However, a reproductive cycle (adult to adult) was only completed on alfalfa and bean. Although eggs and nymphs were observed on *V. vinifera* cv. Syrah, nymphs did not survive and never reached adulthood. In addition, most adults and nymphs died on *V. vinifera* within 3-10 weeks. Therefore, the population of *S. festinus* exposed to *V. vinifera* did not survive whereas those on alfalfa and bean did, and the reproductive rate on these herbaceous hosts was high.

To address objective #4 and disseminate research results to the grape and wine industry, and to farm advisors, research results were communicated to 440 growers, farm advisors, extension educators, crop consultants, researchers, vineyard managers and regulators at the following grower meetings and conventions in California, New York and Ontario, Canada:
Fuchs, M. 2019. Ecology of grapevine red blotch virus. Sonoma Technical Working Group, March 7, Santa Rosa, CA (participants = 150).
Fuchs, M. 2019. Ecology and management of grapevine red blotch virus. Napa Technical Working Group, March 6, Napa, CA (participants = 120).
Cieniewicz, E. and Fuchs, M. 2018. Ecology of grapevine red blotch virus. Cornell Recent Advances in Viticulture and Enology (CRAVE) conference, December 12, Ithaca, NY (participants = 60).
Fuchs, M. 2018. Virus diseases: Why should I care and what can I do? California State University - Fresno, October 3, Jordan College of Agriculture Sciences and technology, Department of Viticulture and Enology, Fresno, CA (participants = 30).
Fuchs, M. 2018. Grape virus research updates. Biennial Grape Research Tailgate Tour, August 30, Niagara-on-the-Lake, Ontario, Canada (participants = 80).

Publication produced and pending, and presentations made that relate to the funded project
Cieniewicz, E., Flasco, M., Brunelli, M., Onwumelu A., Wise, A. and Fuchs, M. 2019. Spread dynamics of grapevine red blotch virus in relation yo *Spissistilus Festinus* populations in vineyards. Viruses, in review.
Cieniewicz, E., Wise, A., Smith, R., Cooper, M, Martinson, T. and Fuchs, M. 2019. Studies on red blotch ecology inform disease management recommendations. Wine Business Monthly, March Issue, pp. 92-102.
Setiono, F.J., Chatterjee, D., Fuchs, M., Perry, K.L. and Thompson, J.R. 2018. The distribution and ability to detect grapevine red blotch virus in its host depends on time of sampling and tissue type. Plant Disease 102:2187-2193.

Cieniewicz, E., Thompson, J.R., Mclane, H., Perry, K.L., Dangl, G.S., Corbett, Q., Martinson, T., Wise, A., Wallis, A., O'connell, J., Dunst, R., Cox, K. and Fuchs, M. 2018. Prevalence and diversity of grabloviruses in free-living *Vitis* spp. Plant Disease 102:2308-2316.

Yepes, L.M. Cieniewicz, E., Krenz, B., Mclane, H., Thompson, J.R., Perry, K.L. and Fuchs, M. 2018. Causative role of grapevine red blotch virus in red blotch disease. Phytopathology 108:902-909.

Perry, K.L., Mclane, H., Thompson, J.R. and Fuchs, M. 2018. A novel grablovirus from non-cultivated grapevine (*Vitis* Sp.) in North America. Archives of Virology 163:259-262.

Cieniewicz, E., Pethybridge S.J., Loeb, G.M., Perry, K.L. and Fuchs, M. 2018. Insights into the ecology of grapevine red blotch virus in a diseased vineyard. Phytopathology 108:94-102.

Cieniewicz, E. and Fuchs, M. 2018. Red blotch disease ecology and management. Appellation Cornell, Research Focus 4:1-7.

Relevance statement, indicating how this project will contribute towards finding solutions to, or dealing with, the pest or disease being addressed

Red blotch was described for the first time on Cabernet Sauvignon at the UC Oakville Research Field Station in 2008 (Calvi 2011; Cieniewicz et al., 2017a; Sudarshana et al., 2015). It is one of the most important viral diseases of grapevine in the United States, contributing to higher acreage being re-planted and shorter re-plant intervals, especially in California. Red or chlorotic blotches on leaves of red- and white-berried *Vitis vinifera* vines, delayed fruit ripening and reduced fruit quality are characteristic of red blotch disease. Poor fruit quality results from interference with the transcriptional and hormonal regulation of ripening (Blanco-Ulate et al., 2017). The estimated economic impact of red blotch ranges from $2,213 to $68,548 per hectare over a 25-year lifespan of a vineyard (Ricketts et. al., 2017).

GRBV is the type member of the recently ratified genus *Grablovirus* in the plant virus family *Geminiviridae* (Varsani et al., 2017). It has a single-stranded DNA genome that codes for six open reading frames (Cieniewicz et al., 2017a; Sudarshana et al., 2015). Analysis of the genetic diversity among GRBV isolates indicated segregation into two phylogenetic groups (Krenz et al., 2014). The majority of isolates belong to the predominant clade 2 and recombination is underlying some of the variation seen among GRBV genomes within clade 1. The two groups of isolates are involved in the etiology of the disease (Yepes et al., 2018).

GRBV is transmissible by grafting, which is likely the most significant mode of dispersal. Since its discovery in 2011, GRBV has been detected throughout the United States (Brannen et al., 2018; Krenz et. al., 2014; Yao et al., 2018), Canada (Poojari et. al., 2017), Switzerland (Reynard et al., 2018), South Korea (Lim et. al., 2016), Mexico (Gasperin-Bulbarela et al., 2018), and India (GenBank accession no. KU522121.1). GRBV was also isolated from numerous table grape accessions at the USDA germplasm repository in Davis, CA (Al Rwahnih et al., 2015a; Thompson et al., 2018), from a herbarium specimen at UC-Davis (Al Rwahnih et al., 2015b), and from free-living grapes in Napa County (Badher et al., 2016a; Cieniewicz et al., 2018b; Perry et al., 2016). While long distance dispersal is attributed to dissemination of infected propagation material, short distance spread within vineyards has thus far only been observed in the western U.S. (Cieniewicz et al., 2017b; Cieniewicz et al., 2018).

GRBV is transmitted by *Spissistilus festinus* [Say] (Membracidae), the three cornered alfalfa treehopper, from infected to healthy vines under greenhouse conditions (Bahder et al., 2016b). The risk this insect poses to vineyard profitability due to virus transmission warrants investigation into *S. festinus* as a vector of GRBV, especially since the extent to which *S. festinus* transmits GRBV in vineyards is poorly understood. Recently, we documented GRBV spread in a 5-acre Cabernet franc vineyard planted in 2008 in Napa County, California, where red blotch symptoms were first observed in 2012 in the vineyard area most proximal to a riparian area. The incidence of diseased plants increased by 1-2% annually from 2014 to 2017. Spatial analysis of diseased plants in each year demonstrated extensive aggregation and strong localized (within vineyard) spread (Cieniewicz et al., 2017b).

In spite of tremendous progress in recent years on the biology and spread of GRBV, research on ecological aspects of the disease is needed. For example, a knowledge of factors affecting disease epidemiology such as the transmission mode, the dynamics of GRBV spread in vineyards, and potential role of legumes commonly used in vineyard middle row cover crop mixes is limited. This research is important to not only better understand disease epidemiology but also to know how insects transmit the virus and to identify alternate plant hosts, particularly among vineyard cover crops. This is a prerequisite for the development of optimal disease management strategies.

Our research provided new insights into the ecology of GRBV. We documented an association between the dynamics of GRBV spread and the population of *S. festinus* by comparatively analyzing the distribution of infected vines overtime and carrying out insect surveys in a 4-acre Cabernet Sauvignon vineyard (0.1% spread annually with a large initial inoculum source estimated at 48% at planting) and a proximal 5-acre Cabernet franc vineyard (2% spread annually and 10% between 2014-2018 and an initial low inoculum source estimated a 1% at planting). In a 3-acre Merlot vineyard in New York, there was no evidence of secondary spread of GRBV over a five-year period (2014-2018), despite a large initial source of inoculum (estimated 40% at planting), and no *S. festinus* was found. None of the legume cover crop species, i.e. bell beans, field peas, vetch, clover, etc., from 13 diseased California vineyard middle rows was positive for GRBV in 2014 and 2018, and no *S. festinus* or other vector candidate species was caught by sweep netting of cover crops in the same 13 diseased vineyards. These results indicated that legume cover crops do likely not serve as reservoir of GRBV and, therefore, do not likely substantially contribute to disease epidemiology in a vineyard ecosystem. The transmission of GRBV by *S. festinus* is nonpropagative, as shown by qPCR in specimens that ingested the virus on infected grapevines and were transferred on alfalfa for gut clearing. This research is essential to inform disease management recommendations. Altogether, our findings suggest that disease management strategies should be aimed at reducing the virus inoculum in vineyards rather than populations of *S. festinus*. Information from this project was disseminated to the wine and grape industry at various venues.

Layperson summary of project accomplishments
Grapevine red blotch virus (GRBV) is a new threat to grape production in the United States. Limited information is available on the spread of GRBV in vineyards. We documented an association between spread dynamics of GRBV and abundance of the three cornered alfalfa hopper (*Spissistilus festinus*) populations in vineyards with a higher rate of spread (2% annually) in a Cabernet franc vineyard with a high population of *S. festinus*, and a substantially lower rate of spread (0.1% annually) in an adjacent Cabernet Sauvignon vineyard with a 10-fold lower

population of *S. festinus*. No evidence of spread was obtained in a Merlot vineyard in New York in 2014-2018 where no *S. festinus* was found. None of the legume cover crop samples, i.e. bell beans, field peas, vetch, clover, etc., collected from 13 diseased California vineyard middle rows tested positive for GRBV in spring 2017-2018. These results indicated that legume cover crops do likely not contribute to red blotch disease epidemiology. Additional work indicated that GRBV does not multiply in *S. festinus* during the transmission process. This research provided insights into the ecology of GRBV that are essential to inform disease management strategies. Based on our current findings, we recommend disease management efforts to focus on the removal of virus inoculum sources rather than a reduction of *S. festinus* populations. Information from this project was disseminated to the wine and grape industry at various venues.

Status of funds
Approximately 65% of the allocated budget has been expended at the time of the interim report. Funds were spent for salaries of key personnel (graduate student assistant and technician) involved in the research, supplies, growth chamber and greenhouse rent, travel from labs to and from vineyards for sample collection, and travel to grower's meetings and other venues to present research progress.

Summary and status of intellectual property associated with the project
No intellectual property is associated with the project.

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