ECOLOGY OF GRAPEVINE RED BLOTCH VIRUS

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ABSTRACT
Limited information is available on the ecology of grapevine red blotch disease, a recently recognized new threat to the grape and wine industry. We characterized attributes of spread of grapevine red blotch virus (GRBV), the causal agent of red blotch disease, in three distinct vineyards: a 5-acre Cabernet franc vineyard in California with an 14% disease incidence 10 years post-planting, an adjacent 4-acre Cabernet Sauvignon vineyard in California with a 2% disease incidence 10 years post-planting, and a 2-acre Merlot vineyard in New York with a 40% disease incidence 10 years post-planting. Analysis of the spatiotemporal distribution of infected vines from 2014 to 2018 was consistent with a 2.5%, 0.5% and 0% increase of infected vines annually in the Cabernet franc, Cabernet Sauvignon and Merlot vineyards, respectively. An analysis of populations of *Spissistilus festinus* - the three-cornered alfalfa hopper (TCAH), the only known vector of GRBV of epidemiological importance so far - over two consecutive growing seasons indicated a 10-fold difference in abundance between the Cabernet franc and Cabernet Sauvignon vineyards. In contrast, no TCAH was found in the Merlot vineyard in New York. These results suggest an association between the rate of GRBV spread, and relative abundance of TCAH populations. Virulifeous specimens of *S. festinus* peaked in late June-July in 2015-2018 in the Californian vineyards. Since legumes, not grapes, are preferred hosts of the TCAH and are often used as cover crops in vineyard middle-rows, we surveyed vineyard cover crops for GRBV. None of cover crop samples collected in diseased vineyards in 2014-2018 tested positive for GRBV, suggesting no major role as virus reservoirs. Among the herbaceous plants that were artificially inoculated with infectious GRBV clones in the laboratory, *Phaseolus vulgaris*, tomato and *Nicotiana benthamiana* were identified as local hosts. Additionally, TCAH was able to establish on *P. vulgaris* and alfalfa, as expected. To characterize the transmission mode of GRBV by TCAH, gut cleansing experiments revealed that the majority of TCAH that fed on infected grapevines tested positive for GRBV following a 2-week feeding period on alfalfa, suggesting a persistent transmission. Furthermore, GRBV was detected in the hemolymph and salivary glands of TCAH one to two weeks post acquisition, suggesting a circular transmission; and quantification of GRBV in TCAH that fed on infected vines showed a decline in virus titer over time, suggesting a non-propagative transmission mode. Collectively, these findings provide a strong foundation for disease management recommendations based on a reduction of virus inoculum sources in diseased vineyards through rogueing and block removal rather than the control of TCAH populations.

LAYPERSON SUMMARY
Red blotch is a recently recognized viral disease of grapevines that is widely distributed in U.S. vineyards. Limited information is available on the spread of grapevine red blotch virus (GRBV), its causal agent. Studying changes in disease incidence over time in selected vineyards in California and New York revealed an increase of diseased vines in the two California vineyards, although at distinct rates (2.5% vs 0.5% annual increase of diseased vines), but not in the New York vineyard. The differential dynamic of GRBV spread in two Californian vineyards is possibly associated with a 10-fold lower abundance of the three-cornered alfalfa hopper (TCAH), the only known vector of GRBV so far, in the vineyard where spread is limited compared to the vineyard where spread is readily occurring. No TCAH was found in the New York study vineyard where GRBV spread is not occurring. Surveys of vineyard middle-row cover crops revealed that none of the plants tested, especially legume species, during Spring of five consecutive years were positive for GRBV, suggesting no major role as virus reservoirs. However, a few experimental plants (tomato, snap bean and *Nicotiana benthamiana*) were found as hosts of GRBV; these plants will facilitate studies of virus-vector-host interactions in the future. Finally, preliminary information revealed a circulative, non-propagative transmission mode of GRBV by the TCAH. Together, our findings stress the need to reduce virus inoculum sources in vineyards for effective red blotch disease management rather than the control of TCAH populations.

INTRODUCTION

Red blotch was described for the first time on Cabernet Sauvignon at the UC Oakville Research Field Station in 2008 (Al Rwahnih et al., 2013; Calvi 2011; Cieniewicz et al., 2017a; Sudarshana et al., 2015). Reductions of 1-6°Brix have been consistently reported in diseased vines, 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; Reynard et al., 2018; Sudarshana et al., 2015). Poor fruit quality results from interference with the transcriptional and hormonal regulation of ripening (Blanco-Ulate et al., 2017) and poor carbon translocation (Martínez-Lüscher et al., 2019). The economic cost of the disease 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 US.

Grapevine red blotch virus (GRBV) was documented in all major grape-growing US States (Brannen et al., 2018; Krenz et al., 2014; Poojari et al., 2013; Seguin et al., 2014; Schoelz et al., 2019; Sudarshana et al. 2015; Yao et al., 2018). GRBV was also isolated from numerous accessions at the USDA germplasm repository in Davis, CA (Al Rwahnih et al., 2015; Thompson et al., 2018), hybrid accessions at the USDA germplasm repository in Geneva, NY (Perry et al., unpublished), and wine and table grapes in Canada (Poojari et al., 2017; Xiao et al., 2015), Argentina (Luna et al., 2019), and Mexico (Gasperin-Bulbarela et al., 2019). GRBV was even found in an archival *Vitis* sample from Sonoma County in California (Al Rwahnih et al., 2015). The widespread occurrence of GRBV in the Americas 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 (Marwal et al., 2019). Additionally, GRBV was identified in free-living *Vitis* spp. in California (Bahder et al., 2016a; Cieniewicz et al., 2018; Perry et al., 2016) but not in New York (Cieniewicz et al., 2018).

GRBV is a member of the genus *Grablovirus* in the family *Geminiviridae* (Varsani et al., 2017). It has a circular, single-stranded DNA genome that codes for seven open reading frames (Al Rwahnih et al., 2013; Cieniewicz et al., 2017a; Krenz et al., 2012; Vargas-Asencio et al., 2019). We recently showed the causative role of GRBV in the etiology of red blotch disease using agroinoculation of tissue culture-grown grapevines with infectious clones (Yepes et al., 2018).

The three-cornered alfalfa treehopper (TCAH, *Spissistilus festinus* [Say]) has been shown to transmit GRBV from infected to healthy vines under greenhouse conditions (Bahder et al. 2016a). The epidemiological significance of this finding was recently documented in a diseased vineyard in California (Cieniewicz et al., 2017b). Spread of GRBV was also reported in some vineyards in Oregon although the insect vector remains elusive (Dalton et al., 2019). Nonetheless, limited infection is available on the ecology of red blotch disease, stressing the need to carry out studies in diseased vineyards. Similarly, information on the transmission mode of GRBV by the TCAH is scarce although the seasonal dynamics (Preto et al., 2019), and feeding and reproductive hosts of TCAH have been identified in Californian vineyards (Preto et al., 2018a). The overarching goal of our research is to advance our understanding of the ecology of red blotch disease with a major emphasis on attributes of GRBV spread and the potential epidemiological role of vineyard cover crops, as well as dissemination science-based information to the grape industry.

OBJECTIVES

Our specific objectives were to:

1. Characterize attributes of the spread of grapevine red blotch virus (GFBV) by *Spissistilus festinus*
    a. Describe the transmission mode of GRBV by *S. festinus*
    b. Test sentinel vines established in a diseased vineyard where spread is documented for the presence of GRBV
    c. Investigate the seasonal diversity and distribution of vector candidate populations in a diseased vineyard for which there is no evidence of spread
2. Determine if vineyard cover crops can host GRBV and/or *S. festinus*
    a. Survey cover crops in Napa Valley vineyards for *S. festinus*
    b. Survey cover crops in Napa Valley vineyards for GRBV

3. Determine the experimental host range of grapevine red blotch virus and *S. festinus*
    a. Agroinoculate commonly used vineyard cover crop species with infectious GRBV clones and assess virus infection
    b. Examine the reproductive potential of *S. festinus* on commonly used vineyard cover crop species
4. Disseminate research results to the grape and wine industry, and to farm advisors

This set of objectives is an amalgamation of objectives initially set for a project funded in 2017 and for a follow-up project funded in 2018.

RESULTS AND DISCUSSION

To address objective #1 and characterize attributes of the spread of GRBV, three distinct vineyards were selected for this study: a 5-acre Cabernet franc vineyard in California, an adjacent 4-acre Cabernet Sauvignon vineyard in California and a 2-acre Merlot vineyard in New York. The three study vineyards were planted in 2008. Foliar symptoms were first noticed in 2012 in the Cabernet franc vineyard, in 2009 in the Cabernet Sauvignon vineyard and in 2011 in the Merlot vineyard. The presence of GRBV was confirmed in the three study vineyards in 2013 and 2014, providing a foundation to investigate the spatiotemporal increase in GRBV incidence.

In the Cabernet franc vineyard in California, an analysis of the number of symptomatic vines showed a disease incidence of 4% (305 of 7,691 vines) in 2014, 6% (461 of 7,691 vines) in 2015, 7% (547 of 7,691 vines) in 2016, 9% (696 of 7,691 vines) in 2017 and 14% (1,058 of 7,691 vines) in 2018 (Figure 1). These results were consistent with a 10% increase in disease incidence from 2014 to 2018 and a 2.5% annual increase in disease incidence over five consecutive years, likely as a result of TCAH-mediated transmission of GRBV.

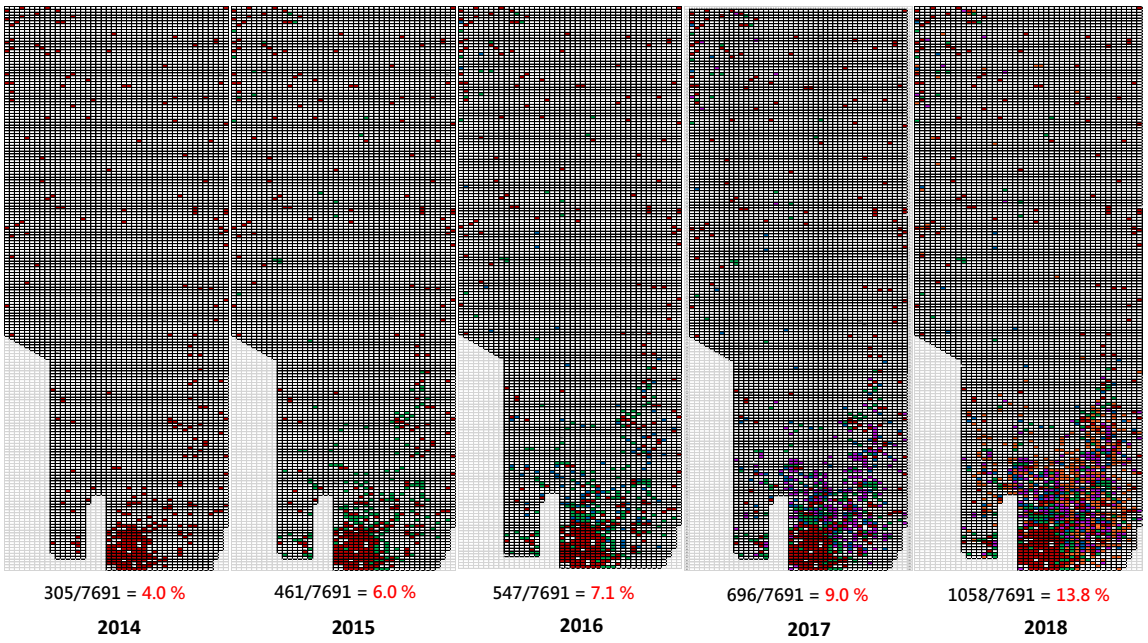

Fig. 1. Red blotch disease progress in a 5-acre Cabernet franc vineyard in California vineyard. Each cell represents a single vine that is asymptomatic (blank) or symptomatic (red in 2014, green in 2015, blue in 2016, purple in 2017 and peach in 2018).

An investigation of the spatial distribution of symptomatic vines through an ordinary runs analysis, a statistical test for randomness of infected plants, revealed disease clustering in the majority of rows in the study area within the Cabernet franc vineyard (Cieniewicz et al., 2017b). Additionally, an analysis of the distribution of diseased vines across rows illustrated a distinct dynamic of spread in the area of the Cabernet franc vineyard proximal to a riparian area versus the reminder of the vineyard (Figure 2). In the area proximal to the riparian area, disease incidence increased from 30% in 2014, 46% in 2015, 48% in 2016, 52% in 2016, and 71% in 2018. In contrast, in the remainder of the vineyard, disease incidence increased from 4% in 2014 to 6% in 2018 (Cieniewicz et al., 2019a). This represents a 10% and 0.5% annual increase of virus infected annually in the area of the vineyard close to the riparian area versus the remainder of the vineyard (Figure 2).

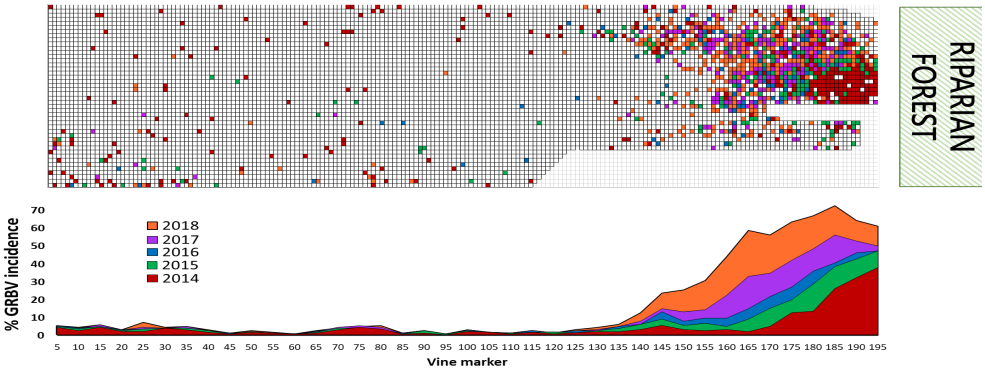

Fig. 2. GRBV spread in a 5-acre Cabernet franc vineyard in California over 5 years. The top graph shows the entire study vineyard with individual cells representing a single vine that is asymptomatic (blank) or symptomatic (colored). The bottom graph shows the distribution of diseased vines in 5-vine panels across rows.

Probability-based modeling using the Markov Chain Monte Carlo algorithm, which integrates the spatial pattern and distance between newly infected vines to determine whether new infections are due to influx of inoculum from within- or outside-vineyard sources of inoculum, suggested that spread in the Cabernet franc vineyard was primarily due to localized, within-vineyard sources (Cieniewicz et al., 2017b). This prediction was confirmed by characterizing the genetic variability of GRBV isolates from infected vines in the aggregated area of the Cabernet franc vineyard by PCR and sequencing. Indeed, the majority of the virus isolates analyzed were genetically identical or nearly identical, and grouped with phylogenetic clade 2 isolates, validating the within-vineyard spread prediction (Cieniewicz et al., 2018a).

Adjacent to the Cabernet franc vineyard is a 4-acre Cabernet Sauvignon vineyard (Figure 4). The Cabernet Sauvignon vineyard is established with two distinct clones: clone 4 and 169. Most vines of clone 4 were symptomatic following establishment, as indicated by the vineyard manager and confirmed by mapping of diseased vines (Figure 5). This is clearly indicated that clone 4 vines were heavily infected with GRBV when planted. In contrast, vines of clone 169 were clean when the vineyard was established and remained asymptomatic for several years (Cieniewicz et al., 2019a).

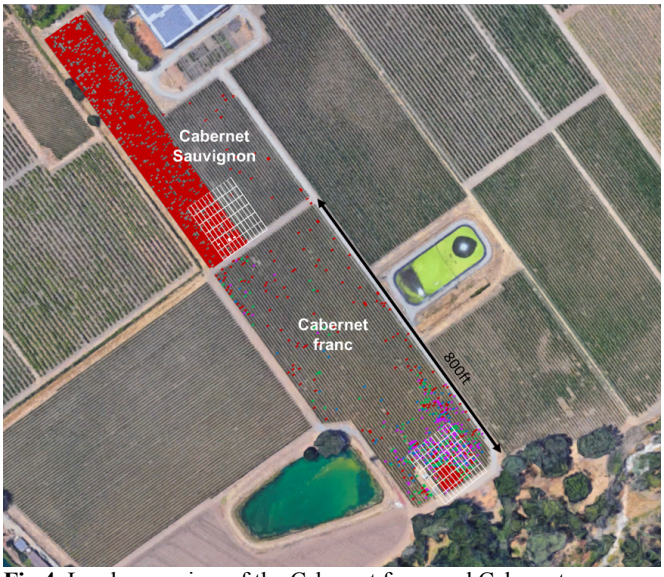

Fig 4. Landscape view of the Cabernet franc and Cabernet Sauvignon vineyards in California. White grids indicate area of surveys for insects in 2015-16 in the Cabernet franc vineyard and 2017-18 in Cabernet Sauvignon vineyard.

An analysis of GRBV incidence in the section of the vineyard established with clone 169 showed a disease incidence of 1% (25 of 1,819 vines) in 2017 and 2% (36 of 1,819 vines) in 2018 (Figure 5). This is consistent with a 1% increase in disease incidence in the Cabernet Sauvignon vineyard from 2017 to 2018, likely as a result of TCAH-mediated transmission of GRBV (Cieniewicz et al., 2019a).

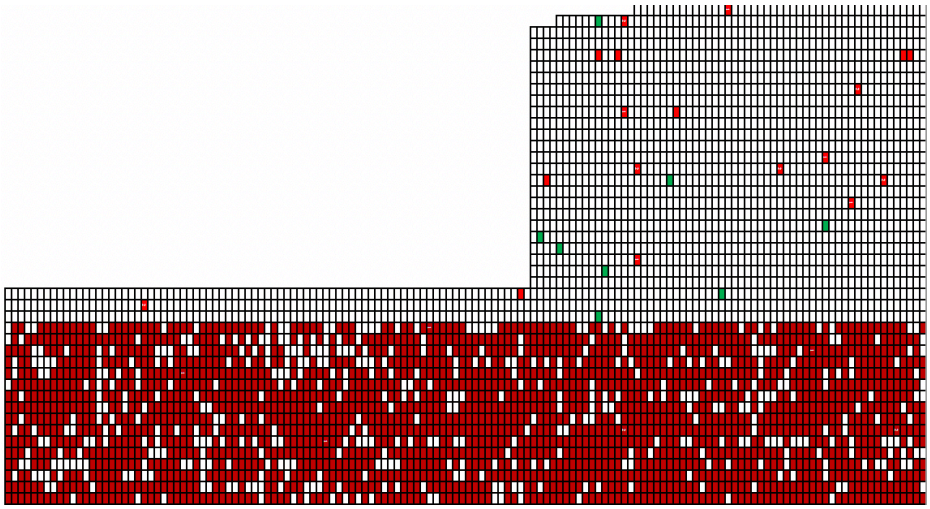

Fig. 5. Map of GRBV incidence in the Cabernet Sauvignon vineyard in California. Almost all vines of clone 4 are symptomatic (bottom). Vines of clone 169 that become infected in 2017 and 2018 are shown in red and green, respectively (top).

The 2-acre Merlot vineyard selected for this study in New York showed a high incidence (40% overall incidence) of red blotch disease following establishment, suggesting that the plant material was highly infected with GRBV. A spatiotemporal analysis of diseased vines in the Merlot vineyard in 2014-2018 did not provide any evidence of an increased incidence of GRBV over time. In indeed, over five years of sampling and GRBV testing in this vineyard, negative vines consistently tested negative with no vines that tested negative one year, testing positive in a subsequent year (Cieniewicz et al., 2019a). This indicated no evidence of secondary spread although GRBV is prevalent in this vineyard with a 40% overall incidence. These data suggested that a GRBV vector does not exist in the Merlot vineyard or it eventually exists in the ecosystem at a very low population abundance or it exists but does not visit the vineyard. Alternatively, the plant protection program used by the vineyard manager in New York is effective at reducing the vector population.

In summary, analyzing the spatiotemporal distribution of read blotch diseased vines in three distinct vineyards showed a difference in spread dynamic of GRBV. A relatively high rate of spread was documented in a Cabernet franc vineyard in California, a limited rate in an adjacent Cabernet Sauvignon vineyard in California and no spread in the Merlot vineyard in New York. This prompted us to ask why is there a differential spread of GRBV in the study vineyards. In other words, why is GRBV readily spreading in the Cabernet franc vineyard but not much in Cabernet Sauvignon in spite of the availability of a very low inoculum source (1%) in the former and a very high inoculum source (40%) in the latter following vineyard establishment? And, why is GRBV apparently not spreading in the Merlot vineyard in New York? Since GRBV shows equally striking symptoms in Cabernet franc, Cabernet Sauvignon and Merlot, we hypothesized that a difference in population or behavior of the TCAH vector (or other potential vectors) in these three vineyards could result in the observed differential GRBV spread. These issues are addressed in objective 1c below.

To address objective #1a and describe the transmission mode of GRBaV by Spissistilus festinus, we hypothesized that the transmission mode is circulative and non-propagative by analogy with other virus species of the family *Geminiviridae*. To address the circulative mode, TCAH were allowed to feed on GRBV-infected grapevines for 5-8 days and then transferred on alfalfa, a non-host of GRBV. These gut cleansing experiments revealed that the majority of TCAH (18 of 28) tested positive for GRBV following a 2-week feeding period on alfalfa, suggesting a persistent, circular transmission. Additionally, TCAH specimens were dissected under a stereoscope to isolate different organs (gut, salivary glands, and hemolymph) for GRBV testing by PCR following a 1-week feeding period on GRBV-infected grapevines in the greenhouse (Figure 6). Results showed the presence of GRBV in the salivary glands (4 of 4), hemolymph (7 of 8) and gut (8 of 8) of dissected TCAH. None of the organs of TCAH that fed on healthy vines tested positive for GRBV in PCR. These observations supported the hypothesis that

GRBV is transmitted in a circulative mode. Replicated experiments to verify a circular transmission mode are under way.

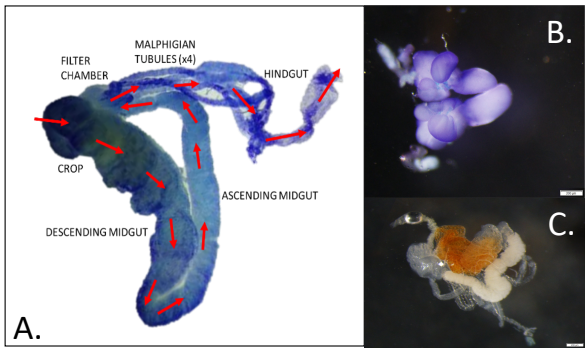

Fig. 6. Description of the *S. festinus* alimentary canal morphology. Guts were dissected in 1X PBS and stained with toluidine blue dye (A). Dissected salivary glands (B, stained) and gut (C, unstained). The scale bar represents 200 µm.

To test whether the transmission mode of GRBV by *S. festinus* is non-propagative, a time course experiment was conducted with 32 adult TCAH 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 $1x10^9$ to $1x10^2$ 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. Results showed that both quantification methods showed a decreasing GRBV titer over time in *S. festinus* after GRBV-exposure is removed (Figure 7). These preliminary results revealed that GRBV does not replicate in *S. festinus* following ingestion, indicating a non-propagative transmission mode.

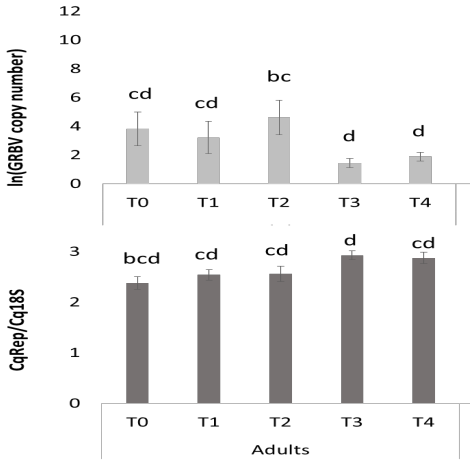

Fig. 7. Reduction in GRBV DNA in *S. festinus* over time, as shown by two qPCR methods. (Top) GRBV copy number compared to external reference; and (Bottom) Comparative Cq method of Rep GRBV target and 18S *S. festinus* target. Following feeding on GRBV-infected grapevine for two weeks, specimens were 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.

To address objective 1b and test sentinel vines established in a diseased vineyard where spread is documented for the presence of GRBV, close to 100 sentinel vines, i.e. healthy vines for which the mother stocks from which scion budwood and rootstock canes were tested and shown to be negative for GRBV, were planted in spring 2015 in the area of the Cabernet franc vineyard in California that is close to the riparian area where infected vines are highly aggregated (Figure 4) and spread is readily occurring. These vines were used to gain direct evidence of insect-mediated GRBV spread. Sentinel vines replaced existing vines that were weak, regardless of their GRBV infectious status. The presence of GRBV was tested in sentinel vines in 2015-2018 by PCR. None of the sentinel vines tested positive for GRBV in 2015-2017, however, a single vine tested positive for GRBV in 2018. Interestingly, this GRBV positive sentinel vine was asymptomatic in 2015-2018. This suggested that three years were necessary for a sentinel vine to become infected, likely as a result of a TCAH-mediated transmission of GRBV, in an area of the study vineyard where infected vines are highly aggregated and the annual increase of disease incidence is 10%. It will be interesting to continue monitoring the virus status of sentinel vines to determine temporal incidence, and check whether the single infected vines found in 2018 will become symptomatic over time.

To address objective 1c and investigate the seasonal diversity and distribution of vector candidate populations in a diseased vineyard for which there is no evidence of spread, insect sticky traps were placed in the area of the selected Cabenert franc vineyard in California where extensive clustering of diseased vines is occurring. Traps were placed on diseased and healthy vines from early April to late November in 2015 and 2016 with the goal of catching insects visiting the vineyard (Cieniewicz et al., 2018a). Traps were rotated on a weekly basis. Each trap was analyzed for the presence of insects to establish a census population and identify them at the species level, if possible, by using morphological parameters as well as genetic markers. 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 PCR. Results indicated that specimens of four species, among more than 40 species/taxa of Dipetra, Apocrita, Coleoptera, Cicadellidae, Thysanoptera, Aphidae, Fulgoroideae, Phylloxera, Aleyrodidae, Membraciade, Blissidae/Lygaeidae, Psyloidea, Psocopetra and Miridae that were caught on sticky traps, consistently carried GRBV (Cieniewicz et al. 2018a). The four species that consistently tested positive for GRBV are TCAH, currently the only known vector of GRBV (Bahder et al., 2016b), two leafhoppers (*Colladonus reductus* and *Obsornellus borealis*), and a planthopper (*Melanoliarus* spp.) (Cieniewicz et al. 2018a). Populations of the four insect vector candidates caught on sticky traps were very low (~5-40 individuals per year) compared to populations of some typical grape pests, such as phylloxera, western grape leafhopper, variegated leafhopper, and thrips (~500 to 1,500 individuals per year) (Cieniewicz et al. 2018a). The vector candidate populations peaked in July (TCAH and Cixiidae species) and September (*Colladonus reductus* and *Osbornellus* sp.) (Figure 3a). The four vector candidates are phloem-feeders, as would be expected for a GRBV transmitter. Of the four species that are able to acquire GRBV in the vineyard, none is considered a pest of grapevines and the TCAH is the only known vector of GRBV so far (Bahder et al., 2016b). The GRBV transmission capacity of *C. reductus*, *O. borealis,* and the *Melanoliarus* spp. is not known.

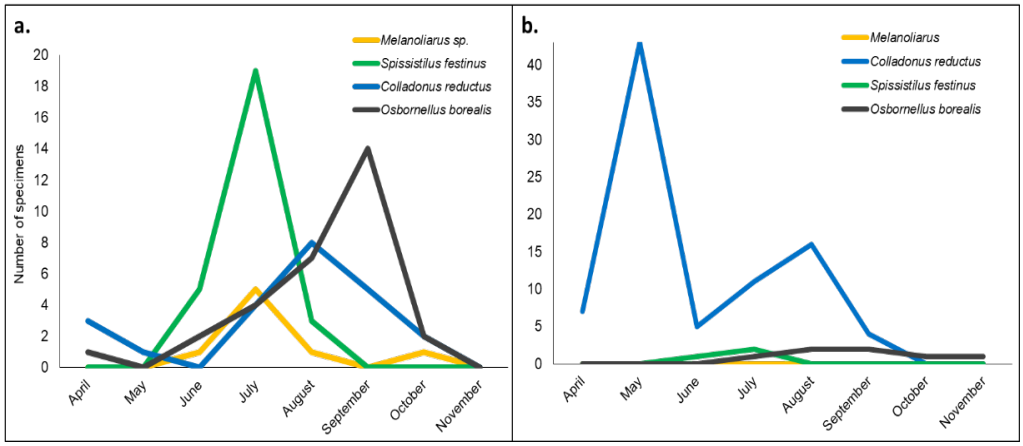

Fig. 3. Populations of vector candidates in (a) a Cabernet franc vineyard where GRBV is readily spreading and (b) an adjacent Cabernet Sauvignon vineyard where limited spread of GRBV is occurring.

Insect vector surveys were expanded to the Cabernet Sauvignon vineyard in California and the Merlot vineyard in New York in 2017-2018. Results showed that although many of the same insects were present in the Cabernet franc and Cabernet Sauvignon vineyards in California, and the four vector candidate species of interest peaked more or less at the same period during the growing seasons (Figure 3), the relative abundance of many of the species/taxa differed. For example, 25 TCAH were found in the Cabernet franc vineyard both in 2015 and 2016, but only three and two TCAH were found in the Cabernet Sauvignon vineyard in 2017 and 2018, respectively (Figure 3b). Similarly, there were fewer *O. borealis* and *Melanoliarus* spp. in the Cabernet Sauvignon vineyard compared to the Cabernet franc vineyard, however, there was a greater abundance of *C. reductus* in the Cabernet Sauvignon vineyard compared to the Cabernet franc (Cieniewicz et al., 2019a). Additionally, 25 of 50 (50%) of the TCAH caught in the Cabernet franc vineyard (Cieniewicz et al. 2018a) and one of five (20%) of the TCAH caught in the Cabernet Sauvignon vineyard carried GRBV, as shown by PCR. A difference in insect vector community dynamic, particularly of the TCAH, including specimens carrying GRBV, could explain the differential spread of GRBV in the two study vineyards in California (Cieniewicz et al., 2019a). Looking at the vineyard ecosystem, there is no major difference between the two study vineyards, except that the Cabernet franc vineyard is proximal to a riparian area and the Cabernet Sauvignon is about 800 feet from the riparian habitat (Figure 4). Could the degree of proximity to the riparian area explain a difference in TCAH population abundance that is visiting the two study vineyards? More work is needed to test this hypothesis. Insect vector surveys in the Merlot vineyard in New York revealed several phloem-feeding leafhoppers and treehoppers, but not the TCAH, and none of them consistently tested positive for GRBV. As expected, most species/taxa of leafhoppers and treehoppers in the New York vineyard were distinct from those in the California vineyards (Cieniewicz et al., 2019a). This suggested that the absence of potential vectors of GRBV in this vineyard likely explains a lack of virus spread.

To address objective #2a and survey cover crops in Napa Valley vineyards for S. festinus, middle row cover crops in eight vineyards of Sauvignon blanc, Cabernet franc, Merlot and Cabernet Sauvignon in Napa Valley (Figure 8) were surveyed for *S. festinus* by sweep netting in March and April of 2018. Based on visual assessment of red blotch disease, four of the surveyed vineyards were heavily (>80%) symptomatic, three were moderately (10-50%) symptomatic, one was mostly asymptomatic (<5%). Two of the vineyards were near water sources (<10 m away from a river or pond), and six of them were adjacent to forested habitats. Time spent sweep-netting ranged from 15 to 50 minutes in duration for each vineyard, totaling over 7 hours of sweep-netting time carried out usually early to mid-morning. In spite of extensive efforts, no *S. festinus* was caught by sweep netting in cover crops of these diseased vineyards.

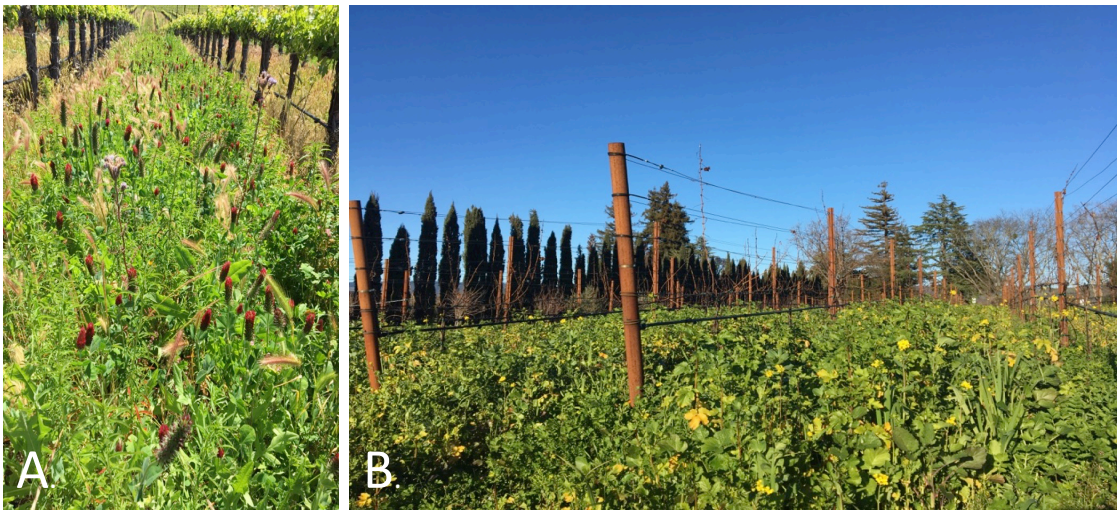

Fig. 8. (A) Red clover and other cover crop species in middle rows of a diseased vineyard, and (B) vetch, birdsfoot trefoil and other cover crop species in vineyard middle rows. Photos were taken in spring 2017.

To address objective #2b and survey cover crops in Napa Valley vineyards for GRBV, we surveyed cover crop species, particularly legume species, in 13 vineyards of Sauvignon blanc, Cabernet franc, Merlot and Cabernet Sauvignon in Napa Valley for GRBV by PCR. These vineyards were selected for this study because they are

infected with GRBV or proximal to vineyards infected with GRBV. A total of close to 500 legume samples including fava beans (*Vicia faba*), purple vetch *(Vicia americana),* red and white clover (*Trifolium spp.*), field peas (*Pisum sativum* subsp. *Arvense)* and other non-leguminous species (barley, oats, rye and grasses) were collected in early March in 2017 and 2018. Results showed that none of the samples tested was positive for GRBV in PCR (Cieniewicz et al., 2019a). These findings were consistent with similar work that did not yield any positive for GRBV in 2014-2016. Together, 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.

To address objective #3a and agroinoculate commonly used vineyard cover crop species with infectious GRBV clones and assess virus infection, we agroinoculated seedlings of clover, vetch, bean (Figure 9), Medicago and peas by needle pricking or syringe infiltration using infectious GRBV clones (Yepes et al., 2018). *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) were agroinoculated as previously described (Yepes *et al.,* 2018). Negative controls included a mock inoculated (sterile needle) and non-inoculated plants. 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).

Fig. 9. Snap bean leaves agroinoculated with a GRBV infectious clone by syringe infiltration.

Results showed accumulation of GRBV spliced transcripts in inoculated leaves of bean, *N. benthamiana* and *S. lycopersicum* 'Florida Lanai' by seven dpi but not in apical (uninoculated) leaves. This suggested that GRBV is replicating locally in inoculated leaves of these three herbaceous plant species but not moving systemically. None of the legumes used as cover crops in vineyard middle-rows that were tested sustained the replication of GRBV. These results provide compelling evidence that legume species used in vineyard cover crop mixes are unlikely involved in red blotch disease epidemiology as virus reservoirs. Nonetheless, snap bean, tomato, and *N. benthamiana* can facilitate studies of virus-host interactions in the future.

To address objective #3b and examine the reproductive potential of *S. festinus* on commonly used vineyard cover crop species, groups of 10-20 TCAH female and male adults were placed on clover, vetch and bean in cages in the greenhouse. Alfalfa and *Vitis vinifera* 'Syrah' were used as controls. Hoppers were evaluated for feeding behavior, oviposition, and reproduction, particularly for the emergence of eggs and nymphs. Results showed that TCAH reproduces on all the species tested. However, a reproductive cycle (adult to adult) was only completed on

alfalfa and bean, not on grapes. 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. These results are consistent with those of Preto et al. (2018b).

To address objective #4 and disseminate research results to the grape and wine industry, and to farm advisors, presentations were delivered on red blotch disease ecology to 710 growers, farm advisors, extension educators, crop consultants, researchers, vineyard managers and regulators at the following grower meetings and conventions in California, New York, North Carolina, and Ontario, Canada in 2018-2019:

- Fuchs, M. 2019. Biology of grapevine viruses. Mealybug and virus outreach meeting, April 4, Stockton, CA (participants = 250).
- Fuchs, M. 2019. Impact of leafroll and red blotch diseases. Vinedresser meeting, March 28, Dobson, NC (participants = 20).
- 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).
- Cieniewicz, E. and 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).

These presentations provided opportunities to communicate on research progress and discuss optimal red blotch disease management tactics that primarily focus on the elimination of virus inoculum sources in vineyards (Cieniewicz et al., 2019b).

CONCLUSIONS

Characterizing the spatiotemporal distribution of infected vines in two vineyards in California and one vineyard in New York documented distinct spread patterns of GRBV, ranging from a relatively high rate of spread (an average of 10% increase in infected vines annually) to no spread. Populations of TCAH peaked in July in the two study vineyards in California, although their abundance was relatively low in both vineyards. However, a higher TCAH population was found at the edge of the California vineyard proximal to a riparian area where spread is readily occurring, highlighting the likely importance of riparian areas for the TCAH. In addition, an association was found between the rate of GRBV spread in the two California vineyards and the abundance of TCAH populations with higher rates of spread correlated to higher TCAH populations. No TCAH was found in the New York vineyard where spread is not occurring. Preliminary work suggests that the transmission mode of GRBV by the TCAH is circulative and non-propagative. Surveys of vineyard middle-row cover crops, particularly of legumes, for GRBV in Spring of 2014-2018 revealed that none of the samples tested was infected with the virus, suggesting that cover crops, including legumes, have limited, if any, role in disease epidemiology as virus reservoirs. Nonetheless, plants of *Phaseolus vulgaris*, tomato and *Nicotiana benthamiana* were identified as local hosts following agroinoculations with infectious GRBV clones in the greenhouse. These herbaceous plants will facilitate virus-host interaction studies in the future. Additionally, the TCAH was able to establish on *Phaseolus vulgaris* but not on *Vitis vinifera.* Research findings on the ecology of red blotch were communicated to the wine and grape industry during winter grower conferences.

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