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BIOLOGY AND SPREAD OF GRAPEVINE RED BLOTCH-ASSOCIATED VIRUS

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ABSTRACT

Limited information is available on the ecology of grapevine red blotch virus (GRBV), the causal agent of red blotch disease. The goals of this project were to advance our understanding of epidemiological aspects of red blotch disease and disseminate information to the wine and grape industry. First, we analyzed the spatial incidence of GRBV over a four-year period (2014–2017) in a Cabernet franc vineyard in California and documented the occurrence of virus spread with a 1-3% annual increase of disease incidence. By contrast, no evidence of spread was obtained in a Merlot vineyard in New York. To determine the diversity and distribution of potential vector candidates in California, sticky cards were placed from March to November in the Cabernet franc vineyard area where disease incidence increased by nearly 20% between 2014 and 2016. GRBV was consistently detected in four species caught on traps in 2015 and 2016: *Spissistilus festinus* (Membracidae), *Colladonus reductus* (Cicadellidae), *Osbornellus borealis* (Cicadellidae) and a *Melanoliarus* species (Cixiidae), suggesting that these four insect species can ingest GRBV. Populations of the four candidate vectors peaked from June to September with viruliferous *S. festinus* culminating from late June to early July in both years. An assessment of co-occurrence and covariation between the spatial distribution of GRBV-infected vines and viruliferous insects identified a significant association only with viruliferous *S. festinus*, revealing the epidemiological relevance of *S. festinus* as a vector of GRBV. In contrast, none of the four California vector candidates were found on sticky traps from May to August 2017 in the Merlot vineyard in New York, and none of the insect species that were caught, including Membracidae, Cercopidae and Cicadellidae, consistently tested positive for GRBV. Second, a search of alternate hosts of GRBV in vineyard ecosystems revealed a high virus incidence in free-living grapes in California but not in New York. The incidence of GRBV in free-living vines was significantly higher in samples from California counties with high compared to low grape production, and in samples near (< 5km) to compared to far (> 5km) from vineyards. These results suggested a directional spread of GRBV inoculum predominantly from vineyards to free-living *Vitis* species. The prevalence of GRBV in California free-living vines highlights the need for vigilance regarding potential GRBV sources in order to protect new vineyard plantings and foundation stock vineyards in California. Third, none of the nearly 500 legume cover crop samples, i.e. bell beans, field peas, vetch, clover, etc., from diseased California vineyard middle rows that were surveyed in spring 2017 and 2018 were positive for GRBV. These results indicated that free-living *Vitis* species but not the legume cover crops serve as reservoir of GRBV. Our insights into the spread of GRBV and population dynamics of *S. festinus* informed epidemiological features of red blotch disease. These findings were used to develop optional disease control recommendations based on vineyard management, i.e. roguing or vineyard removal, depending on the level of disease incidence, and removal of free-living vines proximal

to vineyards. Information from this project was communicated to the wine and grape industry at various venues.

LAYPERSON SUMMARY

This project was designed to advance our understanding of the ecology of grapevine red blotch virus (GRBV) and disseminate information to the wine and grape industry. Analysis of GRBV incidence over a four-year period (2014–2017) in a California vineyard indicated a 1-3% annual increase. By contrast, no evidence of spread was obtained in a New York vineyard. To identify potential vector candidates, sticky cards were placed from March to November in the California vineyard where spread was documented. GRBV was consistently detected in four species caught on traps in 2015 and 2016: *Spissistilus festinus* (Membracidae), *Colladonus reductus* (Cicadellidae), *Osbornellus borealis* (Cicadellidae) and a *Melanoliarus* species (Cixiidae). Populations of these four candidate vectors peaked from June to September with viruliferous *S. festinus* culminating from late June to early July in both years. A significant association was found between the spatial distribution of GRBV-infected vines and of viruliferous *S. festinus*, indicating that *S. festinus* is a vector of epidemiological relevance. None of the four vector candidates was found on sticky traps placed in the New York vineyard selected for this study from May to August 2017 and none of the insects caught on traps consistently tested positive for GRBV. To search for alternative host of GRBV, populations of free-living grapes in California and New York were surveyed for GRBV. Results showed a high virus incidence in free-living grape samples from California and not from New York. Knowing that legumes are preferred hosts of *S. festinus* and commonly used as vineyard cover crops in middle rows, we surveyed legume cover crops, i.e. bell beans, field peas, vetch, clover, etc., for GRBV in diseased California vineyards in spring 2017 and 2018. None of the nearly 500 legumes samples tested positive for GRBV. Collectively, our work provided insights into the spread of GRBV and population dynamics of *S. festinus*, and informed epidemiological features of red blotch disease. Our findings were helpful to devise disease control recommendations based on vineyard management based on roguing or vineyard removal, depending on the level of disease incidence, and removal of free-living vines proximal to vineyards to protect new plantings and foundation stock vineyards in California.

INTRODUCTION

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). 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; Sudarshana et al., 2015). Poor fruit quality results from interference with the 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 in New York (Ricketts et al., 2017). These estimates highlight the economic impact of red blotch disease in different grape-growing regions of the U.S.

Disease 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 (Sudarshana et al., 2015). Grapevine red blotch virus (GRBV), the causal agent of red blotch disease (Yepes et al., 2018), is

documented in all major grape-growing US States (Krenz et al., 2014) and Canada (Poojari et al., 2017). GRBV is also present in numerous table grape accessions at the USDA germplasm repository in Davis, CA (Al Rwahnih et al., 2015) and hybrid accessions at the USDA germplasm repository in Geneva, NY (Perry and Fuchs, unpublished). 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., 2017), South Korea (Lim et al., 2016) and India (GenBank accession number KU522121).

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). 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.

In spite of tremendous progress in recent years on the biology and ecology of GRBV, research on spread in vineyards is needed. This research is important not only to document the extent of vector-mediated transmission of GRBV but also to identify insect vectors. This is a prerequisite for the development of optimal disease management strategies. Also, limited information is available on the role of alternate hosts in disease epidemiology. Wild grapes have been identified as potential reservoirs of the virus in some locations in Napa Valley (Badher et al., 2016b; Perry et al., 2016) but the extent of infection remains to be determined in riparian areas. Similarly, the occurrence of other alternate hosts, particularly of legumes that are commonly used as vineyard cover crops, needs to be evaluated. Finally, disseminating information to the industry is essential to extend research and share the latest knowledge on the ecology of GRBV.

OBJECTIVES

The overarching goal of this project is to advance our understanding of red blotch disease and its causal agent, GRBV. Our specific objectives are to:

1. Monitor the spread of GRBV in selected vineyards in California and New York
    - Evaluate the presence of vector candidates in a selected vineyard in which spread of GRBV is documented in Napa Valley
    - Carry out controlled transmission experiments of GRBV with potential vector candidates
    - Determine the extent to which wild grapes and vineyard cover crops in Napa Valley harbor and serve as reservoirs of GRBV
2. Develop an innovative inoculation methodology of grapevines with GRBV in order to assess symptoms and the impact of clade I and II isolates of the virus
3. Disseminate research results to farm advisors and to the grape and wine industry

RESULTS AND DISCUSSION

Objective 1: *Monitor the spread of GRBV in selected vineyards in California and New York.* The goal of this objective is to quantitatively evaluate spread of GRBV in diseased vineyards, identify a vector of epidemiological importance for GRBV, and identified host reservoirs of GRBV in distinct vineyard ecosystems.

To quantitatively measure spread of GRBV, a 5-acre Cabernet franc vineyard and a 1-acre Merlot vineyard were selected for this study in California and New York, respectively. The two vineyards were chosen based on a relatively low disease incidence. The California and New York vineyards were planted in 2008. In 2013 and 2014, virus prevalence was determined in the two selected vineyards by visual monitoring of diseased vines and testing randomly selected vines for GRBV by multiplex PCR (Krenz et al., 2014). This information was used as a baseline to determine the spatiotemporal incidence of GRBV. Of importance, the California Cabernet franc vineyard was located in proximity to a riparian area.

Disease incidence was 4% (305/7,691 vines) in 2014, 6% (461/7,686 vines) in 2015, 7% (547/7,679 vines) in 2016 and 9% (696/7,670 vines) in 2017 throughout the entire 5-acre Cabernet franc vineyard in California. This result revealed an overall annual 1-3% increase in disease incidence in 2014-2017. Noteworthy, the magnitude of the annual increase in number of symptomatic vines was most substantial in the section of the vineyard adjacent to the riparian habitat (**Fig. 1**).

Plotting disease incidence along the long axis of the vineyard for each year highlighted two major points: i) the absolute magnitude of increase in red blotch incidence slightly varied between 2014 and 2015 (2.1%), 2015 and 2016 (1.1%) and 2016 and 2017 (2%), and ii) the annual increase in incidence was primarily localized to the section of the vineyard nearest the riparian area (**Fig. 1, right**). In this section, disease incidence increased by 16% from 2014 to 2015, to 4.8% from 2015 to 2016, and 12% from 2016 to 2017 (Cieniewicz et al., 2017b).

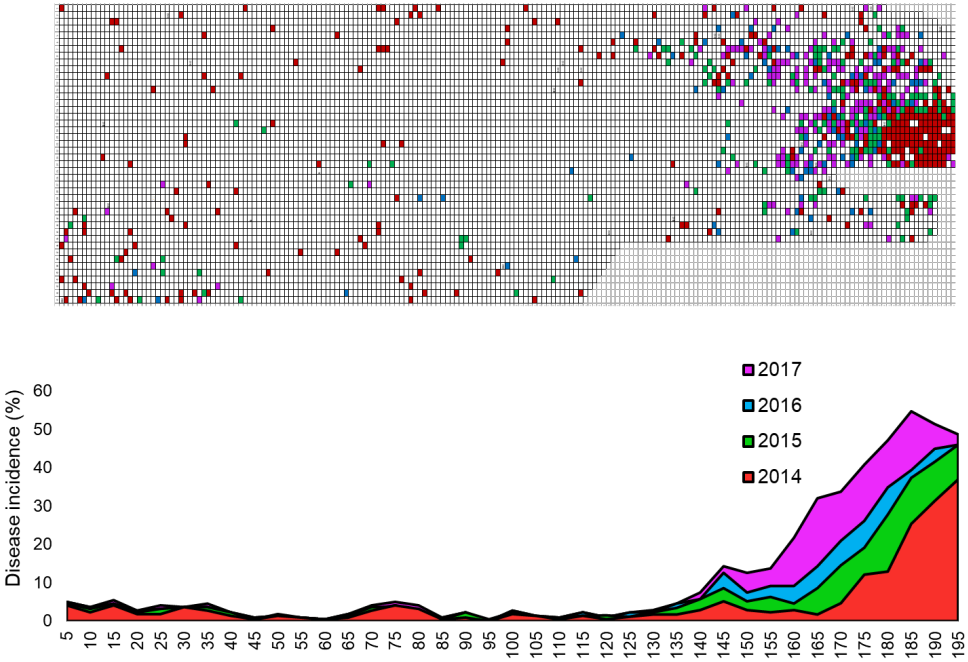

Figure 1. Spatial pattern of diseased vines over a four-year period (2014-2017) in a 5-acre Cabernet franc vineyard affected by red blotch in California. (a) Colored cells indicate diseased vines in 2014 (red), 2015 (green), 2016 (blue) and 2017 (purple). (b) Annual disease incidence plotted over the long axis in 5-vine panel increments.

Similar work in a Merlot vineyard in New York provided no evidence of GRBV spread from 2014 to 2017 (data not shown). These findings suggested that an hemipteran GRBV vector does not exist in the New York vineyard ecosystem or it eventually exists but at a very low population density 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 the Cabernet franc vineyard in California, ordinary runs analysis in 2014-2016 indicated a significant aggregation ($Z_u \leq$ -1.64) of diseased vines in at least two of the three years in rows #16, 21-33, 35, and 38, as well as randomly distributed diseased vines in the remaining rows. Aggregation was observed in 23%, 36% and 39% of the rows in 2014, 2015, and 2016, respectively. The level of aggregation of diseased vines, as indicated by the magnitude of the *Z*-statistic, was higher in rows #21 through 33 than in the other rows analyzed. Based on the results of ordinary runs analysis, the spatial pattern of diseased vines in rows #21 through 33 was selected for analysis using SADIE (Cieniewicz et al., 2017b).

Spatiotemporal analysis between consecutive years within the association function of SADIE revealed a strong overall association among all three years (X = 0.874-0.945). In addition, significant spatial associations ($P < 0.001$) were detected between the local clustering indices between successive seasons, suggesting the degree of spatial aggregation of diseased vines was associated with the spatial position of diseased vines in the previous year. This result also indicated that GRBV can spread over time within and between rows in a vineyard area where diseased vines are aggregated. Analysis of epidemic spread fitting a stochastic spatiotemporal model using the Monte Carlo Markov Chain method identified strong evidence for localized (within vineyard) spread. Altogether, a spatial pattern consisting of a combination of strongly aggregated and randomly isolated symptomatic vines within 8-years post-planting suggested unique epidemic attributes compared to those of other grapevine viruses vectored by mealybugs and soft scales or by dagger nematodes for which typical within-row spread and small-scale autocorrelation are well documented. These findings were consistent with the existence of a new type of vector for a grapevine virus (Cieniewicz et al., 2017b).

Close to 100 sentinel vines, i.e. healthy vines for which the mother stocks from which scion budwood and rootstock canes were collected from tested negative for GRBV were planted in the Cabernet franc vineyard in California in spring 2015. None of these vines flagged for GRBV in 2016 and 2017. These vines will be further monitored in 2018 and tested for GRBV to gain direct evidence of insect-mediated GRBV spread if they become infected. Sentinel vines replaced existing vines that were weak, regardless of their GRBV infectious status.

To evaluate the presence of vector candidates in a selected vineyard in which spread of GRBV is documented in Napa Valley, insect yellow sticky traps were placed at the edge of the selected vineyard in California where clustering of diseased vines is occurring proximal to the riparian area (**Fig. 1, right**). Traps were positioned on the middle trellis wire throughout a sampling area that spanned 12 rows, and six 4-vine panels per row.

Sticky cards were removed weekly, placed in plastic bags, shipped overnight from the vineyard to the laboratory in Geneva, NY for evaluation, and replaced with new sticky cards. The survey was conducted from April to November 2015, and March to November 2016 to span the entire growing season (Cieniewicz et al., 2018b). Insects caught on sticky card traps were identified to genus and species where possible based on morphological characteristics. Specimens were identified and counted while still impacted on sticky cards. The number and identity of specimens was recorded for each sticky card to evaluate the abundance and diversity of flying insects.

Of approximately 134,000 insects caught on yellow sticky traps in both years, 960 (700 and 260 in 2015 and 2016, respectively) were tested for GRBV by multiplex PCR (Krenz et al. 2014). Specimens were individually removed from sticky cards using Goo Gone liquid degreaser to dissolve the adhesive and loosen the specimens. Individual specimens were stored at -20°C until testing by multiplex PCR for GRBV detection (Krenz et al. 2014) and/or species identification by sequencing of the mitochondrial DNA barcode region. GRBV was detected in at least 40% of *S. festinus* (Membracidae), *C. reductus*

(Cicadellidae), *O. borealis* (Cicadellidae) and a *Melanoliarus* species (Cixiidae) by multiplex PCR. This result revealed that specimens of these four hemipteran species visited the study vineyard and ingested GRBV over two consecutive years. GRBV was not found by multiplex PCR in the majority of other insects tested over two consecutive years, or it was found in only a few specimens (3 to 8%) of a limited number of insects (Cieniewicz et al., 2018a). The four insect vector candidates (*S. festinus, C. reductus, O. borealis* and a *Melanoliarus* spp.) collectively comprised only 0.14% (87 of 62,128 in 2015 and 99 of 72,242 in 2016) of specimens on sticky cards in both years, and 0.4% (87 of 18,525) and 0.6% (99 of 16,060) of Hemiptera on sticky cards in 2015 and 2016, respectively (Cieniewicz et al., 2018a).

The relative abundance of the four vector candidates captured on sticky cards was low with only 87 and 99 specimens in 2015 and 2016, respectively. Populations of the four vector candidates peaked between June and September during both years. Populations of *S. festinus* peaked during early July 2015 (**Fig. 2A**) and late June 2016 (**Fig. 2B**), with populations quickly tapering after July. Populations of *C. reductus* peaked in August 2015 (**Fig. 2A**) and in April and September 2016 (**Fig. 2B**). Populations of *Melanoliarus* sp. peaked in June and July, while *Osbornellus borealis* was captured infrequently in June and July, and increased in August and September of both years (**Fig. 2**) (Cieniewicz et al., 2018a).

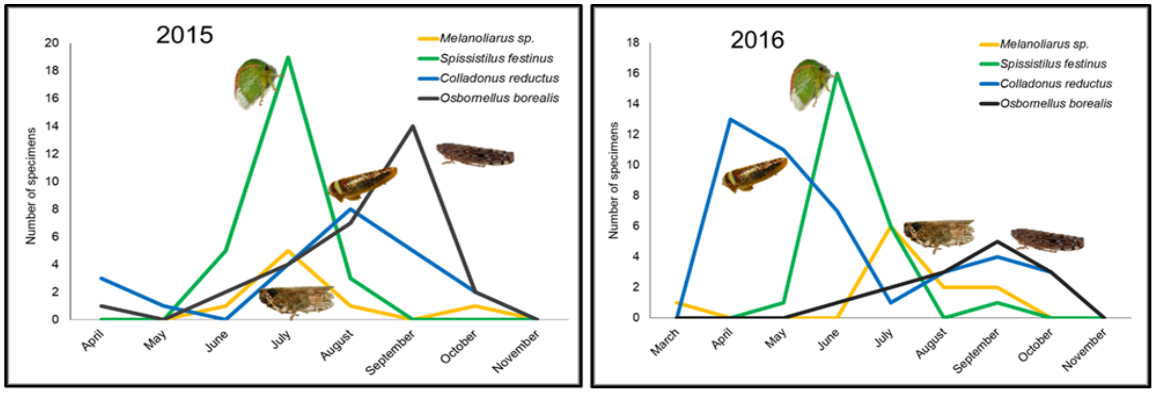

Figure 2. Seasonal population dynamics of candidate insect vectors of GRBV based on specimens captured on sticky cards in a diseased Cabernet franc vineyard in (A) 2015 and (B) 2016.

GRBV was not detected in vector candidates until June, with the exception of one *C. reductus* specimen that tested positive for GRBV in early May 2016. The incidence of viruliferous *S. festinus* was highest in July in 2015 and June in 2016. Viruliferous *O. borealis* were detected from July to November in 2015 and 2016, while viruliferous *Melanoliarus* sp. were captured on sticky cards only from July to September (Cieniewicz et al., 2018a).

Spatial pattern analyses indicated aggregated patterns of GRBV-infected vines and populations of *S. festinus* and *O. borealis*. No significant aggregation was found for *C. reductus* and the *Melanoliarus* sp. (data not shown). Moreover, there was a significant spatial association between the distribution of infected vines and viruliferous *S. festinus* (Cieniewicz et al., 2018a). No significant spatial associations were identified between populations of alternative insect vector candidates and GRBV-infected vines. The spatial distribution of vector candidates on sticky cards also indicated a gradient of higher *S. festinus* (N= 50) and *O. borealis* (N=42) at the edge of the vineyard next to a riparian area and decreasing *S. festinus* and *O. borealis* populations distant from the edge. Additionally, both *S. festinus* and *O. borealis* populations near the edge of the vineyard (0 to 10 m from the edge of the vineyard) had a higher proportion of viruliferous insects than the inner-vineyard (10 to 20 m or 20 to 30 m within the vineyard), in which the proportions of viruliferous specimens were lower. The spatial distribution of *C. reductus* (N = 64) and *Melanoliarus* (N = 20) was not dependent upon proximity to the edge of the vineyard (Cieniewicz et al., 2018a).

To carry out carry out controlled transmission experiments of GRBV with potential vector candidates, specimens of *S. festinus* from alfalfa fields in Yolo and Kern counties in California were collected, transferred to Cornell University, and established on alfalfa plants in a growth chamber with controlled temperature, humidity and photoperiod. Alfalfa is a host of *S. festinus* but not of GRBV (Cieniewicz et al., unpublished). Conditions to rear *S. festinus* colonies were optimized so that a full development cycle, including oviposition, and the production of nymphs (**Fig. 3**) and adults, could be completed within two months.

Figure 3. Nymphs of *S. festinus* on alfalfa plants in a growth chamber.

The transmission mode of GRBV by *S. festinus* is hypothesized to be nonpropagative, circulative. To address this issue, *S. festinus* were allowed to feed on GRBV-infected grapevines for 48-72 h. Then, groups of 2-4 individuals were transferred to alfalfa and allowed to feed for two weeks. These assays were duplicated. Subsets of *S. festinus* were tested for the presence of GRBV after the acquisition and alfalfa feeding steps. After the acquisition period, 6 out of 8 *S. festinus* in experiment #1, and 3 of 5 *S. festinus* in experiment #2 were positive for GRBV in multiplex PCR, confirming that *S. festinus* can ingest GRBV. After feeding on alfalfa, most specimens tested (12 of 20 in experiment #1 and 6 of 11 in experiment #2) were positive for GRBV, revealing that *S. festinus* is capable of keeping the virus even after a gut-clearing episode on a nonhost plant of GRBV. These findings suggested a persistent transmission of GRBaV. To further our understanding of the transmission mode of GRBV, additional work is under way to localize the virus in organ tissue of *S. festinus.*

S. festinus specimens reared on alfalfa were collected and deposited on GRBV-infected vines in insect-proof cages in the greenhouse. *S. festinus* were allowed to feed for 2-6 days. After the virus acquisition access period, individual *S. festinus* were moved to healthy vines (2-3 specimens per vine) for a 4-6 day transmission access period. Individual insects were then collected and tested for GRBV for multiplex PCR and vines were monitored for symptom expression and presence of GRBV. Preliminary results showed that *S. festinus* transmits GRBV to healthy vines in the grenhouse, confirming previous findings (Badher et al., 2016a). Additional transmission experiments are underway to validate preliminary findings.

To determine the extent to which wild grapes and vineyard cover crops in Napa Valley harbor and serve as reservoirs of GRBV, the distribution and diversity of GRBV was determined in free-living *Vitis* species in northern California and New York from 2013 to 2017. These surveys were justified because the occurrence of GRBV in free-living grapes was previously documented (Bahder et al., 2016a; Perry et al. 2016). Survey outputs revealed that GRBV was detected by PCR in 21.5% (43/203) of samples from California but in none of the 163 samples from New York counties such as Suffolk (0%, 0/21), Ulster (0%, 0/31), Clinton (0%, 0/20), Ontario (0%, 0/20), Chautauqua (0%, 0/20), Tompkins (0%, 0/6), Yates (0%, 0/18), Steuben (0%, 0/14) and Seneca (0%, 0/25). GRBV-infected California samples were found in Napa County (28%, 24/87), Sonoma County (22%, 5/23), Solano County (15%, 3/20), Sacramento County (29%, 9/31), Sutter County (5%, 1/19), Butte County (0%, 0/15) and Glenn County (0%, 0/12).

Noteworthy, the incidence of GRBV in free-living vines was significantly higher in samples from California counties with high compared to low grape production ($\chi 2 = 83.09$; $P < 0.001$) (**Fig. 4**), and in samples near (< 5km) to compared to far (> 5km) from vineyards ($\chi 2 = 57.58$; $P < 0.001$) (Cieniewicz et al., 2018b).

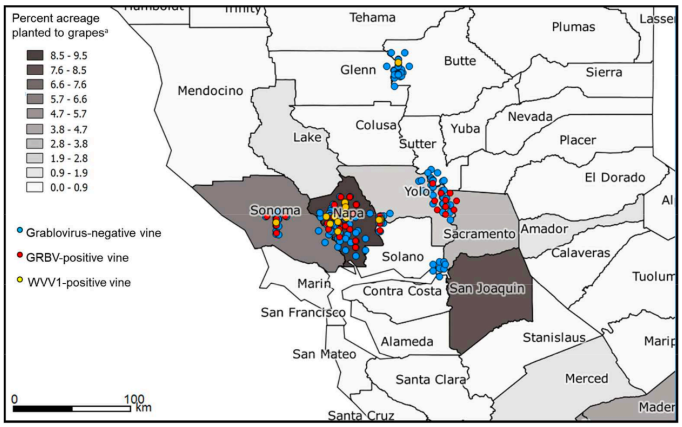

Figure 4. Map of Northern California counties showing the density of acreage planted to grapevine, along with the location and infectious status of free-living *Vitis* vines tested for GRBV and WVV1.

Additionally, the distribution of the recently identified wild Vitis virus 1 (WVV1) was also investigated in populations of free-living *Vitis* spp. in California (Cieniewicz et al., 2018b). WVV1 was recently found in free-living grapevines in Napa County, California but has yet to be identified in vineyards (Perry et al., 2018). This virus belongs to the genus *Grablovirus* and is closely related to GRBV (Perry et al., 2018). Survey outputs revealed the presence of WVV1 in wild vines in California but not in New York. WVV1 incidence was significantly higher in areas with higher grape production acreage ($\chi 2 = 16.02$; $P < 0.001$) (**Fig. 4**). However, in contrast to GRBV, no differential distribution of WVV1 incidence was observed with regard to distance from vineyards ($\chi 2 = 0.88$; $P = 0.3513$) (Cieniewicz et al., 2018b).

Analyzing the genetic diversity of GRBV populations from California free-living *Vitis* samples revealed two distinct phylogenetic clades (Cieniewicz et al., 2018b) (**Fig. 5**), as previously shown for isolates from vineyards (Krenz et al., 2014) and vector candidates (Cieniewicz et al., 2017b).

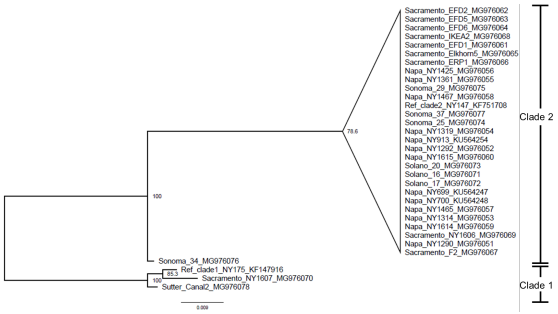

Figure 5. Maximum likelihood phylogeny of grapevine red blotch virus (GRBV) diversity fragment nucleotide sequences of isolates from free-living *Vitis* spp. in California. Numbers at branches indicate bootstrap support (1000 bootstrap replicates, random seed=3). Branches with less than 70% bootstrap support were collapsed.

Two genetic clades were also identified for WVV1 from free-living *Vitis* species in California (Cieniewicz et al., 2018b). (**Fig. 6**).

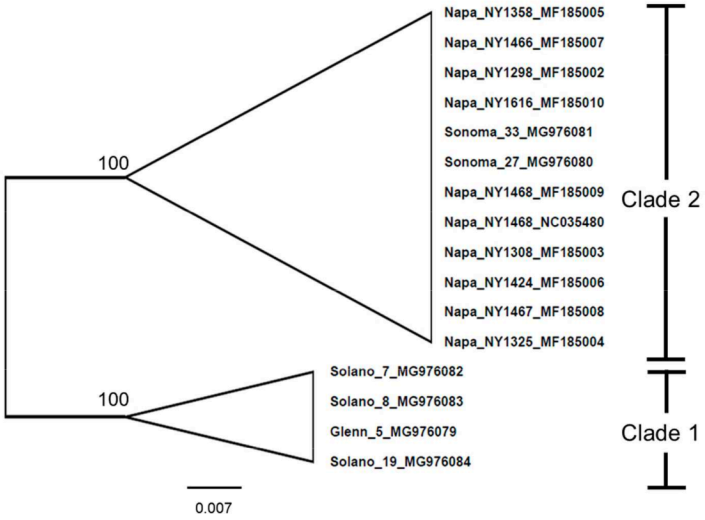

Figure 6. Maximum likelihood phylogeny of wild Vitis virus 1 (WVV1) diversity fragment nucleotide sequences of isolates from free-living *Vitis* spp. in California. Numbers at branches indicate bootstrap support (1000 bootstrap replicates, random seed=3). Branches with less than 70% bootstrap support were collapsed.

Interestingly, the nucleotide sequence variability was higher for WVV1 (94.3-99.8% sequence identity within clade 1 isolates; 90.1-100% within clade 2 isolates) than GRBV (98.3% between clade 1 isolates; 96.9- 100% within clade 2 isolates) (Cieniewicz et al., 2018b). Moreover, evidence for intra-specific recombination events was found in WVV1 isolates and confirmed in GRBV isolates (Cieniewicz et al., 2018b). The prevalence of grabloviruses in California free-living vines highlights the need for vigilance regarding potential inoculum sources in order to protect new vineyard plantings and foundation stock vineyards in California.

A total of 13 California vineyards of Sauvignon blanc, Cabernet franc, Merlot and Cabernet Sauvignon was selected for surveys of cover crops, particularly legume species. These surveys are justified because legumes, i.e. bell beans, field peas, vetch, clover, alfalfa, medicagos, etc., are preferred hosts of *S. festinus* and commonly used as cover crops in vineyard middle rows. The 13 California vineyards are infected with GRBV or proximal to vineyards infected with GRBV. In addition, they carry legumes in their cover crop stands sown in November 2016 (**Fig. 7**). Three additional vineyards were selected because they are not infected with GRBV and carry legumes species in their cover crops stands. Alfalfa samples from unmanaged areas proximal to GRBV-infected vineyards were also sampled.

A total of over 500 legume samples from vineyard middle-row cover crops have been collected in early March in 2017 and 2018 for GRBV testing by multiplex PCR (Krenz et al., 2014) or real time PCR (Setiono et al., 2018). Results showed that all the samples were negative for GRBV in PCR, indicating that legume cover crop species are not infected with GRBV in diseased vineyards. Thus, legumes or other cover crop mixes are unlikely to serve as reservoirs of GRBV and unlikely to contribute to the epidemiology of red blotch disease.

Figure 7. Cover crops in a GRBV-infected vineyard surveyed for GRBV and *S. festinus.*

Objective 2: *Develop an innovative inoculation methodology of grapevines with GRBV in order to assess symptoms and the impact of clade I and II isolates of the virus.* The goal of this objective is to develop a biolistic inoculation method of grapevine with GRBV to facilitate the evaluation of vines singly infected with GRBV, while avoiding manipulations using recombinant DNA. Two approaches to infect grapevines with GRBV were tested: 1) direct mechanical inoculation of grape seedlings with GRBV DNA amplified from infected vines using rolling circle amplification (RCA), and 2) biolistic bombardment of grape seedlings with GRBV DNA from RCA. Among plants mechanically inoculated with GRBV DNA, none were infected at 6 months post inoculation. Biolistic bombardment conditions for seedlings are being established using a reporter gene construct. Grapevine seedlings were agroinoculated with bitmer constructs and will be tested for infection at 6 months post-inoculation. Preliminary experiments did not suggest infection. More efforts are needed to address the technical limitations of our approaches.

Objective 3: *Disseminate research results to farm advisors and to the grape and wine industry***.** The goal of this objective is to raise awareness of the impact of red blotch and to inform stakeholders of research progress on the ecology of GRBV. Research results were communicated to 1,904 growers, extension educators, farm advisors and service providers at the following venues in California, New York, Missouri, Oregon 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).

Fuchs, M. 2017. Updates on leafroll and red blotch diseases. Eastern Winery Exposition, March 23, Syracuse, NY (participants = 40).

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Cieniewicz, E.J. and Fuchs, M. 2016. Spatiotemporal spread of Grapevine red blotch-associated virus, Cornell Recent Advances in Viticulture and Enology (CRAVE) conference, November 2, Ithaca, NY (participants = 60).

Fuchs, M. 2016. Research updates on leafroll and red blotch diseases. Grape Growers of Ontario, August 16, Brock University, St Catharines, Ontario, Canada (participants = 25).

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Conclusions

Our research provided new insights into the ecology of grapevine red blotch virus (GRBV). We documented the spread of GRBV and the epidemiological significance of *S. festinus* as a vector of GRBV. Populations of *S. festinus* peaked from late June to early July in two consecutive growing seasons in a diseased Cabernet franc vineyard in Napa Valley. A high incidence of GRBV was found in free-living grapes in California but not in New York. The incidence of GRBV was significantly higher in free-living vines from California counties with high compared to low grape production, and in samples near (< 5km) to compared to far (> 5km) from vineyards. The prevalence of GRBV in California free-living vines highlights the need for vigilance regarding potential GRBV sources in order to protect new vineyard plantings and foundation stock vineyards in California. Interestingly, mone of the close to 500 legume cover crop samples, i.e. bell beans, field peas, vetch, clover, etc., from California vineyard middle rows that were surveyed in spring 2017 and 2018 were positive for GRBV. These results indicated that free-living *Vitis* species but not legume cover crops serve as reservoir of GRBV. Information on GRBV ecology was used to develop optional disease control recommendations based on vineyard management, i.e. roguing or vineyard removal, depending on the level of disease incidence, and removal of free-living vines proximal to vineyards. Information from this project was disseminated to the wine and grape industry at various venues.

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