INTERIM PROGRESS REPORT FOR CDFA AGREEMENT NUMBER 17-0517-000-SA

Title of Project: Ecology of grapevine red blotch virus

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Introduction

Red blotch disease 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). Diagnosis based on symptoms can be challenging because of several confounding factors, including striking similarities between foliar symptoms elicited by red blotch and leafroll diseases, as well as several other biotic and even abiotic factors. Because symptom variation makes visual diagnosis of diseased vines difficult, only DNA-based assays are reliable for accurate diagnosis (Cieniewicz et al., 2017a; Sudarshana et al., 2015)

Fruit ripening issues have been documented with diseased wine grapes. Reductions of 1-6°Brix have been consistently reported, as well as lower berry anthocyanin and skin tannins, particularly in red wine grapes such as Cabernet franc and Cabernet Sauvignon (Calvi 2011; Cieniewicz et al., 2017a; Sudarshana et al., 2015). The disease is affecting berry development by interfering with transcriptional and hormonal regulation of ripening (Blanco-Ulate et al., 2017). Based on the effect of the virus on fruit quality and ripening, numerous vineyard managers are culling infected vines and replacing them with clean, virus-tested ones. The economic cost of GRBV is estimated to range from $21,833 (for a 5% initial infection in year 3 and a 25% price penalty for infected grapes) to $169,384 (for a 60% initial infection in year 3 and a 100% price penalty for the proportion infected grapes) per acre in Napa Valley; from $12,023 to $93,067 per acre in Sonoma; and from $5,468 to $39,140 per acre on Long Island in New York (Ricketts et al., 2017). These estimates highlight the economic impact of red blotch disease in different grape-growing regions in the U.S. (Cieniewicz et al., 2017a).

GRBV was documented in all major grape-growing US States (Krenz et al., 2014). GRBV was also isolated from numerous table grape accessions at the USDA germplasm repository in Davis, CA (Al Rwahnih et al., 2015) and in Canada (Poojari et al., 2017). GRBV was also reported in *V. aestivalis*, *V. nesbittiana*, *V. biformis*, *V. monticola*, *V. blancoii*, *V. bloodworthiana*, and *V. amurensis* from the USDA germplasm repository in Davis, CA (Thompson et al., 2018). The widespread occurrence of GRBV in North America suggests that propagation material has played a significant role in its dissemination. The virus was also described in Switzerland (Reynard et al., 2018), South Korea (Lim et al. 2016) and India (GenBank accession number KU522121).

Grapevine red blotch virus (GRBV) is a member of the genus *Grablovirus* in the family *Geminiviridae* (Varsani et al., 2017). It has a circular, single-stranded DNA genome that codes for six open reading frames (Al Rwahnih et al., 2013; Cieniewicz et al., 2017a; Krenz et al., 2012; Sudarshana et al., 2015). We recently showed the causative role of GRBV in the etiology of red blotch disease using agroinoculation of tissue culture-grown grapevines with partial dimer or bitmer constructs of the GRBV genome (Yepes et al., 2018).

The Virginia creeper leafhopper (*Erythroneura ziczac* [Walsh]) (Poojari et al. 2013) and the three cornered alfalfa hopper (*Spissistilus festinus* [Say]) (Bahder et al. 2016a) have been shown to transmit GRBV from infected to healthy vines under greenhouse conditions. The epidemiological significance of these findings is unknown, stressing the need to carry out studies in diseased vineyards for vector identification. Interestingly, the transmission ability of *E. ziczac* was refuted (Bahder et al. 2016ba), highlighting the need for additional studies, particularly to determine the role of *S. festinus* in GRBV transmission in vineyards and assess whether any other insects can vector GRBV.

The overarching goal of this proposal is to advance our understanding of the ecology of red blotch disease and its causal agent, grapevine red blotch virus (GBRV), with a major emphasis on transmission attributes and the epidemiological role of vineyard cover crops.

List of Objectives

The specific objectives are to:

1. Characterize the spread of grapevine red blotch-associated virus (GRBV)
2. Determine if vineyard cover crops can host GRBV and/or *S. festinus*
3. Determine the experimental host range of GRBV and *S. festinus*
4. Disseminate research results to farm advisors and to the grape and wine industry

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

1. To address objective #1 - *Characterize the spread of grapevine red blotch virus (GRBV)* -, we selected a 4-acre Cabernet Sauvignon vineyard in Napa Valley, CA. Previous work documented the spread of GRBV in a nearby 5-acre disease Cabernet franc vineyard where red blotch symptoms were first observed in 2012 in the vineyard area most proximal to a riparian area (Perry et al., 2016; Cieniewicz et al., 2018a; Cieniewicz et al., 2017b). 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). Investigations into vector candidates of GRBV in the diseased Cabernet franc vineyard revealed four species of interest among more than 40 different insect species identified on insect sticky cards that were placed from March to November in 2015-2016 on symptomatic and asymptomatic vines (Cieniewicz et al., 2018a). The four species of interest are members of the Membracidae (*S. festinus*), Cicadellidae (*Colladonus reductus* and *Osbornellus borealis*) and Cixiidae (a Melanoliarus sp.). The majority of specimens of these four vector candidates tested positive for GRBV in PCR, suggesting that they can ingest the virus in the vineyard. By contrast, most of the others insects evaluated in this study tested negative for GRBV in PCR (Cieniewicz et al., 2018a). Populations of the four candidate vectors peaked from June to September, with viruliferous *S. festinus* peaking from late June to early July in both years. A significant association was found between the spatial distribution of GRBV-infected vines and viruliferous *S. festinus* (Cieniewicz et al., 2018), revealing the epidemiological relevance of *S. festinus* as a vector of GRBV in a vineyard.

Close to 100 sentinel vines consisting of healthy (GRBV negative in PCR) Cabernet franc grafted onto healthy rootstock 3309C (GRBV negative in PCR) were planted in spring 2015 in the Cabernet franc vineyard. These vines were used to provide direct evidence of insect-mediated GRBV spread if they become infected. None of the sentinel vines became infected at detectable levels with GRBV in 2016 and 2017. Similar work will be carried out in 2018.

Contrary to the Cabernet franc vineyard, the adjacent Cabernet Sauvignon vineyard (to the southwest) selected for this study does not show strong evidence of GRBV spread (**Figure 1**). This vineyard was planted in 2008 with Cabernet Sauvignon clones 4 and 169. Almost every vine of clone 4 exhibited typical red blotch symptoms soon after planting whilst vines of clone 169 remained asymptomatic (**Figure 2**). Vines of the two Cabernet Sauvignon clones were purchased from two different nurseries. GRBV isolates belonging to clade 1 and clade 2 are present in clone 4 vines (Cieniewicz and Fuchs, unpublished).

Fig. 1. Proximity of a Cabernet franc vineyard for which spread of GRBV was reported in 2015-2017) and a Cabernet Sauvignon vineyard for which an extremely limited evidence of spread is available. Red blotch diseased vines are depicted in red.

From 2008 to 2016, GRBV was not observed in any of the clone 169 vines. In fall 2017, 0.9% (25 of 2,796) of clone 4 vines exhibited suspicious red blotch symptoms whilst 96% (2,657 of 2,768) of clone 4 vines were symptomatic (**Figure 2**). Testing symptomatic clone 4 vines for pathogens indicated that 16 of them were infected with GRBV, seven with Pierce disease and two with grapevine leafroll-associated virus 1. This was the first evidence of the presence of GRBV in clone 169 vines (0.6%, 16 of 2,796) nine years post-planting.

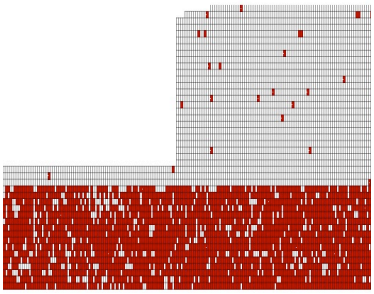

Fig. 2. Distribution of diseased vines in a Cabernet Sauvignon vineyard with clone 4 (bottom) and clone 169 (top).

To advance an understanding of the apparent low level of GRBV spread in the Cabernet Sauvignon vineyard, yellow sticky cards were placed at the east end (closest to the Cabernet franc vineyard) from April thru November in 2017 on diseased and asymptomatic vines of clones 169 and 4, across six rows of each clone (**Figure 1**, 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 Cicadellidae (*Colladonus reductus* and *Osbornellus borealis*) and Cixiidae (unidentified species).

Results indicated a substantially lower population of *S. festinus* in the Cabernet Sauvignon (N = 3) vineyard 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 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 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 compared to the Cabernet franc vineyard (Cieniewwicz et al., 2018a). Similar work with insect traps will be carried out in the Cabernet Sauvignon vineyard in 2018. It will be interesting to see whether a differential number of insect vector candidates will be confirmed between the two study vineyards. This will put us in a unique position to validate our hypothesis about an association between GRBV spread and the dynamics of *S. festinus* in a vineyard ecosystem.

To describe the transmission of GRBV by S. festinus, preliminary work revealed that GRBV is detected in the majority of *S. festinus* specimens (7 of 10) that were tested 10 to 15 days after they were transferred onto alfalfa (GRBV-negative) following a 5-day GRBV acquisition access period on infected grapevines. This result documented the ability of *S. festinus* to retain GRBV for several days following a gut cleansing experiment. It is supportive of a circulative transmission mode of GRBV by *S. festinus*. Replications of this experiment are under way.

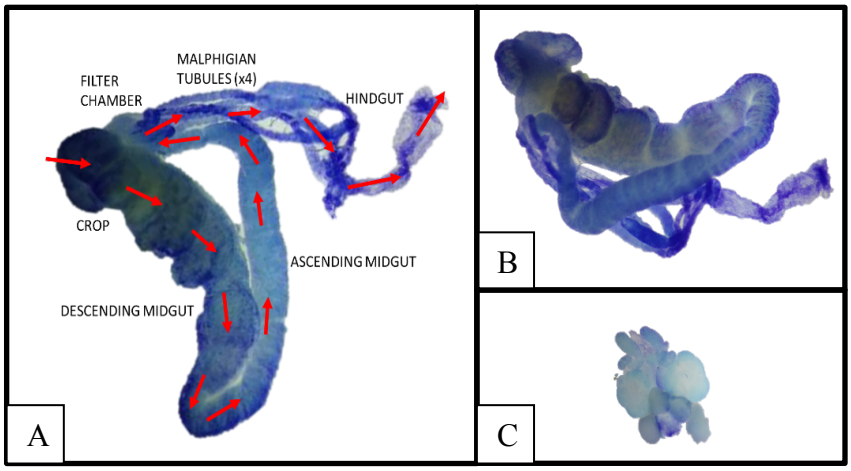

Fig. 3. Description of *S. festinus* alimentary canal morphology. Guts were dissected in 1X PBS and stained with toluidine blue dye (A) with alternative view shown in (B). Salivary glands (C). Organs are not shown to scale.

To characterize the gut morphology of *S. festinus*, preliminary work showed that the esophagus is usually detached during dissection, but the food path would follow into the crop and foregut, descending midgut, followed by the ascending midgut where it meets the filter chamber and descends to the hindgut (**Figure 3**). The malphigian tubules (x4) attach anteriorly at the filter chamber and posteriorly at the hindgut for expulsion of waste (**Figure 3**).

Individual specimens were similarly dissected under a stereoscope to isolate different organs (mid gut, hind gut and salivary glands, as well as hemolymph, for testing by multiplex PCR. Results revealed GRBV in the salivary glands (8 of 14), hemolymph (13 of 14) and gut (14 of 14), showing that transmission if circulative.

Additional work using, fluorescence *in situ* hybridization (FISH) experiments will be carried out to localize GRBV in *S. festinus* rogans and illustrate the transmission pathway. Lack of replication in *S. festinus* will be tested by real-time PCR and RT-PCR.

To address objective #2 - Determine if vineyard cover crops can host GRBV and/or S. festinus.-, legumes (Fabaceae) such as vetch, peas, bean, clover and Medicago sp. were surveyed for GRBV. Alfalfa, a species that is not part of vineyard cover crop mixes but belongs to the legume family and is a reservoir for *S. festinus*, was also surveyed in unmanaged areas proximal to GRBV-infected vineyards as a negative control. A total of 13 California vineyards of Sauvignon blanc, Cabernet franc, Merlot and Cabernet Sauvignon was selected for these surveys (**Figure 4**). The 13 California vineyards are infected with GRBV or proximal to vineyards infected with GRBV. Three additional vineyards were selected because they are not infected with GRBV and carry legumes species in their cover crops stands.

Fig. 4. Red clover and other cover crop species in middle rows of a diseased vineyard in spring 2017.

A total of over 500 legume samples) and other species (barley, oats, rye and grasses) (**Figure 5**) from vineyard middle-row cover crops were collected in early March in 2017 and 2018. Results of multiplex PCR (Krenz et al., 2014) or real time PCR (Setiono et al., 2018) for GRBV showed that none of the samples tested was positive for GRBV in PCR. These findings are consistent with similar work done in 2014-2016 that did not yield any positive findings of GRBV. This work revealed 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.

Fig. 5. Vetch, dirdsfoot trefoil and other cover crop species in vineyard middle rows in spring 2017.

The same 13 diseased California vineyards were surveyed for *S. festinus* and the other three candidate vectors, i.e. *Colladonus reductus* and *Osbornellus* sp. and the unidentified Cixiidae, by sweep netting. In spite of extensive sweep netting in March of 2017 and 2018, no specimen of *S. festinus* or any of the other candidate vectors was caught. This result indicates that the vector and candidate vectors of GRBV are not present in vineyard middle rows in spring or that sweep netting is not appropriate to catch these insects in spring.

To address objective #3 - *Determine the experimental host range of GRBV and S. festinus* -, GRBV infectious clones was used to agroinoculate by needle pricking and vacuum-assisted infiltration seedlings of clover, vetch, bean, Medicago and peas. One to four weeks post-agroinoculation plant samples were assayed by PCR for GRBV and by RT-PCR using primers designed to detect the accumulation of spliced transcripts. 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. Results indicate infection of bean only; infection on bean was asymptomatic **(Figure 6).** None of the other herbaceous host tested sustained the replication of GRBV. Additionally, transmission assays with *S. festinus* specimens from the colony maintained on alfalfa showed transmission of GRBV from infected bean to healthy bean.

Fig. 6. Bean leaves agroinoculated with a GRBV infectious clone by syringe infiltration.

To examine the reproductive potential of *S. festinus* on different host plans, 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. Preliminary work showed that *S. festinus* reproduced on the three 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, adults all 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 - *Disseminate research results to farm advisors and the industry* -, research results were communicated to 663 growers, farm advisors, extension educators, crop consultants, researchers, vineyard managers and regulators at the following winter school meetings and conventions in California, New York, Missouri and Ontario, Canada:

Fuchs, M. 2018. Leafroll and red blotch: What should I be aware of and what can I do? Show me grape and wine conference, March 7, Columbia, Missouri, (participants = 52).

Cieniewicz, E. and Fuchs, M. 2017. Grapevine red blotch virus in free-living *Vitis* sp. Cornell Recent Advances in Viticulture and Enology (CRAVE) conference, November 14, Ithaca, NY (participants = 60)

Fuchs, M. 2017. Update on the ecology of red blotch virus. Sustainable Ag Expo on Nov. 14, San Luis Obispo, CA (participants = 500).

Fuchs, M. 2017. Leafroll and red blotch viruses. Open house, Sept. 8, Niagara-on-the-Lake, Ontario, Canada (participants = 51).

Publication produced and pending, and presentations made that related to the funded project

Publications

Cieniewicz, E., Pethybridge S., Loeb, G., Perry, K.L., and Fuchs, M. 2018a. Diversity and spatial distribution of vector candidates of grapevine red blotch virus in a diseased vineyard. Phytopathology,108:94-102.

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Cieniewicz, E., Pethybridge S., Gorny, A., Madden, L., Perry, K.L., McLane, H. and Fuchs, M. 2017b. Spatiotemporal spread of grapevine red blotch-associated virus in a California vineyard. Virus Research 230:59-62.

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.

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Presentations

Fuchs, M. 2018. Leafroll and red blotch: What should I be aware of and what can I do? Show me grape and wine conference, Columbia, Missouri.

Choi, J., Osterbaan, L. and Fuchs, M. 2017. Effect of single amino acid mutations in the RNA-dependent RNA polymerase of grapevine fanleaf virus on pathogenicity in *Nicotiana benthamiana*. Summer Scholars Poster Presentation, July 28, Geneva, NY.

Cieniewicz, E., Perry, K.L. and Fuchs, M. 2018. Ecology of grapevine red blotch virus in US vineyard ecosystems. 19th Meeting of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine, April 9-12, Santiago, Chile.

Cieniewicz, E. and Fuchs, M. 2017. Grapevine red blotch virus in free-living *Vitis* sp. Cornell Recent Advances in Viticulture and Enology (CRAVE) conference, Ithaca, NY

Cieniewicz, E. and Fuchs, M. 2017. Ecology of grapevine red blotch disease. APS Annual Meeting, August 8, San Antonio, TX.

Fuchs, M. 2017. Update on the ecology of red blotch virus. Sustainable Ag Expo, San Luis Obispo, CA

Fuchs, M. 2017. Leafroll and red blotch viruses. Open house, Niagara-on-the-Lake, Ontario, Canada

Onwumelu, A., Cieniewicz, E. and Fuchs, M. 2017. Investigating potential insect vectors of grapevine red blotch virus in a Napa County, CA vineyard. Summer Scholars Poster Presentation, July 28, Geneva, NY.

Research relevance statement, indication how this research will contribute towards finding solutions to the pest and disease being studied

Our research provided new insights into the ecology of grapevine red blotch virus (GRBV). We documented a lower population of *S. festinus* (N= 3) in a Cabernet Sauvignon vineyard in which spread of GRBV is limited in comparison to higher populations (N = 25) in a proximal Cabernet franc vineyard for which a 1-3% annual increase in diseased vines is reported. This result suggested an association between the dynamics of vector populations and rate of virus spread. None of the legume cover crop species, i.e. bell beans, field peas, vetch, clover, etc., from 13 diseased California vineyard middle was positive for GRBV in 2017 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 host *S. festinus* in a vineyard ecosystem. The transmission of GRBV by. *festinus* is circulative, as shown virus detection by PCR in salivary glands, gut and hemolymph of specimens that were exposed to infected vines. This research provided insights into the ecology of GRBV that are essential to inform management recommendations. Our findings suggested that disease management strategies should be aimed at reducing the virus inoculum in vineyards and that non *Vitis* species used as cover crops, particularly legumes, should not be targeted for disease management purposes. Information from this project was disseminated to the wine and grape industry at various venues.

Layperson summary of project accomplishments

Populations of *S. festinus* caught in a Cabernet Sauvignon vineyard in 2017 in which spread of grapevine red blotch virus (GRBV) is limited were extremely low (N= 3) in comparison to higher populations (N = 25) in a proximal Cabernet franc vineyard for which a 1-3% annual increase in diseased vines is documented. None of more than 500 legume cover crop samples, i.e. bell beans, field peas, vetch, clover, etc., from 13 diseased California vineyard middle rows that were surveyed in spring 2017 and 2018 were positive for GRBV. In addition, no *S. festinus* and no 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 host *S. festinus* in a vineyard ecosystem. Agroinoculation experiments suggested that GRBV can multiply on bean, in addition to grape, but not on any of the other legume species tested. GRBV can also be transmitted by *S. festinus* from infected to healthy bean. Preliminary work indicated that *S. festinus*-mediated transmission of GRBV is likely circulative with the virus detected in various insect organs such as salivary glands, gut and haemolymph. This research provided insights into the ecology of GRBV that are essential to inform management recommendations. Information from this project was disseminated to the wine and grape industry at various venues.

Status of funds

Funds were spent for salaries of key personnel (postdoctoral associate graduate student assistant and technicians) 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.

Literature cited

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