

## **Interim Progress Report for CDFA Agreement Number 18-0398-000-SA**

**Title of project:**      **Biology and Role of Treehoppers in Grapevine Red Blotch Disease**

**Project Leader:**      Dr. Frank G. Zalom,  
Department of Entomology and Nematology  
University of California, Davis

**Co-Project Leader:** Dr. Mysore R. Sudarshana,  
USDA-ARS, Department of Plant Pathology  
University of California, Davis

**Cooperators:**      Dr. Kent Daane  
Dept. of Environmental Science, Policy, and Management  
University of California, Berkeley

Dr. Kaan Kurtural  
Department of Viticulture and Enology  
University of California, Davis

Ms. Rhonda Smith  
UC Cooperative Extension, Sonoma County  
Santa Rosa, CA 95403

Dr. Vaughn Walton  
Professor of Entomology  
Oregon State University

Ms. Lynn Wunderlich  
U.C Cooperative Extension, Central Sierra  
Placerville, CA 95667

**Time period covered by the report:** February 23, 2019 – June 30, 2019

### **Introduction:**

A ssDNA virus, Grapevine red blotch virus (GRBV; family Geminiviridae), associated with Grapevine red blotch disease (Al Rwahnih et al. 2013; Sudarshana et al. 2015), is now recognized as the causal agent of red blotch disease (Cieniawicz et al. 2017). Because of its adverse effect on wine quality and resulting revenue loss, GRBV is becoming one of the most intensely studied grapevine viruses in California. A recent analysis on the economic impact indicated that the disease can cause economic losses of as much as \$30,000 per acre in North Coast vineyards (Rickett et al. 2016).

Among the several insect species found in commercial vineyards with red blotch disease, the three-cornered alfalfa treehopper (3CAH), *Spissistilus festinus* Say, was found to be capable of transmitting GRBV under greenhouse conditions (Bahder et al., 2016). In studies conducted in California by Cornell University virologists, spatial patterns of red blotch distribution and *S.*

*festinus* adults caught on yellow sticky traps that tested positive for GRBV by PCR indicated that this membracid is the most likely vector of significance to virus epidemiology (Cieniewicz et al. 2018). However, our studies on GRBV transmission using 3CAH have not produced consistent results. We are curious whether our inconsistency in getting transmission by 3CAH might be due to the fact that different sources of field collected insects have been used in different studies and different years, raising the possibility of differential transmission specificity among 3CAH populations or even the existence of potential cryptic species such as is case for the sweetpotato whitefly, *Bemisia tabaci*, and the Geminivirus Tomato yellow leaf curl virus, that it transmits (Polston et al. 2014). In addition, we are also continuing to search for additional vectors among related treehopper species (especially in the treehopper genus *Tortistilus* which have been found in vineyards where virus spread is occurring in Oregon and California (Dalton et al. 2019) and other members of the Hemiptera suborder Auchenorrhyncha.

In summary, this proposal is intended to unravel the role of treehoppers (Hemiptera: Membracidae) and related Auchenorrhyncha found in California vineyards in the spread of GRBV, and developing sustainable management guidelines by building on our recently completed studies involving the population dynamics of 3CAH in vineyards (Preto et al., 2019), the suitability of grapevines as a reproductive host for 3CAH (Preto et al. 2018b), and cover crops and common weeds that serve as their feeding and reproductive hosts (Preto et al. 2018a).

### **Objectives:**

1. Determine timing and distribution of treehopper girdling in relation to red blotch incidence in vineyards.
2. Conduct field and greenhouse GRBV transmission studies using three cornered alfalfa hopper (3CAH) and *Tortistilus* sp. collected from vineyards with red blotch disease, and detect GRBV presence in the salivary glands of insects collected.
3. Confirm taxonomic identification and monitor *Tortistilus* sp. populations in California vineyards and determine their seasonal biology in vineyards and surrounding landscapes.

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

**Objective 1:** Determine timing and distribution of treehopper girdling in relation to red blotch incidence in vineyards.

The activities for this objective were completed by November, 2018, and results from our studies were presented in our Renewal Progress Report submitted in February 2019.

We continued to sample vineyard groundcover for treehoppers during winter and early spring in our study sites at UC Davis Oakville Experimental Station and a commercial Cabernet Sauvignon vineyard located along Oakville Cross Rd. just east of Oakville, but very few 3CAH or other treehoppers were found this year, perhaps due to the rainy winter conditions that was experienced this year as opposed to the last several years. One reason for continuing these collections into spring 2019 was to attempt to determine if GRBV could be detected in the overwintering treehoppers. In the end, we were only able to collect about 31 adult 3CAH from these vineyards between February and April. The salivary glands have been removed from these insects and are awaiting testing for presence of GRBV. This will be useful information to determine if the overwintering adults present in vineyards through the winter could be a source of GRBV for the following year.

**Objective 2.** Conduct field and greenhouse GRBV transmission studies using three cornered alfalfa hopper (3CAH) and *Tortistilus* sp. collected from vineyards with red blotch disease, and detect GRBV presence in the salivary glands of insects collected.

**a) 2019 *Tortistilus albidosparsus* Gordon Valley Rd. (Napa Co.) cage study:**

Our collections of insects found in vineyards with confirmed GRBV spread indicated that another treehopper *Tortistilus albidosparsus* would be of interest as a potential GRBV vector. This species tends to be more abundant in cooler hillside areas of vineyards in contrast to 3CAH which tends to be in warmer vineyard areas. Little is known about the biology of this species, but we have observed that it appears to have only a single, short-lived generation per year. To this time, it has been both seasonally and geographically difficult for us to collect in suitable numbers required for vector transmission studies. *Tortistilus* sp. treehoppers have also been reported in vineyards with reported GRBV spread in Oregon, and are being studied by Dr. Vaughn Walton at Oregon State University.

In our Renewal Progress Report, we mentioned that In June 2018 we began working in a Sauvignon Blanc vineyard in the Gordon Valley Rd. area of Napa County that had a large resident population of *T. albidosparsus* treehoppers. We qPCR tested all of the vines at the easternmost edge of the most heavily insect-infested of the vineyard blocks for GRBV infection, and mapped which vines tested positive for the virus in preparation for an onsite transmission study we anticipated conducting at this site in spring 2019.

In early May 2019, 450 third and fourth instars of *T. albidosparsus* were sweep netted off of vetch growing approximately 2,000 feet from a vineyard located in Pope Valley in Napa County. All nymphs were immediately placed inside a 2 ft x 2 ft mesh insect cage containing pots with mature vetch grown from seed. These insects were then transported to our greenhouse and allowed to mature into adults. These nymph-to-adult reared insects were analyzed for the nucleotide sequence of Cytochrome oxidase 1 (CO1) gene, a mitochondrial gene used in insect taxonomy and identification, and confirmed to be the same species (*T. albidosparsus*) collected in our Gordon Valley Rd. field site. These insects reared from nymphs on vetch served as uninfected controls for our field study because *T. albidosparsus* collected at the study site itself could have potentially already fed on GRBV infected vines and therefore become infected. The insects used for our transmission study were sweep-netted directly off of GRBV positive vines. Our study used 15 tested GRBV-free field grapevines as replicates for untreated controls and 15 GRBV-positive field grapevines as source vines for acquisition by the treehoppers. Individual mesh cages containing 10 insects each were placed onto each of our healthy (Figure 1A) and GRBV-positive block vines (Figure 1B). All insects were given a 48 hr acquisition access period (AAP) and then immediately placed onto potted mesh-caged, tested GRBV-free recipient vines that were placed directly beneath all negative control and treatment vines. Insects were then given a 48 hr inoculum access period (IAP), afterwards, all vines were brought back to our greenhouse. These will be tested for GRBV infection status by qPCR in 6 months.



**Figure 1.** qPCR tested grapevines that were uninfected (left) or GRBV-infected (right) showing AAP and IAP cages with *T. albidosparsus*.

To confirm that GRBV was present on the canes where the insects were caged for acquisition, we removed the nearest distal leaves to the cages and tested them using qPCR. All leaves from the 15 GRBV-positive vines tested GRBV positive and all leaves from the 15 GRBV-negative vines tested GRBV negative except one that had a high CT value which might be interpreted as a "potential" positive.

Another transmission study was simultaneously conducted during this experiment that did not have a set AAP for *T. albidosparsus*, and instead involved the direct transfer of adult insects feeding on tested GRBV-infected and uninfected field vines. The status of GRBV vines had been confirmed by qPCR tests in April 2019. The rationale for this study was to reduce the amount of handling of the insects to avoid injuring their mouthparts or otherwise harming them in such a way that could potentially inhibit virus transmission. This study also used 10 adult insects from either tested GRBV-infected and uninfected field grapevines transferred to mesh caged recipient plants representing 15 replicates of both GRBV-infected and 15 uninfected (control) vines. These recipient grapevines will also be tested 6 months post 48hr IAP.

#### **b) GRBV detection in salivary glands:**

Detection of GRBV directly through the dissections of insect abdomens, mouthparts, and salivary glands followed by highly sensitive diagnostic testing using qPCR test is a very useful tool in assessing where GRBV is detectable inside insects. If insects are actively feeding on known GRBV-infected vines, then detecting the virus in these body parts, would assist in defining circulative or noncirculative transmission, and propagative or non-propagative pathways. In our Renewal Progress Report, we described the methods that we are using which we developed and confirmed using beet leafhopper, *Circulifer tenellus*, adults transmitting beet curly top virus (BCTV), a ssDNA geminivirus.

In order to address the GRBV detection and possible virion pathways, 20 adult *T. albidosparsus* were collected directly off of the tested GRBV-negative controls and GRBV-positive grapevines at the Gordon Valley Rd. field site. These insects were then brought back to the lab, dissected and each insect separated into abdomen, mouthpart, and salivary glands. The qPCR test results of this experiment indicated that one of the 20 adult *T. albidosparsus* collected grapevine was GRBV-positive within its abdomen and salivary glands, resulting in a 5% possibility of GRBV vector competence. Furthermore, none of the 20 adult treehoppers collected

from the GRBV-negative grapevines had detectible amounts of GRBV in any of the dissected body parts. Current CO1-barcoding of this potential GRBV-vectored insect is underway and will be provided in our next report on this project.

**c) Conduct field and greenhouse GRBV transmission studies using three cornered alfalfa hopper (3CAH):**

**3CAH greenhouse transmission study using GRBV positive clade-I and clade-II isolates from Foothills area in Amador County, CA, as source vines:** We have monitored a Zinfandel vineyard in Amador County from 2015 to 2017 in which GRBV infected grapevines doubled in number after two seasons (Wunderlich et al., 2017) indicating a very rapid spread of the virus as had been observed in some Oregon vineyards in a collaborative study (Dalton et al. 2019). Ten months ago, 500 adult 3CAH's were collected in an organic alfalfa field near Davis, CA. These insects were then divided into three groups and transferred to insect cages. One cage contained a GRBV clade-I vine (ACU-I), one cage contained GRBV clade-II (ACU-II), and one cage contained a tested (GRBV-free) vine. After 48 hr AAP, these insects were transferred individually into clip cages fastened onto the oldest leaf of tested GRBV-negative recipient vines. Ten replicate grapevines for each of the three treatments were established. These vines are currently being maintained and will be sampled at 6 months post AAP.

**Armstrong 3CAH field transmission study using two GRBV positive source vines.** As mentioned in our Renewal Progress Report, we have previously established a Cabernet Sauvignon vineyard using qPCR-tested GRBV negative vines at the UC Davis Armstrong Tract in Solano County for use in field transmission experiments. The vineyard consists of 30 vine rows with 55 vines per row and is oriented east to west, and has an established alfalfa strip along the southern edge of the vineyard that became naturally infested with 3CAH. Twenty grapevines each infected with GRBV clade-I (CS-337-1) and clade-II (CF-214-1) isolates were planted along the southernmost vine row when the vineyard was established 3 years ago. Samples have been collected from the vines in the vineyard in June 2019, and are being qPCR tested for GRBV. No virus spread has been detected in prior years.

On September 8, 2018, we planted rooted Zinfandel vines from the Amador County vineyard mentioned above that were infected with clade I (ACU-I) and clade II (ACU-II) GRBV isolates between our established Cabernet Sauvignon GRBV-free vines in the third vine row north of the alfalfa strip in order to provide another virus source within the established vineyard to monitor possible infection and spread. Five plants of each GRBV clade were interplanted in this manner. Samples have been collected from the neighboring established vines and are being qPCR tested for GRBV.

Results from both of these studies will be presented in our next progress report.

***Tortistilus* sp. transmission study using GRBV clade Ghv-392 and Ghv 377 as GRBV source vines:**

Two years ago, 500 adult *T. albidosparsus* were collected from vetch growing 100 ft away from GRBV-infected grapevines in Pope Valley Ca. These insects were separated into four groups and transferred into four separate insect cages. One cage contained a GRBV-infected Ghv-392 vine, one cage contained a GRBV-infected Ghv-377 vine, one cage contained a tested GRBV-positive wild grapevine, and one cage contained a tested GRBV-negative vine. These insects

were given 48 hr AAP, then transferred to tested GRBV-negative recipient vines and allowed a 48 hr IAP. There were 15 replicates from each of the four groups, and samples from all 60 of these recipient vines have been collected and are being qPCR tested for GRBV. The results will be presented in our next progress report.

**d) Napa County Cixiid Collection and Dissection to Detect GRBV:**

Cixiids (planthoppers) are of interest as GRBV vectors as our studies prior to the identification of 3CAH as a vector of GRBV in the greenhouse (Bahder, et al. 2016) indicated that a cixiid as well as a psyllid and two species of phloem-feeding leafhoppers collected from vineyards where GRBV spread had been confirmed also had GRBV in their bodies when tested by qPCR. In addition, cixiids are closely related to beet leafhopper which transmit Beet curly top virus (BCTV) and treehoppers which transmit Tomato Pseudo Curly Top Virus (TPCTV). Both are geminiviruses. In late February, 350 adult cixiids (determined to be *Cixius praecox* based on CO1-barcoding), were sweep-netted off of grass growing in a former Napa Co. grapevine block that was completely removed in 2018 due to a very high incidence of GRBV. After collection, cixiids were divided into three groups and immediately placed onto a single GRBV- clade I (ACU-1) grapevine, a GRBV- clade (ACU-2) grapevine, or a tested GRBV-negative grapevine that were taken to the field site that day. All three groups of cixiids were given a 48 hr AAP, then removed. Only 8 cixiids survived. All 60 cixiids were dissected into abdomens and heads due to difficulty in removing salivary glands from dead insects and qPCR tested for GRBV presence. Molecular detection assays on these insects showed that none of these individuals had acquired GRBV. The inability of these cixiids to survive or reproduce on grapevines and the lack of presence of the virus in them makes us question this species' potential as GRBV vectors.

**e) Insect Collections in Vineyards for GRBV Detection:**

During 2014 and 2015, we collected insects by sweep-netting and light trapping at the UC Davis Oakville Experimental Station in Napa Co., and tested the captured insects for GRBV presence by molecular tests. Transmission studies were conducted when possible for those insect species that had GRBV present in their bodies. These studies provided initial clues indicating that 3CAH was a candidate as a vector of GRBV (Bahder et al., 2016). Because of our inability to date to confirm transmission of GRBV by 3CAH in the field, we reinitiated day and night insect collections in vineyards where GRBV spread is occurring with a specific focus on insects in the Hemiptera suborder Auchenorrhyncha (which includes treehoppers, leafhoppers, planthoppers, and psyllids) in June 2019. Locations where we will be collecting include vineyards where we have worked previously near Paso Robles (San Luis Obispo Co.), Buellton (Santa Barbara Co.), Oakville (Napa Co.), and Winters (Solano Co.). Our collection methods include sweep-netting of vineyard vegetation by day and using a mercury halide lamp with long (360 nm) UV wavelength) by night during the 'new moon' cycles.

Although most of our June and July collections remain to be processed, we present the following table (Table 1) to illustrate the number of leafhopper and planthopper species and individuals collected, and the associated test results for GRBV presence in the abdomens. The insects were collected at a vineyard in Paso Robles for two hours during the day and two hours at night. The insects were placed inside a vial containing 95% ethanol immediately following their capture, returned to the laboratory and dissected to remove the heads (and salivary glands) from the abdomen. The abdomens are tested initially for GRBV presence, and any insects that test positive for GRBV presence will also be processed for presence of GRBV in salivary glands. In

this example, 89 leafhoppers representing 17 species were collected and tested for GRBV. One specimen, a sharp-nosed leafhopper species, tested positive for GRBV in its abdomen. This individual represented one of the 2 specimens of that species that was captured and its salivary glands are now being tested for GRBV. Test results from an additional 14 specimens were inconclusive, and are currently being evaluated using different primers. Further results from our collections will be presented in our next progress report.

**Table 1.** Number of Auchenorrhyncha insects collected in a Paso Robles vineyard, and their GRBV status.

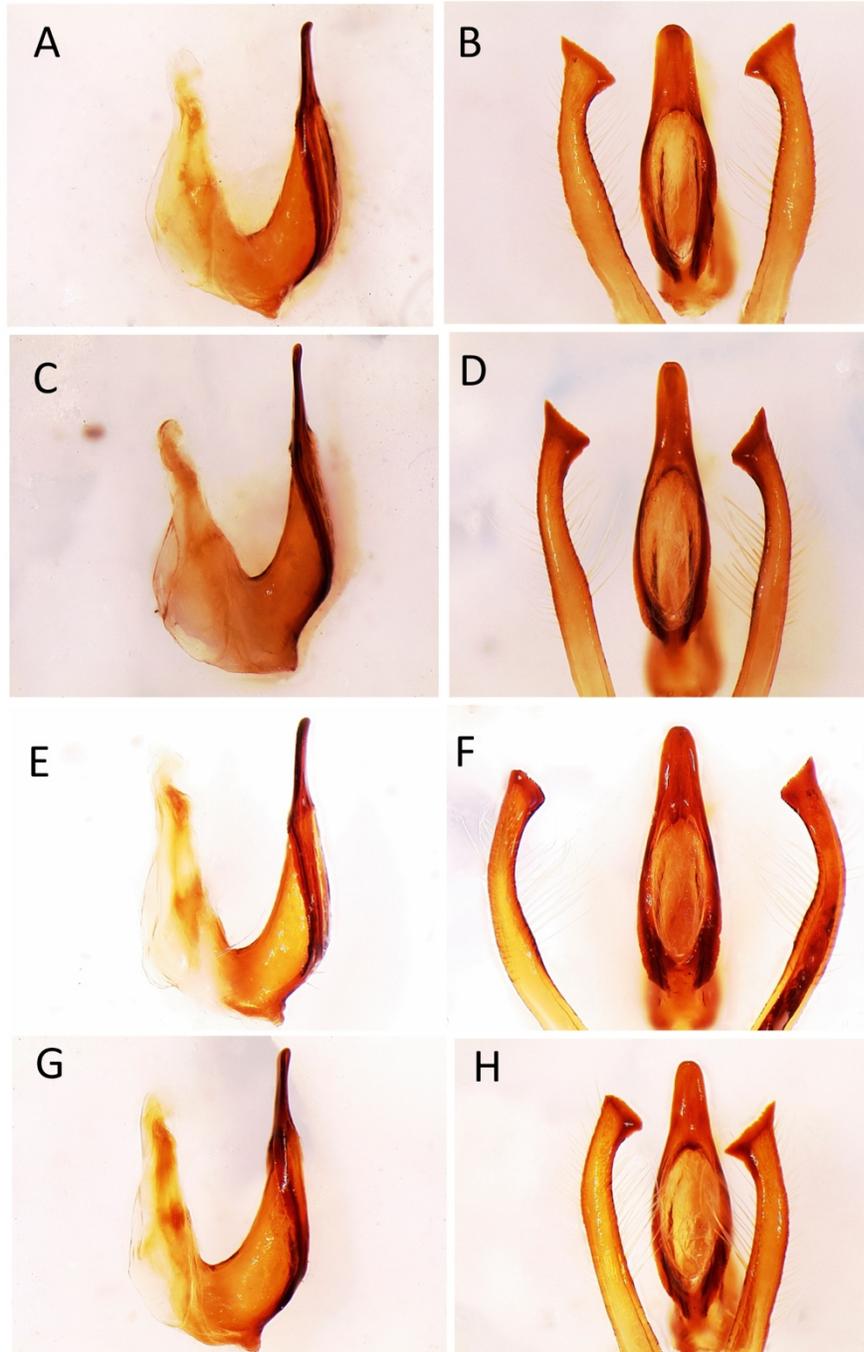
Family	Number of species collected	# of individuals collected and tested	# with GRBV in abdomen	# with inconclusive results for GRBV
Cicadellidae (leafhoppers)	17	89	1	9
Fulgoridae (planthoppers)	2	19	0	5

**Objective 3.** Confirm taxonomic identification and monitor *Tortistilus* spp populations in California vineyards and determine their seasonal biology in vineyards and surrounding landscapes.

**a) Taxonomic Identification of male *Tortistilus albidosparsus* via Auto-montage**

In spring 2016, we found colonization of grapevines by treehoppers that we identified to belong to the genus *Tortistilus* in vineyards where virus spread was occurring. *Tortistilus* treehoppers had not been associated with grapevines prior to that time, although there was mention of the ‘buffalo treehopper’ which actually belongs to a different treehopper genus, as feeding on California grapevines in Smith (2013). Later that year, Dr. Vaughn Walton’s lab at Oregon State University also found *Tortistilus* treehoppers in Oregon vineyards where Grapevine red blotch disease was spreading (Dalton et al. 2019). Both 3CAH and *Tortistilus* spp. belong to the Ceresini tribe of Membracidae. In spring 2017, we made an extensive collection of *Tortistilus* adults from a Napa Co. vineyard and found morphs of brown and green color both with and without horns from the same host plants on the same day. These insects were tentatively identified as *Tortistilus albidosparsus*, *Tortistilus pacificus* and *Tortistilus wickhami* based on the presence or absence of a suprahumeral horn characteristic and to some extent their coloration. That three closely-related species would seemingly occupy the same feeding niche at the same time and location seemed odd to us so we sent them to a specialist on the family Membracidae, Dr. Dennis Kopp at the Smithsonian Natural History Museum in Washington DC. He identified the four morphs; brown horned, green horned, brown unhorned and green unhorned, as being the same species based on microscopic observations of genitalia and the characteristic spots on the front of their head. As reported in our Renewal Progress Report in February, we performed shotgun DNA sequencing on some of the voucher specimens of these morphotypes, all collected on the same hosts on the same date and from the same Napa Co. vineyard, and found them to possess the identical CO1 gene, indicating that they are the same species.

Traditional identification of *Tortistilus* sp. was made exclusively by morphological characters of male genitalia. However, taxonomic determination of the *Tortistilus* sp. of interest to us because of their possible role in the transmission of GRBV was met with challenges as the



**Figure 2.** Plates A, C, E, and G are profiles of male *Tortistilus albidosparsus* aedeagus posterior and anterior arms. Plates B, D, F, and H are caudal views of male *Tortistilus sp.* posterior aedeagus and posterior style arms. Plates A & B are Horned Brown, Plates C & D are Horned Green, Plates E & F are Hornless Brown, and Figs. G & H are Hornless Green. All *Tortistilus* male genitalia were dissected and lysed using 180uL ATL buffer and 20uL proteinase K in a 1.5 ml centrifuge tube at an incubation temperature of 80C for 40 minutes. Images were taken with a digital JVC camera mounted onto a Leica MZ 16A dissecting microscope at 110X magnification.

original descriptions of these particular insects were only accompanied by hand-drawings. In order to examine the male genitalia more thoroughly, we used high resolution Leica auto-montage to create photoimages. The four morphotypes of *Tortistilus* we found in California vineyards with documented GRBV spread, consisted of brown horned, green horned, brown un-horned, and green un-horned (Figure 2, A-H), and the auto-montage images revealed that they had identical genitalia, confirming our biological observations as well as the results of CO1 sequencing, and are thus confirmed to belong to a single species *Tortistilus albidosparsus*.

**b) *Tortistilus* sp. Breeding Experiment Using the *Tortistilus* sp. Morphotypes**

On April 25, 2018, we collected third and fourth instar *T. albidosparsus* nymphs from common vetch growing in a riparian area 30 m removed from a confirmed GRBV infested vineyard in Pope Valley, Napa County. The nymphs were then returned to UC Davis where they were transferred into individual clip cages placed on potted vetch plants placed within field cages (Figure 3), and allowed to reach maturity. As the treehoppers emerged as adults, they were placed into separate cages according to their color and suprahumeral horn morphotypes. These morphotypes were subsequently placed in various combinations according to their morphotype in cages on oak plants that were grown from acorns in 1 gallon pots to test the heritability of the color and suprahumeral horn characteristics. The acorns were collected in 2017 from Napa Co.



**Figure 3.** Individually isolated *Tortistilus* sp. nymphs inside yellow clip cages attached to vetch grown from seed.

Of six successful mating pair combinations, 3 samples had oviposition scars (Figure 4) that could indicate successful mating and egg-laying by the treehoppers. In late April, the mating pairs consisting of 4 hornless males and 5 horned females produced nymphs that were reared to

adults with 1 horned brown female and 1 hornless brown female morphotypes. The mating pairs consisting of four hornless males and 5 hornless females produced nymphs one of which survived to emerge as an adult horned brown female.

This is the first report of successful mating of the horned and hornless morphs and the production of offspring.



**Figure 4.** Scar on oak tree due to oviposition by *Tortistilus* sp in a mating study using horned and unhorned female and male insects.

**Publications Produced and Pending, and Presentations** (since Renewal Progress Report):

Preto, C.R., B.W. Bahder, E.N. Bick, M.R. Sudarshana, and F.G. Zalom. 2019. Seasonal dynamics of *Spissistilus festinus* (Say) (Hemiptera: Membracidae) in a Californian vineyard. *J. Econ. Entomol.* 112(3): 1138-1144. <https://doi.org/10.1093/jee/toz022>

Preto, C.R., M.R. Sudarshana, and F.G. Zalom. 2019. A Grapevine red blotch virus vector: three-cornered alfalfa hopper in Napa vineyards. *American Vineyard Magazine.* 28(2): 14-18.

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**Relevance statement:** The primary aim of this proposed study is to gain a better understanding of the ecology and epidemiology of Grapevine red blotch virus (GRBV) in California vineyards so that appropriate measures for preventing infection and spread of the virus can be developed. This will be accomplished by documenting the role of treehoppers (Hemiptera: Membracidae) and other insects in Hemiptera suborder Auchenorrhyncha in the spread of GRBV in California vineyards, and how their use of the surrounding landscape influences the spread of the virus

**Layperson summary of project accomplishments:** The results of this project are expected to better define the possible role of the three-cornered alfalfa hopper (3CAH) and other vineyard treehoppers in the epidemiology of GRBV, including management of virus spread, by

determining feeding on grapevines seasonally and their phenology in relation to cover crops and non-crop vegetation in and around vineyards. Possible transmission by other treehoppers, planthoppers, psyllids and phloem-feeding treehoppers found in vineyards where GRBV is spreading will also be confirmed. This essential information will contribute to the management of red blotch disease by cultural methods such as reducing plant hosts favorable to sustaining vector populations or precise treatment timings based on treehopper biology in vineyards and when transmission is most likely to occur.

**Status of funds:** At the end of year 1, as of June 30, 2019, the projected balance is \$25,255.73 out of \$119,900 received at the start of the project.

**Summary and status of intellectual property associated with the project:** None

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