Project Title: X-disease Vector Identification and Acquisition From Low Titer Trees

Report Type: Final Project Report

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Cooperators: Rodney Cooper, Louis Nottingham, Garrett Bishop

Project Duration: 2-Year, no-cost extension

Total Project Request for Year 1 Funding: $ 55,266
Total Project Request for Year 2 Funding: $ 55,304
Total Project Request for Year 3 Funding: $0

Other related/associated funding sources: Awarded
Funding Duration: 2020 - 2022
Amount: $249,360
Agency Name: WSDA/USDA Specialty Crop Block Grant
Notes: USDA SCBG funding to identify strains of phytoplasma in cherries and other stone fruit as well as weedy plants, and to conduct molecular gut content analysis on X-disease vectors. PI: Harper, co-PI's: Northfield, Cooper, DuPont

Other related/associated funding sources: Awarded
Funding Duration: 2021 - 2023
Amount: $244,750
Agency Name: WSDA/USDA Specialty Crop Block Grant
Notes: USDA SCBG funding to evaluate selective broadleaf herbicides as a management option for X-disease vectors. PI: Northfield, co-PI: Harper.

Other related/associated funding sources: Awarded
Funding Duration: 2022 - 2024
Amount: $295,376
Agency Name: USDA Crop Protection and Pest Management
Notes: USDA funding to develop phenology models for phytoplasma prevalence in plants and vectors to integrate into phenology models for leafhopper abundance (WTFRC project led by Nottingham). PI: Northfield, co-PI: Nottingham (WSU), Harper (WSU), Adams (OSU), Galimba (OSU).

Other related/associated funding sources: Awarded
Funding Duration: 2021 - 2023
Amount: $164,765
Agency Name: USDA AFRI
Notes: USDA postdoctoral fellowship awarded to Adrian Marshall (mentors: Northfield, Harper, and Cooper) to precisely estimate the time between acquisition to transmission for leafhoppers to better inform timing of control measures.

Other related/associated funding sources: Awarded
Funding Duration: Ongoing
Amount: $2 million per year
Agency Name: USDA ARS congressional funding
Notes: Cooperative research project between USDA ARS and WSU to better understand little cherry disease (caused by X-disease phytoplasma and Little cherry virus). PI: Cooper, co-PIs: Northfield, others.

Other related/associated funding sources: Awarded
Funding Duration: 2021-2022
Amount: $40,000
Agency Name: WSU BioAg grant
Notes: Coordinate efforts with this project to test X-disease vectors for three potential biological control agents: a parasitic fly, a parasitic wasp, and an entomopathogenic fungus using molecular methods. This collection, by Cesar Reyes Corral has been conducted alongside the collection in this grant to share resources and gain synergistic insights. PI: Northfield, co-PIs: Harper, Cooper.

WTFRC Collaborative Costs: None

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Item	2021	2022	No-cost extension
Salaries	$28,260.00	$29,390.00	
Benefits	$10,206.00	$10,614.00	
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$13,362.00	$11,862.00	
Travel	$3,438.00	$3,438.00	
Plot Fees			
Miscellaneous			
Total	$55,266.00	$55,304.00	$0.00

Footnotes:
[1] New postdoctoral researcher position (50% FTE)
[2] 36.1% (postdoctoral researcher)
[3] Fieldwork consumables, X disease tests, and extension supplies
[4] Domestic travel for research and extension

Objectives

1) Evaluate leafhoppers as potential X-disease phytoplasma vectors.

While all known leafhopper vectors of X-disease phytoplasma are in the subfamily Deltocephalinae, worldwide there are 6,683 species in the subfamily (Zahniser and Dietrich 2013), and more than 20,000 species of leafhoppers across the 30 leafhopper subfamilies. Therefore, we will narrow down our search to leafhoppers that commonly occur in Pacific Northwest cherry orchards, to limit the number of leafhoppers tested. As part of our Specialty Crops Block Grant project we will conduct surveys of phytoplasma strains in leafhoppers, and here we will pair these surveys with molecular analyses of salivary glands to evaluate phytoplasma presence. We will conduct surveys of ground cover and cherry trees using sweep nets and insect vacuums (D-vacs), as each method may collect different leafhoppers better (Purcell and Elkinton 1980). Sampling trees and ground cover will account for our findings that leafhoppers often spend a great deal of time in the groundcover, but regularly move into the trees to feed (TD Northfield, personal observation). We will sample from 20 orchard blocks in each period of leafhopper abundance: May/June, and August/September. During the two-year survey period, a total of 1000 (non-*Colladonus*) leafhoppers feeding on groundcover and surrounding extra-orchard vegetation will be screened for phytoplasma presence, and if found to be positive, the phytoplasma will be genotyped. Because many of the leafhoppers will test negative, we will combine 10 leafhoppers of the same species into one sample, such that if a single leafhopper carries the phytoplasma the entire sample will be identified as positive. It is possible that non-vector leafhoppers have fed on the phytoplasma, but the phytoplasma is not able to make it through the leafhopper gut and to the salivary glands to be transmitted during feeding. Therefore, we will dissect and evaluate the presence of the phytoplasma only in the salivary glands to determine which leafhoppers have the ability to transmit rather than just acquire the phytoplasma.

2) Assess potential for vectors to acquire X-disease phytoplasma from trees with low titer levels.

To evaluate the effects of low titer levels on acquisition rates of X-disease phytoplasma, we will capitalize on within-season and between tree variation in X-disease phytoplasma titer (i.e. concentration) levels. Co-PI Harper's research suggests that phytoplasma titers increase over the course of the year. Therefore, we will place 5 phytoplasma-free *Colladonus* sp. leafhoppers from a laboratory colony in a sleeve cage on known X-disease infected trees at three periods: April (low phytoplasma levels), July (high phytoplasma levels), and September (lower phytoplasma levels). After 1 week of allowing the leafhoppers to feed on the branch, we will store the leafhoppers for molecular phytoplasma detection, and use qPCR to evaluate titer level within the branch. This molecular measure of phytoplasma titer level will allow us to ensure that we do have seasonal differences over the course of the year, as well as evaluate the effect of variation between trees within a given time point on the acquisition of phytoplasma by the leafhopper. We will set up 10 leafhopper sleeve cages at each time point, for a total of 30 sleeve cages and 150 leafhoppers per year. To analyze the data we will conduct a regression of phytoplasma titers (combining all data points) and acquisition rate.

Note: Due to problems rearing leafhoppers in colony to ensure uninfected leafhoppers to evaluate these tests, we were unable to conduct these experiments in 2021. Initial trials in 2022 were also unsuccessful in leading to acquisition, and therefore, we changed our methods for 2023.

3) Develop a website at treefruit.wsu.edu updating the list of known leafhopper vector status, organized by subfamily.

A gallery of leafhoppers will be created, which will list vector status on the treefruit.wsu.edu website. High quality images will be taken of leafhopper species screened using microscope camera and 2x macro-lens with image stitching technology. Images will also be obtained from existing resources. Images will be marked with a easy to read symbol to indicate vector status. The gallery will be organized by subfamily in order for viewers to be able to view the relationship between leafhoppers with known vector status and leafhoppers with negative vector status. For each leafhopper a description will be

included which designates vector status and other relevant details. Untested common leafhoppers will also be included prior to testing.

Significant findings

- 82% of the leafhoppers collected from our 22 sites in August 2021 were not *Colladonus* spp. leafhoppers
- We found no evidence that any other leafhoppers were transmitting X-disease phytoplasma. In contrast, we did identify *C. m. reductus* and *C. geminatus* collected from the same sites with high X-disease phytoplasma titers in their salivary glands/mouthparts.
- *Euscelidius variegatus* can transmit X-disease in the laboratory, but with a ~50% longer latency period than *Colladonus* species. Here we tested more than 300 *E. variegatus* over the course of two seasons and never found evidence that X-disease phytoplasma was replicating in their mouthparts.
- In the first generation (May/June) some *Colladonus* spp. (*C. m. reductus* and *C. geminatus*) leafhoppers had X-disease phytoplasma in their guts during the first generation, but none had phytoplasma in their heads. This suggests they can acquire phytoplasma in the first generation, but the long latency prohibits transmission.
- The likelihood that a *Colladonus* spp. leafhopper had X-disease phytoplasma replicating in its mouthparts was highest in the second (August) and third generation (October).
- Evaluation of X-disease acquisition with highly infected cherry trees for 3 days led to short latency periods, suggesting that the latency period is dose dependent.

Methods

1) Evaluate leafhoppers as potential X-disease phytoplasma vectors.

2021 Sampling. We collected leafhoppers from 22 sites from 8 different Central Washington orchards, ranging widely in management regime from the Wenatchee, Yakima, and Mattawa regions. We also collected from another site in Pasco, but did not find any leafhoppers. We collected the leafhoppers by sweep netting during the peak of the second generation of leafhoppers to determine leafhopper abundance and species composition. We targeted three time periods each focusing on a different generation of *Colladonus* adults (May 18-26, August 4-17, and September 13-October 7) to focus on the time when phytoplasma titers are highest, providing the greatest change of collecting phytoplasma in leafhopper salivary glands. Samples consisted of 20 sweeps in 10 rows at each site (200 sweeps per site). The contents of the sweeps were transferred to mesh bags and brought back to the lab for sorting and recording by species (*C. m.. reductus*, *C. geminatus*, *Scaphytopius acutus*, other leafhoppers, and nymphs). Other leafhoppers primarily consisted of a small unidentified brown species, and the larger *Euscelidius variegatus*. We primarily targeted cherry and stone fruit blocks, but we also included apple blocks in the vicinity of cherry/stone fruit blocks to broaden the range of leafhoppers we could find.

2022 Sampling. In the 2022 field season we expanded our range to 30 sites for generation 1, and 24 sites in *C. m. reductus* generations 2 and 3. For generations 2 and 3 we prioritized the 22 locations that had higher leafhopper numbers in our first round of sampling and added in 2 sites in Tonasket to increase our sample range. Compared to 2021 sampling in 2022 we were able to obtain more information, targeting blocks with high X-disease prevalence, and reaching farther north, to include 2 sites in the Chelan region, 2 sites in the Omak region, and 2 sites in the Tonasket region. We also included 6 sites in the Cashmere to Rock Island region, one in Mattawa, and the rest in the corridor from Yakima to Pasco. In 2022, because we were better at identifying *E. variegatus*, we tested them individually, analyzing entire bodies and heads together.

2) Assess potential for vectors to acquire X-disease phytoplasma from trees with low titer levels.

To develop methods, on Aug 1, 2022 branches were collected from an X-disease-infected cherry tree (CT 28 [tested in 2021], equivalent to less than 5,293 phytoplasma cells/sample) in the Rock Island

area. Branches were returned to the lab and placed in a deli cup with water in a mesh cage with an infected (CT 35, equivalent to less than 51 phytoplasma cells/ sample) dandelion plant in the greenhouse. The next day, we collected *C. m. reductus* leafhoppers from an apple block. Leafhoppers were sorted at the lab and all adults were placed in the mesh cage with the infected plant material. After one week of feeding, we collected the remaining alive adults (11) and transferred them into ethanol and whole bodies were tested by qPCR for X-disease phytoplasma. However, no X-disease phytoplasma was detected in any of the leafhoppers.

2023 study. On August 1st 2023, we evaluated X-disease acquisition using colony *C. m. reductus* leafhoppers. A test of 29 of the colony leafhoppers found no X-disease presence in the colony. We collected X-infected cherry branches from trees that were highly infected in a block in the Yakima region and placed the branches in three cages with colony *C. m. reductus*. Infected branches were removed after 3 days and replaced with clean celery root, and the branches were tested for X-disease. After 3 days of feeding on celery root, 10 *C. m. reductus* were collected, dissected in half, and tested for X-disease using qPCR. A second group of 12 *C. m. reductus* were collected, dissected, extracted, and tested for X-disease ten days after switching to celery root. Finally, a third group of 15 leafhoppers was collected 17 days post feeding and were dissected, extracted, and tested.

3) Develop a website at treefruit.wsu.edu updating the list of known leafhopper vector status, organized by subfamily.
We are using a microscope with a camera attachment to carefully photograph each type of leafhopper being evaluated.

Results and Discussion

1) Evaluate leafhoppers as potential X-disease phytoplasma vectors.

Of the 1844 adult leafhoppers collected from the 22 sites in August 2021, 257 (14%) were *C. m. reductus*, 4 were *C. geminatus*, and 64 (3.5%) were *Scaphytopius acutus*. The remaining were a combination of *Euscelidius variegatus* and a diverse group of brown colored leafhoppers that resemble *E. variegatus*, but appear to be different species (Figure 1). In addition, we collected 406 nymphs for which the species is unknown. We have extracted DNA from the "other" species to determine phytoplasma presence. Because *E. variegatus* has proven to be a vector in laboratory experiments, but has a 50% longer incubation period than *Colladonus* species (Jensen 1969), we will also measure the proportion of *E. variegatus* that have phytoplasma in their salivary glands. We have also been using a microscope with a camera attachment to take photographs of the various leafhoppers that we are testing so they can be shared in a webpage. We present some of those photographs in Figures 2 and 3.

2021 Sampling. In 2021 we tested 28 groups of 10 *E. variegatus* (280 total whole-bodies tested) and never detected X-disease phytoplasma from them. We also tested 6 other leafhopper species that look similar to *E. variegatus* and never found X-disease phytoplasma in their heads. These were tested in 5, 6, 5, 34, 2, 3, 1, and 1 groups of 10, respectively. All batches of leafhoppers tested included the same species from the same site. We also tested 17 groups of 10 green leafhoppers (comprising 4 species) and found no distinguishable X-disease.

For other known X-disease vectors (*C. m. reductus*, *C. geminatus*, and *S. acutus*), we tested them individually, evaluating their heads and guts. In some cases, we identified very low titers (*Ct* scores > 38, or less than 7 phytoplasma cells per sample) in the heads with no phytoplasma present in the rest of the body, likely representing a tiny amount of phytoplasma passing through the mouthparts. Four out of the 332 (1.2%) *C. m. reductus* heads tested positive, with *Ct* scores 35 or less (equivalent to less than 51 phytoplasma cells/ sample), all of which had positive tests for the bodies too, suggesting phytoplasma had successfully integrated through the body. 15 of the 332 *C. m. reductus* leafhoppers had *Ct* scores greater than 35 (equivalent to 51or more phytoplasma cells per sample), mostly with the body testing negative, suggesting they were not infective. All *C. m. reductus* that tested positive were collected in August and

October, with none collected in May or June coming back positive. The other key vector, *C. geminatus* was rarely collected, and the mouthparts of two of the 38 collected tested positive with *Ct* scores less than 35 (51 or more phytoplasma cells per sample). Again, these leafhoppers had phytoplasma in their bodies as well, whereas the two other leafhoppers with mouthpart *Ct* scores greater than 35 did not. We also collected and tested 42 *S. acutus*, which is a known vector, but no leafhopper heads tested positive with a *Ct* score of 35 or less.

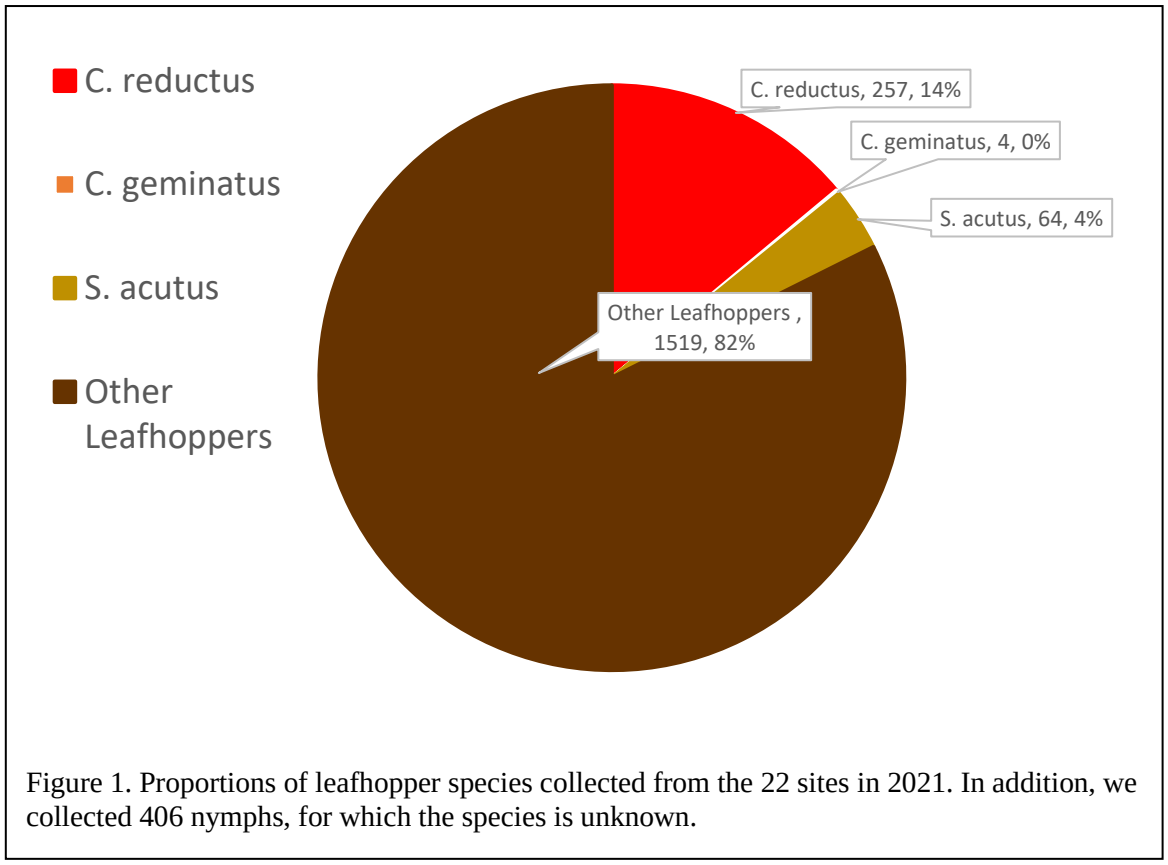

Figure 1. Proportions of leafhopper species collected from the 22 sites in 2021. In addition, we collected 406 nymphs, for which the species is unknown.

2022 Sampling. Interestingly, we found very few *Colladonus* spp. leafhoppers in northern growing regions, with only a few *C. geminatus*, and 1 *C. m. reductus* across our 6 sites in Chelan, Omak, and Tonasket. In the first generation 18% (13/71) of the *C. m. reductus* bodies and 5% (2/44) of *C. geminatus* bodies tested positive, but none appeared to be actively transmitting (high titers in mouthparts). In the second generation 9% (7/75) of the *C. m. reductus* bodies and 0% (0/6) *C. geminatus* bodies tested positive. Only 1 of the 75 tested *C. m. reductus* had high titers in the mouthparts, suggesting it was actively transmitting phytoplasma. In the third generation 25% (13/53) of the *C. m. reductus* bodies and 24% (4/17) of *C. geminatus* bodies tested positive, with only 1 *C. m. reductus* and no *C. geminatus* having high titers in the mouthparts. The low detection in mouthparts in 2022 may be influenced by the cold spring weather that year.

In the first generation, 2 of the 55 *E. variegatus* bodies tested positive for X-disease phytoplasma with *Ct* scores less than 35 (51 or more phytoplasma cells per sample), but the phytoplasma was not present in their mouthparts. We have continued to evaluate all collected leafhoppers, including 2 other brown species and 3 green species of leafhopper, but none of these have tested positive for X-disease phytoplasma. We also detected high levels of phytoplasma in the body of a treehopper at a site with highly infected trees, but no phytoplasma in the mouthparts, suggesting it was not able to transmit. In addition, because we did not find white apple leafhoppers in our cherry or stone fruit blocks, we placed white apple leafhoppers on

infected trees to see if they could acquire X-disease. However, all leafhoppers died within a matter of hours, suggesting they starved and did not feed on the cherry trees.

Figure 2. Variability in *E. variegatus* collected. Research on Italian populations suggested pigmentation can vary with sex and infection with other phytoplasmas (Galetto et al. 2018)

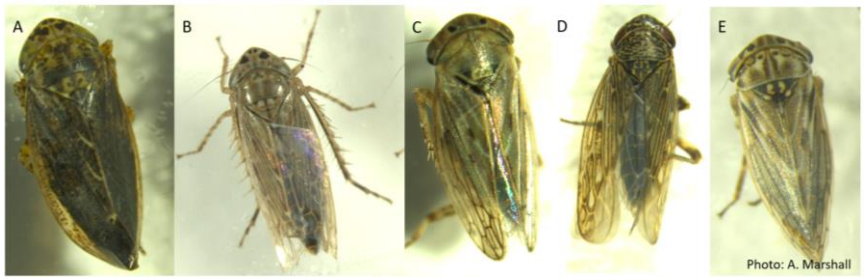

Figure 3. *E. variegatus* (A), and four other types of leafhoppers commonly collected in surveys. Leafhoppers are shown in order of abundance, with A being the highest abundance, and E being the least abundant. Note especially variation in the head shape and patterns, as well as the pronotum (the pirate hat-shape just behind the head). Pictures are not to scale: *E. variegatus* is larger than the other four leafhoppers.

2) Assess potential for vectors to acquire X-disease phytoplasma from trees with low titer levels.

Preliminary evaluations of adult leafhoppers acquiring X-disease phytoplasma from low titer trees in 2022 proved unsuccessful (all leafhoppers tested negative), and we retooled our approach to evaluating acquisition in 2023.

When caging leafhoppers to highly infected cherry branches (CT 22, equivalent to approximately 279,000 phytoplasma cells/sample) for 3 days, we found much shorter latency periods than previously reported, suggesting that the latency period may be dose-dependent. At 6 days post initial access (3 days post switch to celery root) 50% of leafhopper bodies tested positive with low titers and no heads tested

positive, showing they acquired the phytoplasma but were not transmissive. At 13 days post access (10 days post switch) ~25% of leafhopper bodies tested positive with high titers and ~20% of leafhopper heads tested positive with low titers. This demonstrates accumulation and replication of phytoplasma in leafhopper gut and the beginning of colonization of salivary glands and possible transmission. This was supported by the finding that all 3 celery roots had a positive testing leaf; however, with very low titers. At 20 days post access (17 days post switch) 50% of leafhopper bodies tested positive with high titers, and 50% of leafhopper heads tested positive with high titers. At this point the phytoplasma appeared to have colonized and replicated in the leafhoppers gut and salivary glands and they have become fully transmissive. This was shown by all three celery roots testing positive with slightly higher titers, and one leaf in particle having extremely high titers. In conclusion, if given only access to a high titer plant for 3 days, *C. m. reductus* can acquire and begin transmitting two weeks after initial access with a high likelihood of transmitting after 20 days post initial access. Now that the methods are worked out these tests need to be conducted with varying titers to determine the effect on acquisition and transmission time.

3) Photos and website development. A gallery of leafhoppers known to transmit X-disease was developed at https://treefruit.wsu.edu/vector-gallery/ This gallery contains images of the seven known vectors for X-disease phytoplasma.

A gallery of known leafhopper vectors and non-vectors has also been developed as part of a downloadable App for Iphone and Android. The app can be previewed here https://littlecherryguide.treefruit.wsu.edu/leafhopper-vectors/ The App is available in both English and Spanish. In 2023 leafhoppers tested as negative for X-disease phytoplasma vectors were added to the gallery. Currently 18 examples of non-vectors are included which allows growers to see some of the diversity of leafhoppers that are easily confused with vectors.

Figure 4. Examples of photos from the vector gallery of *C. geminatus* (top) and *C. m. reductus* (bottom).

A summary of known vector information was also updated at the main X-disease page https://treefruit.wsu.edu/crop-protection/disease-management/western-x/ receiving 1703 pageviews from 1032 individuals over the past year. We have used the app to train growers and consultants at field days in July 2022 and 2023 and September 2022. In 2023 we also distributed 50 business-card sized leafhopper identification cards with a QR code leading to the app for further information.

References

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EXECUTIVE SUMMARY

Project Title: X-disease Vector Identification and Acquisition From Low Titer Trees

Keywords: X-disease, leafhoppers, vectors

A large part of X-disease management has been focused on controlling X-disease vectors. In the 1950s in Wenatchee, WA Homer Wolfe tested the vector capacity of 200 species of leafhoppers before identifying *Colladonus geminatus* as the key vector in Washington, and later *Colladonus montanus reductus* was identified as a key vector in California. However, given the time that has past since that research, it is important to search again for unknown vectors. In our survey of 22 (in 2021) and 30 (in 2022) sites throughout the Washington cherry growing region, the vast majority of leafhoppers were not *Colladonus* spp. leafhoppers. However, we found no evidence of any unknown vectors transmitting X-disease phytoplasma. Previous research has demonstrated that *Euscelidius variegatus* can transmit X-disease in the laboratory, but with a longer latency period than *Colladonus* species. Here we tested more than 300 *E. variegatus* over the course of two seasons and never found evidence that X-disease phytoplasma was replicating in their mouthparts. In the first generation (May/June) some *Colladonus* spp. (*C. m. reductus* and *C. geminatus*) leafhoppers had X-disease phytoplasma in their guts, but none had phytoplasma in their heads. This suggests they can acquire phytoplasma in the first generation, but the long latency prohibits transmission then. The likelihood that a *Colladonus* spp. leafhopper had X-disease phytoplasma replicating in its mouthparts was highest in the second (August) and third generation (October). Evaluation of X-disease acquisition by leafhoppers feeding on highly infected cherry trees for 3 days led to shorter time to transmission than previously reported, suggesting that the latency period is dose dependent. Further analysis of leafhoppers feeding on plants varying in phytoplasma titer and for different time periods will help to better understand the latency period in different scenarios.