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Investigating the Latency Period of Grapevine Red Blotch Virus in a Diseased Cabernet franc Vineyard Experiencing Secondary Spread

Madison Flasco [1,2*], Elizabeth J. Cieniewicz [1,3], Monica L. Cooper [4], Heather McLane[1], and Marc Fuchs [1]

Abstract

Background and goals

Grapevine red blotch virus (GRBV) causes red blotch disease and is a threat to vineyard sustainability in some areas. Advances in disease epidemiology have been made, but information on the latency period of GRBV is lacking. This work aimed to estimate red blotch disease latency (the time between a vine first testing positive for GRBV and the onset of disease symptoms).

Methods and key findings

Sentinel vines consisting of GRBV-negative Cabernet franc buds grafted onto GRBV-negative 3309 Couderc cuttings were planted in June 2015 in a red blotch-diseased Cabernet franc vineyard experiencing secondary spread of GRBV. Sentinel vines were monitored for disease symptoms from October 2015 to 2023, and were sampled annually for GRBV testing from October 2015 to 2023 and from June 2021 to 2022. The first sentinel vine tested positive for GRBV by polymerase chain reaction (PCR) in October 2018; this vine became symptomatic the following October. An increasing number of sentinel vines tested positive for GRBV by PCR and exhibited foliar disease symptoms either when the virus was detected, or four to 12 months later.

Conclusions and significance

Of those sentinel vines that became infected (69%, 25/36), three distinct patterns of initial GRBV detection and disease symptom onset were observed. The first applied to sentinel vines (20%, 5/25) that were both GRBV positive by PCR and symptomatic for the first time in October. The second pattern applied to sentinel vines (56%, 14/25) that were GRBV positive by PCR for the first time in June and symptomatic the following October. The third pattern applied to sentinel vines (24%, 6/25) that were GRBV positive in PCR for the first time in October and not symptomatic until the following October. Estimating a four-to-12-month latency period enables a better understanding of red blotch disease trajectories in vineyards showing signs of secondary spread.

Key words: geminivirus, red blotch disease, *Spissistilus festinus,* symptoms, three-cornered alfalfa hopper

Introduction

Red blotch disease was described for the first time more than 15 years ago (Calvi 2011). This disease is caused by grapevine red blotch virus (GRBV) (Yepes et al. 2018), a threat to grape production and vineyard sustainability in some areas of North America (Sudarshana et al. 2015, Cieniewicz et al. 2017a, Rumbaugh et al. 2021). GRBV elicits red or chlorotic blotches on leaves of red- and white-berried *Vitis vinifera* cultivars, respectively (Sudarshana et al. 2015, Cieniewicz et al. 2017b, Rohrs et al. 2023). GRBV is a member of the genus *Grablovirus* in the plant virus family *Geminiviridae* (Varsani et al. 2017, Rojas et al. 2018), and analysis of the genetic diversity among GRBV isolates indicated two phylogenetic clades with up to 8.5% nucleotide sequence divergence (Krenz et al. 2014). Isolates of both phylogenetic clades are involved in the etiology of red blotch disease (Yepes et al. 2018).

The widespread distribution of GRBV in North American vineyards results from the dissemination of infected propagation material (Sudarshana et al. 2015, Cieniewicz et al. 2017a), with secondary spread documented in some vineyards in northern California (Cieniewicz et al. 2017b, 2018, 2019, Flasco et al. 2023b) and southern Oregon (Dalton et al. 2019, KC et al. 2022). Implicated in secondary spread of GRBV in northern California vineyards is the three-cornered alfalfa hopper, *Spissistilus festinus* (Flasco et al. 2021). Transmission of GRBV by

[1]Cornell University, School of Integrative Plant Science, Plant Pathology and Plant-Microbe Biology, Geneva, NY 14456; [2]present address, Plant Pathology and Crop Physiology, Louisiana State University, Baton Rouge, LA 70803; [3]present address, Plant and Environmental Sciences, Clemson University, Clemson, SC 29634; and [4]University of California, Cooperative Extension, Napa, CA 94559.

*Corresponding author (mflasco@agcenter.lsu.edu)

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S. festinus is circulative, non-propagative, and more importantly, inefficient among winegrape cultivars (Flasco et al. 2021, 2023a, Hoyle et al. 2022). Furthermore, *S. festinus* is the only vector candidate for which GRBV acquisition to the salivary glands and GRBV transmission in vineyard settings has been documented across multiple experimental replicates (Flasco et al. 2023d).

When GRBV is introduced to a vineyard via infected planting material, the onset of disease symptoms is correlated to the source of infection. Indeed, when the infection source is the winegrape scion material, red blotch disease symptoms are apparent one year post-planting, whereas when the infection source is the rootstock, red blotch disease symptoms are seen three to five years post-planting (Cieniewicz et al. 2019, Flasco et al. 2023b). The incubation period (the time between the exposure of a grapevine to GRBV following inoculation by viruliferous *S. festinus* and the onset of red blotch disease symptoms) is hypothesized to be at least 16 months (Flasco et al. 2023a). The latency period (the time between a grapevine first testing positive for GRBV and disease symptom onset) when infection is due to S. festinus-mediated spread remains unknown. In this study, we produced GRBV-negative sentinel vines, planted them in a red blotch-diseased Cabernet franc vineyard experiencing secondary spread of GRBV, and regularly monitored them to determine the latency period following S. festinus-mediated inoculation.

Materials and Methods

Vineyard site

A 2-ha Cabernet franc vineyard established in 2008 in Napa County, CA was selected for this study. This vineyard consisted of Cabernet franc clones 214 and 623 grafted onto 101-14 Mgt with spacing of 1.2 m and 2.1 m within and between rows, respectively. Throughout the duration of this work, a vertical shoot positioning system was used and drip irrigation and pest management practices conventional for vineyards in Napa County were applied. The Cabernet franc vineyard was selected for this study because the occurrence of GRBV was previously documented, and the spatial distribution of diseased vines was mapped during surveys from 2014 to 2023 (Cieniewicz et al. 2017b, 2019, and this study). In addition, from 2014 to 2018, annual increases of newly symptomatic vines (1 to 4%) were documented in this vineyard (Cieniewicz et al. 2017b, 2019). Furthermore, the presence of *S. festinus* was reported in the vineyard in 2015 and 2016 (Cieniewicz et al. 2018).

Plant material

GRBV-negative sentinel vines were used in this study to monitor both the early stages of GRBV infection following *S. festinus*-mediated inoculation, and the onset of disease symptoms. To produce sentinel vines, buds of GRBV-negative Cabernet franc (unknown clone) were grafted onto cuttings of GRBV-negative 3309 Couderc (*Vitis riparia* × *Vitis rupestris*). During two consecutive growing seasons prior to collecting buds and cuttings for grafting in 2013, the mother vines that supplied the scion buds and rootstock cuttings were confirmed to be GRBV negative by repeated virus testing via multiplex polymerase chain reaction (PCR). The scion buds and rootstock cuttings were collected and delivered to a local nursery for grafting. Grafted vines were callused in a growth chamber and grown in pots in a greenhouse at Cornell AgriTech in Geneva, New York. Then, potted sentinel vines were shipped from Geneva, NY to Napa County, CA with a phytosanitary certificate delivered by the New York State Department of Agriculture and Markets. A total of 36 sentinel vines were planted in June 2015 in the red blotch-diseased Cabernet franc vineyard to replace dead vines, with most vines placed in the eastern area of the vineyard with documented secondary spread (Cieniewicz et al. 2017b) (Figure 1).

Survey for red blotch disease

Sentinel vines were visually monitored annually for red blotch disease symptoms, i.e., red foliar blotches, from October 2015 to 2023. Each year, six leaves and petioles were collected from the base of the canopy, three from each cordon close to the trunk—regardless of the presence or absence of disease symptoms—to determine the GRBV infection status via multiplex PCR (Krenz et al. 2014). No tissue collection was conducted in 2020 because of institutional travel restrictions related to the COVID-19 pandemic. Tissue was similarly sampled from each sentinel vine in June 2021 and 2022, when GRBV-infected vines were asymptomatic (Setiono et al. 2018, Kahl et al. 2021, DeShields and KC 2023, Flasco et al. 2023a, 2023c, Rohrs et al. 2023).

Detection of GRBV and isolate characterization

Collected petioles were processed as previously described (Flasco et al. 2023c). DNA extractions were conducted using the MagMAX-96 Al/ND Isolation Kit (Thermo Fisher Scientific) on a KingFisher instrument. The presence of GRBV in the nucleic acid samples was determined via multiplex PCR using primer pairs to amplify the regions of the open reading frames to code the coat protein and RepA replication-associated protein (Krenz et al. 2014, Cieniewicz et al. 2017a, 2019). DNA amplicons were analyzed via electrophoresis on agarose gels and visualized using ultraviolet illumination after staining with GelRed (Biotium). Samples testing positive for GRBV in PCR underwent further characterization via restriction digestion of DNA amplicons that corresponded to the RepA replication-associated gene of the GRBV genome, to determine the phylogenetic clade of the isolate infecting the sentinel vines, as previously described (Flasco et al. 2023a).

Results and Discussion

Thirty-six GRBV-negative sentinel vines were planted in June 2015, primarily in an area of a red blotch-diseased Cabernet franc vineyard, where diseased vines were aggregated and secondary spread was previously documented

(Cieniewicz et al. 2017b) (Figure 1). Therefore, sentinel vines were at risk of becoming infected with GRBV following natural *S. festinus*-mediated inoculations. Using sentinel vines was paramount to estimate the latency period because their GRBV-negative status was confidently ascribed at planting. As may be expected, the GRBV-negative status of vines already established in the study vineyard (originally planted in 2008) was less certain because the GRBV status of the mother stocks from which the buds and cuttings were sourced for grafting was unknown. Additionally, established vines may be infected with GRBV but not exhibit disease symptoms (Flasco et al. 2023a, 2023c), thus confounding our study.

The first sentinel vine to test positive for GRBV by multiplex PCR was identified in October 2018, but this vine was not symptomatic until October 2019. This vine was growing in the eastern vineyard area experiencing pronounced secondary spread (Cieniewicz et al. 2017b). The fact that the first GRBV-infected sentinel vine was found three years post-planting is consistent with an inefficient GRBV transmission rate from winegrape to winegrape (Flasco et al. 2021, 2023a, Hoyle et al. 2022). Similarly, the first GRBV-infected sentinel vine displayed disease symptoms four years post-planting, one year after testing positive for GRBV, which is consistent with previous observations from controlled *S. festinus* releases in the vineyard (Flasco et al. 2023a). Following 2018, more sentinel vines tested positive for GRBV and expressed foliar symptoms (Table 1), with most infected vines identified within or near the eastern area of the vineyard, where secondary spread has been previously documented (Cieniewicz et al. 2017b) (Figure 2). By 2023, more than two-thirds (69%, 25/36) of the sentinel vines were infected with GRBV (as shown by PCR) and exhibited red blotch disease symptoms (Figure 3 and Table 1).

If a sentinel vine tested positive for GRBV via PCR in October, it also tested positive the following June (Table 1). Additionally, if a sentinel vine displayed foliar disease symptoms (i.e., foliar red blotches), it remained symptomatic through the remainder of the study, although the level of reddening observed on individual leaves and throughout the canopy varied between seasons (Figure 3). This observation on variable disease symptoms is consistent with a recent report (Rohrs et al. 2023). Environmental factors and/or viticultural practices such as water imbalance and fertilizer use may contribute to this variation in disease symptoms (Copp and Levin 2021). Together, our work conducted from 2015 to 2023 identified three distinct patterns occurring between the first instance of a vine testing positive for GRBV by PCR and the first appearance of foliar disease symptoms. The first pattern applied to sentinel vines that tested GRBV positive in PCR and were also symptomatic for the first time in October (20%, 5/25), perhaps suggesting no latency. The second pattern applied to sentinel vines that tested GRBV positive in PCR for the first time in June and became symptomatic the following October (56%, 14/25), indicating a four-month latency period. The third pattern applied to

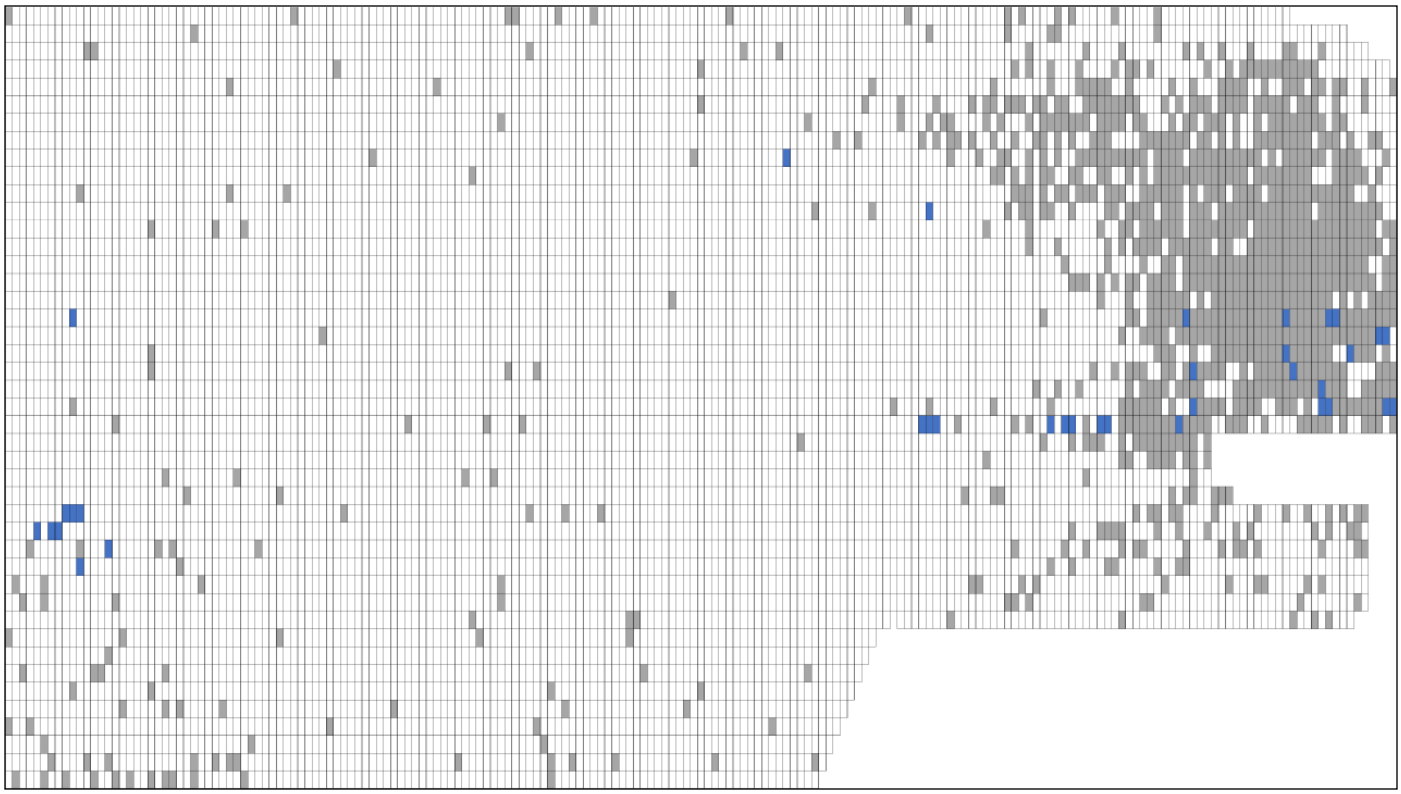

Figure 1 Spatial location of sentinel vines planted in June 2015 in a red blotch-diseased Cabernet franc vineyard. Grapevines exhibiting red blotch disease symptoms (i.e., foliar red blotches) in October 2015 are shown in gray, and the placement of sentinel vines is shown in blue.

sentinel vines that tested GRBV positive in PCR for the first time in October and became symptomatic only the following October (24%, 6/25), illustrating a 12-month latency period. It would be interesting to investigate whether similar trends are observed in other viticultural regions where climate conditions and viticultural practices differ from those in Napa County, CA.

The lack of apparent latency observed for some sentinel vines (20%, 5/25) is difficult to understand because it took months, or even years, for a vine to display disease symptoms following *Agrobacterium tumefaciens*-delivery of an infectious GRBV clone in the laboratory (Yepes et al. 2018). Similarly, vines that tested GRBV positive in PCR following *S. festinus*-mediated inoculations were not symptomatic 16-months postexposure to viruliferous insects (Flasco et al. 2023a). A plausible explanation for the apparent lack of latency is that the GRBV status of these sentinel vines may have been misdiagnosed in June, as previously reported (Setiono et al. 2018, DeShields and KC 2023). Furthermore, some GRBV-positive vines in June may have been overlooked from 2018 to 2020, and in 2023, when sampling was only conducted in October of these years. More work is needed to thoroughly address this apparent lack of latency. Similarly, although visual surveys were conducted in October when symptoms were readily apparent, additional refinements are needed to verify our estimated four-to-12-month latency period because red blotch disease symptom development varies between and within vineyard blocks (Rohrs et al. 2023), and GRBV detectability varies early in the growing season (Setiono et al. 2018, DeShields and KC 2023). Monthly or bimonthly PCR testing and monitoring of sentinel vines

Table 1 Grapevine red blotch virus (GRBV) and red blotch disease symptom status of sentinel vines from 2015 to 2023.

Date[a]	GRBV (+)[b]	Symptomatic[c]
October 2015	0/36	0/36
October 2016	0/36	0/36
October 2017	0/36	0/36
October 2018	1/36	0/36
October 2019	2/36	2/36
October 2020	ND[d]	6/36
June 2021	14/36	NA
October 2021	16/36	13/36
June 2022	22/36	NA
October 2022	24/36	22/36
October 2023	25/36	25/36

[a]Month and year in which survey and/or petiole tissue collection was conducted.
[b]Proportion of sentinel vines testing positive for GRBV via multiplex polymerase chain reaction over the number of sentinel vines tested.
[c]Proportion of sentinel vines exhibiting red blotch disease symptoms over the total number of sentinel vines visually monitored.
[d]ND, not determined; NA, not applicable.

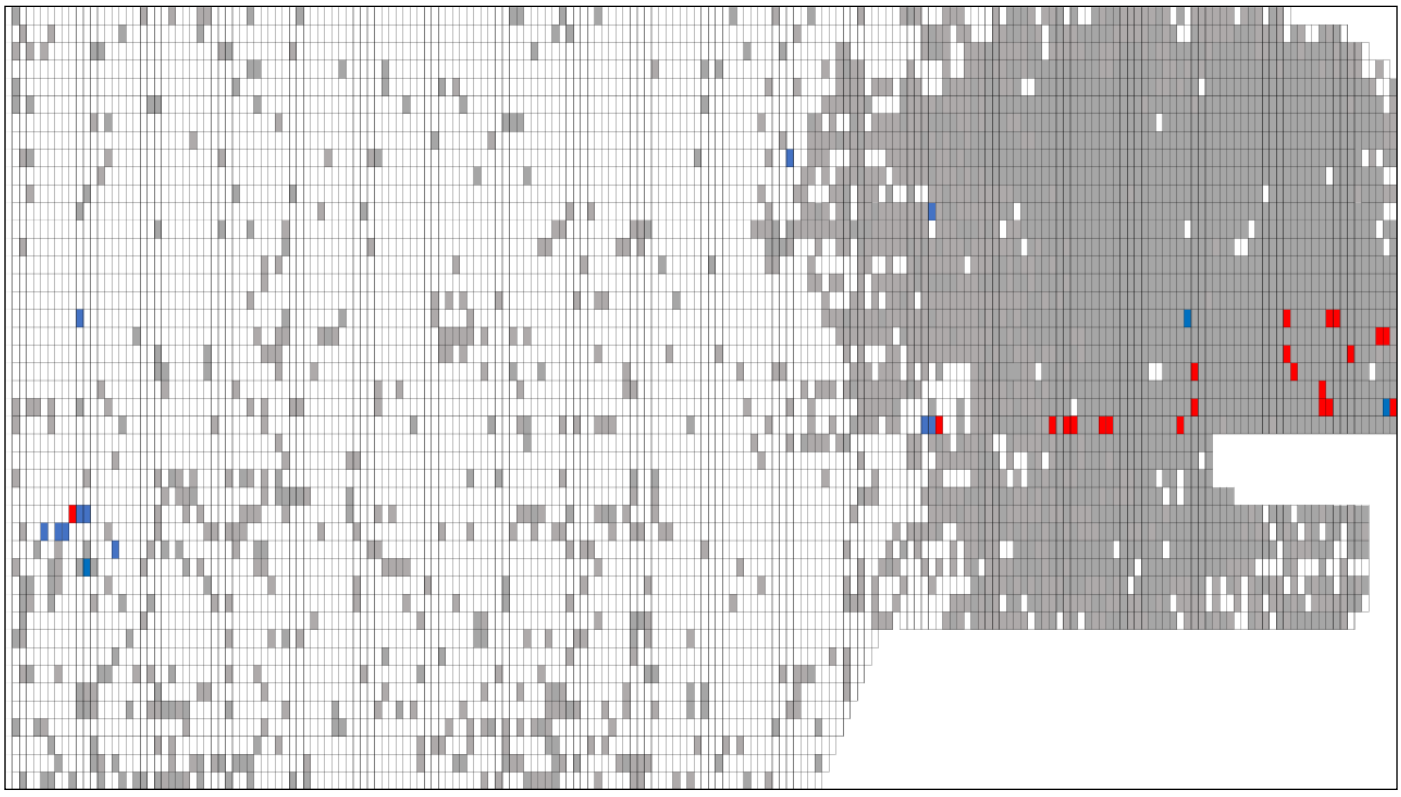

Figure 2 Distribution of sentinel vines testing positive for grapevine red blotch virus (GRBV) via polymerase chain reaction in a red blotch-diseased Cabernet franc vineyard, in October 2023. Grapevines exhibiting red blotch disease symptoms (i.e., foliar red blotches) are shown in gray, sentinel vines testing positive for GRBV are shown in red, and uninfected sentinel vines are shown in blue.

for disease symptoms of the virus may enable verification of this estimated latency period.

To determine the phylogenetic clade to which the GRBV isolates infecting the sentinel vines belonged, additional assays were performed. Results showed that most sentinel vines were infected with GRBV isolates of phylogenetic clade 2 (92%, 23/25) (versus those infected with a GRBV isolate of phylogenetic clade 1 [8%, 2/25]). The skewed occurrence of GRBV clade 2-infected vines coincides with the greater incidence of clade 2 infections in the Cabernet franc study vineyard, particularly in the area with an aggregation of diseased vines and previously documented secondary spread (Perry et al. 2016, Cieniewicz et al. 2017a, 2019). The aggregation of diseased vines was previously speculated to be due to the presence of GRBV in the rootstock of the planting material because it took four years for disease symptoms to be apparent after planting (Cieniewicz et al. 2017b, 2019). Furthermore, all aggregated vines were infected with GRBV isolates of phylogenetic clade 2, and their genomic sequence was identical (Cieniewicz et al. 2017b, 2019). The presence of the same GRBV isolate in different vines can only result from the vegetative propagation of a single source of infected material. Nevertheless, a few GRBV isolates of phylogenetic clade 1 were found in infected vines (Flasco et al. 2023c and this study) and in viruliferous S. *festinus* caught on sticky cards in the Cabernet franc study vineyard (Cieniewicz et al. 2018). Together, these data showed that the type and distribution of GRBV isolates in infected sentinel vines mirrored the composition and distribution of GRBV isolates in the Cabernet franc vineyard.

Because the sentinel vines were free of GRBV prior to and at planting, their infection in 2015 to 2023 can be assumed to be from S. *festinus*-mediated inoculations. However, exactly when sentinel vines were inoculated by viruliferous S. *festinus* remains unknown. Interestingly, previous controlled-insect release experiments revealed that vines inoculated by viruliferous S. *festinus* did not show symptoms the same year of inoculation, although PCR testing showed that some vines tested positive for GRBV in the inoculated leaf tissue,

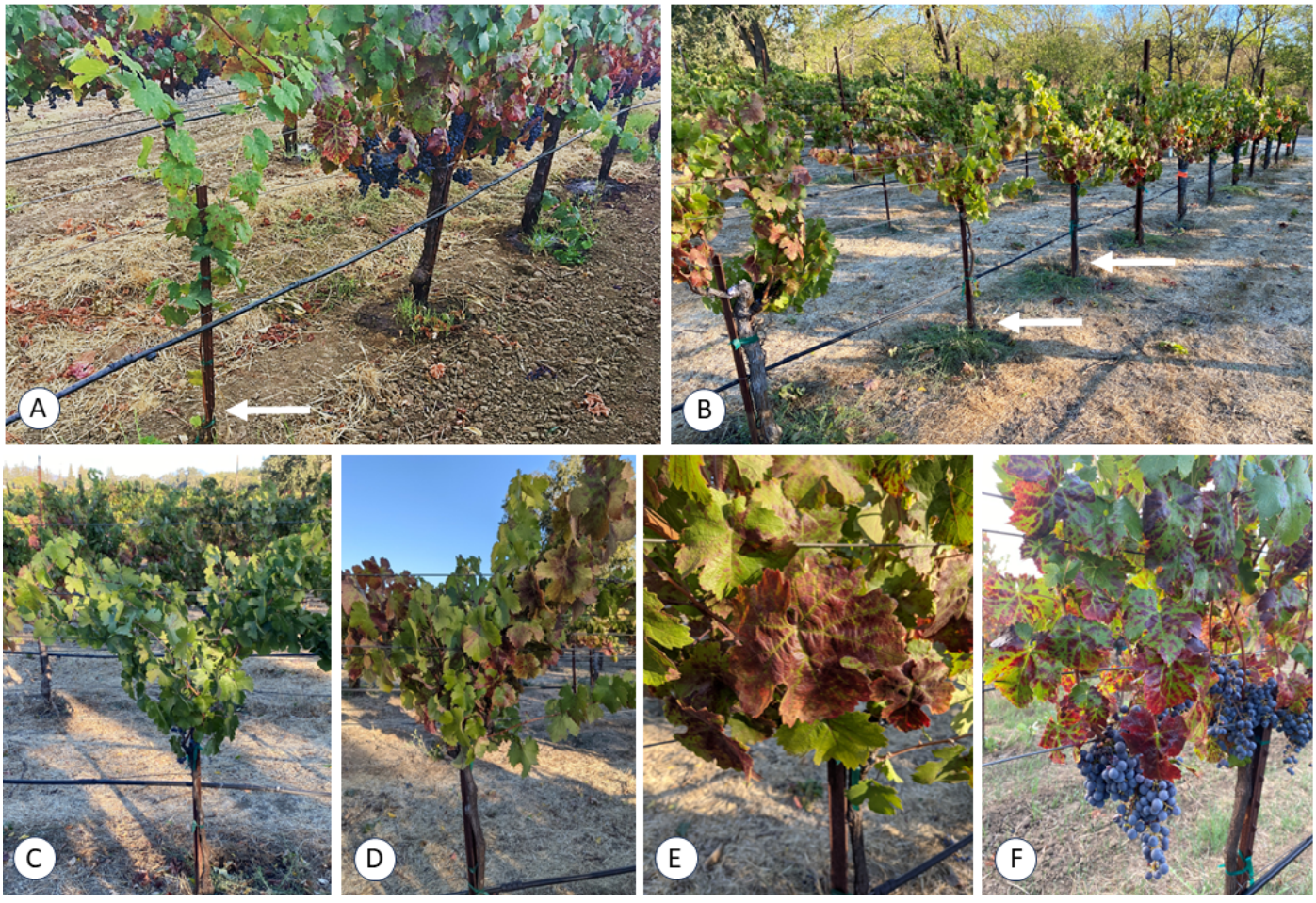

Figure 3 Sentinel vines infected with grapevine red blotch virus in a diseased Cabernet franc vineyard experiencing secondary spread. (**A**) The first symptomatic (i.e., foliar red blotches) sentinel vine surrounded by diseased vines in October 2019, shown by a white arrow; (**B**) two symptomatic sentinel vines surrounded by symptomatic vines in October 2022, indicated with white arrows; (**C**) an infected but asymptomatic sentinel vine surrounded by diseased vines in October 2021; (**D**) a symptomatic sentinel vine in October 2021; (**E**) a closeup of foliar red blotch symptoms displayed by the sentinel vine shown in (**D**); and (**F**) a symptomatic sentinel vine in October 2023.

but not in the mature leaves, such as those used in this study (Flasco et al. 2023a). Similarly, vines that tested positive the first time at 12-months postinoculation via viruliferous S. *festinus* were not yet symptomatic at 16-months postinoculation (Flasco et al. 2023a). By analogy, when considering the sentinel vine that was first confirmed as positive via PCR in 2018 and symptomatic in 2019, it is reasonable to speculate that this vine could have been inoculated by viruliferous S. *festinus* in June or July of 2017, or even in June or July of 2016.

GRBV-infected grapevines are asymptomatic in June (Setiono et al. 2018, Kahl et al. 2021, DeShields and KC 2023, Flasco et al. 2023a, 2023c), however, such vines can act as virus reservoirs and contribute to secondary spread mediated by S. *festinus* (Flasco et al. 2023a). With the new understanding that GRBV can be detected in grapevines that may not show symptoms until later in the growing season, or until the following growing season, symptomless GRBV-infected vines may serve as hidden contributors to secondary spread by S. *festinus* for an extended time, and on a large scale. Factoring in the estimated incubation time may extend this timeline and complicate disease management. Such information is essential to model spread trajectories, fine tune red blotch disease management recommendations, and better understand disease epidemiology.

Conclusions

In a red blotch-diseased Cabernet franc vineyard experiencing secondary spread, otherwise healthy sentinel vines required at least three years to test positive for GRBV via PCR, and at least four years to exhibit foliar disease symptoms. The previously hypothesized 16-month minimum incubation time (Flasco et al. 2023a) combined with an estimated four-to-12-month latency period highlights the complexity of GRBV-S. *festinus*-*Vitis* interactions. The information gained through this research will be considered for modeling disease epidemiology and to better inform red blotch disease management responses.

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ORCID

Madison Flasco 0000-0003-1841-8301
Elizabeth J. Cieniewicz 0000-0002-2620-4672
Monica L. Cooper 0000-0003-1465-6142
Marc Fuchs 0000-0001-5332-6766

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Data Availability

The data underlying this study are available on request from the corresponding author.

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