Interim Progress Report for CDFA Agreement Number 17-0460-000-SA

TITLE OF PROJECT: BIOLOGY AND ROLE OF TREEHOPPERS IN GRAPEVINE RED BLOTCH DISEASE

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REPORTING PERIOD: The results reported here are from work conducted July 2017 through early-March 2018, and includes data from relevant related studies that began earlier in the 2017 season prior to when this project was funded.

INTRODUCTION: A grapevine disease with symptoms that resembled those of leafroll was found in Napa County vineyards in 2007 (Calvi 2011). The disease was named red blotch disease and further investigations revealed a new DNA virus initially named Grapevine red blotch-associated virus (GRBaV) that was tentatively grouped in the family Geminiviridae (Al Rwahnih et al., 2013; Sudarshana et al. 2015). The virus was also found independently in grapevines in New York, Oregon and Washington (Krenz, et al. 2012; Poojari et al., 2013; Seguin et al., 2014), and is now known to be widely distributed across the US. California vineyards with the disease, especially those planted to red varieties, are known to impact quality of the grapes, substantially reducing their value.

Red blotch disease epidemiology is not well known. Although some researchers initially believed that the virus did not spread to or within established vineyards, observations by growers, consultants, and other researchers strongly suggested spread was occurring in some vineyards that appeared to be consistent with that of a motile insect vector. The virus has been isolated from wild grapevines, mainly open-pollinated *Vitis californica* and hybrids (Bahder et al., 2016; Perry et al., 2016), even at a considerable distance from commercial vineyards. After surveying many of the hemipteran insect species found in commercial vineyards where there was evidence of the spread of red blotch disease, the three-cornered alfalfa hopper (3CAH), *Spissistilus festinus* Say, (Hemiptera: Membracidae) was found capable of transmitting GRBV under laboratory conditions by Bahder et al. (2016). Subsequently, other treehoppers of the genus *Tortistilus* were observed feeding on grapevines where red blotch disease was believed to be spreading in California, southern Oregon and the Willamette Valley (Zalom and Sudarshana, unpublished; Walton, unpublished), but the status of these species as GRBV vectors has yet to be confirmed. Although some aspects of 3CAH biology is mentioned in the scientific literature, the majority of this information comes from legume cropping systems such as soybean, peanut and alfalfa where it is considered to be a pest. The biology of 3CAH and more especially the other

treehoppers in vineyards is little known. A better understanding of their seasonal biology in and around vineyards and their role in virus transmission is essential for developing management guidelines to prevent spread of red blotch disease within and between vineyards. The research objectives addressed through this grant began in 2016 with funding from the CDFA Specialty Crops Block Grant Program to Sudarshana and Zalom, USDA-ARS National Program funds to Sudarshana, and the American Vineyard Foundation to Daane. All of these grants ended in June 2017. Funding for this Pierce's Disease Program grant was finally received on October 10, 2017, due to complications in contracting between UC and CDFA, however we continued many elements of the proposed research prior to formally receiving funding. Therefore some of the results reported hereafter chronologically precede the initiation of this grant.

OBJECTIVES: The long-term objectives of this proposed study address improved 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 red blotch disease can be developed. The primary goal is to document the presence of treehoppers focusing on the three-cornered alfalfa hopper and *Tortistilus* species in California vineyards and the surrounding landscape, and to understand their role in the spread of GRBV between grapevines and regionally.

The specific objectives of this project are:

1. Monitor the population dynamics of 3CAH in vineyards and surrounding landscapes over the season.
2. Conduct GRBV transmission studies using treehoppers collected from vineyards with red blotch disease, and detect GRBV in the salivary glands of insects collected. Monitor field transmission by 3CAH.
3. Determine transmission efficiency of 3CAH to identify virus acquisition periods and persistence in the insect.
4. Evaluate the role of cover crops on the 3CAHs in vineyards.
5. Determine status of common weed and cover crops as feeding and reproductive hosts for 3CAH.

DESCRIPTION OF ACTIVITIES TO ACCOMPLISH OBJECTIVES:
Monitor the population dynamics of 3CAH in vineyards and surrounding landscapes over the season. This objective was addressed by both the Zalom and Sudarshana labs at UC Davis, and by the Daane Lab at UC Berkeley.

In the study by the Zalom and Sudarshana labs and primarily conducted by UC Davis PhD candidate Cindy Preto, ground cover located in and around a 53-row Cabernet Sauvignon block at the UC Davis Oakville Research Station and the perimeter of the reservoir pond at that site was sampled weekly by sweep net since March 2016. All odd numbered rows were tilled late March and were therefore not sampled. Each even numbered row was subdivided corresponding to the 6 proximal vines on each row border and the middle 18 vines, and ground cover within these areas were sampled separately for treehopper adults and nymphs. The adults were sexed. Sampling will continue through March 2018 when the vineyard is scheduled for removal due to increasing red blotch disease incidence. The first 3CAH adult collected for 2017 was on February 15. We now believe that this marks the initiation of activity of the overwintering generation in the vineyard. Bud break occurred on April 6, seven weeks after the first 3CAH adult was found in the vineyard. The first nymphs were collected on May 23 coinciding with an increase in adult 3CAH captures (Figure 1) and the grapevine phenological marker of bloom.

Increase in captures of later-instar nymphs increased in concert with adult captures, and we posit that this indicates the first in-vineyard generation of 3CAH. Subsequent 3CAH generations overlap one another. Veraison was noted on August 3 and seasonal vineyard weeds, which constitute the ground cover sampled at Oakville, started to noticeably dry by August 10, corresponding with a drop in adult 3CAH. The weeds in the vineyard and surrounding the irrigation pond were mowed by August 31 and no 3CAH adults were collected from ground cover thereafter. Vineyard floor weeds have not regrown since mowing, probably due to lack of rain. The first 3CAH adult collected for 2018 was on February 1 (Figure 2) which is 2 weeks earlier than in 2017. Bud break has yet to occur for 2018.

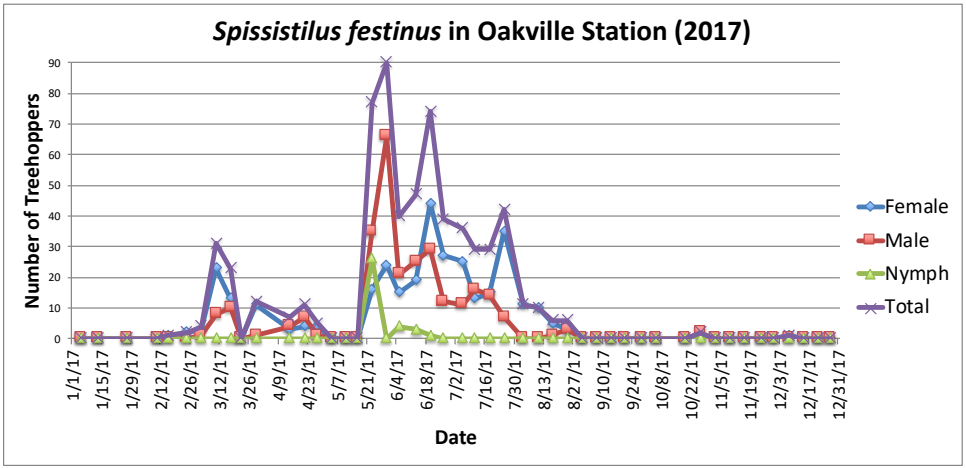

Figure 1. Weekly sweep net sampling of vineyard ground cover for three cornered alfalfa hopper at Oakville, 2017.

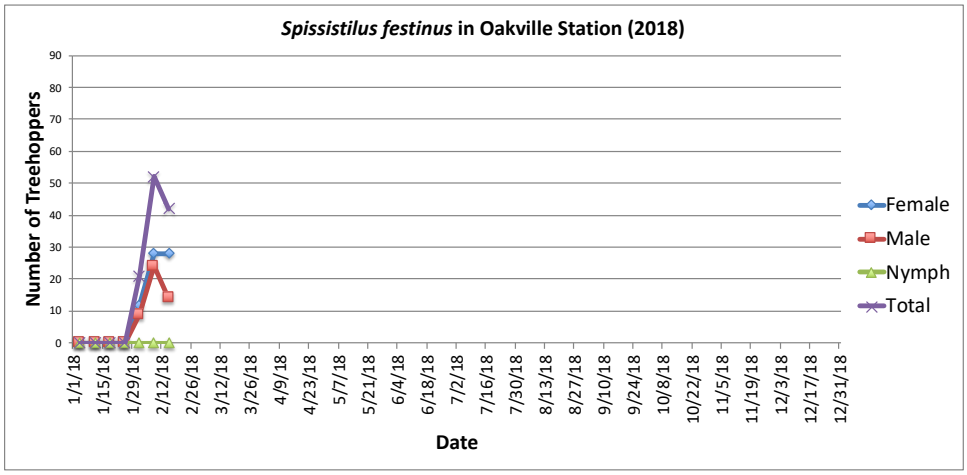

Figure 2. Weekly sweep net sampling of vineyard ground cover for three cornered alfalfa hopper to date at Oakville, 2018.

Salivary glands were extracted from the 3CAH collected at the Oakville vineyard to test for presence of GRBV biweekly beginning March 3, 2017, just prior to bud break. A total of 96 usable samples were collected. Salivary glands from 3CAH reared from eggs were dissected on each collection date, and these will serve as negative controls. The salivary glands were removed, placed in 180 uL ATL and 20 uL proteinase K incubated 4 h at 56ºC, and are currently stored in a -80ºC freezer at UC Davis awaiting GRBV detection.

In a related study conducted by Dr. Houston Wilson of the Daane lab, changes in 3CAH populations and crop damage along transects that extend out from natural habitats into vineyards were evaluated at approximately 2 week intervals between March 2017 and January 2018 using a combination of yellow sticky-traps, sweep-nets and beat-sheet sampling. Field sites consisted of vineyard blocks >2 acres in size adjacent to riparian and/or oak woodland habitat located in Napa and Sonoma counties. At each site, insects were sampled along five parallel transects (positioned 20 m apart) that extended out from the riparian or oak woodland habitat (i.e. "natural habitat") into the vineyard. Each transect was 160 m long – going 10 m into the natural habitat and 150 m into the vineyard. Along each transect, samples were taken at the interior of the natural habitat (10 m into the habitat) as well as at the edge and interior of the vineyard (10 and 150 m into the vineyard, respectively). There were 5 total study sites in all, and all vineyard blocks were red varietals that were at least 5 years old and located on level ground with similar trellis and irrigation systems. All plots were maintained insecticide free throughout the course of the study. Two yellow sticky-traps (16 x 10 cm, Seabright Laboratories, Emeryville, CA) were placed at each transect point in the vine canopy and on the drip irrigation line at ~0.3 m above the soil surface. In the natural habitat, two sticky-traps were hung from a pole at each transect point at a height above the ground surface equivalent to those in the vineyard. On each sampling date, proportion of ground cover to bare soil was recorded along with species composition and ground cover status. At each transect point, a set of 30 sweep net samples were used to sample the ground cover. A modified beat-sheet was used at each transect point to sample the canopy of grape vines (in the vineyard) and non-crop species (in the natural habitat). The beat-sheet consisted of a 1 m^2 nylon funnel that fed into a detachable 1gallon plastic bag. For each sample, the funnel was held beneath the canopy while vigorously shaking the plant (or vine) for 30 sec. in order to dislodge insects into the funnel and plastic collection bag.

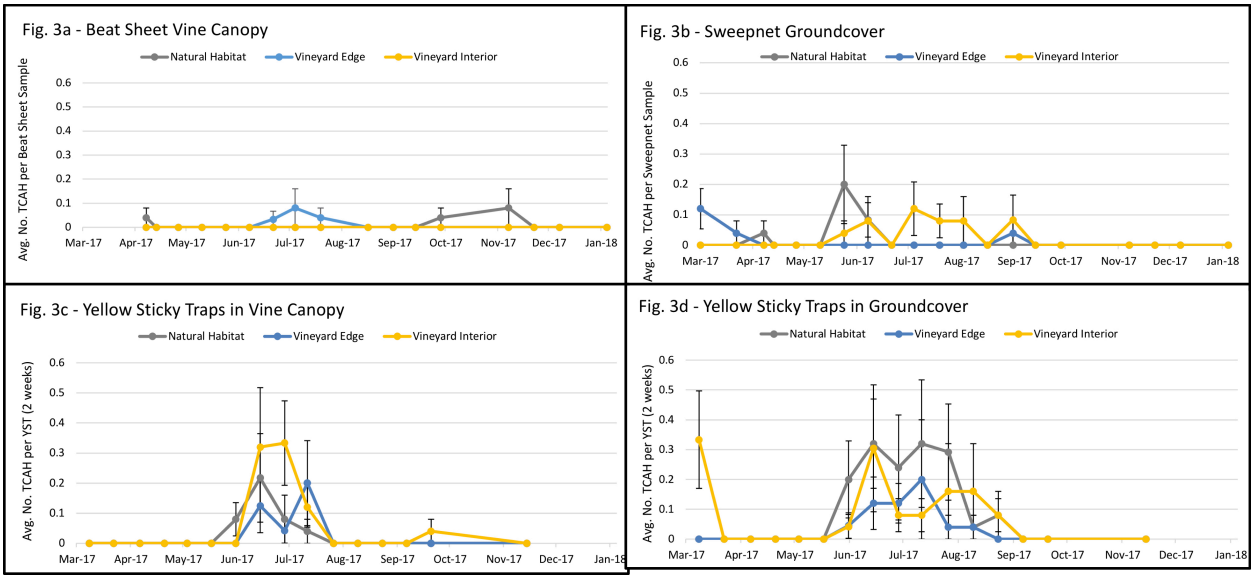

Figure 3. Three cornered alfalfa hopper densities sampled along the transect using (1a) beat sheet in the vine canopy or perennial vegetation canopy; (1b) sweep-net on ground covers; (1c) yellow sticky traps in the vine canopy or at vine canopy height; and (1d) yellow sticky traps at ground cover height (~0.3 m).

Each month, vines along each vineyard transect point were evaluated for signs of TCAH feeding damage (i.e. girdling of leaf petioles). At each vineyard transect point, 1 cane from each of 10 randomly selected vines was visually inspected for leaf girdling. Total leaf nodes and leaf girdles per cane were recorded for each vine. Petiole girdling became apparent in August 2017 with a

higher proportion of girdles located at the vineyard interior. This increase in girdling in August follows increased 3CAH densities observed in the vine canopy between June and August.

Preliminary findings indicate that 3CAH activity showed a strong temporal trend, with densities generally increased between June and August along with some activity observed in March and October/November (Figure 3). Comparing the different sampling techniques, the highest TCAH densities were recorded on yellow sticky traps followed by sweep-nets and then beat sheets While there was no clear gradient of 3CAH activity across the transect points, densities on the yellow sticky traps were slightly elevated in natural habitats in early June just prior to increases observed in the vine canopy at both the vineyard edge and interior in the following round of sampling (Figures 3c and 3d). Comparing the different sampling techniques for 3CAH from the

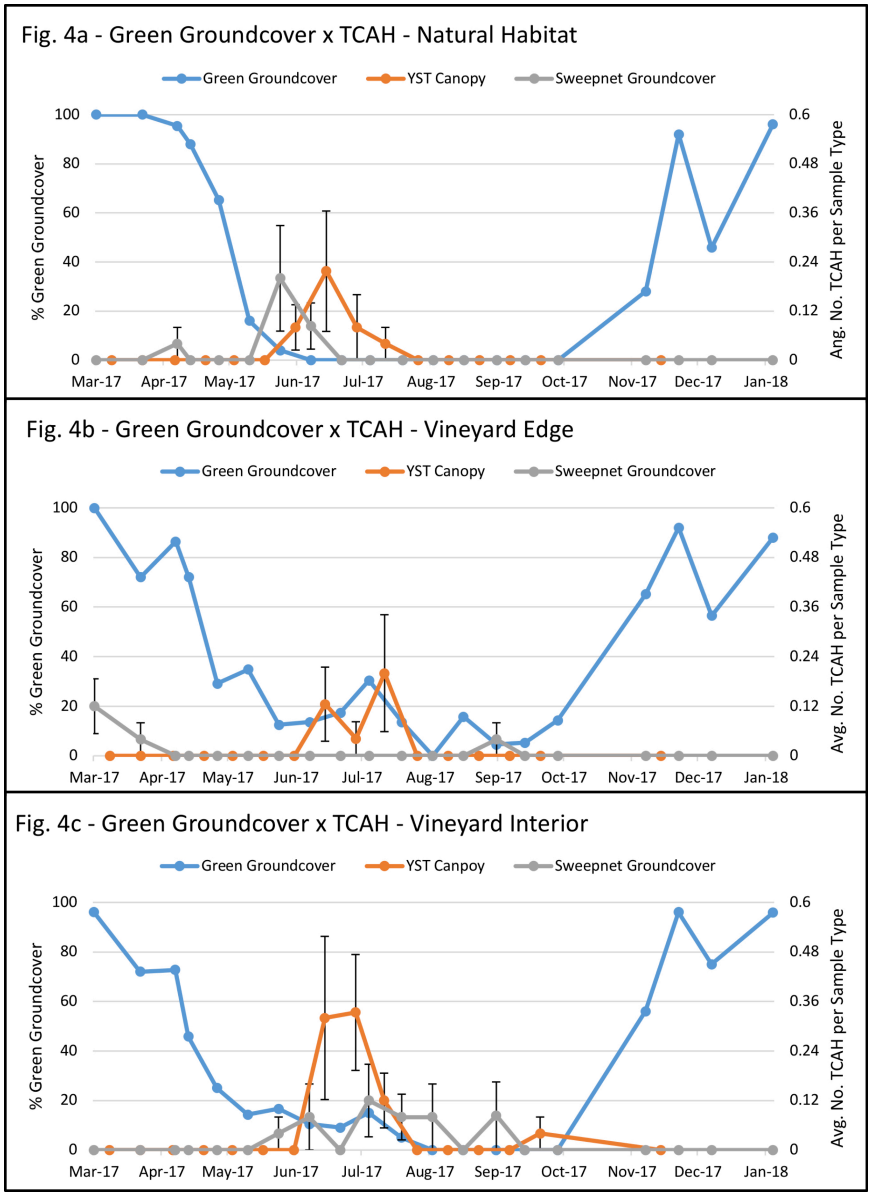

Figure 4. Three cornered alfalfa hopper densities in the vine canopy increased as the proportion of healthy/green ground covers diminished although some 3CAH persisted on ground covers late into the season. Treehoppers sampled on natural habitat nearby vineyards (4a), at the vineyard edge (4b), and at the vineyard interior (4c),

vine canopy and natural habitat, the highest 3CAH densities were recorded on yellow sticky traps, followed by sweep-nets and beat sheets. Changes in 3CAH densities between the ground covers and vine canopy were not always clearly reflected in the data. While densities in the vine canopy did increase as the proportion of healthy/green ground covers diminished (Figure 4), some 3CAH could still be found on the little bit of ground cover that remained in natural areas or at the vineyard edge later in the season (Figures 4a, 4b). Surprisingly, these late season 3CAH adults were most frequently encountered on ground covers in the vineyard interior (Figure 4c).

Changes in 3CAH densities along these transects may provide evidence of seasonal movement of the insect between natural habitats and vineyards, while differences in 3CAH abundance on ground covers and in the crop canopy, along with petiole girdling, may indicate the timing of vine colonization and feeding.

Conduct GRBaV transmission studies using treehoppers collected from vineyards with red blotch disease and detect GRBaV in the salivary glands of insects collected. Monitor field transmission by 3CAH.

Michael Bollinger of the Zalom lab at UC Davis has been collecting *Tortistilus* treehoppers in Napa and Sonoma County vineyards where GRBV has been occurring since May 2016 when we became aware of a large population of adults present and actively feeding on grapevines, but we have been unable to establish a reproducing colony in the laboratory. We attempted GRBV greenhouse transmission studies with field-collected 'horned' and 'unhorned' *Tortistilus* during 2016 that have yet to confirm transmission by qPCR. A larger study was initiated on May 24, 2017, when a very large population of wild *Tortistilus* was found feeding on vines in a Pope Valley vineyard. *Tortistilus* collected on that date and for several weeks thereafter were separated into 'horned' and 'unhorned' morphs, and individuals of each were placed onto qPCR GRBV confirmed positive Ghv-24-392 (Clade II) and onto Ghv-32-377 (Clade I) Cabernet Sauvignon source vines. qPCR confirmed test healthy Ghv-37 Cabernet Sauvignon source vines served as a negative control. Transmission was attempted both by placing individuals of both morphs that had fed on GRBV infected source vines into clip cages on the uninfected vines or in large cages containing 8 uninfected vines and 20 male and 20 female *Tortistilus* of each morph. qPCR analysis of these plants will begin shortly. Also, In order to test acquisition in a more natural environment, field captured *Tortistilus* collected on May 30, 2017 were placed inside cages wrapped around separate qPCR confirmed positive and negative Cabernet Sauvignon field vines located at the Pope Valley vineyard and similarly on qPCR GRBV confirmed wild grapevine located in the vicinity for an AAP of 6 days then transferred to qPCR confirmed healthy Cabernet Sauvignon recipient vines and allowed an IAP of 6 days. The GRBV testing of these plants will began in November 2017, but qPCR did not indicate infection in any of the samples. Testing will begin again this spring. All qPCR testing for these studies is being done by the Sudarshana lab at UC Davis

All *Tortistilus* removed from the grapevines post-inoculation were placed inside 1.5 ml tubes filled with 95% ethanol for salivary gland removal and GRBV testing. Salivary glands from *Tortistilus* collected from the test positive Cabernet Sauvignon in the field have not yet been tested for presence of the virus.

At three intervals during summer and fall 2016, ten adult 3CAH that were allowed to feed on clade 1 or clade 2 GRBaV infected vines for at least 3 days were caged on each of 5 virus free Cabernet Sauvignon grapevines that had been planted on the UC Davis Plant Pathology Field Station in 2015 by the Sudarshana lab (Figure 5, left photo). Testing of these vines for GRBV presence during 2017 did not indicate presence of the virus, but testing will begin again in June

2018. We also monitored all of the grapevines for 3CAH girdles during 2016 and 2017, and many girdles were found in the planting.

Figure 5. Cabernet Sauvignon grapevines on Freedom planted in 2015 at the UC Davis Plant Pathology Field Station. Above left: Caged grapevines for 3CAH release. Above right: Grapevines showing treehopper feeding damage with girdled shoots that turned red.

Determine transmission efficiency of 3CAH to identify virus acquisition periods and persistence in the insect. Studies related to this objective have been initiated by the Daane lab, but there are no results to present at this time.

Evaluate the role of cover crops on the 3CAHs in vineyards. In Fall 2016, common cover crops were planted in replicated plots at 3 vineyard locations by UC Davis PhD candidate Cindy Preto of the Zalom lab, and sampled by sweep net for presence of treehoppers. Figure 6 shows an example of a grass (left) and legume (right) cover crop replicate at one of the sites. However, few 3CAH were found overwintering in the cover crops and the growth of the different cover crops varied considerably. In fall 2017, we planted cover crops at a vineyard site at UC Davis and will introduce adult 3CAH when we begin finding them in commercial vineyards. Development of the treehopper populations on these cover crops and movement to the vines will be monitored through spring.

Figure 6. A grass (left) and legume (right) cover crop plot from our winter 2016-17 study.

Determine status of common weed and cover crops as feeding and reproductive hosts for 3CAH. Feeding and reproductive weed and cover crop hosts of 3CAH were determined in the greenhouse in a series of no-choice experiments that began in late 2016. This study is part of Cindy Preto's PhD. Three female and three male 3CAH were caged onto individual pots of weeds or cover crops (Figure 8). The cages were opened weekly for 4 weeks to determine adult survival, girdling, oviposition, and nymph emergence. Purple vetch was used as a positive standard in each run of the no choice experiment because of our previous laboratory and field observations of successful feeding and oviposition. The common vineyard weeds and cover crops evaluated as feeding and reproductive hosts of 3CAH are presented in Table 1. The relative reproductive success of 3CAH on each host has been compared to its success on purple vetch at the conclusion of all no choice tests.

Scientific Name	Common Name	Plant Family	No. of Girdles	No. of Nymphs	Percent Survival[a]
Acmispon americanus	Spanish clover	Fabaceae	Yes	Yes	92
Taraxacum officinale	Dandelion	Asteraceae	Yes	Yes	71
Lotus corniculatus	Birdsfoot trefoil	Fabaceae	No	Yes	58
Poa pratensis	Kentucky bluegrass	Poaceae	Yes	No	25
Senecio vulgaris	Common groundsel	Asteraceae	Yes	Yes	21
Plantago lanceolata	Buckhorn plantain	Plantaginaceae	No	No	8
Daucus carota	Wild carrot	Apiaceae	Yes	No	4
Convolvulus arvensis	Field bindweed	Convolvulaceae	Yes	Yes	4
Kickxia elatine	Sharppoint fluvellin	Plantaginaceae	No	No	0
Cynodon dactylon	Bermuda grass	Poaceae	No	No	0
Pisum sativum	Magnus Peas	Fabaceae	Yes	Yes	92
Vicia faba	Bell beans	Fabaceae	No	Yes	83
Bromus hordeaceus	Blando brome	Poaceae	Yes	Yes	33
Vicia benghalensis	Purple vetch	Fabaceae	Yes	Yes	30
Medicago lupulina	Black medick	Fabaceae	Yes	Yes	25
Trifolium subterraneum	Subterranean clover	Fabaceae	Yes	Yes	17
Trifolium incarnatum	Crimson clover	Fabaceae	Yes	Yes	13
Vicia villosa ssp. Varia	Woollypod vetch	Fabaceae	Yes	Yes	13
Brassica sp.	Mustard	Brassicaceae	No	No	0
Avena sativa	California red oats	Poaceae	No	No	0

[a] Survival of adults for first 2 weeks on plants

Table 1: Plant species tested as feeding and reproductive hosts for *Spissistilus festinus* in no choice tests.

In an effort to evaluate preference of 3CAH to confirmed reproductive cover crop and weed reproductive hosts when presented a choice, three groups of 5 plants (Table 2) the included four known reproductive hosts from the completed no-choice experiment were randomly arranged in a large dome-shaped cage in the greenhouse and replicated three times (Figure 9). Purple vetch was included in each evaluation as a standard. Ten male and ten female 3CAH were released into each cage and allowed to freely feed and oviposit. All adults were removed from the cages after one week. Nymphs were counted and collected from individual plants on weeks two and three. Destructive sampling of all plants and collection of nymphs were conducted at week four. Data collected from this experiment is currently being summarized and will then be analyzed.

Figure 8. Weeds and cover crops caged with three cornered alfalfa hoppers in a greenhouse study at UC Davis.

Group one cover crops	Group two cover crops	Group three weeds
Purple vetch	Purple vetch	Purple vetch
Black medick	Blando bome	Field bindweed
Dutch white clover	Crimson clover	Spanish clover
Subterranean clover	Bell beans	Birdsfoot trefoil
Woollypod vetch	Magnus peas	Dandelion

Table 2. Three groups of cover crops and weeds identified as reproductive hosts of three cornered alfalfa hopper that have been compared in a preference study.

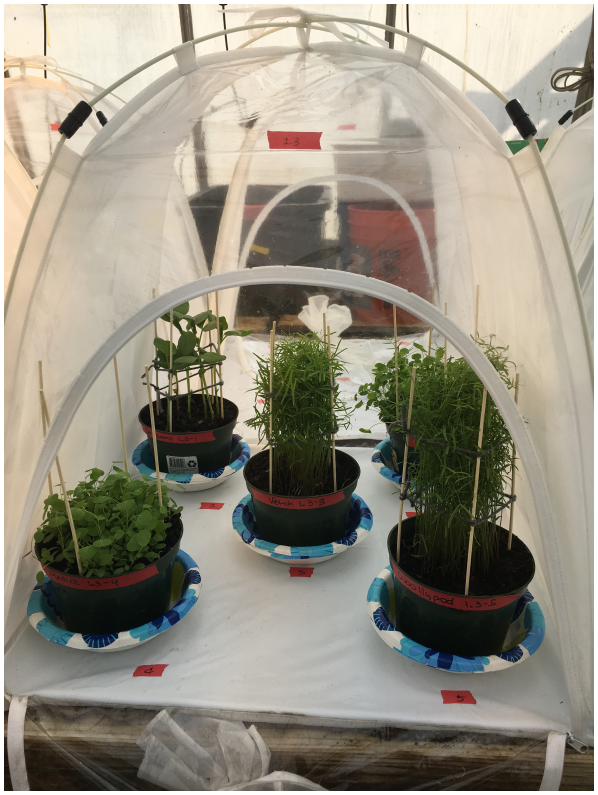

Figure 9. Four reproductive hosts plus purple vetch as a standard caged with three cornered alfalfa hoppers in a greenhouse preference study at UC Davis.

PUBLICATIONS PRODUCED AND PENDING: No publications have been published or are submitted at the time of this report.

RESEARCH RELEVANCE STATEMENT: This newly funded project is intended to address important gaps in knowledge of transmission and spread of GRBV in California vineyards that were identified in our earlier studies. Project co-investigators Zalom and Sudarshana confirmed transmission of the virus by 3CAH and this current project hopes to confirm transmission in the field as well as details of the transmission process. Observations by Zalom and Sudarshana and researchers at Oregon State University suggest that other treehopper species may also transmit the virus, but transmission has not been confirmed to date. This is being addressed by our project as well. Alternate feeding and reproductive hosts of 3CAH in the greenhouse and field that were initiated in the last year will be completed as a result of this project. This information will be directly applicable to management of red blotch disease in California vineyards.

LAYPERSON SUMMARY; The results of this project are expected to better define the role of the three-cornered alfalfa hopper (3CAH) in the epidemiology of GRBV, and to examine the role of grapevines, cover crops, and non-crop vegetation in and around vineyards in sustaining 3CAH populations. Possible transmission by other 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 where nearby GRBV source are known to occur.

STATUS OF FUNDS: The UC Davis/CDFA contract was approved in early October 2017 and funds became available to us on October 17, 2017. Immediately thereafter a UC Multicampus Agreement (MCA) was prepared between UC Davis and co-investigator Daane at UC Berkeley, but was not approved immediately, so funds finally came available to Daane the first week of January 2018. We anticipate that all funds will be expended by the June 30, 2018, end date, but we will likely request a line item shift for funds in the salaries and benefits as well as the supplies budget. This is due to differences in payroll titles of individuals working on this project, and differences in associated benefits rates. We are aware that it is possible to request a no-cost extension, and if we need to do this because of the delays by UC Davis and UC Berkeley in establishing subcontracts and getting funds to the co-investigators we would make the request in a timely manner well before the end date.

SUMMARY AND STATUS OF INTELLECTUAL PROPERTY: We do not anticipate development of intellectual property that will require protection during the course of our studies.

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