Project Title: Dispersive distance of cherry X-disease vector leafhoppers

Report Type: Final Project Report

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Cooperators: Orchard View Orchards

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $22,477
Total Project Request for Year 2 Funding: $23,210
Total Project Request for Year 3 Funding: $22,864

Other related/associated funding sources: None

WTFRC Collaborative Costs:

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Item	2022	2023	2024
Salaries[1]	$7,975.00	$8,215.00	$8,461.00
Benefits	$5,575.00	$5,742.00	$5,914.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies[2]	$4,500.00	$4,500.00	$4,500.00
Travel[3]	$1,000.00	$1,000.00	$1,000.00
Plot Fees			
Miscellaneous			
Total	$19,050.00	$19,457.00	$19,875.00

Footnotes:
[1]Adams lab Faculty Research Assistant at 0.15 FTE, with 3% increase in years 2 and 3; OPE 70%
[2]Research consumables - sticky cards, fluorescent powered,
[3]Travel to field plots

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Item	2022	2023	2024
Salaries[1]	$1,000.00	$1,000.00	$1,000.00
Benefits			
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies[2]	$2,127.00	$2,453.00	$1,679.00
Travel[3]	$300.00	$300.00	$300.00
Plot Fees			
Miscellaneous			
Total	$3,427.00	$3,753.00	$2,979.00

Footnotes:
[1]Galimba lab FRA at 0.01 FTE
[2]Research consumables for ELISA testing
[3]Travel to field plots

Objectives

1) Develop methods for consistently marking vector leafhoppers that does not impede movement and allows for positive identification upon recapture. We explored two marking methods, protein markers (using milk or egg whites) and DayGlo powder.

    Deviations: None. We showed that leafhoppers could be marked.

2) Describe dispersive distance and rate of movement over time of key leafhopper vector species, within cherry orchards.

    Deviations: None. We have measured the dispersive distance of *Euscelidius variegatus* in orchard drive rows.

3) Describe rate of movement relative to prevailing wind direction and outside orchard habitat.

    Deviations: Leafhopper movement is on a small scale. We have no evidence that leafhoppers are migrating from outside orchards or at distances that could be impacted by wind direction.

Significant Findings

Protein Marking.
We broke down Objective 1 into several sub-objectives. Protocols for testing milk and egg proteins in a greenhouse setting were developed to answer the following questions related to Objective 1: Develop methods for marking leafhoppers.

1.1. Do both egg whites and milk work as protein markers for grass and leafhoppers?
1.2. Does trapping with sticky cards work? i.e. can we get a positive signal when insects are collected this way (on glue)?
1.3. Because some insect parts might be left behind, can we hole punch and test insect + card?
1.4. Will samples still test positive after sitting on a sticky card for 1 week?
1.5. Does trapping by other means (sweep netting/vacuum) and allowing the hoppers to comingle with unmarked insects, cause them to cross-contaminate unmarked insects?
1.6. Will marked insects still test positive for protein markers, after 1 week of living on unmarked vegetation, and does method of collection (sticky card or net) differ after this amount of time?

Significant Findings for Objective 1

1.1. Do both egg whites and milk work as protein markers for grass/leafhoppers?
Milk seems to work better than egg whites. The milk ELISA exhibited no false positives, for empty buffer, unsprayed grass, or unmarked leafhoppers but the egg white ELISA exhibited multiple false positives (Table 1). Additionally, while the rates of total positive leafhoppers after 24 hours of exposure to marked grass was the same between both proteins (63%), the milk protein appears to last longer – with greater numbers of positive leafhoppers after 1 and 2 weeks on a sticky card or on clean grass.

1.2. Does trapping on sticky cards work? i.e. can we get a positive protein signal when insects are collected this way?
Yes. There were multiple samples taken from sticky cards that were positive, both from insects that were removed from sticky cards with forceps and from insects left on cut-outs of sticky cards.

1.3. Can we just cut sticky card and wash insect + card?
Yes, though sample sizes were small, results indicate that this method and aspirator collection were similar in the number of positive, marked insects. The cut outs had the assumed added benefit of keeping the hydrophobic insect bodies submerged in buffer during the extraction phase.

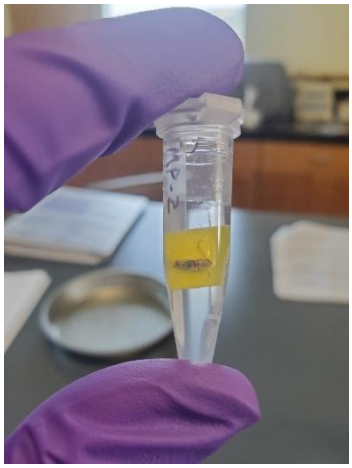

Figure 1. leafhopper + sticky card

1.4. Will samples remain positive after sitting on a sticky card for 1 week?
Yes. It also appears from these data that milk lasts longer than egg white.

1.5. Does trapping by other means (sweep netting/vacuum) and allowing the hoppers to comingle cause them to cross-contaminate unmarked insects?
When 4 marked insects were allowed to comingle with 4 unmarked insects, we never saw cross-contamination. This is likely due to the low concentration of protein that the insects pick up from the marked grass.

1.6. Will marked insects still test positive for protein markers, after 1 week of living on unmarked vegetation? and does method of collection (sticky card or net) affect results?
As in the 24-hour tests, there is no clear superior method of collection – rates do not vary wildly between the two. After 1 week of exposure to unmarked grass after the initial 24 hours on marked grass, positive rates are lower for both proteins, but milk seems to hold up the longest.

Table1: ELISA Protein Marker Results

		Milk		Egg Whites	
Sample	Total #	Positive #	Rate	Positive #	Rate
Negative control: empty extraction buffer	18	0	0%	1	6%
Negative control: unmarked leafhopper	4	0	0%	1	25%
Negative control: unmarked grass	3	0	0%	2	67%
Total negative control	**25**	**0**	**0%**	**4**	**16%**
24 hours - sticky card - removed with forceps	2	2	100%	0	0%
24 hours - sticky card - cut off, card included	2	1	50%	2	100%
24 hours - 4 caught off marked grass and comingled with 4 clean leafhoppers for 2 hours	8	2	50%*	3	75%*
Total after 24 hours	**12**	**5**	**63%**	**5**	**63%**
1 week - sticky card - removed with forceps	2	1	50%	1	50%
1 week - sticky card - cut off, card included	2	1	50%	0	0%
Kept on unmarked grass 1 week - sticky card - removed with forceps	2	1	50%	0	0%
Kept on unmarked grass 1 week - sticky card - cut off, card included	2	0	0%	0	0%
Kept on unmarked grass 1 week - 4 caught of marked grass and comingled with 4 clean leafhoppers for 2 hours	8	1	25%*	0	0%*
Total after 1 week	**16**	**4**	**50%**	**1**	**13%**
Kept on unmarked grass 2 weeks - sticky card - removed with forceps	2	0	0%	0	0%
Kept on unmarked grass 2 weeks - sticky card - cut off, card included	2	1	50%	0	0%
Kept on unmarked grass 2 weeks	3	0	0%	0	0%
Total after 2 weeks	**7**	**1**	**14%**	**0**	**0%**
Positive control: grass marked with milk	3	3	100%	3	100%
Positive control: grass from marked milk cage 1 week after being sprayed	3	3	100%	3	100%
Positive control: grass from marked milk cage 2 weeks after being sprayed	3	3	100%	3	100%
Total positive control	**9**	**9**	**100%**	**9**	**100%**
Grass from unmarked milk cage 1 week after introduction of marked leafhoppers	3	0	0%	0	0%

Table 1. Results from ELISA testing protocol to determine efficiency of milk and egg protein as markers for leafhopper dispersal research. Red numbers indicate false positives. Asterisks indicate that the positive percentage is out of 4, the total number of marked insects before comingling.

Methods for Objective 1

Protein marking

Set up: one replicate consisted of:
4 Cages

1. Grass with milk application.
2. Grass with egg white application.
3. Unmarked grass.
4. Unmarked grass.

Figure 2. Grass sprayed with milk protein marker

Four grass plants in cage 1 were sprayed with 100% whole milk, to saturation. Four grass plants in cage 2 were sprayed with 25% egg white, to saturation. Grass was allowed to dry for one hour. Thirty leafhoppers were added to cages 1 and 2, one hour after milk or egg application, and held for 24 hours. At 24 hours, eight leafhoppers were caught on a sticky trap. Two were removed with forceps and two were removed by cutting out the sticky card around them, and immediately frozen. Four were left on the stick card for one week in the greenhouse, and then removed in the same way. Four leafhoppers were also caught by aspirator and held in a small container for two hours with four unmarked leafhoppers. All eight of these were frozen after two hours. After this 24-hour period, eighteen leafhoppers were transferred to the unmarked (clean) grass cages 3 and 4 and allowed to live for one week. After *one* week on the unmarked grass, the exact same sticky card and aspirator collections were made. After *two* weeks, four leafhoppers were caught on a sticky trap, and two were removed with forceps and two were removed by cutting out the square of card around them. Three leafhoppers were collected from the cage via aspirator. All seven were frozen for processing. Sprayed grass samples were taken at 24 hour, one, and two weeks. Grass samples were collected at one week from the unmarked grass cages 3 and 4.

Controls (for milk protein)

- Extraction buffer negative control was always negative.
- Grass that was sprayed was always positive, up to 2 weeks later.
- Unsprayed grass was always negative.
- 4/4 leafhoppers with no exposure to milk tested negative.

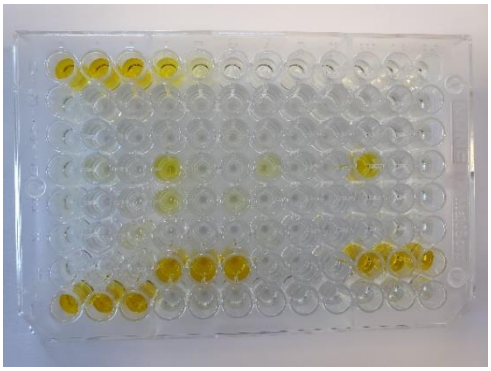

Figure 2. ELISA tray control results

Methods for Objective 1 - continued

Milk Samples

- 63% (5/8) of leafhoppers allowed to behave on sprayed grass, then collected 24 hours later tested positive.
- There was no transference of protein markers to clean leafhoppers in the aspirator.
- After a week on a sticky card, 50% (2/4) leafhoppers caught at 24 hours still tested positive.
- 25% (2/8) of leafhoppers allowed to behave on sprayed grass for 24 hours and then allowed to live on clean grass for one week tested positive, with no transference to clean hoppers.

Egg Whites

Control (for egg protein)

- One extraction buffer negative control was strongly positive. (false positive)
- Grass that was sprayed was always positive, up to 2 weeks later.
- 66% (2/3) unsprayed grass samples were positive. (false positive).
- 50% (2/4) of leafhoppers with no exposure to milk tested positive. (false positive).

Egg Whites

- 63% (5/8) leafhoppers allowed to behave on sprayed grass and then collected 24 hours later tested positive.
- There was no transference of egg protein to clean leafhoppers in the aspirator.
- After a week on a sticky card, 25% (1/4) of leafhoppers caught at 24 hours still tested positive.
- None (0/8) of the leafhoppers allowed to behave on protein marked grass for 24 hours and then allowed to live on clean grass for one week tested positive, no transference to clean hoppers.

Significant Findings Objective 2

The recapture rate of the thousands of dayglow powder marked leafhoppers on caught on yellow sticky cards for these replicates was around 2%. While small, this is in line with previous mark-release-recapture experiments. **The dispersive distance of DayGlow powered marked *Euscelidius variegatus* after one week is only around 10 meters**. This suggests that transmission of cherry-x disease around an infected tree may be quite slow and periodic insect control tactics may be sufficient to slow or stop the spread of further infection.

Extensive sampling efforts have found no leafhoppers outside of managed irrigated orchards. In a separate experiment looking at optimal sticky card height we found that leafhoppers in the Mid-Columbia area are found primarily at ground level, suggesting that leafhoppers are spending most of their time in ground cover within drive rows.

Significant Findings Objective 3

There does not appear to be leafhoppers living in the dryland habitat outside orchards. Long range migration, that could be affected by wind direction, does not appear to be occurring with the key leafhopper vector species we looked at. This is an encouraging finding for insect management and for understanding the rate of spread of cherry x-disease.

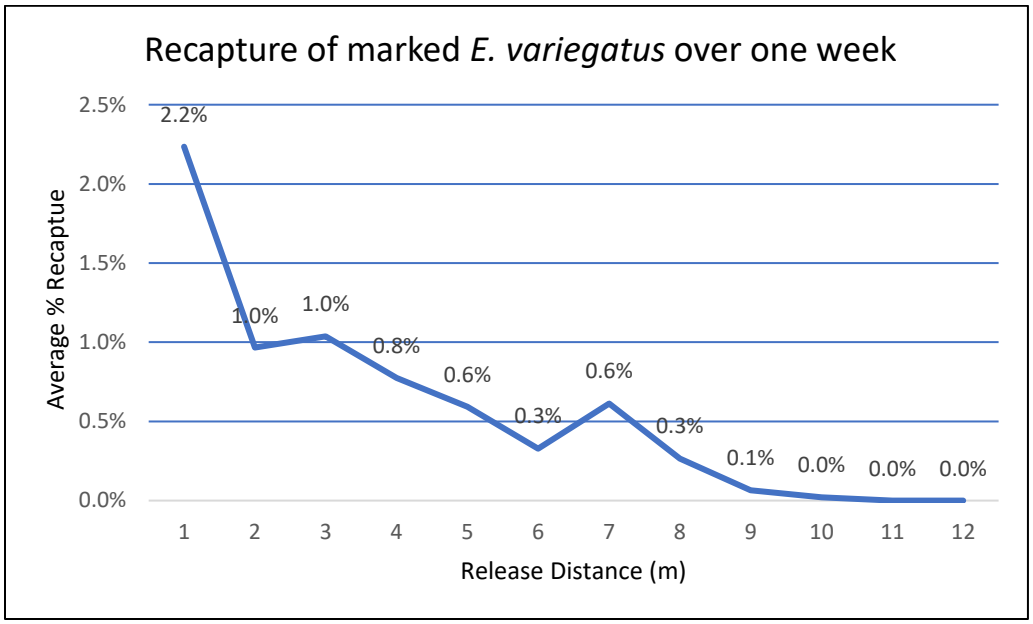

Figure 3. Proportion of released population recaptured over distance.

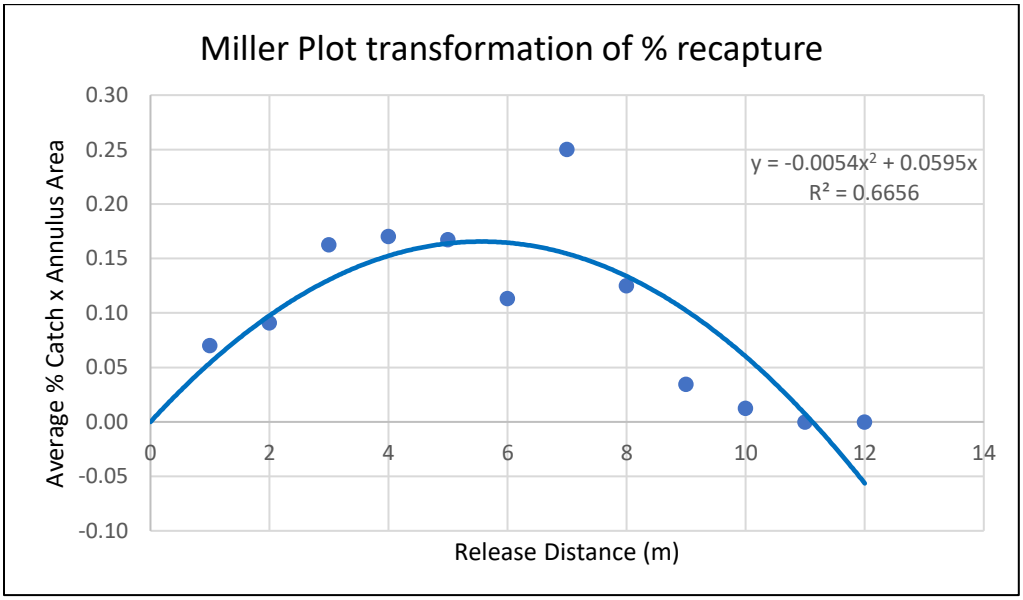

Figure 4. The Miller plot transformation of capture data deals with the low proportion recapture with increasing distance and reveals maximum dispersive distance of the released population. These data indicate that the maximum dispersive distance for *E variegatus* is just over 10 m.

Methods for Objective 2

DayGlow Powder Marking

Concurrently to protein marking we marked leafhoppers with DayGlow powder. This method involved several extra steps. Leafhoppers (and other insects) were captured using sweep nets from inside commercial orchards in The Dalles, Oregon. Insects were transferred to screened insect cages and held within coolers with several ice packs. Insects were transported to the lab and leafhoppers were sorted from all other non-target insects. Leafhoppers were then collected, counted, and placed into cups with freshly cut bouquets of grass as a food source. Cups were labeled with designated release distance and held in the lab at room temperature overnight. The following day, cups of leafhoppers were transported in a cooler back out to the field for release. A single yellow stick card was placed at the center of the releases. Each release distance was marked with a unique color. The experiment was replicated 8 times over two seasons and each experiment used approximately 2,000 leafhoppers.

Figure 5. experimental layout of single trap multiple release experiment. Marked leafhoppers were released from four distances at a time in four directions (north, south, east, west).

Figure 6. populations of leafhoppers marked with unique colors for each relase distance.

Conclusions and Future Directions

Protein marking

Protein markers can be used with limited success for field marking insects. Our 63% positive rate after 24 hours is relatively low, and likely not adequate for use in dispersal research, indicating that this method (spraying proteins on grass and allowing the insects to pick it up through contact) is likely not the most ideal use of these markers. A much more efficient use of proteins like milk might be to spray insects directly, in a mark-release-recapture study. When leafhopper cadavers are sprayed with milk or egg white, they test positive 100% of the time (12/12).

DayGlow powder

Dayglow powder making is a well-established method for marking insects and has been used to effectively mark a number of other insect species. Handling time to capture, transport, and separate leafhoppers from non-target insects is a bottleneck in the system but has been manageable. Initial hypotheses about leafhopper movement included the idea that insects might move over tens or hundreds of meters. We have concluded that the dispersive distance of the key leafhopper vector found in the Mid-Columbia region, E. variegatus, is no more than 10 meters per week. DayGlow powder marking allowed us to uniquely mark and recapture leafhoppers on yellow sticky card and easily identify the distance of release.

Future experiments looking at the movement of other key leafhopper species such as *C. m. reductus* and *C. geminatus* should employe the DayGlow power marking method to measure insect movement.

EXECUTIVE SUMMARY

Project Title: Dispersive distance of cherry X-disease vector leafhoppers

Key words: Leafhoppers, Cherry X-disease, Dispersive distance, *Euscelidius variegatus*

Abstract:

The pathogenic phytoplasma that causes X-disease is vectored by several species of leafhopper including *Colladonus geminatus*, *C. montanus*, *C. reductus*, and *Euscelidius variegatus*. Infected trees produce fruit that is small, discolored, and bitter. Currently there is no cure for X-disease and infected trees must be removed to prevent further spread through the orchard. Estimates of financial impact exceed $100 million here in the PNW alone. The disease is spread when leafhoppers feed on infected trees or weeds and then pass the phytoplasma on through their salivary glands during feeding on the phloem of healthy plants. The rate of spread of the disease through an orchard is related to the movement of the leafhoppers. Currently, little is known about the dispersive capabilities of these key insect vectors. Understanding the spatial and temporal dimensions of insect movement both from surrounding landscapes, such as neighboring orchards or alfalfa fields, as well as the in-orchard movement, is critical to designing effective control programs. Mark-release-recapture experiments have been used successfully to describe dispersive distance and movement patterns over time in several insect species including leafhoppers. Here we proposed to develop methods of consistently marking leafhoppers in a manner that does not impede movement and allows for consistent positive identification of marked individuals upon recapture. Once developed, we used these marking methods to mark, release, and recapture key leafhopper vector species within cherry orchards.

We looked at Milk and egg protein markers as a possible method to mark leafhoppers and grasses, with the intention of spraying drive rows to mark insects in place. We found that these proteins could be detected up to a week later using ELISA tests. The challenge with this technique is that insects must be tested one at a time, they cannot be comingled, and testing can be expensive. We also looked at DayGlow powder for marking insects. Dayglow powder was an effective marking method and does not require expensive additional testing. Marked individuals can be quantified using black light illumination. Mark release recapture experiments were conducted using the DayGlow powder technique.

Surveys of leafhoppers in the Mid-Columbia cherry growing region of The Dalles, Oregon, found that *Euscelidius variegatus,* makes up the majority of the leafhoppers found in managed cherry orchards. Movement experiments were therefore limited to *Euscelidius variegatus*. The maximum dispersive distance of released *E. variegatus*, after one week, was 10 meters. We conclude that the rate of spread of X-disease in the The Dalles cherry growing region by *Euscelidius variegatus* is related this limited movement.