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Research Article

# Identification of Potential Grapevine Red Blotch Virus Vector in Missouri vineyards

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**Abstract:** Grapevine red blotch virus (GRBV), the causal agent of Grapevine red blotch disease, was recently detected in vineyards across the United States and throughout Missouri. Insect transmission of GRBV in cultivated vineyards of Missouri had not been investigated prior to this research. The objectives of this study were to characterize the potential insect vectors present in four commercial vineyards that had previously been determined to be infected with GRBV, test

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potential vectors caught in vineyards and surrounding habitats for the presence of GRBV with the use of PCR, and investigate the ability of candidate vectors to acquire and transmit GRBV in controlled greenhouse experiments. Of the vineyard collected insects tested over the course of this research, one species of the treehopper *Entylia carinata*, tested positive for GRBV. This species and one other treehopper, *Enchenopa binotata*, were selected for direct transmission assays. Both species successfully acquired GRBV from infected grapevines and transmitted GRBV to confirmed GRBV-free grapevines. *Entylia carinata* has been identified as a promising economic vector after insect samples from vineyards tested positive for GRBV, and the monitoring data revealed this species as the second most abundant treehopper captured in traps. We do not consider *E. binotata* to be a likely economically significant vector because our monitoring data showed that this species was rare and only found along edge habitat surrounding vineyards, never inside vineyard rows. Samples of the most abundant treehopper, *Micrutalis calva*, have not tested positive but the vector status remains unresolved. Further research on rates of secondary spread and transmission by *M. calva* are required, but these results provide evidence that insect transmission of GRBV is feasible in the region.

**Key words:** grapevine red blotch disease, insect transmission, PCR detection, virus

## Introduction

Grapevines face a host of abiotic and biotic stressors ranging from soil nutrients to insect pests and disease pressure (Weaver 1976, Cox 2015). Viruses are of particular importance, with nearly 90 viruses and viroids identified from grapevines (Fuchs 2020). Many of these viral diseases shorten the lifespan of vineyards and reduce yield for growers (Maliogka et al. 2015).

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Seventy percent of viral plant diseases rely on an arthropod vector for transmission (Whitfeld et al. 2015), thus understanding the role of vectors in virus transmission is critical to managing these diseases.

Grapevine red blotch disease (GRBD) is a recently discovered disease caused by a single-stranded circular DNA virus called Grapevine red blotch virus (GRBV) (Al Rwahini et al. 2013, Yepes et al. 2018). GRBV is a geminivirus (family *Geminiviridae*) with isolates in two distinct clades placed in a unique genus called *Grablovirus* (Cieniewicz et al. 2017). Symptoms of this disease in European grape cultivars (*Vitis vinifera*) include the characteristic red blotches on leaves of red-berried cultivars or yellow blotches in white-berried cultivars. These foliar symptoms typically arise late in the growing season and first appear on older leaves (Cieniewicz et al. 2017). Alterations in berry chemistry like reduced sugar content (Brix -1 to -4) and anthocyanin concentrations as well as delays in ripening have been documented (Blanco-Ulate et al. 2017, Girardello et al. 2019, Martínez-Lüscher et al. 2019, Bowen et al. 2020).

Viruses in the family *Geminiviridae* are transmitted by hemipteran insect vectors (Whitfeld et al. 2015). GRBV currently has one confirmed vector; the three-cornered alfalfa treehopper (*Spissistilus festinus*) successfully transmitted GRBV to uninfected *V. vinifera* grapevines under greenhouse conditions (Bahder et al. 2016a, Flasco et al. 2021). Other taxa have been implicated as vectors. The Virginia creeper leafhopper (*Erythroneura ziczac*) successfully transmitted the virus under greenhouse conditions, but subsequent assays were unable to replicate these results (Poojari et al. 2013, Bahder et al. 2016a). Several leafhopper and treehopper species collected from *V. vinifera* vineyards in New York and California tested

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positive for the DNA of GRBV, but their ability to directly transmit the virus has not been determined (Cieniewicz et al. 2018, Cieniewicz et al. 2019, Wilson et al. 2021).

Missouri grows ~1,700 acres of wine grapes with a wine industry that contributes 3.2 billion dollars annually to the state (Frank et al. 2015, Dunham et al. 2017). A comprehensive statewide virus survey conducted in 2017 revealed 35% of composite samples were infected with GRBV (Schoelz et al. 2021). Missouri commonly grows hybrid wine grape cultivars, crosses of *V. vinifera* and North American grape species (*Vitis spp.*), and unlike *V. vinifera* cultivars, hybrid vines infected with GRBV are often completely asymptomatic (Atucha et al. 2018, Schoelz et al. 2021). It is unknown whether the negative fruit effects and overall decline in vine health documented with symptomatic *V. vinifera* cultivars occur in these asymptomatic hybrids. It is also unknown whether the confirmed vector, *S. festinus*, or other potential insect vectors contribute to GRBV transmission in Missouri vineyards.

Our objectives were to (1) identify potential GRBV insect vectors in Missouri vineyards, focusing specifically on treehoppers and those leafhoppers that have previously tested positive for the virus, (2) determine if field-collected candidate vectors were carrying GRBV, and determine if candidate vectors found in Missouri vineyards are capable of (3) acquiring GRBV from infected vines and (4) transmitting GRBV to uninfected grapevines. An understanding of the potential role of insect transmission of GRBV in hybrid grape cultivars is essential to developing effective management strategies for this disease.

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## Materials and Methods

**Candidate insect vector collection in vineyards.** Potential insect vectors were collected from four commercial vineyards in central Missouri in 2018 and 2019. Sampled vines were hybrid cultivars commonly grown in Missouri including French-American hybrid cultivars, Chardonel, Chambourcin, Crimson Cabernet and an American grape cultivar, Norton (*Vitis aestivalis*). Vineyard blocks used in this study were confirmed to be infected with GRBV in a 2017 statewide virus survey (Schoelz et al. 2021).

In 2018, vineyards were sampled weekly for a total of 19 consecutive sampling weeks from bud break in April to harvest in early October. In 2019, the sampling window was reduced to 12 consecutive weeks from bud break in April to veraison in late July. The reduced sampling dates correspond with the peak insect abundance measured in 2018. Insects were collected using yellow sticky card traps (Pherocon, No-Bait Traps, 22 x 28 cm, Great Lakes IPM, Vestaburg MI) secured to 1.8 m tall wooden 2.45 x 5.08 cm posts. Initially, three sticky cards were secured to each post at ground level, mid canopy height and within the fruit zone. The sticky card placement was reduced to a single mid canopy level card (approx. 1 m from the ground) after the first year of monitoring. In 2018 grapevine leaves and vineyard debris collected heavily on the ground and fruit zone cards impacting successful collection of insects. Fifteen posts were installed at each vineyard, five in the edge habitats surrounding the vineyards and ten within the vineyard. Edge habitats consisted of tree lines with understory plants or weedy riparian areas and posts were located approximately 5 to 10 m from the nearest grapevine. The dominant plant species surrounding vineyards were recorded (Table 1) and free-living wild grape (*Vitis sp.*) was present in all habitats proximal to vineyards. Vineyard interior samples were located at various

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distances from the vineyard border, including samples near the end of rows and within the middle of the vineyard. All posts were at least 6 m apart. Each of the vineyards sampled utilized high-wire bilateral cordon training systems and had grassed alleyways with 2.43 m in-row spacing and 3.05 m between vines. Vineyard block size ranged from 0.98 ha to 1.57 ha with edge habitat typically within 6 m of cultivated vines. Sticky cards were collected and replaced weekly, placed in a plastic bag and stored in a -4°C freezer prior to processing.

Treehoppers and leafhoppers were identified to the lowest taxonomic level possible using dichotomous keys and voucher specimens (Delong 1946, Kopp and Yonke 1974, Enns Entomological Museum, University of Missouri). Species-level determination of leafhoppers often requires the dissection of male genitalia, which was not feasible with our sticky card sampling scheme, so some determinations were made to genus level. Additional insect specimens were collected using sweep nets and a D-Vac suction sampler (D-Vac Suction Sampler, Model 24, Ventura, CA), secured in plastic bags and stored in a -4°C freezer for molecular testing of GRBV DNA presence. The insects collected using sweep nets and the D-Vac were not included in the statistical analyses.

The main and interactive effects of location (vineyard interior vs. outside of the vineyard) and sample week on (1) total treehopper (Membracidae) abundance, (2) total leafhopper (Cicadellidae) abundance, (3) abundance of *Micrutalis calva* treehoppers, and (4) abundance of *Entylia carinata* treehoppers were determined by repeated measures ANOVA with vineyard included as a random blocking factor (PROC MIXED, SAS version 9.3; SAS Institute). For all analyses, data were logarithmically transformed to fit the assumptions of ANOVA. In all cases,

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compound symmetry covariance structure was determined to be the best-fit using the Bayesian information criterion.

**Acquisition assay of GRBV by candidate vectors.** Eight species of treehoppers and one species of leafhopper were collected at the University of Missouri Baskett Wildlife Research and Education Center (Boone Co., MO), a 917 ha research area. The nearest cultivated vineyard is approximately 8.28 km from our sampling site. Insects were collected in a weedy, riparian area. Some of the foliage identified was giant ragweed (*Ambrosia trifida*), common ragweed (*Ambrosia artemisiifolia*), sunflower (*Helianthus sp.*) and other herbaceous plants at least 2 m from a tree line. The insects were transported live in a cooler to the Curtis Hall Greenhouse on the University of Missouri campus for acquisition studies (photoperiod of 16:8, 24 to 32°C, and 35% RH; University of Missouri, Columbia). Treehoppers and leafhoppers were placed in mesh sleeve cages (sock enclosure, dimensions D25.4 × L50.8 cm, BioQuip Products Inc., Rancho Dominquez, CA) on a GRBV infected Crimson Cabernet grapevine, all contained inside a larger observation cage (model BugDorm-2120, dimensions W60 × D60 × H60 cm, MegaView Science Co. Ltd, Taichung, Taiwan). The grapevines used in the acquisition and transmission assays were one year old and were propagated from cane wood collected from one commercial vineyard identified as GRBV positive in a virus survey in 2016 (Schoelz et al. 2021). Grapevines were tested for GRBV before assays took place to confirm infection. Insects were placed on new tender growth to ensure successful feeding. Insects were allowed to feed on the grapevine for a 72hr acquisition access period. They were then removed from the grapevine and placed in 1.5 mL microcentrifuge vials and stored at -80°C prior to testing for the presence of GRBV DNA.

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Individuals of the same insect species were combined into an aggregate sample of up to 50 mg of tissue. Tissue was homogenized with disposable microtube pestles in 1.5 mL microcentrifuge tubes with 180 µL Phosphate Buffered Saline, pH 7.2 (1×). Total DNA was extracted using the DNeasy Blood & Tissue Kit (Qaigen, Germantown, MD) following the manufacturer's insect specimen protocol. DNA extracted from insect specimens was tested for the presence of GRBV by PCR using GoTaq Polymerase (Promega, Madison WI). The PCR primers (GRBV-For621 5'-TCA ACT GAG TAG ACG CGT TGC-3' and GRBV-Rev1261 5'-TCA ACA TCA TTC CGT CCT CCA-3') amplified a 640-bp DNA segment of the GRBV genome from nucleotide 621 to 1261. PCR primers were synthesized by Integrated DNA Technologies (Coralville, IA). PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 60 sec. At the conclusion of the final cycle, the temperature was held at 72°C for 10 min and then held at 4°C. PCR products were analyzed by gel electrophoresis in a 1.5% agarose gel run in TBE (0.089 *M* Tris, 0.089 *M* Boric Acid, 0.002 *M* EDTA).

To confirm successful DNA extraction from insect specimens, isolated DNA was also tested for the presence of a gene present in the mitochondria of insects, COI, by PCR using GoTaq Polymerase (Promega, Madison WI) (Lunt et al 1996). The PCR primers COI-F (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and COI-R (5'-TAA ACT TCA GGG TGA CCA AAA AAT-3') amplified a 686-bp DNA segment of the COI genome. PCR primers were synthesized by Integrated DNA Technologies (Coralville, IA). PCR conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 40 sec, 47.9°C for 40 sec, and 72°C for 40 sec. At the conclusion of the final cycle, the temperature was held at 72°C for 5 min and then held at 4°C.

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PCR products were analyzed by gel electrophoresis in a 1.2% agarose gel run in TBE (0.089 *M* Tris, 0.089 *M* Boric Acid, 0.002 *M* EDTA).

Whole insect bodies were homogenized. Therefore, this assay does not distinguish insects that test positive due to the presences of GRBV in their gut versus acquisition of the virus in the salivary glands.

**Transmission assay of GRBV by candidate vectors.** Two species of treehoppers, *E. carinata* and *E. binotata*, that tested positive for GRBV in the acquisition assays and for which there were sufficient numbers of wild individuals available were selected for further transmission studies. Treehoppers were collected at the University of Missouri Baskett Wildlife Research and Education Center and the City of Columbia Capen Park (Boone Co., MO), a 12.9 ha municipal park without cultivated vineyards in proximity. Collected insects were transported to the University Curtis Greenhouse in a cooler.

A total of 15 *E. binotata* and 15 *E. carinata* were used in the direct transmission assays. For both species, three groups of five insects were placed inside mesh sleeve bags secured to a Crimson Cabernet grapevine that was previously confirmed positive for GRBV. The insects were allowed to feed on the GRBV positive vine for a 48hr acquisition access period. Two *E. binotata* individuals died during the acquisition feeding period, resulting in two groups of four *E. binotata* and one group of five. There was no mortality of *E. carinata* in the acquisition assay. Insects were then transferred to six different Crimson Cabernet grapevines that were confirmed GRBV free by PCR testing. Treehoppers were secured in mesh sleeve bags on vines with young, tender growth to facilitate successful insect feeding. Insects were allowed to feed on the virus-free vines for a 48hr inoculation access period. There was no mortality of *E. binotata* or *E. carinata* during

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the inoculation period. Insects were then removed and placed into 1.5 mL microcentrifuge tubes and stored in a -80°C freezer. As with the acquisition assay, all individual vines were contained inside of a larger observation cage.

Recipient plants were maintained within the greenhouse (photoperiod of 16:8, 24 to 32°C, and 35% RH; University of Missouri, Columbia) for 4 months to allow GRBV titer to build up before testing. Phloem scrapings from green cambium on canes were collected from each recipient vine. Additionally, tissue from leaf petioles or, if leaves had abscised, dormant buds were collected. Up to 100 mg of each type of plant tissue were processed separately and homogenized for 2 min using 5 mm Tungsten Carbide Beads in 2 mL microcentrifuge tubes in a TissueLyser II (Qiagen, Germantown, MD). Total DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Germantown, MD) following the manufacturer's protocol. DNA extracted from the different plant tissues were then tested for the presence of GRBV as described in the acquisition assay with the same PCR primers and conditions used for detection of GRBV in insects.

**Viral genome sequencing of infected vines used in transmission assay.** GRBV viral DNA was isolated from a GRBV-positive donor vine and one of the recipient vines used for the transmission assay using a DNeasy Plant Mini Kit (Qiagen, Germantown, MD). PCR products were amplified using primers synthesized by Integrated DNA Technologies and PCR conditions described in the previous section, then purified for DNA sequencing using a QIAQuick PCR Purification Kit (Qiagen, Germantown, MD) and submitted for DNA sequencing at the University of Missouri Genomics Technology Core.

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# Results

**Candidate insect vectors collected in vineyards.** Over the two-year monitoring period, 1,787 yellow sticky card traps were deployed and a total of 65,870 individual leafhoppers and treehoppers were collected (Table 2). The samples yielded 12 species of treehoppers, as well as two leafhopper species that have been identified as candidate vectors of GRBV, *Colladonus reductus* and *Osbornellus sp* (Cieniewicz et al. 2019). The previously confirmed insect vector of GRBV, the three-cornered alfalfa treehopper (*S. festinus*), was not found over two years of monitoring.

Treehopper abundance peaked both inside and outside of the vineyard in June 2018 ($F$=2.98, $p$=<0.0001) and 2019 ($F$=451.41, $p$=<0.0001) (Figs. 1A and 2A). There were significantly more treehoppers in the vineyard interiors than vineyard edges in June of both years. Peaks in total treehopper abundance are attributed largely to the most abundant species in our survey, *Micrutalis calva*. *Micrutalis calva* was significantly more abundant inside vineyards than in the edge habitats outside of the vineyards in the month of June (Figs. 1B and 2B). The second most abundant species, *Entylia carinata*, was primarily found outside of the vineyards (Figs. 1C and 2C).

Leafhoppers were more abundant than treehoppers over the course of this study, but leafhoppers overall shared a similar population dynamic trend as treehoppers. There was a population peak in June of 2018 and 2019, followed by a gradual decrease throughout the season (Fig. 3). There was no significant difference between the number of leafhoppers outside the vineyards and in the vineyard interiors in 2018. In 2019 there were significantly more leafhoppers outside of the vineyards. Two leafhopper taxa that are GRBV candidate vectors,

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*Colladonus reductus* and *Osbornellus sp.,* were also present (Cieniewicz et al. 2019). They were found in vineyard interiors as well as in edge habitats outside of vineyards.

A total of 1,168 insects collected from the field were assayed for the presence of GRBV. Of the field-caught specimens tested, two pooled samples of *Entylia carinata* tested positive for GRBV (Table 2). One pooled sample contained eight individual insects with four removed from sticky cards in the edge habitat and four removed from sticky cards in the vineyard interior. The other positive sample contained seven *E. carinata,* all found on one vineyard interior card. The percent of insects that tested positive for GRBV is listed as a range dependent on the number of individuals in one aggregate sample weighing 50 mg (Table 2).

**Acquisition of GRBV by candidate vectors.** Six treehopper species tested positive for GRBV after feeding on infected grapevines for a 72hr acquisition period (Table 3). The leafhopper species, *Graphocephala coccinea*, and one species of treehopper, *Archasia pallida*, tested negative for GRBV. The molecular results of the treehopper *Micrutalis calva* were inconclusive because DNA extracted was not viable as it did not test positive for the control gene.

**Transmission of GRBV by candidate vectors.** Both species of treehoppers selected for direct transmission assays (*E. binotata* and *E. carinata*) successfully transmitted GRBV to virus-free grapevines (Table 4). None of the recipient vines appeared to have symptoms of GRBV at the time when plant material was collected for PCR assays (4 months post inoculation). DNA extracted from grapevine tissue tested positive for GRBV. In some instances, phloem scrapings and petiole tissue tested positive for GRBV while bud tissue did not (Table 4). These inconsistencies may be due to biological reasons such as differences in virus titer in various plant

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tissues (Setiono et al. 2018), which should be further investigated, or it may be due to incomplete disruption of the bud tissue during the extraction protocol.

GRBV DNA was isolated from donor and recipient vines and the nucleotide sequence determined for approximately two thirds of the virus genome of each (2050-bp). The nucleotide sequences were compared using the Global Align program of BLAST (Zhang et al. 2000), showing that they were 100% identical over the 2050 nucleotide stretch. A separate BLAST nucleotide search of the sequences showed that they were 99.90% identical to MO-CC7 (Schoelz et al. 2018), a GRBV isolate recovered from the same Crimson Cabernet vineyard as the donor vine. The comparison of virus sequences recovered from donor and recipient vines is consistent with the hypothesis that the transmitted virus originated from the source vine in our greenhouse and was not the result of contamination by prior insect acquisition of GRBV in the field.

## Discussion

The goal of this research was to identify the insect vectors of GRBV in Missouri vineyards. We sampled the candidate vector community in four vineyards throughout the growing season and tested field-collected individuals for GRBV. Of the 1,168 individuals tested, only 0.06 to 4% were positive for GRBV. All individuals testing positive were of the treehopper species, *E. carinata*. Given the low likelihood of finding GRBV-positive individuals in the field, we directly tested the ability of candidate vectors to acquire the virus by feeding on confirmed GRBV-infected grapevines and to transmit the virus to GRBV-free grapevines in the greenhouse. Six species of treehoppers tested positive for GRBV after an acquisition access period of 72hr on infected Crimson Cabernet grapevines (Table 3), indicating potential acquisition of the virus.

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Because whole insects were homogenized and the salivary glands were not dissected, this result is consistent with, but does not confirm, acquisition of the virus by the treehoppers. Further testing revealed that two species of treehoppers, *E. carinata* and *E. binotata*, successfully infected grapevines through direct feeding, providing clear evidence of acquisition and transmission of GRBV by these species.

*Entylia carinata* is the most promising candidate vector of GRBV identified thus far in Missouri. This species was the second most abundant treehopper in each of the vineyards monitored. Pooled samples of individuals collected from one vineyard tested positive for GRBV providing evidence that these treehoppers are feeding on cultivated grapevines and ingesting GRBV. *Entylia sp*. have also tested positive in vineyards in New York (Cieniewicz et al. 2019). Additionally, direct transmission of GRBV by *E. carinata* was confirmed in our greenhouse assays. *Entylia carinata* is commonly found feeding and reproducing on herbaceous weeds in the family Asteraceae like ragweed (*Ambrosia sp.*), horseweed (*Conyza sp.*) and fleabane (*Erigeron sp.*) (Kopp and Yonke 1974). We found that *E. carinata* is abundant in edge habitats surrounding vineyards, but it is also commonly found in vineyard interiors, especially at the end of vineyard rows near edge habitats. The abundance of *E. carinata* caught in traps in the vineyards reduced by nearly 10% in 2019. This may be due to the reduced sampling period and the reduced number of sticky card traps deployed. Further assays and monitoring should be conducted with this species.

*Enchenopa binotata,* the two-marked treehopper, successfully transmitted GRBV in transmission studies, but we do not consider it to be a likely economic vector. In the monitoring efforts of Missouri vineyards, *E. binotata* was rare and only found in edge habitats outside

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vineyards, never inside vineyards. Of samples collected from edge habitats surrounding vineyards, no individuals tested positive for GRBV. While we do not consider this insect to be a likely economic vector, the ability to transmit GRBV is significant as it demonstrates that the potential vector community may be broad.

Our investigation enabled the exclusion of some insects as vectors of GRBV. One species of treehopper, *Archasia pallidia*, and one species of leafhopper, *Graphocephala coccinea*, tested negative for GRBV in our 72hr acquisition assay. These species either did not feed on the grapevines or did not have the ability to successfully acquire the virus (Whitfeld et al. 2015). The contribution of the most abundant treehopper in Missouri vineyards to GRBV transmission is unresolved. In the two years of community monitoring efforts, 10,280 *M. calva* were collected inside and around vineyards, comprising more than 94% of the treehopper individuals. 1,086 field-collected individuals were tested for the presence of GRBV and none of the pooled samples tested positive, suggesting that this species is not a vector of GRBV. However, we are unable to completely exclude *M. calva* as a potential vector since the molecular results of our direct transmission tests in the greenhouse were inconclusive. The timing of these assays relative to the phenology of *M. calva* precluded our ability to find additional individuals in the field for testing. Future acquisition and transmission assays with the species are needed to confidently exclude this species as a vector.

Understanding whether insect vectors contribute to secondary spread of GRBV is critical for management decisions. Currently, the only disease management option is to test each vine or a subsample of vines for the virus and then remove, or rogue, the entire vineyard if over 30% of a *V. vinifera* cultivar is infected (Ricketts et al. 2017). Grapevines are perennial crops that

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342 require a considerable initial time investment, between three to five years, before a full crop
343 yield can be expected (Cox 2015); therefore, roguing a vineyard of GRBV infected grapevines is
344 a significant cost. However, if secondary spread has been documented in a region, the removal of
345 sources of inoculum in and surrounding cultivated vineyards is vital.

346 Surveys for GRBV in vegetation adjacent to cultivated vineyards in California have
347 found alternate hosts growing in riparian edge habitats (Bahder et al. 2016b, Wilson et al. 2021).
348 Thirteen species of woody herbaceous plants growing around three vineyards were tested for the
349 presence of GRBV. Two species including wild grape (*V. californica* × *V. vinifera*) tested
350 positive for the virus. The candidate insect vectors monitored in our study, including *E. carinata*,
351 were present in the edge habitats surrounding cultivated vineyards as well as vineyard interiors.
352 Wild *Vitis* was present in the edge habitat of all four of the vineyards we surveyed. A 2021
353 survey of the prevalence of GRBV in wild *Vitis sp., Ampelopsis sp.* (a vine in the Vitaceae
354 family), and four species of Roundup® resistant weeds (*Solanum carolinense, Conyza*
355 *canadensis, Ambrosia artemisiifolia, Ambrosia trifida*) in 13 different Missouri vineyards found
356 that 13.24% of 137 samples of wild *Vitis* tested positive for GRBV. In addition, one sample of
357 *Ampelopsis sp.* tested positive for GRBV and no samples of the four weed species tested positive
358 for GRBV (Dean Volenberg, personal communication). The presence of GRBV in riparian edges
359 could provide a reservoir of virus inoculum for a mobile insect vector which may move from
360 these riparian areas into vineyards, indicating spread may be inevitable. Spatial data from this
361 study demonstrates that *E. carinata* is abundant in habitats surrounding vineyards and common
362 along vineyard edge rows, potentially indicating movement from edge habitats to vineyard rows.
363 These insects may feed on alternate GRBV hosts in surrounding habitat while moving between

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edge habitats and cultivated grapevines. Further research on GRBV reservoirs in Missouri vineyards is crucial to ensure vineyards are not reinfected after GRBV positive vines are removed and replaced.

## Conclusion

The results of this study indicate that insect transmission of GRBV in hybrid cultivars is possible in Missouri vineyards. The successful transmission of the virus by two species of treehoppers, *E. binotata* and *E. carinata*, under greenhouse conditions demonstrates that the molecular mechanisms of virus transmission exist. However, the spatiotemporal occurrence of the treehoppers and the presence of infected individuals in vineyards indicate that *E. carinata* is the species that is most likely to play an economic role. Continued monitoring for infected *E. carinata* individuals and a better understanding of phenology and host plants of these treehoppers is necessary. Monitoring individual vines for secondary spread is essential to understanding if these insects are vectors of economic significance. Further research, including an economic impact study on common Missouri cultivars and the effect of GRBV on fruit and wine yield and quality among asymptomatic cultivars is required to develop a management plan to prevent spread of this disease in Missouri vineyards. Additionally, understanding if alternate sources of inoculum exist in the environment surrounding cultivated grapevines will play a crucial role in control of secondary spread of GRBV via insects.

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466 **Table 1** Plant composition of the edge habitat surrounding the four cultivated vineyards
467 surveyed in 2018 and 2019 for potential insect vectors of GRBV.
468

| Vineyard 1, Hermann MO | Vineyard 2, Rocheport MO | Vineyard 3, New Haven MO | Vineyard 4, Berger MO |
|---|---|---|---|
| *Allium stellatum* | *Acer saccharum* | *Ageratina altissima* | *Allium stellatum* |
| *Apocynum cannabinum* | *Ageratina altissima* | *Ambrosia artemisiifolia* | *Ageratina altissima* |
| *Asclepias syriaca* | *Allium stellatum* | *Carduus nutans* | *Ambrosia artemisiifolia* |
| *Brassica kaber* | *Ambrosia artemisiifolia* | *Eutrochium purpureum* | *Asclepias syriaca* |
| *Carduus nutans* | *Ambrosia trifida* | *Festuca sp.* | *Festuca sp.* |
| *Catalpa speciosa* | *Ampelopsis brevipedunculata* | *Ilex decidua* | *Juniperus virginiana* |
| *Ceanothus cuneatus* | *Carduus nutans* | *Juniperus virginiana* | *Lamium sp.* |
| *Cynanchum leave* | *Elymus virginicus* | *Lonicera japonica* | *Lonicera japonica* |
| *Desmodium canadense* | *Euonymus fortunei* | *Quercus sp.* | *Parthenocissus sp.* |
| *Elymus virginicus* | *Eutrochium purpureum* | *Rosa multiflora* | *Phalaris arundinacea* |
| *Eutrochium purpureum* | *Festuca sp.* | *Rubus allegheniensis* | *Quercus sp.* |
| *Festuca sp.* | *Gleditsia triacanthos* | *Setaria pumila* | *Rubus sp.* |
| *Fraxinus pennsylvanica* | *Juglans nigra* | *Solanum carolinese* | *Salsola sp.* |
| *Fraxinus sp.* | *Juniperus virginiana* | *Solidago sp.* | *Setaria pumila* |
| *Gleditsia triacanthos* | *Lamium sp.* | *Stellaria media* | *Setaria viridis* |
| *Juglans nigra* | *Maclura pomifera* | *Symphyotrichum sp.* | *Solidago sp.* |
| *Juniperus virginiana* | *Parthenocissus quinquefolia* | *Vitis sp.* | *Stellaria media* |
| *Lolium perenne* | *Pinus strobus* | | *Symphoricarpos obiculatus* |
| *Lonicera japonica* | *Platanus occidentalis* | | *Symphyotrichum sp.* |
| *Parthenocissus quinquefolia* | *Rosa multiflora* | | *Ulmus americana* |
| *Pinus strobus* | *Rubus allegheniensis* | | *Vitis sp.* |
| *Quercus sp.* | *Rumex crispus* | | |
| *Rosa multiflora* | *Sicyos angulatus* | | |
| *Rubus allegheniensis* | *Solidago sp.* | | |
| *Salix sp.* | *Stellaria media* | | |
| *Salsola sp.* | *Symphoricarpos orbiculatus* | | |
| *Smilax glauca* | *Symphyotrichum sp.* | | |
| *Solanum carolinense* | *Toxicodendron radicans* | | |
| *Solidago sp.* | *Urtica dioica* | | |
| *Stellaria media* | *Vitis sp.* | | |
| *Symphoricarpos orbiculatus* | | | |
| *Symphyotrichum sp.* | | | |
| *Toxicodendron radicans* | | | |
| *Verbascum thapsus* | | | |
| *Vitis sp.* | | | |
| *Yucca smalliana* | | | |

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**Table 2** Abundance of treehoppers (Membracidae) and leafhoppers (Cicadellidae) at monitoring sites in four commercial Missouri vineyards in 2018 and 2019. Samples were collected weekly from bud break to harvest in 2018 and from bud break to veraison in 2019. "Inside" refers to insects trapped on sticky cards placed in interior vineyard rows. "Outside" refers to insects trapped on sticky cards placed along the edge habitats surrounding vineyards. Selected species of insects were tested using standard PCR for GRBV.

| | 2018 Inside | 2018 Outside | 2018 Total | 2019 Inside | 2019 Outside | 2019 Total | Number of individuals tested | Number of positive aggregate samples/ total samples tested | Percent of insects tested positive |
|---|---|---|---|---|---|---|---|---|---|
| **Membracidae** | 5,742 | 2,361 | 8,103 | 2,426 | 391 | 2,817 | 1,168 | 2/77 | 0.17-1.28[c] |
| *Spissistilus festinus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0/0 | 0 |
| *Micrutalis calva* | 5,619 | 1,902 | 7,521 | 2,410 | 349 | 2,759 | 1,086 | 0/54 | 0 |
| *Entylia carinata* | 123 | 438 | 561 | 15 | 38 | 53 | 55 | 2/11 | 3.6-27[c] |
| *Stictocephala sp.* | 0 | 15 | 15 | 0 | 3 | 3 | 5 | 0/5 | 0 |
| *Enchenopa binotata* | 0 | 1 | 1 | 0 | 1 | 1 | 3 | 0/2 | 0 |
| *Campylenchia latipes* | 0 | 2 | 2 | 1 | 0 | 1 | 1 | 0/1 | 0 |
| *Archasia belfragei* | 0 | 3 | 3 | 0 | 1 | 1 | 0 | 0/0 | 0 |
| *Glossonotus turriculatus* | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0/0 | 0 |
| *Acutalis tartarea* | 3 | 9 | 12 | -[a] | -[a] | -[b] | 6 | 0/2 | 0 |
| *Publilia concava* | 44 | 33 | 77 | -[a] | -[a] | -[b] | 0 | 0/0 | 0 |
| *Publilia modesta* | 0 | 6 | 6 | -[a] | -[a] | -[b] | 0 | 0/0 | 0 |
| **Cicadellidae** | 26,760 | 19,135 | 45,895 | 4,675 | 4,380 | 9,055 | 31 | 0/14 | 0 |
| *Osbornellus sp.* | 4 | 19 | 23 | 0 | 0 | 0 | 28 | 0/8 | 0 |
| *Colladonus reductus* | 0 | 3 | 3 | 1 | 0 | 1 | 3 | 0/1 | 0 |
| *Empoasca sp.* | 17,063 | 11,800 | 28,863 | 2,590 | 1,636 | 4,226 | 0 | 0/0 | 0 |

[a] No data available
[b] Species present but total abundance not available
[c] Percent of insects tested positive is shown as a range dependent upon the number of individuals in the aggregate sample.

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479 **Table 3** Insects tested using standard PCR for potential acquisition of GRBV after feeding for
480 72hrs on GRBV infected Crimson Cabernet grapevines under greenhouse conditions.

| Family | Species | Number of insects tested | Number of aggregate samples positive/ total samples tested |
|---|---|---|---|
| Membracidae | | | |
| | *Campylenchia latipes* | 3 | 1/1 |
| | *Entylia carinata* | 4 | 1/1 |
| | *Actualis tartarea* | 5 | 1 /1 |
| | *Publilia reticulata* | 6 | 1 /1 |
| | *Enchenopa binotata* | 3 | 1/1 |
| | *Stichtocephala sp.* | 1 | 1 /1 |
| | *Archasia pallida* | 2 | 0/1 |
| | *Micrutalis calva* | 6 | 0/0[a] |
| Cicadellidae | | | |
| | *Graphocephala coccinea* | 4 | 0/1 |

[a] Indicates inconclusive molecular results, did not test positive for a control gene, COI

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**Table 4** Transmission studies of GRBV were conducted with two species of treehoppers, *Entylia carinata* and *Enchenopa binotata.* PCR assays were performed on grapevine tissue collected from recipient plants four months post-inoculation.

| Treehopper species feeding on vine | Vine number and tissue type | Result |
|---|---|---|
| *Entylia carinata* | 1, phloem scrapings | Negative |
| | 1, leaf petioles | Negative |
| | 2, phloem scrapings | Positive |
| | 2, dormant buds | Negative |
| | 3, phloem scrapings | Positive |
| | 3, dormant buds | Positive |
| *Enchenopa binotata* | 4, phloem scrapings | Positive |
| | 4, dormant buds | Negative |
| | 5, phloem scrapings | Negative |
| | 5, leaf petioles | Negative |
| | 6, phloem scrapings | Positive |
| | 6, leaf petioles | Negative |

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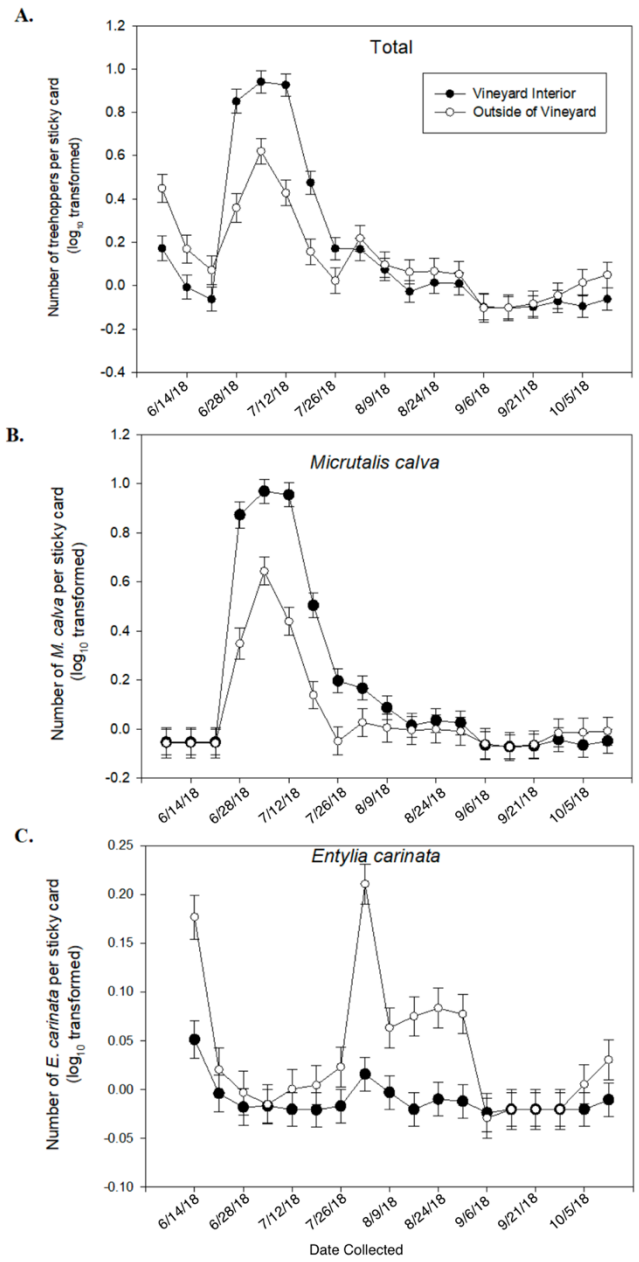


**Figure 1** The average number of total treehoppers (A), *Micrutalis calva* (B) and *Entylia carinata* (C) per sticky card trap over the weekly sampling season in four commercial vineyards in 2018. Vineyard interior indicated by filled circles and outside of the vineyard indicated by open circles. Error bars represent the ±SD; $p$=<0.0001 based on repeated measures ANOVA.

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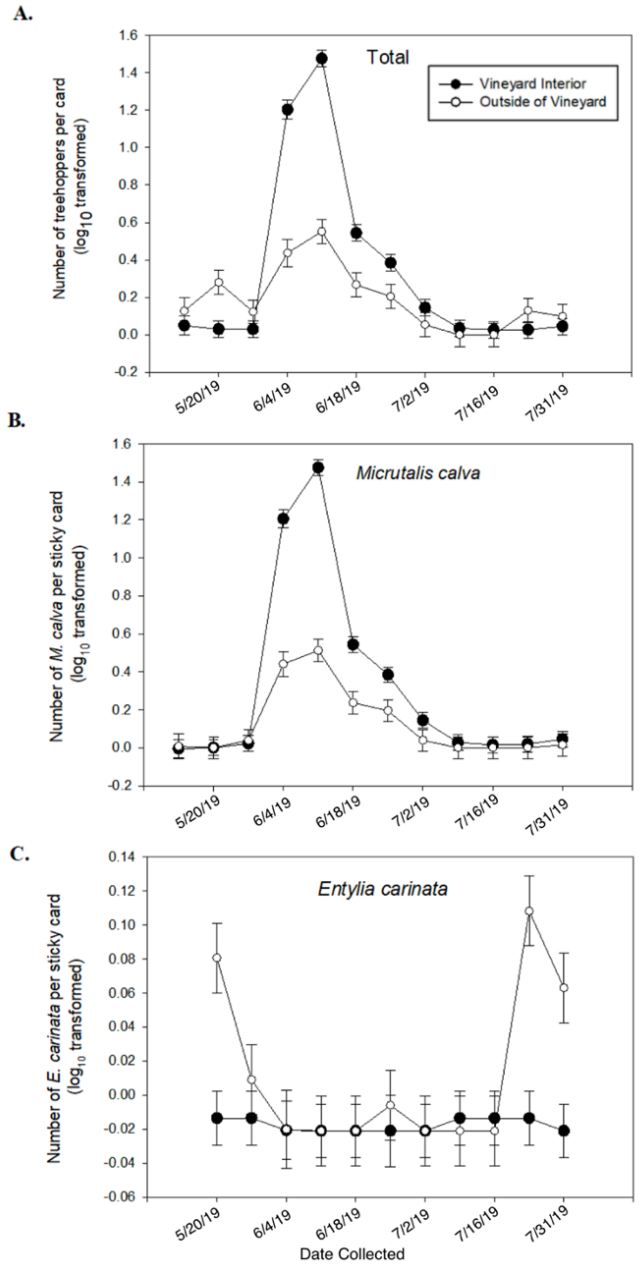


**Figure 2** The average number of total treehoppers (A), *Micrutalis calva* (B) and *Entylia carinata* (C) per sticky card trap over the weekly sampling season in four commercial vineyards in 2019. Vineyard interior indicated by filled circles and outside of the vineyard indicated by open circles. Error bars represent the ±SD; $p$=<0.0001 based on repeated measures ANOVA.

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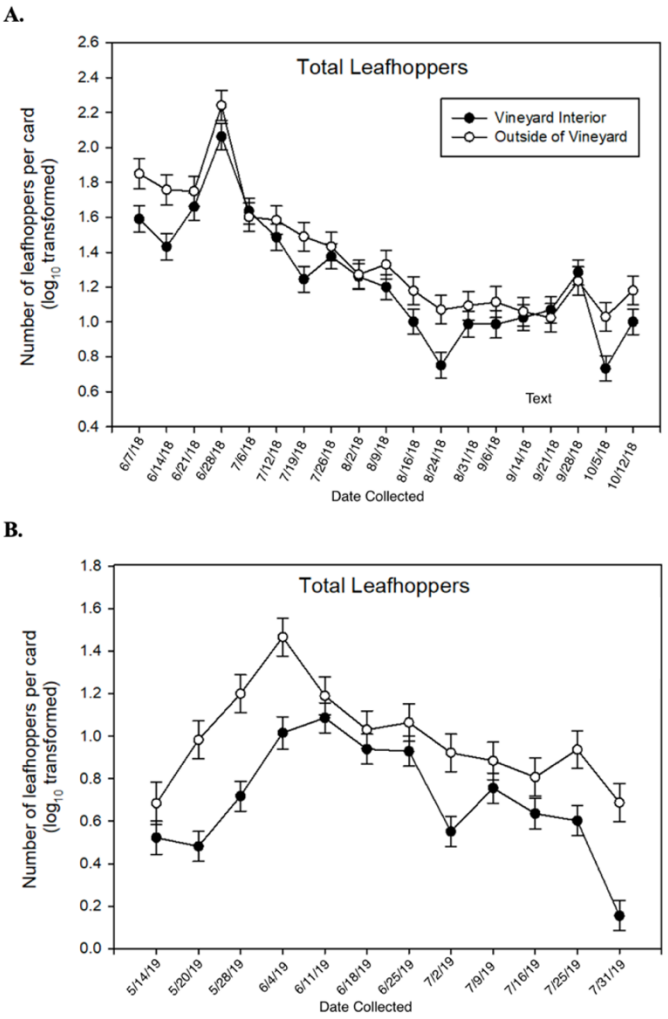


505
506 **Figure 3** The average number of leafhoppers per sticky card trap over the weekly sampling
507 season in (A) 2018 and (B) 2019 from four commercial vineyards. Vineyard interior is indicated
508 by filled circles and outside of the vineyard is indicated by open circles. Error bars represent the
509 ±SD; $p$=<0.0001.
510