2023 NW Cherry and Stone Fruit Research Review

Little Cherry Disease symptoms observed in a Skeena orchard just before harvest.
Photo Source: Corina Serban, WSU ITT Extension Coordinator

November 8, 2022

Hybrid
Yakima, WA

Project/Proposal Title: Understanding little cherry disease pathogenicity
Report Type: Continuing

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Cooperators: Washington cherry growers and extension agents.

Report Type: Continuing Project Report

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $155,882
Total Project Request for Year 2 Funding: $153,942
Total Project Request for Year 3 Funding: $148,198

Other funding sources: None

Budget 1
Primary PI: Dr. Scott Harper
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Item	2020	2021	2022
Salaries[1]	60,528	62,950	65,468
Benefits	23,034	23,956	24,915
Wages[2]	4,650	4,836	5,030
Benefits	745	775	805
Equipment	0	0	0
Supplies	64,850	59,350	49,905
Travel	1,500	1,500	1,500
Miscellaneous	0	0	0
Plot Fees	575	575	575
Total	155,882	153,942	148,198

Footnotes: 1. Salaries and benefits are for a 1.0 FTE Postdoctoral Associate and a 0.25 FTE Technician to conduct research on this project. 2. Wages and associated benefits are 180 hours for a summer student research assistant to assist with sample collection, and for 80 hours for a field maintenance technician.

Objectives:

1. Establish and inoculate a field plot of representative cherry germplasm to screen for little cherry disease induction and potential sources of disease resistance/tolerance.

2. Identify the physiological effects of little cherry disease of different cherry cultivars from experimental plots and field collected samples to determine a) whether there are different symptom patterns, and b) what effect these have on fruit quality and tree health using a physiological and metabolomics approach.

3. Examine the underlying genetic basis of little cherry disease through examination of transcriptomic changes during disease induction and identify potential effectors or interacting genes/proteins at the host level to develop a method to screen germplasm for tolerance/susceptibility.

Significant Findings for 2022:

- In a field site with heavy leafhopper pressure, 11% of trees became infected with *Ca*. P. pruni in less than 18 months.
- There are multiple genotypes of *Ca*. P. pruni in WA & OR, with potentially different virulence and pathogenicity, so assumptions on disease progress and appearance cannot be based off old literature. This finding has also affected experimental design and analysis.

Methods:

Objective 1. We will establish a 1-acre test block at WSU-IAREC consisting of 30 different cherry varieties. This list includes commercially grown varieties, as well as cherries reported to have some level of tolerance or resistance to LChV-2 or X-disease, and several accessions that represent more unique genetic backgrounds. For each variety, we will plant eight trees, three of which will be inoculated with LChV-2 and three with X-disease phytoplasma. The two remaining trees will serve as non-inoculated controls. To promote early fruiting, we will bud the trees on the precocious rootstock Gisela-6. Budding will take place at IAREC in the greenhouse during late winter/early spring. In mid-late spring, the budded trees will be transplanted to the field. Inoculation for both diseases will be via chip budding of infected material in late summer of 2021. Orchard maintenance, including pruning, fertilization, pesticide application, and weed control, will be conducted according to current horticultural practices.

Note: Due to the poor inoculation success rates and natural invasion of the field block, the approach will be assessed and refocused during the winter of 2022/2023.

Objective 2. Knowing how different cultivars respond to both LChV-2 and X-disease phytoplasma is essential to developing an accurate field guide for growers. Therefore, we proposed to collect symptom development observations and physiological data from grower fields throughout the state.

To do so we will focus on two areas. First, recording of symptoms present on known infected trees as the fruit develop from fruit set to harvest, collecting data on fruit size, weight, color (both skin and pulp), and seed size/maturation, and correlating this data with cultivar type. Second, examining the sugar and secondary metabolite content of infected fruit at harvest, including sucrose, fructose, glucose, and sorbitol content as well as citric acid, malic acid, and total phenolic contractions, and comparing to fruit from healthy, uninfected trees. Additional compounds may be examined based on new information from other objectives of this study.

Objective 3. The underlying genetic basis of X-disease development will be examined in parallel with the physiological studies. Samples will be collected from different symptomatic and asymptomatic cultivars in the controlled field trial described in objective 1 as well as from field samples. From the trees in the new research block, three different tissue types (fruit, pedicel, and leaf tissue) will be sampled at three time points (pit hardening, shuckfall, and harvest), macerated and total RNA extracted. Samples will be samples submitted for library preparation and deep sequencing. The resulting data will be analyzed to generate a transcriptome against which individual samples can be compared for differential gene expression analysis. This analysis will be performed to identify transcripts that are upregulated or downregulated between samples. Differentially expressed transcripts will be assigned a function, if possible, based on homology to sequences with known function. These transcripts will be examined to determine which pathways may be altered in cherry when infected with the X-disease phytoplasma, and associated with disease expression, particularly with reference to fruit development.

Symptom development for little cherry disease may be a result of protein-protein interactions between cherry proteins and pathogen proteins. To investigate this, relevant genes identified in the transcriptomics study described above for both cherry and the pathogens will be selected for a yeast two hybrid screen. Yeast two hybrid analysis will be performed to identify proteins that have the potential to interact. The yeast two hybrid system is a relatively quick means of identifying potential protein-protein interactions, however it occurs in an artificial environment. To rule out any false positives, protein-protein interactions identified in the yeast two hybrid assay will be further investigated using bimolecular fluorescence complementation assays. These assays examine protein-protein interactions in plant cells, creating a more realistic environment that the yeast two hybrid assay.

Results and Discussion:

Objective 1

As reported in 2021, the test plot was established in May 2021 at the WSU Pear Acres field site. Scions were grafted onto Gisela-6 rootstock in the greenhouse during 2020 and early 2021, with failures re-grafted in the field after planting in August 2021 and bark-chip grafted with Little cherry virus-2 genotype Rube-74 or *Ca*. P. pruni genotype 3 in September of 2021. Replacement scion grafts were made in late spring of 2022. Due to the departure of the postdoctoral researcher, Dr. Alice Wright, to take up a position with the USDA-ARS, and the early season focus on the risk of potential infections in nursery stock, diagnostic testing of all trees was delayed until September 2022.

There has been significant mortality in the trial block, with deer damage to the trees over the winter of 2021/2022, and again in late summer 2022, as well as vole damage to the roots in spring-summer 2022, and unfortunately a total of 50 (19%) trees across the block have been lost. Inoculation results were disappointing, with only 1 of 72 surviving *Ca*. P. pruni-inoculated trees becoming infected from the grafts, and 0 of the 68 LChV-2 inoculated trees. We suspect that this poor rate was due to the approach, using bark-chip grafts, and grafting the inoculum too late in the season (late September 2021) for good survival rates. This approach will be revised in spring of 2023.

However, despite the poor graft success rates, natural leafhopper transmission has been occurring in the block, resulting in a total of 26 of the 218 trees (12%) being infected with *Ca*. P. pruni since being planted in May of 2021. Interestingly, these new infections were from genotype Group 2, which as we used a Group 3 isolate for graft-inoculation, suggests that this was local, endemic spread from another source. While experimentally is not a desired outcome, it does provide information about the effects of high insect pressure and management. As we had collected data about *Ca*. P. pruni incidence in the block for the SCBG '*Epidemiology of the X-disease phytoplasma' project*, we are able to build a picture of what occurred.

Table 1. Timeline of vectored spread of *Ca*. P. pruni genotype Group 2 from the environment into the trial block.

Timeline	**Weeds Positive**	**Description**
May 2021	N/A	Block Planted
2nd generation 2021 leafhoppers		
September 2021	6 / 15	Inoculations Performed
3rd generation 2021 leafhoppers		
April 2022	12 / 22	Heavy weed growth, little grass.
1st generation 2022 leafhoppers		
May 2021	N/A	Irrigation commenced, weeds sprayed and mown down.
July 2022	3 / 31	Weed growth controlled.
2nd generation 2022 leafhoppers		
September 2022	TBD	Trees tested, 26 / 218 positive.

Given the timing, 2nd or 3rd generation leafhoppers may have transmitted to the trees, and the detection of 1 stronger positive in a non-graft inoculated tree would suggest that at least one infection occurred. The remainder of the infections were at lower concentration, which we suggest may have occurred from the first generation of leafhoppers feeding on overwintered weeds (which contained genotype Group 2) in spring, and by the second generation emerged, incidence in weeds was much lower.

Given this outcome, we aim to reassess the approach for this experiment, either removing contaminated plants, and establishing insect barriers, or moving the entire experiment into greenhouse conditions, space permitting.

Objective 2

This objective was largely completed in 2021, with the description of symptom patterns and effects on fruit quality in multiple cultivars. The discovery of multiple distinct genotypes of *Ca*. P. pruni present in WA during the SCBG project '*Epidemiology of the X-disease phytoplasma*' this year does explain some of the inter-site variation in the time taken for symptoms to express, as well as the severity of symptom expression between different sites. Furthermore, identification of phytoplasma genotypes in Washington and Oregon (Figure 1: Groups 2 and 3) that are distinct from genotyped or sequenced isolates in eastern Canada, California, Utah (Figure 1: Group 1) supports the observation that the biology we are seeing in the current outbreak is not the same as what was reported in the 1930s-1950s in California or the Eastern U.S., or even California in the 1970s.

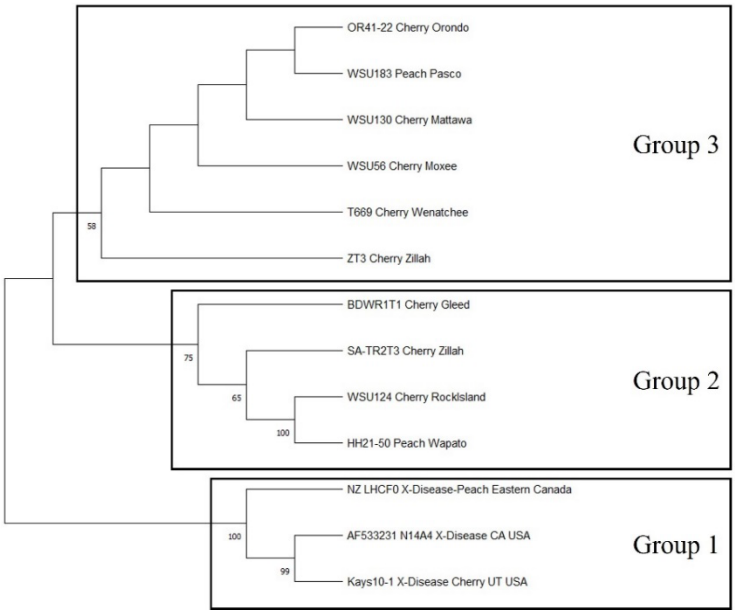


Figure 1. Phylogeny of *Ca* P. pruni genotypic groups identified in the U.S. and Canada.

Objective 3

Due to the departure of the postdoctoral researcher, Dr. Alice Wright, to take up a position with the USDA-ARS, progress on this objective has been slower than anticipated, and efforts have focused on data analysis and sample collection/preparation in anticipation of the arrival of a new postdoctoral researcher in the winter of 2022.

The unusual cold period in spring of 2022 caused differential or delayed bloom on the trees at many of the field sites we intended to collect samples from, with damage to blooms resulting in lower fruit load and delayed development. This cold period also significantly reduced the accumulation of *Ca*. P. pruni in infected plants, making detection and characterization difficult. Therefore, we only collected samples for examination of phytoplasma and cherry gene expression at harvest (June/July 2022) this season. To reduce the effect of different genotypes on differential gene expression, we collected only from plants (Table 2) we were able to characterize as being infected by genotype Group 3, which is being most frequently encountered in new infection and weeds (Shires et al. unpublished data). RNA has been extracted from these samples and was submitted for sequencing in September 2022; we hope to have results for both the host and phytoplasma gene expression to present by the winter meetings.

Table 2. Samples collected during the 2022 field season for i) *Ca*. P. pruni gene expression, and ii) host gene expression in leaf, pedicel, fruit pulp, and stem bark tissues.

Sample ID	Collection Timepoint	Host Cultivar	XDP Genotype
469	Harvest	Bing	3.1
522	Harvest	Cristalina	3.1
531	Harvest	Santina	3.1
1867	Harvest	Coral Champagne	3.1

In 2022 we performed differential expression analysis of the *Ca* P. pruni field samples collected in 2021 (same field, all genotype Group 2). Though comparison of with asymptomatic leaves, fruit, fruit stems, and flowers from bloom to pit hardening we found differential expression (minimum three-fold change

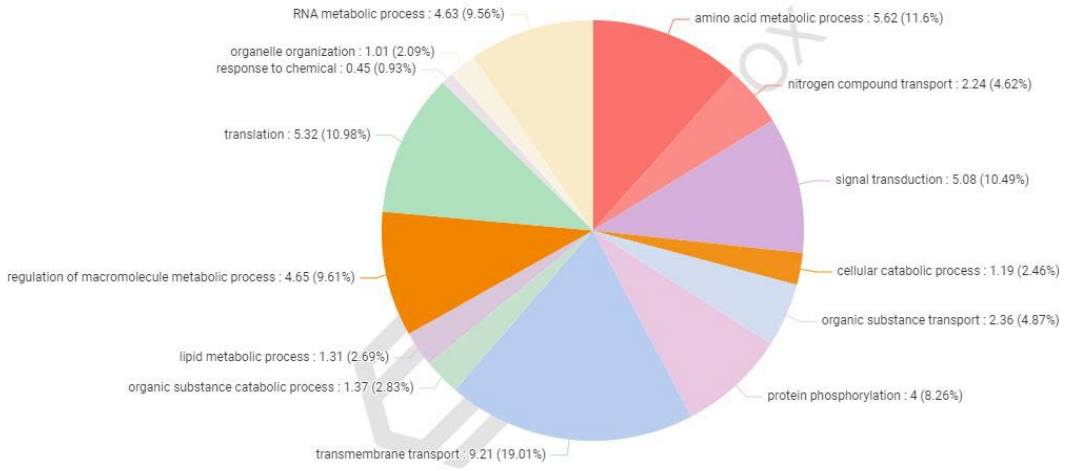
in expression, P < 0.05, FDR < 0.05) of significant numbers of genes in each tissue type and timepoint. Table 3 shows that the phytoplasma infection affects expression right from bloom, and in developing fruit and fruit-associated tissues (flowers, pedicel), whereas it also has broader systemic effects on the plant, causing differential expression of larger numbers of genes in leaves as well.

Table 3. Number of differentially expressed (minimum three-fold change in expression, P < 0.05, FDR < 0.05) *P. avium* genes from bloom to pit hardening in different tissues when infected with *Ca*. P. pruni against asymptomatic 'Bing' cherry during the 2021 field season.

Cultivar	Tissue	Timepoint	No. Upregulated	No. Downregulated
Bing	Leaves	Bloom	154	58
		Shuckfall	118	86
		Pit Hardening	180	245
	Flowers	Bloom	43	28
	Fruit	Shuckfall	126	35
		Pit Hardening	27	26
	Pedicel	Shuckfall	98	145
		Pit Hardening	52	38

For example, in flowers at bloom (Figure 2) we see changes in genes involved in metabolic processes, protein synthesis and transport of macromolecules. But when we examine the resulting fruit at shuckfall (Figure 3a) and pit hardening (Figure 3b), a large number of processes have been disrupted, from gene expression to synthesis of a wide range of compounds that contribute to fruit structural development, sugar content, and flavor compounds. Interestingly, most of the changes occur at shuckfall, supporting our hypothesis that the disease induction occurs early in fruit development.

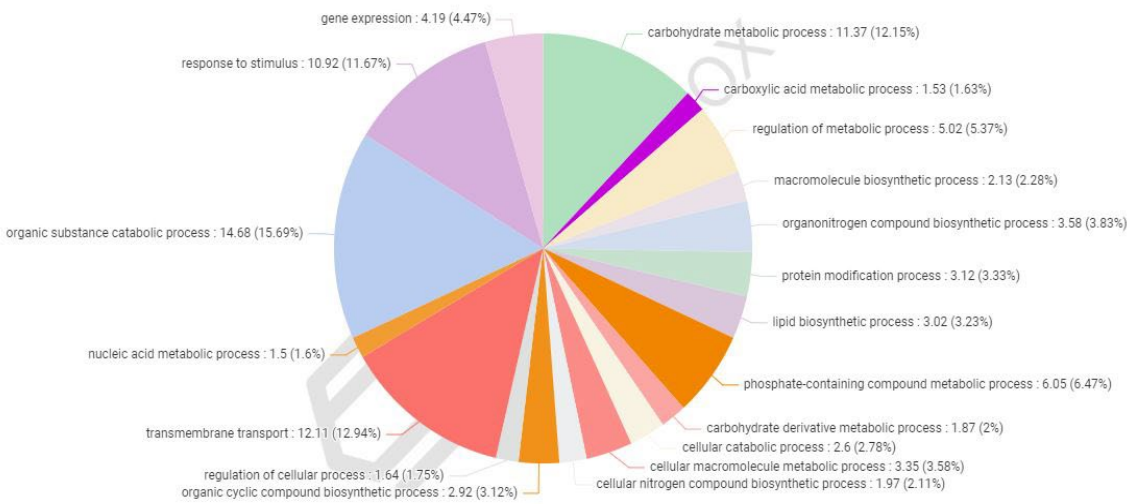
Figure 2. Classes of genes differentially expressed (3-fold, P < 0.05, FDR < 0.05) in *Ca*. P. pruni infected flowers at bloom sorted by biological function



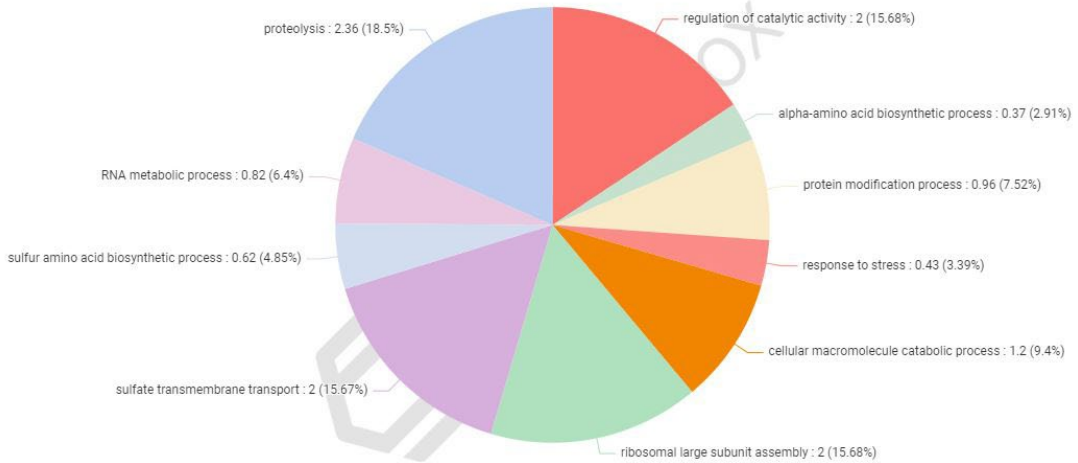
Further study and comparative analysis of the differential expressed genes is needed to map the affected pathways and will be performed by the soon-to-be hired postdoctoral researcher, comparing to data from the 2019 2020, and 2022 datasets.

Figure 3. Classes of genes differentially expressed (3-fold, P < 0.05, FDR < 0.05) in *Ca*. P. pruni infected fruit at bloom a) shuckfall, and b) pit hardening sorted by biological function.

a)



b)



We also examined whether there was differential expression in the phytoplasma genes. To date we have sequenced 9 *Ca*. P. pruni isolates from Washington state, with 14 large contigs each encompassing 588 kb or 87% of the estimated x-disease phytoplasma genome. The first genome draft has been annotated, identifying 469 genes. As it remains fragmented due to long repeat sequences, in the winter of 2022/2023 we aim to use the minIon (Oxford Nanopore) platform to generate longer reads to bridge these gaps and build a complete scaffold.

Nevertheless, we have been able to use the draft genome annotation to look at differential expression of *Ca*. P. pruni genes in different cherry tissues. We examined RNAseq data from the fruit stems and fruit pulp from two infected trees, and although found that 389 genes were differentially expressed, only 11 had changes of greater than 1-fold (log2) (Table 4). Preliminary data suggests that the phytoplasma is behaving differently in different tissues, replicating more in fruit stems, which is supported by qPCR data (Wright et al. 2022), but interestingly in fruit a phase variable surface lipoprotein and HlyC/CorC transporter are upregulated. The former is commonly switched on and off in mycoplasmas and potentially phytoplasmas to change the structure of the envelope in response to antigens or recognition proteins so could be involved in either leafhopper immune system or plant

defense response evasion, while the latter is upregulated in bacteria to tolerate environmental stress or defense responses.

Table 4. Differential expression of *Ca*. P. pruni genes (Log^2, $P < 0.05$) in fruit versus fruit stems of the same plants.

Phytoplasma gene	**Fold change in Fruit vs. Fruit Stems (Log 2)**	**Predicted Function**
Dihydrofolate reductase	2.65	DNA synthesis
Hypothetical protein	2.36	Unknown
Phase variable surface lipoprotein	2.32	Structural
Hypothetical protein	1.68	Unknown
HlyC/CorC-family transporter	1.41	Environmental stress tolerance
Hypothetical protein	1.16	Unknown
Alpha ketoacid dehydrogenase subunit beta	-1.32	Amino acid catabolism
50S ribosomal protein L9	-1.41	Protein synthesis / replication
50S ribosomal protein L21	-1.51	Protein synthesis / replication
Phenylalanine tRNA ligase subunit alpha	-1.58	Protein synthesis / replication
Hypothetical protein	-2.14	Unknown

Additional data on the expression of *Ca*. P. pruni genes in different plant tissues will be generated from harvest timepoint samples collected during 2022 described in table 2. Understanding what genes, proteins and effectors are being expressed by the phytoplasma is an important part of revealing how disease occurs – and how to develop plants that are tolerant or resistant.

Goals for 2023 – No Cost Extension

- Analyze sweet cherry and phytoplasma gene expression data from 2019-2022 samples, and should the weather permit, obtain data from samples collected in 2023. Data will also be compared to sequence collected during the WTFRC funded project '*Understanding phytoplasmas infecting stone fruit trees in Washington State.*' To get a better understanding of the differential response of *Prunus* sp. to this phytoplasma.
- Identify and test for effectors in the phytoplasma-host system.

Publications:

Wright AA, Shires M, Beaver C, Bishop G, DuPont ST, Naranjo R, Harper SJ. 2022. The effect of *Candidatus* Phytoplasma pruni infection on sweet cherry fruit. Phytopathology: 10.1094/PHYTO-03-21-0106-R

Wright AA, Harper SJ (2022) Draft genome of a Washington isolate of *Candidatus* Phytoplasma pruni. *Microbiology Resource Announcements, In review*.

CONTINUING PROJECT REPORT **YEAR:** 2 of 3 **NCE**

PROJECT TITLE: Isolation and in vitro culturing of the X-disease pathogen

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Cooperators: Alexandra Johnson (WSU PhD student, Pullman)

TOTAL PROJECT REQUEST: **Year 1:** $29,000 **Year 2:** $30,000 **Total:** $59,000

Other funding sources

Agency Name: WTFRC/OSCC
Amt. awarded: $539,661 (2019-2021)
Notes: "Supporting a robust PNW sweet cherry breeding and genetics program". PI: Per McCord. Co-PIs: Cameron Peace, Bernardita Sallato, Mateus Pasa.

Agency Name: USDA Germplasm Evaluation Funds, Prunus
Amt. awarded: $28,000 (2020-2021)
Notes: "Germplasm evaluation for sweet cherry genetic diversity and disease resistance".
PI: Cameron Peace. Co-PIs: John Preece, Stijn Vanderzande, Alexandra Johnson.

BUDGET

Organization Name: WSU **Contract Administrator:** Katy Roberts
Telephone: 509-335-2885 **Email address:** katy.roberts@wsu.edu

Item	2021	2022	2023-NCE
Salaries[a]	$15,250	$15,950	$0
Benefits	$2750	$2900	$0
Wages	$5850	$6050	$0
Benefits			
Equipment			
Supplies[b]	$2150	$2100	$0
Travel[c]	$2000	$2000	$0
Plot Fees	$1000	$1000	$0
Miscellaneous			
Total	$29,000	$30,000	$0

Footnotes

[a] Graduate student support for Alexandra Johnson
[b] Single use, disposable materials for sample collection and laboratory assays
[c] Pullman-Prosser return for approx. 4-5 multi-day trips during spring and summer each year

OBJECTIVES

Identify optimum growing conditions for generating and maintaining '*Candidatus* Phytoplasma pruni' colonies

1. Develop a rapid and reliable method for culturing '*Candidatus* Phytoplasma pruni'
2. Optimize the culture medium for year-round live growth of '*Candidatus* Phytoplasma pruni'

SIGNIFICANT FINDINGS

- Bacteria were successfully cultured in a liquid medium and on a solid medium from cherry tissues known to be infected with '*Candidatus* Phytoplasma pruni' (*Ca.* P. pruni)
- Five colonies were isolated from the solid medium that were identified phenotypically as possible *Ca.* P. pruni in 2022; these isolates have yet to be genotyped via PCR testing to putatively determine if they are Ca. P. pruni
- Three of the isolates have yet to be sequenced to confirm identity as *Ca.* P. pruni; one was determined by DNA sequencing in 2021 to not be *Ca.* P. pruni
- All seven colonies that are possibly *Ca*. P. pruni continue to be maintained in pure culture for future use by plant pathologists if they are verified to be *Ca.* P. pruni.

METHODS

Two-year plan: **2021** – Collect infected plant material and begin isolating and identifying colonies produced. Maintain pure isolates of colonies found to respond to current genetic detection methods. **2022** – Collect additional plant material for bacterial isolation and identification. Maintain pure isolates of colonies found to be '*Candidatus* Phytoplasma pruni'.

Experimental material: Field work utilized accessible material from sites throughout central Washington known to be infected with X-disease as determined by WSU plant pathologists. Twenty trees in 2021 and a different set of 20 trees in 2022 were used in this study. All trees were growing in commercial and research orchards, under standard management practices. In 2021 and again in 2022, stems, spurs, and fruit from trees with high to moderate levels of infection were harvested and processed as described below. Additionally, stems, spurs, and fruit from asymptomatic trees were collected and processed. Briefly, infected and asymptomatic plant materials were transported back to the laboratory for surface disinfestation (tissues were soaked in a 10% NaOCl solution for 10 minutes, and then thoroughly rinsed with distilled, sterile water). Once rinsed, plant materials were placed in a sterile laminar flow hood to dry and then dissected. Five 25 mm transverse sections were cut from each stem and spur using a sterile razor blade. Additionally, 25 mm sections of cambium were also harvested from stems. Five symptomatic, as well as five asymptomatic fruit from each infected tree were sliced sagittally into 10 mm sections. Each dissected piece of tissue was placed in a separate, sealable 15 mL vial of liquid phytoplasma growth medium and incubated to room temperature (26 °C) for at least 48 hours.

Bacterial cultures: Stems, spurs, and fruit from infected, symptomatic trees were collected and numerous colonies were produced on phytoplasma-selective medium (Contaldo and Bertaccini 2019) and in a selective environment. Generation of selective medium in 2022 was significantly delayed due to equipment failure, but was resumed before the end of the 2022 growing season. Both liquid and solid phytoplasma growth media contained the broad-spectrum antibiotic ampicillin (25 µg/mL) as well as the antimycotic nystatin (50 µg/mL), which inhibited growth of most bacterial and fungal species. Additionally, cultures were kept in an oxygen-restricted environment, which further limited growth of both obligate anaerobic and aerobic bacterial species. The pH reactive pigment phenol red was added to each vial of phytoplasma liquid growth medium. Tubes inoculated with dissected plant material were

observed to turn yellow after 24 or more hours, indicating acidification of the medium by bacterial metabolic processes, and were considered positive for bacterial growth. These tubes were quantified for degree of color change and 1 mL of broth was removed from each vial and spread aseptically across a new plate of solid phytoplasma growth medium. Plates of solid medium inoculated with bacterial broth were then incubated for at least 48 hours at room temperature (26 °C) in a low oxygen environment. Isolated colonies observed growing after incubation were picked using a sterile loop, introduced to new liquid growth medium, cultured until indication of pH change, and streaked onto a new plate of solid growth medium. Isolated colonies were then sampled for DNA testing.

DNA testing: The existing PCR-based assay described by Kogej et al. (2020) that detects *Ca.* P. pruni-specific DNA sequences was used to putatively determine which colonies were likely *Ca.* P. pruni. Such colonies with the same genotypic signature as *Ca.* P. pruni will be subject to targeted genome sequencing (i.e., specific genomic regions) to confirm identity.

RESULTS & DISCUSSION

Summary: In 2021 and 2022, bacteria were isolated from plant tissues known to be infected with *Ca.* P. pruni, the causative organism in X-disease. For both years, most colonies generated were determined to not be *Ca.* P. pruni. However, in 2021, four colonies were putatively identified as *Ca.* P. pruni by PCR-based DNA assays. Genome sequencing of one of these colonies indicated it was not *Ca.* P. pruni. The remaining three colonies that were also putatively identified as *Ca.* P. pruni were transferred to a fresh liquid medium and then a solid growth medium, to generate additional pure colonies. These colonies have been maintained in pure culture, in growth medium over 2021 and 2022. Phenotypic observation in 2022 identified five additional colonies as possible *Ca*. P. pruni. These newly identified colonies were isolated, are being maintained in pure culture, and will be genotyped via PCR assay in late 2022. For all of the possible *Ca*. P. pruni colonies isolated from the 2021 and 2022 seasons that are then putatively identified as Ca. P. pruni via PCR testing, portions of their genomes will be sequenced in late 2022 to verify if any are *Ca.* P. pruni.

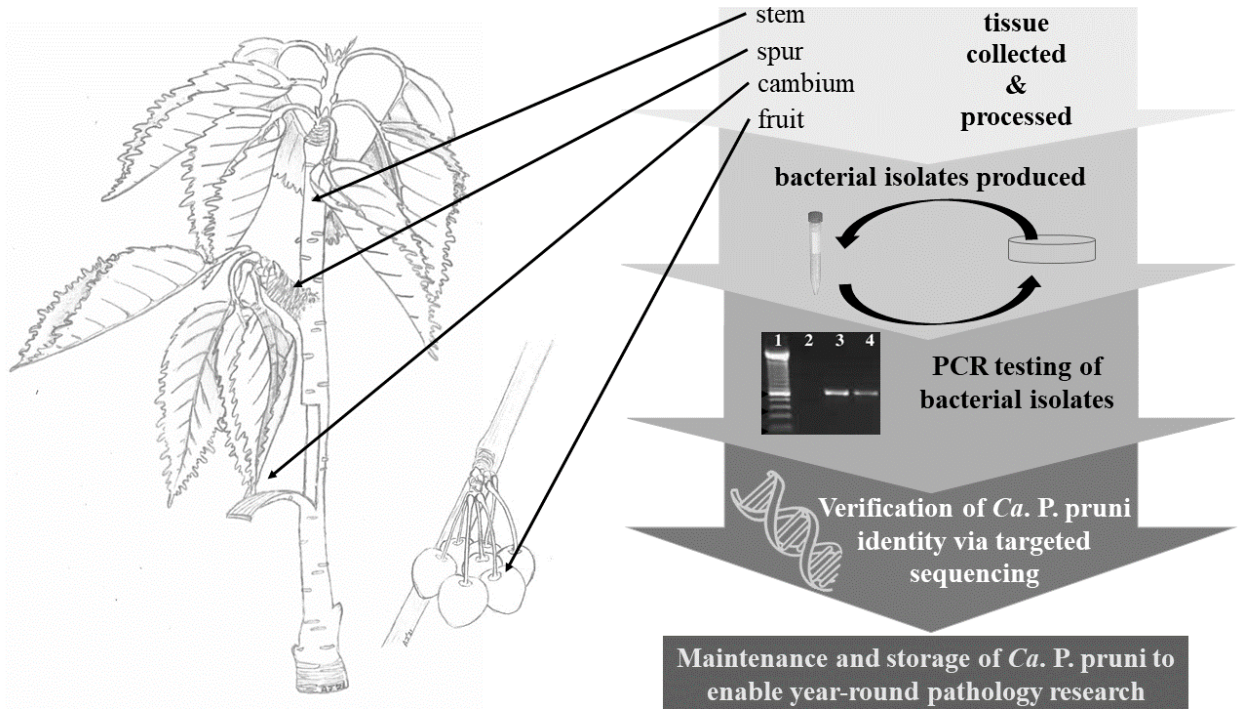

Figure 1: Collection, generation, and identification of bacterial isolates found in trees infected with *Ca.* P. pruni

Bacterial isolation: In 2021 and again in 2022, initial plates generated from broth cultures produced numerous individual colonies. Five to six species of bacteria were identified morphologically in both years. Broths from asymptomatic tissues did not produce any bacterial colonies, as expected. Colonies of different bacterial species cultivated in 2022 will be phenotypically compared with those produced in 2021 to investigate the hypothesis that a specific subset of other bacteria must be present with *Ca.* P. pruni for X-disease symptoms to appear in sweet cherry trees.

Bacterial identity confirmation: From PCR-based assays of 60 colonies in 2021, four were putatively identified as *Ca*. P. pruni. One was sequenced to date and was found to be a contaminant. PCR testing of the five colonies produced in 2022 will be conducted in late 2022 and sequencing of all putatively identified colonies will confirm if any are *Ca.* P. pruni.

Bacterial storage: The eight colonies identified as possible *Ca.* P. pruni have been maintained on solid phytoplasma growth medium in a low-oxygen environment. If any are verified by targeted genome sequencing to be *Ca.* P. pruni, growth of cells from these colonies will be evaluated on several growth media and at several temperatures, including testing ultra-low-temperature (-80 °C) for long-term storage, and the culture medium will be optimized for year-round live growth.

Project/Proposal Title: Studying the infection progression of LCD pathogens in young trees.
Report Type: Continuing

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Total Project Request for Year 1 Funding: $65,656
Total Project Request for Year 2 Funding: $62,017

Other related/associated funding sources: None.
WTFRC Collaborative Costs: None.

Budget 1
Primary PI: Dr. Scott Harper
Organization Name: Washington State University
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Station Manager/Supervisor: Naidu Rayapati
Station manager/supervisor email address: naidu@wsu.edu

Item	2022	2023
Salaries	24,916	25,913
Benefits	9,079	9,443
Wages		
Benefits		
Equipment		
Supplies	31,661	26,661
Travel		
Miscellaneous		
Plot Fees		
Total	65,656	62,017

Footnotes:

Objectives:

1. Determine how rapidly diverse isolates of LChV-1, LChV-2, and/or the X-disease phytoplasma can infect young trees and establish a systemic infection after inoculation.

2. Determine the effect of coinfection with LChV-1, LChV-2, and/or the X-disease phytoplasma on infection progression and plant health.

Significant Findings:

- *Ca*. P. pruni and LChV-2 are found in both seeds and pollen from infected trees BUT they are not transmissible through to the resulting seedlings.
- Infected planting stock is a risk factor that needs to be accounted for in new plantings and experiments.
- In field trees, *Ca*. P. pruni appears to outcompete or indirectly interfere with LChV-2's ability to move and accumulate.

Methods:

Objective 1

a) Identification and establishment of inoculum sources

Tissue from potential inoculum sources were collected from commercial orchards in Washington state in late 2021 and early 2022, total nucleic acids extracted as per established protocols, and the samples were tested for LChV-1, LChV-2, and *Ca*. P. pruni using validated assays (Katsiani et al. 2018; Kogej et al. 2020; Shires et al. 2022). Those samples positive for LChV-2 were further tested by an assay capable of discrimination between the 'LC5' and 'Rube-74' genotypes (Shires et al., unpublished), while *Ca*. P. pruni positives were genotyped using a high-resolution melt marker system developed during the SCBG project '*Epidemiology of the X-disease phytoplasma*' (Shires et al., unpublished).

b) Inoculation of seedlings for pathogen accumulation trials

Budwood from selected inoculum sources was collected in April, and again in July (due to the cold spring weather altering patterns of pathogen accumulation), and two rounds of grafting performed onto 12-month-old *P. avium* cv. 'Mazzard' seedlings. In each case, a single bud from the virus or phytoplasma inoculum source was t-grafted at approximately halfway up the stem of the rootstock, and bud survival assessed at 4- and 12-weeks post-grafting. Monitoring is scheduled to being in late October of 2022.

c) Testing of new planting stock

Combined root and cuttings from the top of the main stem/trunk of tree were collected from between 50-72 individual trees from 3 new cherry and 4 new peach orchards before the trees were plantings in the spring of 2022. Samples were extracted and tested for by qPCR (Kogej et al. 2020) for the presence of *Ca*. P. pruni.

d) Risk of seed transmission of pathogens into planting stock

As *P. avium* cv. 'Mazzard' seedlings obtained from a commercial nursery in the Pacific Northwest were being used as receptor plants to be inoculated in these experiments, we examined whether seed transmission was a potential risk that could disrupt the experiments. Seeds collected from known *Ca*. P. pruni or LChV-2 positives trees showing different degrees of symptom severity and pathogen titer during the 2021 field season were surface sterilized in 10% bleach, dried and processed as follows:

i) Subsets from each seed lot were dissected, separating the seed coat and embryo, and total nucleic acids were extracted from each and tested separately. While LChV-2 samples were tested for virus directly using a published assay (Shires et al. 2022), *Ca*. P. pruni samples were first tested for phytoplasma DNA presence using the standard assay (Kogej et al. 2020), then to determine whether the phytoplasma was alive, the samples were DNAse-treated and tested using an RNA-based assay targeting one of the highest-expression genes of the phytoplasma (Harper et al., unpublished).

ii) The majority of the seeds were cold stratified and germinated in moist vermiculite for a period of six months, survival assessed, and viable seeds planted in soil. Seedlings were grown on a mist-bench for three months, with gradual reduction in watering, then transferred to larger pots and moved to a greenhouse environment. Plants were tested for pathogen presence as above at three months and six months post-germination.

Objective 2

Since this project was proposed we have determined that the diversity of *Ca*. P. pruni genotypes is greater than was previously known and so we will expand the inoculation series in 2023. In lieu, we examined mixed infections of Ca. P. pruni with LChV-2, or the two LChV-2 genotypes together, in orchard trees. Sample collection, nucleic acid extraction, and PCR was performed as described above.

Results and Discussion:

Objective 1

Progress on this project was slower than anticipated due to: 1) The cold spring weather suppressing *Ca*. P. pruni titer early in the season, making identification and collection of inoculum material difficult. 2) The laboratory participated a preliminary study of the potential risk of nursery stock, which generated valuable data for the industry, and produced a need for preliminary experiments for this project. 3) The primary researcher, Dr. M. Shires, left WSU in August for a faculty position at SDSU; efforts are underway to replace this position.

a) Identification and establishment of inoculum sources

During the winter of 2021/2022 we reviewed data on potential inoculum sources to use in this project, selecting isolates for confirmation in the spring of 2022. We found that the unusually cold spring weather significantly hampered *Ca*. P. pruni accumulation making the collection of viable budwood with high pathogen titer difficult. We also found that several potential LChV-2 genotype LC5 and Rube-74 positive trees had subsequently been infected with *Ca*. P. pruni, presumable by leafhoppers late in the previous season. This reduced our inoculum pool significantly, therefore for this first season we proceeded with Rube-74 inoculations only; we aim to continue with LC5 in 2023 from test plants we graft-inoculated this year.

In addition, since this project was proposed, data from our research as part of the SCBG project '*Epidemiology of the X-disease phytoplasma*' has shown that the diversity of *Ca*. P. pruni genotypes present in the Pacific Northwest is greater than was previously known. We have identified three genotypes (named as Group 1, 2, and 3) that appear to have differences in pathogenicity and virulence, and further, our data suggests that what we thought was the dominant genotype (Group 2) is being overtaken in frequency by a different type (Group 3). Therefore, we propose to expand the inoculation series below and study the virulence and infectivity of three genotypes individually rather than focus on what was previously thought to be the dominant genotype.

b) *Inoculation of seedlings for pathogen accumulation trials*

The first attempt at inoculation in April 2022 was unsuccessful, with poor graft survival due to low-quality budwood as a result of the cold spring weather that delayed both plant growth and pathogen accumulation. A second attempt in July 2022 was more successful, with 34 of 67 LChV-2 strain Rube-74 and 42 of 75 *Ca*. P. pruni genotype 3 inoculated *P. avium* cv. 'Mazzard' seedlings showing viable graft survival at two months post-inoculation.

While the original plan was to begin destructive sampling at six weeks post-inoculation, the departure of Dr. Shires prevented this from occurring, therefore it will begin at three months (late October), and data from that timepoint will be presented at the research review in November.

c) *Testing of new planting stock*

In spring of 2022 we tested young trees being planted in four new peach and three new cherry orchards in Washington state. All four of the peach and two of the three cherry plantings had *Ca*. P. pruni positive plants, with infection frequencies ranging between 5-12%. While there were outliers with higher titer in some cases that may have been indicative of the use of infected budwood, most positives were at low concentration, suggestive of leafhopper transmission. These results were concerning and led us to question other routes by which a new cherry plant could become infected. We have also transplanted several of the positive peach trees, thanks to the generosity of the orchard owners, into contained conditions to monitor how rapidly the pathogen accumulates.

d) *Risk of seed transmission of pathogens into planting stock*

An important if unanticipated consideration that needed to be examined before commencing this project in earnest was the cleanliness of the starting material, *P. avium* cv. 'Mazzard' seedlings obtained from a commercial nursery in the Pacific Northwest. It has been assumed, but not confirmed that neither LChV-2 or *Ca*. P. pruni are seed transmissible. Given that another *Prunus* infecting phytoplasma, *Ca*. P. prunorum has been reported to seed transmissible, and given the high incidence in *Ca*. P. pruni in trees that may be used for seed collection in the northwest, we thought it necessary to answer this question. In addition, seed that drop from unpicked fruit, germinate, and grow into volunteer plants could present a major risk for further spread in orchards.

i) *Little cherry virus-2*

We detected LChV-2 in both the seed coat and embryo from seeds that were obtained from known virus infected trees, although frequency was very low, and while we did get 3 very weak seedling positive, these could not be confirmed months later, and we concluded that the virus did not transfer across to the seedlings produced from these pools (Table 1). Pathogen concentration was low in both seed tissues, with less than approximately 100 virus particles per sample (data not shown). More surprisingly, we also detected the virus in pollen samples, sometimes at high concentration (between 500 to 6000 virus particles) in four of seven sample pools tested. While it is unlikely that LChV-2 is pollen transmitted, it does present a potential contamination risk during testing, suggesting the need to surface-sterilize tissue samples before processing.

Table 1. Incidence of LChV-2 in seed samples collected from infected trees, and in resulting seedlings produced from seed pools.

Source Cultivar	Seed Coat Positives	Embryo Positives	Seedling Positives
Coral Champagne	1 / 20	4 / 20	NA
Bing	0 / 20	2 / 20	NA
Skeena	2 / 20	3 / 20	0 / 42
Other Cultivars	NA	NA	0 / 56

ii) *Ca. P. pruni*

In contrast to the LChV-2 seeds, there were marked physiological differences between seeds taken from asymptomatic, mild and severely disease trees infected with Ca. P. pruni. Overall seed weight was increased by 7% and seed viability was severely impacted as disease increased. Few seeds from the heavily infected plants germinated, while those from mild or asymptomatic plants were near normal with good survivorship. *Ca*. P pruni was found in both the seed coat and embryo of seeds from all levels of infected trees, and with thorough testing of RNA, was confirmed to be alive. Interestingly, *Ca*. P. pruni DNA was also found in a handful of seedlings and 3- and 6-months post-germination, but RNA testing suggested that it was carryover and not actually live, viable phytoplasma cells (Table 2). *Ca*. P. pruni was also found in pollen, with a variable concentration of between a low of 10 to 100 cells and a high of ~1000 cells, and confirmation using the RNA-specific assay after DNAse treatment confirmed that the phytoplasma was alive and replicating. This is a new finding as phytoplasma are generally not known to infect pollen.

Table 2. Incidence of *Ca*. P. pruni in seed samples collected from infected trees, and in resulting seedlings produced from seed pools

Source Cultivar	Description	Seed Coat Positives		Embryo Positives		Seedling Positives	
		DNA	RNA	DNA	RNA	DNA	RNA
Benton	Asymptomatic	12 / 15	N/A	8 / 15	N/A	01/ 22	0/1
	Mild	15 / 15	N/A	15 / 15	N/A	4 / 92	0 / 3
	Severe	9 / 15	N/A	15 / 15	N/A	2/16	0/2
Coral Champagne	Severe	N/A	16 / 20	N/A	19 / 20	N/A	N/A
Bing	Severe	N/A	19 / 20	N/A	20 / 20	N/A	N/A
Skeena	Severe	N/A	13 / 20	N/A	12 / 20	NA	N/A
Other Cultivars	Mild-Severe	N/A	N/A	N/A	N/A	3/59	0/3

Objective 2

Since this project was proposed, we have determined that the diversity of *Ca*. P. pruni genotypes is greater than was previously known and so we will expand the challenge-inoculation series in 2023. In lieu, we examined mixed infections of *Ca*. P. pruni with LChV-2, or the two LChV-2 genotypes together, in orchard trees.

a) *Ca. P. pruni and LChV-2*

Mixed infection between *Ca*. P pruni and LChV-2 were found to be common in diseased or symptomatic trees, occurring in approximately 30% of targeted samples collected in 2021/2022. We

were able to discriminate between trees that previous had LChV-2 and were recently infected with *Ca*. P. pruni, and those where *Ca*. P. pruni was more established. To gather preliminary data on their interaction, we sampled trees from commercial orchards that previous studies has shown to have both pathogens present in the orchard. We found that infection followed three patterns (Figure 1). The first had established LChV-2, present for a long time, with new infections of *Ca*. P. pruni, the second where *Ca*. P. pruni had accumulated to the same level as LChV-2 and the third where *Ca*. P. pruni was at high concentration and LChV-2 was low. While it is possible that the latter represents new virus infection, this is unlikely due to the slow rate of spread of this pathogen in-state, and we know from previous work that the virus was at higher titer in the trees in previous seasons. This suggests that the phytoplasma may outcompete or interfere with virus accumulation. This effect has been seen previously with citrus greening (*Candidatus* Liberbacter asiaticus) and Citrus tristeza virus (from the same family as LChV-2) in citrus, where the bacterial infection induced host plant defenses that interfered with the virus's ability to move systemically and accumulate (Harper, unpublished). It may also partly explain why detection of LChV-2 in commercial orchards is less frequent that five years ago. In the next performance period we hope to replicate these effects experimentally.

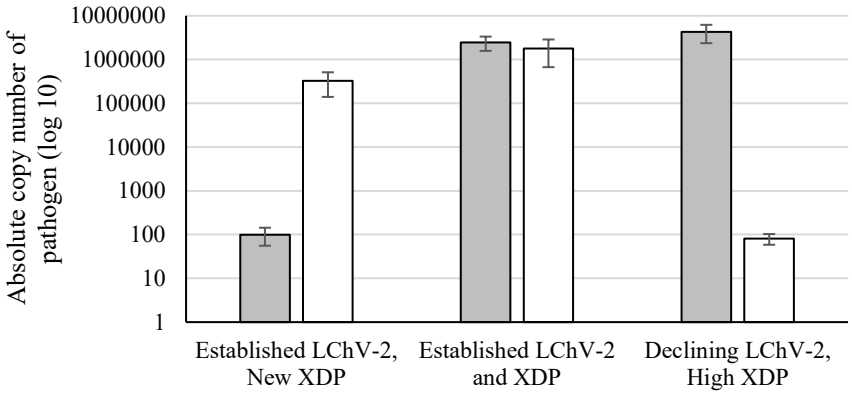

Figure 1. Quantification of the concentration of *Ca*. P. pruni and LChV-2 in mixture together in orchard trees in Washington state.

b) LChV-2 Genotypes LC5 and Rube-74

Comparison of the concentration of the LChV-2 genotypes LC5 and Rube-74 from field trees showed interesting patterns depending on the presumed progress of the infection. Most LC5 single infections were well established, and at high concentration, while most Rube-74 infections were newer and at low(er) titer (Figure 2), which fits with our hypothesis that this genotype is newer and spreading. Mixed infections also reflecting this patten, although curiously in many samples Rube-74 appeared to be outcompeting LC5 as the infection was moving systemically (data not shown), although in longer-term established infections, the titers had largely equilibrated. Rube-74 accumulated at a statistically significant higher concentration than LC5 ($F(1,112)=4.051$, $p=0.0465$) (Figure 2), suggesting that this is more virulent than the LC5 genotype that has long been present in the Northwest.

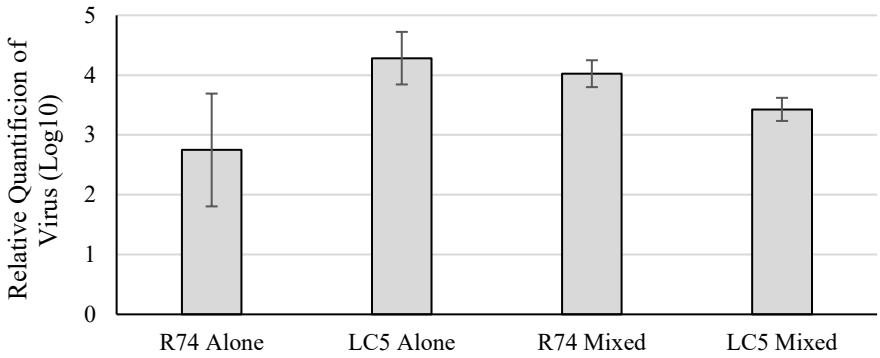

Figure 2. Quantification of the titer of the LChV-2 genotypes LC5 and Rube-74 alone, or in mixture together in orchard trees in Washington state.

Changes to approach and goals for the next performance period

- Expand the inoculation series to examine the three newly identified *Ca*. P. pruni genotypes for differences in virulence and ability to infect.
- Use *Ca*. P. pruni genotype group 3 isoalte for challenge inoculations versus LChV-2.

Publications:

Shires, M.K., Molnar, C., Wright, A.A., Bishop, G., Harper S.J. (202X) Discrimination of Little cherry virus-2 genotypes in *Prunus* species. *Manuscript in preparation.*

Shires, M.K., Molnar, C., Johnson, A., Harper, S.J. (202X) Little cherry disease-causing pathogen accumulation and impacts on reproductive tissues of sweet cherry. *Manuscript in preparation*.

Project Title: Experimental Orchard for X-Disease and Little Cherry Disease Research

Report Type: Final Project Report

Primary PI: William Rodney Cooper
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City/State/Zip: Wapato, WA 98951

Cooperators: Tobin Northfield, Scott Harper, Louis Nottingham

Project Duration: 1-Year

Total Project Request for Year 1 Funding: $ 65,586

Other related/associated funding sources: None

Budget 1
Primary PI: William Rodney Cooper
Organization Name: USDA-ARS
Contract Administrator: Mara Guttman
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Contract administrator email address: mara.guttman@usda.gov

Item	2022	2023	2024
Salaries	$35,265.00		
Benefits	$2,821.00		
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$26,500.00		
Travel			
Plot Fees			
Miscellaneous			
Total	$64,586.00	$0.00	$0.00

Footnotes:

Objectives
1) Develop and maintain an experimental cherry orchard for X-disease/little cherry disease research.

Significant Findings
A cherry orchard consisting of 37 Bing trees and 38 Gabrielle trees was planted at the USDA Experimental Farm near Moxee, WA. The new orchard will be used exclusively for research on Little Cherry Disease/X-disease by researchers at the USDA and Washington State University. The farm is located about 5 miles from the nearest commercial cherry orchard, so presence of infected cherry trees will not be a major threat to commercial production.

Results and Discussion

The current epidemic of X-disease or little cherry disease is causing substantial economic losses to the cherry production in the Pacific Northwest. Symptoms often don't manifest for several years after initial infection. Visible disease symptoms begin with development of small, off-color, bitter-tasting cherry fruits, eventually leading to tree death (Uyemoto et al. 1991). Other stone fruits, including peach, nectarine, and plum, are similarly affected, but development of symptoms and tree death occurs more rapidly in other stone fruits than in cherry (Marcone et al. 2014).

X-disease is caused by a phloem-limited bacterium called "Candidatus Phytoplasma pruni" (16SrIII). This pathogen is transmitted by several different leafhopper species, but Colladonus reducutus (=montanus) and C. geminatus appear to be among the most important vectors in the Pacific Northwest (Wolfe et al. 1950, Kaloostian 1951, Wolfe et al. 1951, Harper et al. 2020, Prengaman 2020). Both leafhopper vectors feed on a wide range of weedy host plants within at least 14 families (Severin and Frazier 1945, Severin and Klostermeyer 1950, Jensen 1953, Nielsen 1957). The X-disease phytoplasma also has a wide host range that includes many herbaceous weeds that the leafhoppers develop on as nymphs. The leafhoppers acquire the pathogen from these weedy herbaceous hosts or from infected cherry trees as nymphs, then transmit the pathogen to uninfected cherry trees. There are no cures for X-disease, so growers rely primarily on regular use of insecticides to reduce vector populations and culling of infected trees or entire orchards (DuPont 2020, Harper et al. 2020).

Previous research on X-disease and associated leafhopper vectors was conducted nearly half a century ago in California, leaving many unknowns related to the current X-disease epidemic in the Pacific Northwest. It is still unclear which leafhoppers, other than those already identified, are vectors of the X-disease phytoplasma in the Pacific Northwest, or from which weedy host species leafhoppers primarily acquire the pathogen. It is also unclear how development of disease symptoms is affected by cherry cultivar or rootstock. Identification of factors related to the spread of X-disease phytoplasma in the Pacific Northwest is considered high priority. A major challenge to the conduct of this research is the lack of a suitable research orchard where infected trees can be maintained for experiments without risking infection of commercial orchards.

Research is currently underway to identify methods to cure trees of X-disease phytoplasma or to prevent trees from becoming infected. This work is being conducted in cooperation with funded research on a similar group of pathogens called "Candidatus Liberibacters". Like phytoplasmas, Liberibacters are phloem-limited bacteria that are transmitted by insect vectors. Liberibacter asiactus is the pathogen that causes citrus greening disease (Huanglongbing) resulting in substantial economic losses for citrus growers in Florida, Texas, and California. Liberibacter solanacearum is a related pathogen that infects potato, tomato, and other crops and weeds within the Solanaceae. PI Cooper collaborates with citrus researchers on the development of novel therapies for Liberibacters (Shatters and Heck 2020). Some of these therapies developed for Liberibacters may be effective against X-disease Phytoplasma.

The development of a designated orchard to conduct research on X-disease and little cherry disease where trees can be infected without threatening commercial orchards was listed as a high research priority for 2022 by the Washington Tree Fruit Research Commission and Oregon Sweet Cherry Commission. ***The objective of our project was to plant an experimental orchard at the USDA experimental farm near Moxee, WA to support new and ongoing research on management of X-disease***. The experimental orchard is located about 5 miles from the nearly commercial cherry orchard (Figure 1). This isolation reduces the likelihood that the experimental plot will threaten commercial orchards with X-disease.

Figure 1. Site of the proposed research orchard (top), and general vicinity of the research farm (bottom). The star marks the orchard location while the circle shows a 5-mile radius.

We established an 80-tree block of cherry trees in spring of 2022. The orchard consists of 37 Bing and 38 Gabriel trees. Bing trees were certified disease-free, but Gabriel is a new variety and was not certified. The orchard is irrigated with sprinkler irrigation. Broadleaf weeds were managed with herbicides and orchard row middles were planted with perennial rye grass and fescue to reduce populations of the vector within the orchard. All trees survived and are available for research in spring of 2023.

A few challenges were encountered that required modification of the original plan. Desired cherry cultivars were not available at the time of purchase, so we purchased trees of the Gabriel variety. Bing trees became available prior to planting, so we established a mix block of 37 Bing on Mazzard rootstock and 38 Gabriel on GIS-12 rootstock. The orchard also includes 5 Attika on Mazzard rootstock as pollinator trees. Our proposal included cages to confine and protect trees from vectors. Our source of cages – BioQuip – went out of business in early spring of 2022, and we were unable to find an alternative source of cages until late summer. We have purchased 21' x 328' roll of Protek Net-Insect Exclusion netting and 1" snap clamps to build A-frame cages with 1" PVC pipe in spring of 2023. Browsing by deer and porcupines were a constant challenge in summer of 2022. Four porcupines were removed from the orchard, and the gate and perimeter fence has been repaired to exclude deer in 2023.

Figure 2. Newly planted experimental cherry plot to be used for research on biology and management of Little Cherry Disease/X-disease.

All trees were tested for presence of X-disease phytoplasma using real time PCR. Two trees – 1 Bing and 1 Gabriel – were infected with X-disease phytoplasma. Titers were relatively high, suggesting that the trees were infected at the nursery level, not infected after planting. Both trees will be caged and maintained to determine how soon symptoms are observed in nursery-infected trees.

The USDA-ARS Temperate Tree Fruit and Vegetable Research Unit budget was increased by $2 million in 2022 to support research on Little Cherry Disease/X-disease. These funds are to be used in part to maintain the experimental orchard at the Moxee Farm. ***No new funds for the orchard are requested from Washington Tree Fruit Research Commission in 2023.*** We plan to use USDA funds to increase our well capacity so that the orchard size can be increased as needed. We also plan to install new wind machines at the orchard site to provide frost protection and to purchase and install a modular workspace to provide USDA and WSU researchers with a clean and air-conditioned place to meet, sort samples, and take lunch breaks. Initial funds from WTFRC to establish the orchard provided evidence for stakeholder support helps us justify to ARS Administration the infrastructure improvements at the USDA experimental farm.

Executive Summary

Project Title: Experimental Orchard for X-Disease and Little Cherry Disease Research

Key words: Little Cherry Disease, X-Disease, Phytoplasma pruni, experimental orchard

Abstract

The pathogens that cause X-disease and little cherry disease are primary threats to stone fruit production in the Pacific Northwest. There are currently no methods to directly control these pathogens, so management of X-disease and little cherry disease relies upon removal of infected orchards and the use of insecticides to suppress populations of the insect vectors. Very little is known of the basic biology of these pathogens and their insect vectors. The development of a designated orchard to conduct research on X-disease and little cherry disease where trees can be infected without threatening commercial orchards was listed as a high research priority for 2022 by the Washington Tree Fruit Research Commission and Oregon Sweet Cherry Commission. We planted an experimental cherry orchard at the USDA experimental farm near Moxee. The orchard consists of 37 Bing trees on Mazard rootstock 38 Gabriel trees on GIS-12 rootstock, and Attika trees on Mazard rootstock for pollinators. The USDA experimental farm is located at least 5 miles from the nearest commercial cherry orchard, so the presence of infected cherry trees would not be a major threat to commercial production. Real time PCR revealed that two trees – 1 Bing and 1 Gabriel – were infected with X-disease phytoplasma at planting. Both trees will be caged and maintained to determine how quickly disease symptoms are observed in trees that are infected at nurseries. This new orchard will be dedicated to basic and applied research on X-disease and little cherry disease USDA and WSU researchers and will support ongoing research to better understand epidemiology of X-disease and to screen experimental products to reduce pathogen infection in trees.

CONTINUING REPORT (YEAR 2) **PROPOSED DURATION**: 3 Years

Project Title: Evaluating Replant Strategies for X-disease Infected Orchards

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Cooperators: Stacey Cooper, Casey Pink, John Byers, Craig Harris, Washington Fruit, Danny Messimore, Keith Vaselka, and Casey Hubbs, Garret Bishop (GS Long).

Total Project Request: **Year 1:** $43,388 **Year 2:** $45,207 **Year 3:** $27,494

Budget 1
Organization Name: Oregon State University ARF **Contract Administrator:** Dan Arp
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Supervisor: Nicole Strong **Email address:** Nicole.strong@oregonstate.edu

Item	2021	2022	2023
Salaries			
Benefits			
Wages[1]	3,864	3,864	3,864
Benefits[2]	2,704	2,704	2,704
Equipment			
Supplies[3]	11,480	6,000	6,000
Travel	660	660	660
Miscellaneous			
Plot Fees			
Total	$18,708	$13,228	$13,228

Footnotes: [1]Wages for a Bioscience Technician II (10 hours a week x 24 weeks x $16.10). [2]OPE calculated at 20%. [3]Testing new plants (160 plants x $25), testing soil, roots, and suckers (160 samples x $25), testing plants in year two (80 x $25), trees (40 x $12), and netting. [4]Travel to and from research plots ($0.54 a mile).

Budget 2

Organization Name: Washington State University
Contract Administrator: Katy Roberts
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Station director: Naidu Rayapati
Email address: naidu.rayapati@wsu.edu

Item	2021	2022	2023
Salaries			
Benefits			
Wages[1]	5,400	5,616	5,841
Benefits[2]	522	543	565
Equipment			
Supplies[3]	17,500	24,560	6,600
Travel[4]	700	700	700
Plot Fees[5]	560	560	560
Miscellaneous			
Total	24,682	31,979	14,266

Footnotes: [1]Wages for a non-student temporary hire (24 weeks x 15 hours/week x $15). [2]Benefits for non-student temporary hire calculated at 9.7%. [3]Testing new plants (320 plants x $25), testing soil, roots, and suckers (320 samples x $25), testing plants in year two (160 x $25), trees (80 x $12), and netting. [4]Travel to and from research plots ($0.54 a mile). [5]Fees for using the Roza block at IAREC.

OBJECTIVES:

1. *Evaluate the survival of roots in the soil under three common replant strategies.*

   A detailed list of replant strategies observed in this study are listed in Table 1.
   In Washington and Oregon, we found no evidence of live roots from the previous planting in 2021. The majority of the roots found in Oregon in 2021 and 2022 were small diameter roots, less than 0.5 inched. In 2022, a total of seven live roots were found across the three Oregon sites. These roots were sent to Dr. Harper for LCD testing. Roots were not sampled in 2022 in Washington since no live roots were found the previous year. Root suckers were monitored and not found in any of the sites, except for site WA 6, however the root suckers belonged to the new planted trees. In 2022 we included an additional experimental trial to assess herbicide application method and temperature effect on tree death on a Skeena/Gi 6 block.

2. *Evaluate the rate of infection of replanted trees under three common replant strategies*.

   Initially, in 2021 we were going to select the sites with infected trees and follow through with removal and replanting strategies to start monitoring replanting trees in 2022. However, we decided to change the strategy and include sites that were replanted in 2020, others that were being replanted in 2021 and the initially proposed ones to be replant in 2022. This modification enabled us to obtain a wider range of conditions and monitor trees for longer period of time after replanting (up to four years). Washington added two additional site (WA 7 and WA 8) in 2022. Samples from leaf of caged trees were taken during August (2021), July and September (2022) in Washington and September in OR, when titer levels were increased according to Harper. Collecting spurs and wood tissue was challenging in newly planted trees in 2021, thus samples mostly containing leaves were collected from the base of the trees (older possible spurs and leaves) to increase detection probability. We altered the protocol in 2022 to collect more woody tissue from near the base of the tree to further increase detection probability.

3. *Provide orchardists with science-based replant strategies for X-disease infected orchards.*

In 2021 WSU hired a new Little Cherry Disease (LCD), Information Technology Transfer (ITT) Extension, Corina F. Serban. Corina has been incorporated as a Co-PI and lead the outreach and extension effort of the project starting in 2022.

SIGNIFICANT FINDINGS:

- In individual tree replanting sites:
    - Root suckers were not observed at Washington or Oregon sites.
    - In Oregon, the individual tree replanting site had a greater number of roots than the entire removal blocks. However, no live roots were found in the individual replant site.
- Entire block removal:
    - In Washington at all sites of entire block removal there were no roots or root suckers, regardless of the tree removal method, fumigation rate, removal method and fallow period.
    - In Oregon, 70 roots were found at between the entire removal sites. The majority of these roots were under 0.5 inches in diameter, and 90% of the roots were found in a state of decay.
- In Washington, the 40% of all trees sampled (controls and netted trees) tested positive for X-disease in 2022. With titer levels varying between 16 and 39 indicative of a variety of infection sources (according to Co-PI Harper). This is an increase compared to 2021 when half of the orchards, one of ten sampled trees were positive to X-disease.
- In Oregon, 10% of all trees samples (controls and netted trees) tested positive for X-disease in 2022. Titer levels varied from 32 to 39. In 2021, only one tree tested positive for X-disease. This tree was at a whole orchard removal site.

METHODS:

1. Evaluate the survival of roots in the soil under three common replant strategies.

The blocks were monitored for root suckers bi-weekly starting May 1st. If suckers were detected, they were removed and tested for LCD pathogens.

In Oregon, a 1ft x 1 ft x 1ft volume of soil was excavated approximately two feet from each caged tree. Soil was sieved through a 10 mm mesh soil sieve to separate the roots from the soil. Roots were brought back to the lab and measured using a caliper. Any root that appeared to be living was tested for LCD by Dr. Harper.

In the Sept 2021 we included an additional experiment where we evaluated three methods of herbicide application including complete cut, half saw cut and high branch application at two temperatures (75 and 85 F) were tested in three replicated Skeena on Gi 12 trees (all in the same planting row).

2. Evaluate the rate of infection of replanted trees under three common replant strategies.

Four orchard blocks near The Dalles, OR and eight orchards blocks in the Yakima valley, WA were monitored for LCD infection after replanting (Table 1).

Table 1. Removal and replanting conditions

Location-Cutivar/Rootstock (previous root)	Herbicide/Tree cut	Removal/Soil	Fumigation	Period to replant
WA 1* Skeena/Gi.12 (G.12)	August - Stump herbicide	Spring - Stump removal. Replace soil in the planting hole	NO fumigation	Next spring
WA 2 Suitenote/Gi.12 (Mazzard)	August - Stump herbicide.	Winter- excavator	Fall Fumigation	One year fallow
WA 4 Chelan/Mazzard 1	August - Stump herbicide.	Fall -excavator	Spring Fumigation 1rate	One year fallow
WA 5 Chelan/Mazzard 2	August - Stump herbicide.	Fall -excavator	Spring Fumigation x2 rate	One year fallow
WA 3 Chelan/Gi.12 (Cherry)	No herbicide	Fall-excavator	Spring Fumigation	Next spring
WA 7 Chelan/Gi.12 (Peach)	No herbicide	Fall-excavator	Spring Fumigation	One year fallow
WA 6 Coral/Mazzard (K5)	No herbicide	Fall -excavator	Spring Fumigation	Next spring
WA 8 Skeena/Gi.12 (G.12)	No herbicide	Summer remove large	NO fumigation	Same fall
The Dalles 1 (Mazzard)	Stump herbicide	Fall- Ripping and large root removal	Cover cropping	Undecided
The Dalles 2* Bing/K.6 (Mazzard)	No herbicide	Fall - large root removal	NO fumigation	Following spring
The Dalles 3 Benton/K.6 (Mazzard)	August- Stump herbicide	Fall-excavator. Ripped.	NO fumigation	Three years fallow
The Dalles 4 Suite Note/K.6 (Mazzard)	Herbicide applied to root suckers for two years	Fall-excavator. Removed large roots. Ripped.	NO fumigation	Two years fallow

* Individual tree removal in a block with high pressure and confirmed X-phytoplasma.

In 2021, initial qPCR of 10 replant trees was done to ensure free virus trees were selected for netting, to discard possible infection associated to the nursery stock or early infection prior to the onset of the trial. Results from this test showed that three out of six replanting sites had 1 of 10 (10%) trees infected with X phytoplasma, and no positives to LChV1 and LChV2. These trees were immediately removed by the grower.

In 2021 the shade net (OVS, 7% shade) was installed around the selected trees (10 total for each site). The nets were removed after leaf drop to prevent damage by snow or wind, and allow management

(training and pruning). In 2022, netting was installed during the spring, between May 27 and June 2nd. In Oregon, nets remained in place over winter. Installation characteristics vary widely between orchards, according to the tree high, system and grower needs. Figure 1. Yellow sticky traps were installed inside the cages to monitor for leaf hoppers, and effectiveness of the netting system. When present, the inside of the cage was cleaned of weeds.

In 2022, samples from 10 netted and 10 neighboring controls at the Washington sites were obtained twice (July 7th and September 25th) and roots were collected once (September) for X phytoplasma, LChV 1 and LChV2 testing via qPCR in Co-PI Harper's laboratory. In Oregon, woody tissue was collected for LCD testing from near the base of the netted and control trees on August 28.

Figure 1. Examples of netting strategies depending on tree high and system.

3. *Provide orchardists with science-based replant strategies for X-disease infected orchards.*

RESULTS AND DISCUSSION

1. *Evaluate the survival of roots in the soil under three common replant strategies.*

In WA sites, in 2022 there were no live roots associated to the previous planting, regardless of the removal method. The lack of roots in whole block removal could've been further promoted by the lack of water or management between removal and planting (one or two year of dry soils), impacting root survival. These results suggest the importance of irrigation, weed control, and nutrient management in the soil where trees have been removed, especially in individual tree removal sites, as these management will likely continue and can benefit root survival and suckering. In addition, for whole tree removal sites, prolonged fallow periods increase the likelihood of root mortality.

In Oregon, we did not observe live roots at the individual tree removal site in 2022. We did excavate nine roots that appear to be alive from The Dalles 3 location, which was fallow for two years following whole block removal. The live roots were small in diameter, but averaged 5 inches in length. We are awaiting the results of LCD testing on the live roots. Oure preliminary results suggest the importance of removing large roots with an excavator if possible. The Dalles 3 is the only site where an excavator was not used with the explicit intent to remove large roots from the soil.

In the additional trial implemented in the fall of 2021, all herbicide methods killed the aerial portion of the trees in approximately 10 days, only one neighboring tree (of the 18 treated) showed symptoms of herbicide damage in the lower branches, suggesting the presence of root grafting. Root death monitoring of the treated trees is underway.

2. Evaluate the rate of infection of replanted trees under three common replant strategies.

According to PI Harper, Ct values indicative of infection correspond to values below Ct 40. Above Ct 35 infection likely occurred the current year, Ct values between 30 and 35, infection likely occurred the previous year, while values below 30, infection likely occurred two or more years ago. In 2022, the number of XDP positive trees was 32 – 40 % in Washington site trees that tested negative in 2021 (Table 2). However, we obtained contradictory results when comparing between the first sampling date in July 2022 and the second sampling date in September 2022 (Table 2). Only one tree, located at The Dalles 4, at any of the Oregon sites, tested very weakly positive for X-disease (Ct= 37.1) in 2021. In 2022, this tree had an X phytoplasma Ct of 38.4.
WA 1 site, the only site with individual tree replanting, samples collected in July reported two control and three netted trees positive to XDP (Ct 30.8 - 36.5) and three (different trees) were positive to LChV2 with Ct values above 36. However, all the trees came back negative to XDP in the second sampling date (September). Similarly, WA 2, a block replanted in 2021, reported 80% and 40% XDP infection in the netted and control trees respectively, and 5% positive to LChV2 in the first sampling, with Ct values above 32, however only one positive in the control (Ct 39.5) in the second sampling.

Table 2. Percent of X phytoplasma detection and range of Ct values when detected in control (C) or netted (N) trees for WA sites.

Site	Netting	XDP July	Ct Value range	XDP Sept	Ct Value range
WA 1	C	20%	34 – 31	0%	-
	N	30%	34 – 36	0%	-
WA 2	C	40%	35 – 39	10%	39.5
	N	80%	32 – 39	0%	-
WA 3	C	0%	-	70%	36 - 38
	N	30%	39 - 40	20%	38
WA 4	C	100%	21 - 39	75%	20 - 38
WA 5	N	75%	16.5 - 38	100%	21 - 37
WA 6	C	0%	-	0%	-
	N	0%	-	0%	-
WA 7	C	20%	38 - 41	30%	34 - 38
	N	50%	38 – 41	60%	33 - 38
Grand Total		**40%**		**32%**	

In WA 4 and 5, both located in the same block, but treated with two different rates/timing of fumigation. According to the results obtained this year, one or two rates of fumigation had not difference on LCD infection. For simplification, we combined both site for reporting. This block was the one with higher level of infection, with only two trees (n=16) negative to XDP, and two trees were positive also to LChV2. The Ct values obtained in this orchard ranged between 16.5 and 39, (11 below Ct 35 and 7 below 30), regardless of being netted or not. These results suggest that several trees were infected prior to planting in 2021. These blocks don't have cover crop and had an intensive program for weed control by burning. PI Harper also reported that in 2022 the orchard had no XDP inoculum pressure (by measuring weeds and trees from a neighboring block), confirming the likelihood of infection prior to planting.

WA 6 is the oldest orchard (planted in 2019) had no XDP positive trees, however two of the controls came back positive to LChV2, with Ct values above 36, suggesting that infection occurred this year

(2022). This orchard is the only site with bearing fruit in 2022, thus netting needed to be removed during harvest.

Sites WA 3 and WA 7 are located near Wapato in a hill slope with trees planted in 2019 and 2020 respectively. In WA 3 first sampling, the control had no XDP infected trees and four LChV2 infected trees, while under the net there were three XDP positive (Ct values above 39.4), and two trees LChV2 positive (Cts above 36.4). Surprisingly, in the second sampling (two month later) seven control trees were positive to XDP (previously none), four of which the LChV2 positive observed in July, and one of the three initial positives under the net confirmed to be positive in the second sampling (Ct 37.8). In WA 7, the control had two XDP and one LChV2 positives in the first sampling (Ct above 37.5). In the second sampling, only one of the XDP was confirmed positive, plus two new other trees came back positive. Under the netting, initial test reported five XDP positive (Cts 37.7) and no LChV2, and three of the five were confirmed positive in the second sampling, plus three others. Given that Ct values range between 33.4 to 40.6, infection must've occurred after planting (in 2021 or 2022). These two sites were netted in the spring of 2022 (May 27), after confirming negative qPCR test in Sept 2021. Thus, a. Infection between 2020 and 2021 (prior to stablishing the nets) and was not detected due to low infection levels, b. The netting system failed to control leafhoppers, c. The infection can be attributed to remaining infected roots in the soil (although site WA 7 was left fallow for one year).

Oregon had fewer trees test positive for X-disease in 2022 than Washington; however, our local incidence increased from 1.6% to 10% in 2022. Ct values in Oregon ranged from 32-39, suggesting that the majority of the infections we identified likely occurred within the last two years.

3. Provide orchardists with science-based replant strategies for X-disease infected orchards.

Project details were shared by Co-PI Serban at the **Wilbur Ellis Grower Meeting, Jan 4, 2022, Yakima WA (65 participants – in person)**, **Cherry Institute Annual Meeting, Jan 7th, 2022, Yakima WA (100 participants – in person) and Northwest Wholesale Grower Meeting, Feb 11, 2022, in Wenatchee WA (80 participants – in person)**. Preliminary results were shared by PI Thompson and Co-PI Sallato at the **Little Cherry Disease Day, February 16, 2022, in Ellensburg WA (178 participants – Hybrid)**, Project details were shared by Co-PI Serban at the Tree Fruit Endowment Advisory Committee Meeting, March 15, 2022, Prosser WA *('Little Cherry Disease Extension & Outreach Program'; **30 participants**),* and at **the Little Cherry & X-disease Field Day, June 21, 2022, in Buena WA (57 participants – in person)**, both organized by Co-PI Corina Serban. Preliminary results were shared in the **Columbia Basin Tree Fruit Club – October meeting** organized by Co-PI Sallato. Two articles in the Good Fruit Grower, July 2022. "Managing little cherry disease in an unconventional way" (https://www.goodfruit.com/managing-little-cherry-disease-in-an-unconventional-way/), and "Getting the X out" (https://www.goodfruit.com/getting-the-x-out/#:~:text=Getting%20the%20X%20out%20Removing%20infected%20trees%20is,in%20Devon%20Wade%E2%80%99s%20orchard%20in%20The%20Dalles%2C%20Oregon). One interview with Co-PI Serban in YaktriNews.Com, June 2022 (https://www.yaktrinews.com/researchers-use-dogs-to-sniff-out-infected-cherry-trees-in-eastern-washington/).

CONSIDERATIONS FOR 2023

Lost confidence on outcomes and conclusions we can provide with the current methodology

The methodology proposed in this project was based on the initial premise that qPCR testing of newly planted trees could confirm XDP or LChV infection, accurately. Thus, we tested all the trees prior to selecting the controls and to-be netted trees. In 2022, we learned (Harper's research) that early infection levels might not be detected with qPCR, providing false negatives. Thus, with the current methodology, we no longer can conclude with certainty that the infection occurred: a. before planting (when Ct values are below 35) or b. before or after installing the netting, and thus the risk of soil/root infection versus leaf hopper infection.

Information we can provide

We will advice tree removal, based on sampling results. The grower will decide on removal strategy. We propose to continue monitoring these sites to provide case study examples of XDP progression, with or without removal (depending on the grower decision), and differences between removal and replanting scenarios. We propose to remove netting, as we no longer can provide certainty of clean trees upon planting.

Project Title: Identifying sources of X disease in cherry orchards
Report Type: Continuing Project Report.

Primary PI: Tobin Northfield
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Cooperators: Garrett Bishop, Scott Harper, Tianna DuPont

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 58,400
Total Project Request for Year 2 Funding: $ 55,849
Total Project Request for Year 3 Funding: $ 53,707

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.

Budget 1
Primary PI: Tobin Northfield
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Item	2020	2021	2022
Salaries	$39,629.00	$41,214.00	$42,863.00
Benefits	$4,478.00	$4,657.00	$4,844.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$7,000.00	$4,000.00	$4,000.00
Travel	$2,000.00	$2,000.00	$2,000.00
Plot Fees			
Miscellaneous			
Total	$53,107.00	$51,871.00	$53,707.00

Footnotes:

Budget 2
Co PI 2: W. Rodney Cooper
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Station Manager/Supervisor: Rodney Cooper
Station manager/supervisor email address: Rodney.Cooper@usda.gov

Item	2020	2021	2022
Salaries			
Benefits			
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$5,293.00	$3,978.00	
Travel			
Plot Fees			
Miscellaneous			
Total	$5,293.00	$3,978.00	$0.00

Footnotes:
[1] new student position
[2] 11.3%
[3] Research consumables (e.g., cages, pots, soil), + molecular tests for disease presence
[4] In state travel

Objective Recap, Goals, and Anticipated Accomplishments:

Objectives

1. Conduct oviposition tests and life cycle analysis on leafhoppers on five host plants (cherry, clover, dandelion, peach, alfalfa).

In 2021 we sequenced key genes of *Colladonus reducutus* and *Colladonus montanus* and have determined that the two "species" are nearly identical for the genes sequenced, supporting a 1957 USDA bulletin (Nielsen 1957) we have found recently suggesting they are the same species (identified as subspecies in the bulletin). Since that time it has been determined that the genitalia Nielsen used to distinguish the subspecies varies with daylength, further suggesting they are members of the same species (A. Purcell, personal communication). In light of this recent finding, we now refer to *C. reductus* as *C. montanus reductus*, and here summarize the research conducted on this species in California that was previously thought to be a different species. In particular, this allows us to use the detailed life cycle description provided by Severin and Klostermeyer (1950) to inform *C. m. reductus* management (Table 1). We find that the life cycles conducted by researchers on *C. m. reductus* and *C. geminatus* in the 1940s in California are quite similar to those conducted in Oregon in the 1950s, providing confidence in the values.

Table 1: Life cycle analysis conducted by Mervin Nielson (Nielson 1968), or Severin and Klostermeyer (Severin & Klostermeyer 1950) for *C. geminatus* and *C. montanus reductus*.

Life stage	***C. geminatus*** (days) (Nielson 1968, peach host, OR)	***C. geminatus*** (days) (Severin & Klostermeyer 1950, Celery host, CA)	***C. m. reductus*** (days) (Severin & Klostermeyer 1950, Celery host, CA)
Egg	20	17.6	14.3
1st instar larva	4.0	7.1	5
2nd instar larva	5.0	4	4.6
3rd instar larva	8.0	4.3	4.3
4th instar larva	6.0	3.5	3.6
5th instar larva	9.0	7.4	5
6th instar larva	N/A (Only 5 instars)	N/A (Only 5 instars)	7.5
Total nymph stages	32.0	26.6	27.6
Total egg, nymph	52	44.6	42
Pre-oviposition stage	8	7-13 (only range given)	13.9
Total generation time	60 days	~55.6 days	55.9 days

Despite detailed life cycle numbers, it has been unclear what plants leafhoppers feed on or how other hosts affect their growth and reproduction. In particular, *C. m. reductus* was not included in the extensive *C. geminatus* study by Mervin Nielsen study in Oregon, but is often far more abundant than *C. geminatus* in Washington orchards (*C. m. reductus* made up 97.5% of the *Colladonus* spp. in our surveys of Wenatchee and Yakima region orchards). Here, we originally set out to build on this research by evaluating the generation time for *C. m. reductus* and *C. geminatus* on 5 plant species: cherry, white clover (*Trifolium repens*), dandelion (*Taraxacum officinale*), peach and alfalfa. Understanding host plant use will help inform management plans. In our surveys of cherry farms in the Wenatchee and Yakima regions in this project and in the project title, "Field evaluation of

leafhopper controls for X-disease management" we rarely observed *C. geminatus*, with *C. m. reductus* being >95% of *Colladonus* spp. individuals collected by sweep nets and sticky traps, and even fewer in 2021. In response to the abundance of *C. m. reductus* and lack of knowledge, we focused our trials on this species. Furthermore, when collecting leafhoppers, we noticed they were commonly found on mallow plants, so we included mallow in our trials. In two attempts to start a colony of *C. m. reductus* with a diverse offering of plants (attempt 1: pea plants, clover, alfalfa; attempt 2: alfalfa, clover, mallow) the leafhoppers died as older nymphs or newly emerged adults, often with deformations (Figure 1). One potential reason is there was something missing in their diet, and that they may need a diverse diet. Follow up attempts with high diet diversity have shown that the leafhoppers are still dying mid-molt in growth rooms (but not outdoor field cages), potentially due to high humidity, and most recently died with fungus emerging from the leafhoppers. We are currently evaluating whether this is due to fungal infection from an entomopathogenic fungus (*Hirsutella*) that we have identified infecting field-collected leafhoppers. We are focusing *C. m. reductus* leafhopper rearing efforts on a combination of peach trees, mallow, alfalfa, dandelion, and clover. Given an apparent need for a diverse diet, we have focused trials on feeding behavior, and used an oviposition test to determine the number of generations per year for *C. m. reductus*, which is unknown (2 reported for *C. geminatus* in the 1950s) and is unclear from sticky trap data.

***Figure 1. Nymph deformation in colony**. Example of leafhopper deformation (left) compared to healthy (right) C. m. montanus in leafhopper colonies. It is not clear what is causing these abnormalities in colonies.*

2. Evaluate incubation time and acquisition probability for leafhoppers feeding on each, cherry and peach trees and transmission likelihood to cherry, clover, dandelion, peach, and alfalfa.
In our evaluation of acquisition and transmission studies we will follow the methods of previous studies (Jensen 1971, Suslow and Purcell 1982), with the addition of molecular techniques to better evaluate acquisition and transmission success. While cherry has long been known to transmit X-disease to other trees, a 1951 study was unable to get *C. geminatus* to acquire X-disease in 17 symptomatic peach trees (Nielson and Jones 1954). These peach trees were likely infected with peach yellow leaf roll phytoplasma (a.k.a. pear decline, transmitted only by pear psylla), rather than X-disease, given the common misidentification at the time. However, we aim here to evaluate the potential for vectors to acquire X-disease phytoplasma from peach. To evaluate acquisition in year 2 of the project we will identify cherry and peach trees exhibiting X-disease symptoms during harvest, and place *C. geminatus* and *C. m. reductus* leafhoppers in sleeve cages on the diseased trees. After 1 week of feeding (the maximum time needed according to previous research) we will cut the branch off the tree, keeping the sleeve cage intact and place the sleeve cage and branch immediately into a cooler with ice for transport back to the WSU TFREC without allowing leafhopper escape. The leafhoppers collected from cherry trees will then be transferred to greenhouse cages containing one of five potential host plants: cherry, peach, alfalfa, dandelion, or white clover, and replicated 8 times (40 total cages). Each cage will include 3 *C. geminatus* and 3 *C. m. reductus* leafhoppers, to focus on the potential of the plant to host the disease and allow for either leafhopper species to transfer the disease. Note: we have been able to obtain successful X-disease transmission in growth rooms using field collected leafhoppers that came in infected, so we have been able to adapt these methods to determine

host capability. We now have potted, infected peach trees, and plan to conduct the acquisition tests to demonstrate that peach is not a dead-end host as soon as we have a clean colony.

3. Use molecular analysis on leafhoppers raised on different host plants to evaluate the reliability of gut content analysis to identify previous hosts of leafhoppers collected in orchards.

Research conducted by co-PI Rodney Cooper and colleagues on purple top disease in potatoes (Horton et al. 2018, Cooper et al. 2019), caused by a phytoplasma vectored by beet leafhoppers has included the development of molecular methods to identify previous plant hosts of leafhoppers collected from crops. While the methods have been focused on beet leafhoppers, rather than the *Colladonus* spp. that vector X-disease, we expect the methods to be directly applicable to identifying non-cherry plants as sources of leafhoppers. Here, we will use leafhoppers arising from experiments described in objective 1 as a cost-effective evaluation of such methods for cherry-X-disease research. These data can then be used as pilot research justifying federal funding identifying alternative leafhopper hosts and their potential importance for disease transmission in cherry orchards. Thus, at the end of the life cycle analysis in year 1 we will send leafhoppers from the field trials to the USDA lab in Wapato for molecular analysis to identify the host plant within the insect's gut. Assuming identification success in year 1, in year 2 we will collect adult leafhoppers from the end of experiments and place them on cherry seedlings, raised separately for each host plant. We will then collect 5 leafhoppers from each seedling at 0, 1, 2, and 3 weeks to identify the timeframe in which the previous host plant can be detected. We have stored leafhoppers from feeding trials and will conduct gut content analysis over the winter months.

Objectives timeline

Objective	Y1	Y2	Y3
1 Life history tests	x	x	
2 Transmission tests		x	x
3 Gut content analysis	x	x	

Significant Findings:

- We have conducted preliminary (only 3 replicates so far) oviposition studies on second generation leafhopper adults, and they readily laid eggs on broadleaf weeds and grasses. However, all adult leafhoppers in the 3 cages containing only grasses died before the end of the experiment.
- Leafhopper eggs developing in grasses and broadleaf weeds did not survive when cut from the plant. Further studies are needed to see if this suggests mowing after oviposition reduces egg survivorship.
- We have developed and published methods for molecular gut content analysis for leafhoppers, including *C. m. reductus* and *C. geminatus* (Cooper et al. 2022).
- Field-collected *C. m. reductus* successfully transmitted X-disease phytoplasma to each, mallow (*Malva neglecta*) and alfalfa (*Medicago sativa*). These have not previously been reported as hosts. The other broadleaf plants (dandelion and white clover) did not test positive, but were less preferred feeding hosts and have been reported as phytoplasma hosts elsewhere (https://www2.ipm.ucanr.edu/agriculture/cherry/X-disease-cherry-buckskin/)
- Potted alfalfa plants infected in fall feeding trials were left outside for the winter again, and again tested positive for X-disease phytoplasma the following spring, suggesting they can host the phytoplasma from year to year.

- *C. m. reductus* and *C. montanus* are the same species (genetically indistinguishable), allowing us to use early *C. montanus* research on life histories and incubation period to inform management of *C. m. reductus*
- Of the plants included in the trials (cherry, peach, mallow, alfalfa, white clover, and dandelion), *C. m. reductus* have a strong affinity for mallow and alfalfa. Given how common these plants are in orchard groundcover, these hosts should be considered in management strategies. *C. m. reductus* may also benefit from a diverse diet, that includes tree feeding.
- Leafhoppers feeding rates on cherry trees ranged from 14% to 51% of the observed feeding, depending the available herbaceous plants, with highest feeding when mallow was not present. Rates were highest when mallow was not present.
- Leafhopper feeding rates on peach trees ranged from 22% to 41% of the observed feeding, depending on the available herbaceous plants. Peach feeding was highest when mallow was not present.
- We conducted molecular gut content analysis on 5 *C. m. reductus* and 5 *C. geminatus* leafhoppers from a commercial orchard in Wapato and found all *C. m. reductus* had fed primarily on dandelion, with little else in their guts. Four of the five *C. geminatus* had fed on dandelion as well, demonstrating the importance of ground cover broadleaf weeds as leafhopper feeding hosts. Dandelion was the dominant weed at the location and time sampled.
- In the second generation (August) leafhoppers in growth rooms deposited eggs on the underside of the leaves of cherry, mallow, and clover.
- *C. m. reductus* leafhoppers collected during the final generation (late September) laid eggs on grassy weeds in potted plants, but not broadleaf weed or trees, suggesting they may overwinter as eggs on tall grasses.

Methods:

Feeding trials

We initiated feeding trials in 24in × 24in × 56in (w × w × h) cages with a combination of white clover, alfalfa, dandelion, mallow, Early Red Haven peach trees, and/or Bing cherry trees, with each plant in a separate pot (Figure 1). Each trial lasted 5 days and each cage contained 10-15 leafhoppers, depending on mortality after collection. In the first trial, we conducted observations every two hours from 8am to 11pm. However, leafhoppers rarely moved in the span of the two-hour intervals and did not appear active in observations made at 9pm and 11pm, which were in the dark and made with red headlamps to avoid disturbing insects.
Therefore, in subsequent trials, observations were made at 8AM, 1PM, and 6PM, doing 3-minute time searches in each cage. Trials were conducted in environmentally controlled growth rooms set at 75F, with a 16:8 L:D daylength. During each observation, we counted how many leafhoppers were on each plant, what plants they were on and if actively feeding or not by visually observing stylets piercing the plant. We present data only on actively feeding leafhoppers summarized across the insects within a cage.

Figure 1 Feeding trial cages in the growth room.

The trials included the following treatments:

- 2 trials of cherry, alfalfa, clover, dandelion; each with 2 cages
  - Initiated June 11 and August 3, 2020
- 2 trials of peach, alfalfa, clover, dandelion; each with 2 cages
  - Initiated June 11 and August 3, 2020
- 1 trial of cherry, clover, mallow, dandelion; each with 2 cages
  - Initiated September 22, 2020
- 1 trial of peach, clover, mallow, dandelion; each with 2 cages
  - Initiated September 22, 2020
- 1 trial of peach, alfalfa, mallow, dandelion; each with 3 cages
  - Initiated August 22, 2020
- 1 trial of cherry, alfalfa, mallow, dandelion; each with 3 cages
  - Initiated October 6, 2020

Transmission tests

After the completion of the feeding trials, the Northwest Clean Plant center tested the plants for X-disease phytoplasma.

Field oviposition test

Based on yellow sticky card data, in the Pacific Northwest *Colladonus* species leafhoppers typically have three periods of abundance: May, late July/early August, and October. However, it is difficult to determine the number of generations per year from yellow sticky card data. This is because the October generation may be the same generation as the August generation, just moving into orchards after loss of alternative host plants. Because leafhoppers overwinter as dormant eggs, we evaluated the potential for eggs laid in field conditions in August to hatch into nymphs. Development of these eggs would then suggest that the August adults represent a distinct generation that gives rise to the adults collected in October. Therefore, during the first week of August 2020 we collected *C. m. reductus* and placed them in cages 24in × 24in × 24in mesh cages with combinations of herbaceous plants next to the Brunner building at the WSU Tree Fruit Research and Education Center. The cages were monitored periodically to identify the emergence of nymphs and/or adults.

Second generation oviposition test

2021 field season. Second generation oviposition trials took place in 2021 within two growth rooms under two different temperatures: 60°F, 30% relative humidity (RH) and 80°F, 30%RH with a 16:8 L:D daylength. Due to a growth room malfunction in the first replication, the first room fluctuated around 75-80°F with about 70% RH in the beginning. High humidity was corrected in subsequent trials by placing a dehumidifier in the rooms and set to 30%RH, but the temperature could not be corrected. This took place from July 22nd to July 29th 2021. For both temperatures, four rearing observation cages (24x24x56"; BioQuip) were set up individually with two cages with Bing cherry, two cages with Early Red Haven peach, and each with Dutch white clover (*Trifolium repens*), alfalfa (*Medicago sativa*), dandelion (*Taraxacum sp.*), and common mallow (*Malva sp.).* Two additional cages of only clover, alfalfa, dandelion, and mallow were set up to test preference without the presence of fruit trees. Two rearing observation cages (24x24x56"; BioQuip) were placed in field conditions outside of the lab with clover, alfalfa, dandelion, and mallow as well to serve as a control. To each cage we introduced 5 male and 5 female field-collected *C. m. reductus*. Sex determination was conducted by anesthetizing them with CO2 using a modified sparkling water maker (SodaStream Inc.), and a microscope for identification. Two days were given before the start, and timed checks happened twice a day at 8-9AM and 5-6PM for 3 minutes. Leafhoppers were counted and recorded

what plants they were on, and if they were actively feeding. We made oviposition observations using the Simplified Leafhopper Egg Detection by Autofluorescence method, also known as the Blue Light Detection Method, to detect eggs within the plants (Hermann and Boll 2003; Yao et al. 2020). Using a blue LED flashlight with a 455-460nm wavelength (LEDwholesalers; Amazon) and wearing blue light blocking computer glasses (UVEX; Amazon), we scanned each plant for eggs. Plants that had eggs were recorded as well as where on the plant they were laid.

2022 field season. We conducted the same experiment from August 9-12th, and from September 1-9 2022. For each experiment, we set up a total of 9 cages to evaluate oviposition in 3 treatments: broadleaf plants only (2 pots of alfalfa, 2 pots of clover), broadleaf plants and grasses (1 pot with alfalfa or clover, 1 pot containing perrennial ryegrass or creeping red fescue), or grass only (2 pots containing perennial rye grass and two creeping red fescue), with 3 replicates per treatment. The key difference between the August and September experiments is that in the August experiment we realized there was too much plant material to search for eggs, making them difficult to find. Therefore, in the September experiment, we switched to seedling trays to reduce the amount of plant material in cages and improve egg identification. Cages were kept in a growth room set to 16:8h Light:Dark, 70°F (21°C), and 30%RH (controlled with a dehumidifier). To each cate, we added 5 females, field-collected *C. m. reductus* leafhoppers and began observations same day. We searched for eggs daily over 7 days, scanning each plant using a blue light and blue light filtering classes to find eggs. On each day, we removed plant material containing eggs when found and place in small deli cup on top of soil labeled by cage number and what plant it was found on. Because the blue light detection method can also confuse thrips feeding with eggs, we confirmed eggs in plant material under microscope. For each egg, we recorded the number of eggs, when they were found, and what plant they were on. Deli cups with eggs were kept in the same growth room that cages were in previously (16:8 L:D, 70°F (21°C), 30%RH) to monitor for nymph emergence. We also monitored each plant in separate cages and checked for nymph emergence after observations were finished.

Overwintering oviposition test

To see if eggs laid by the third generation that overwinter as eggs are laid in a different location, we set up an oviposition test that was similar to the "second generation" oviposition test. These trials took place from September 20th to September 24th, 2021, and then repeated again from September 28th to October 1st, 2021. The two growth rooms were maintained under two different temperatures: 70°F, 30% RH and 80dF, 30%RH with a 16:8 L:D daylength. For both temperatures in both replicates, two rearing observation cages (24 × 24 × 56"; BioQuip) were set up with two cages of Bing cherry, each with Dutch white clover (*Trifolium repens*), alfalfa (*Medicago sativa*), dandelion (*Taraxacum sp.*), and common mallow (*Malva sp.).* Two additional cages of only clover, alfalfa, dandelion, and mallow were set up to test preference without the presence of fruit trees. Two rearing observation cages (24 × 24 × 56"; BioQuip) were placed in field conditions outside of the lab with clover, alfalfa, dandelion, and mallow as well to serve as a control. To each cage we introduced 10 females and at least 5 field-collected *C. m. reductus*. The leafhoppers that were placed in the second replication were put into the same cages as the first. Additionally, half of the cages (one with weedy hosts in the growth rooms and the field, and one with cherry in the growth rooms) were used to test a method of inducing oviposition in leafhoppers (Tipping *et al*. 2005). To do this, we placed the 10 females and around 5 males in a plastic tube with mesh secured on both ends to allow airflow and ran a hairdryer through both ends on cool for 2 minutes, flipping the side half way through. Sex determination for both replicates was done by anesthetizing them with CO2 using a modified sparkling water maker (SodaStream Inc.), and a microscope for identification. Two days were given before the start of the first replicate, and checks happened once a day over a 5-day period for however long was needed for a thorough search of the plants (around 5-10 minutes). For the second replicate we allowed 24 hours for leafhopper acclimation before observations were initiated, which included one check for the same amount of

time, and halfway through, barley was added for additional observations. Egg detection was conducted using the Blue Light Detection (Simpliefied Leafhopper Egg Detection by Autofluorescence) method by using a blue LED flashlight (LEDwholesalers; Amazon) and wearing blue light blocking computer glasses (UVEX; Amazon) (Herrmann and Boll 2003; Yao et al. 2020). Plants were scanned for eggs using this method and plants with eggs were recorded.

We plan to conduct another overwintering oviposition experiment in October 2022, but the experiment will not be completed in time for this report.

Results & Discussion:

Feeding trials. We observed active feeding on all plants offered during the feeding trials (Figure 3). In the feeding trials that included cherry trees, the order of *C. m. reductus* preference appeared to be: mallow, alfalfa, cherry, white clover, and dandelion. Indeed, when offered mallow, alfalfa and a cherry tree we did not observe feeding on dandelion. In the feeding trials that included peach trees, the order of preference appeared to be: mallow, alfalfa, peach, white clover, and dandelion. However, interestingly, when offered mallow, alfalfa and peach together they fed more on peach than alfalfa. The fact that leafhoppers always fed on cherry or peach trees, regardless of what herbaceous plants were there begs the question of whether there is something important about feeding on trees that provide important nutrients to leafhoppers. However, future research is needed to determine whether this is the case.

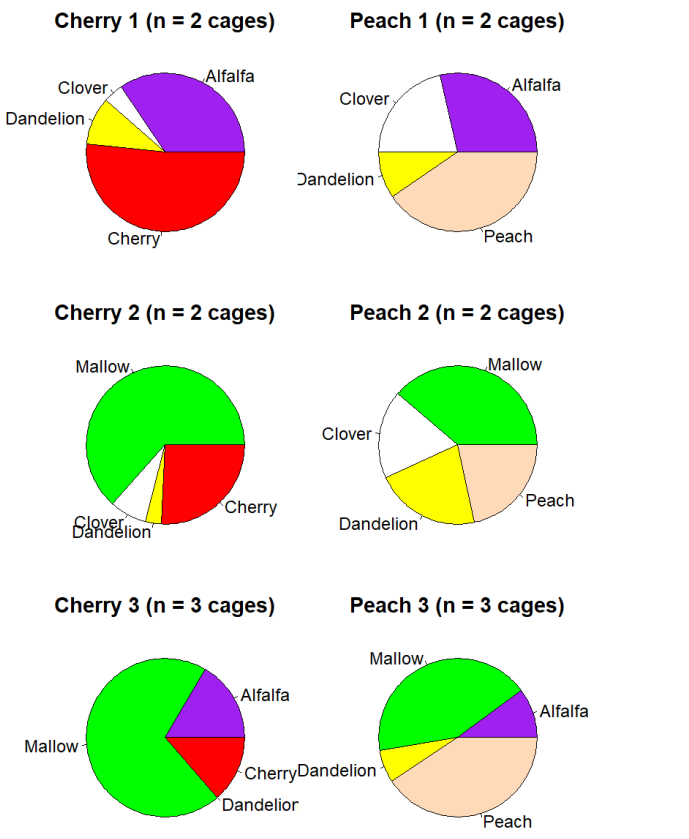

Figure 3 *Proportion of feeding observations made on each of the different plant species in cages: cherry (red), white clover (white), dandelion (yellow), alfalfa (purple), peach (peach), or mallow (green) during feeding observations.*

Field oviposition tests: Adult *C. m. reductus* leafhoppers collected in the first week of August and introduced to outside cages with mallow and clover readily laid eggs that hatched into nymphs and began reaching the adult stage in October, suggesting that the August generation is a separate generation from the first generation that emerges in May from overwintering eggs and from the October generation that lays eggs that remain dormant for the winter. Given that these two later generations typically occur after cherry harvest, leafhopper control after harvest is likely critically important.

Transmission test

Of the plants from the feeding trials, alfalfa and mallow tested positive for X-disease phytoplasma following the experiment. One of the two alfalfa plants tested positive with a Cq score of 36.82, and two of the three mallow plants tested positive with Cq scores of 38.71 and 38.29. In addition, one alfalfa, one mallow, and one dandelion plant was kept outside all winter and tested again the following April to see if the phytoplasma could survive the winter in the roots. Of those, the alfalfa tested positive with a 39.31 Cq score. Therefore, we found that alfalfa and mallow can host X-disease, and that it can survive the winter in broadleaf roots.

Second generation oviposition test

2021 experiment. During this experiment, most of the leafhoppers died within the acclimation period so there were fewer feeding results. From the data collected, there were no records of feeding on dandelion, cherry, or peach, but they did feed on (in order of preference) clover, mallow, and alfalfa. In the cages without a fruit tree, there were more observations on clover than there were on mallow, alfalfa, and dandelion. Overall, the feeding proportion for clover was 53%, for mallow was 37%, for alfalfa was 11%, with no feeding observations on either dandelion and cherry/peach. During this experiment we were able to find some eggs deposited in the first growth room (75-80°F conditions) despite the lack of feeding data. In the cages that had fruit trees, we found eggs deposited on the underside of the leaves of mallow, and clover, and did not observe any eggs on alfalfa and dandelion. Although no eggs were found in the cages without fruit trees, we were able to observe young instar nymphs on clover, as well as other nymphs on cherry, mallow, and clover within the fruit tree cages. Due to lack of leafhoppers in the field by the end of this experiment (being at the end of the second generation), we were not able to replicate this experiment before the final generation began.

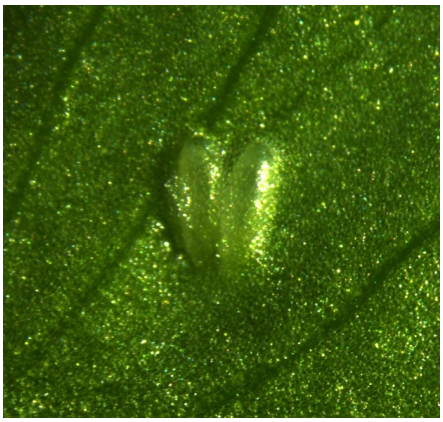

Figure 4. Leafhopper eggs in alfalfa (approximately 1mm long)

2022 experiments. In the August experiment, no eggs were found using the blue light detection method. At the end of the observation period, all plants were taken out of the cages and searched for eggs. The first set of plants were sorted through for about 1 hour, and found 4 total eggs (cage B1: 1 clover, 2 alfalfa; cage BG2: 1 clover). Due to the large amount of plant material to sort through, egg searching was reduced to 15 minutes per plant, and no other eggs were found. Plants were then separated into individual cages and checked for nymph emergence, but no nymphs were found. This could be because the nymphs were too small to observe, the plants died before finding nymphs, or no/very few eggs were actually laid.

In the September experiment, plants were planted in seedling trays together for each cage to reduce amount of plant material needed to go through. Eggs were first found on 9/6, mostly with plain sight. On the last day, all trays were removed, and plants were sorted through to find eggs with no time restrictions. Overall, 48 eggs were found on barley, 28 on perennial ryegrass, 20 on creeping red fescue, 5 on clover, and 2 on alfalfa. A majority of the eggs were found in the cages that had only grass in them, and the eggs found in the broadleaves were found in the mixed broadleaf/grass cage. At the end of the trial, it was noticed that the leafhoppers in the grass cages had all died, and the leafhoppers in the cages with broadleaves had mostly survived. This suggests that *C. m. reductus* relies on broad-leafed weeds as feeding hosts and only uses grass as reproductive hosts. Other leafhopper species have been shown to lay all eggs in their ovaries when there is significant mortality risk (Tipping et al. 2005), and this may have been the reason for high numbers of eggs produced in grass only-cages. Thus, it could be that the leafhoppers in the grass cages laid most of the eggs due to dumping their eggs to have the best chance of their offspring surviving. From all of the eggs removed by cutting leaves from plants, none of them emerged, and either developed partially (exhibiting eye spots in developing nymphs) before dying or had eye spots beginning to form.

Final generation oviposition test

No eggs were laid in any of the broadleaf plants in any of the cages. However, one of the pots with the dandelion plants had grass growing from the pots and we found 100 leafhopper eggs deposited in the grass. While it was a single cage that had eggs deposited, it suggests that leafhoppers may lay

eggs in grass in the final generation to overwinter. This would make logical sense, given that they may be more likely to persist throughout the winter than tree leaves, or even leaves on perennial broadleaf weeds. With this observation on grass, barley was added halfway through the experiment, finding that they would feed on the barley, but no eggs were laid on it in any of the cages.

Gut content analysis:
We conducted gut content analysis on 5 *C. m. reductus* and 5 *C. geminatus* collected on May 22, 2020 from our control blocks in the WTFRC project "Field evaluation of leafhopper controls for X-disease management." The block had many dandelions in the ground cover, and dandelion comprised the vast majority of plant DNA in all five *C. m. reductus*. Dandelion species included both common, and red-seeded dandelion. We did not detect cherry in the guts, but did identify small amounts of clover, alfalfa, and chickweed. Dandelion also dominated the plant species within guts of *C. geminatus* but they also had a more diverse group of plants in their guts, including mallow, chickweed, an *Oxybasis* species, and cherry. These findings support the feeding trial data that ground cover is an important part of the diet of these key X-disease vectors. These results will be built on by a WSDA/USDA Specialty Crop Block Grant to Scott Harper (PI), Tobin Northfield (co-PI), Rodney Cooper (co-PI), and Tianna DuPont (co-PI) that includes gut content analysis for known vectors. Furthermore, these results are documented in a recently published scientific publication describing methods for leafhopper gut content analysis (Cooper et al. 2022).

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Project Title: X-disease Vector Identification and Acquisition From Low Titer Trees
Report Type: Continuing 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.

Primary PI: Tobin Northfield
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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	$0.00	$0.00	
Benefits	$0.00	$0.00	
RCA Room Rental	$0.00	$0.00	
Shipping	$0.00	$0.00	
Supplies	$13,362.00	$11,862.00	
Travel	$3,438.00	$3,438.00	
Plot Fees	$0.00	$0.00	
Miscellaneous	$0.00	$0.00	
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, so we hope to change 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. For an example of a related gallery see http://treefruit.wsu.edu/crop-protection/disease-management/western-x/symptoms-gallery/

Significant findings

- 82% of the leafhoppers collected from our 22 sites in August 2021 were not *Colladonus* spp. leafhoppers, and molecular analyses conducted in the fall/winter of 2021 did not identify X-disease phytoplasma above detection thresholds. In contrast, we did identify X-disease phytoplasma from *C. m. reductus* and *C. geminatus* collected from the same sites.
- *Colladonus* spp. (*C. m. reductus* and *C. geminatus*) vectors fell into four categories: leafhoppers with no X-disease phytoplasma (by far the most common occurrence), low titers in their guts, low titers in their heads, or high titers in both, heads and guts. The low titer scenarios are thought to represent tiny bits of phytoplasma found in either the mouthparts or gut.
- *Colladonus* spp. leafhoppers did not have phytoplasma in their heads in the first generation (May and June) of 2021. Heads for 2022 have not yet been tested, but no bodies came back positive, suggesting they are not infective.
- *C. geminatus* were rare in 2021, and out of 332 *C. m. reductus*, 1% of their heads tested positive for X-disease phytoplasma. Evaluation of 2022 data are ongoing.
- In 2021 all of the 28 sets of 10 *E. variegatus* bodies (280 total) tested negative for X-disease phytoplasma. In 2022, 2 out of 55 *E. variegatus* bodies tested positive for X-disease phytoplasma, although it is not clear if the phytoplasma had reached the salivary glands.

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 this time period (August 4- 17, 2021) 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]) 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) 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.

Given that the methods described above were unsuccessful, we are planning to attempt acquisition studies with nymphal leafhoppers. This is to account for the fact that these are the most likely to transmit as adults, given the long latency period between acquisition and transmission that may not allow adults to transmit after acquiring phytoplasma. Thus, doing acquisition trials with nymphs will be significantly more challenging than with adults, but we feel this is an important step to understanding acquisition in the field that ultimately results in transmission. This will require a strong colony and diseased plants. After extensive shipping delays, we now have humidity-controlled growth chambers needed for colony maintenance and have developed a colony. We have also identified 18 young *Prunus persica* trees that tested positive immediately after being planted in a Washington orchard and plan to conduct such experiments on these trees, as well as from broadleaf weeds during the 2022-2023 winter and following spring.

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 were never able to detect X-disease phytoplasma from. 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 groups 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) in the heads with no phytoplasma present in the rest of the body, likely representing a

tiny among 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 (lower *Ct* scores represent higher phytoplasma titer), 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, 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 two of the 38 collected tested positive with *Ct* scores less than 35. Again, these leafhoppers had phytoplasma in their bodies as well, whereas the two other leafhoppers with *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.

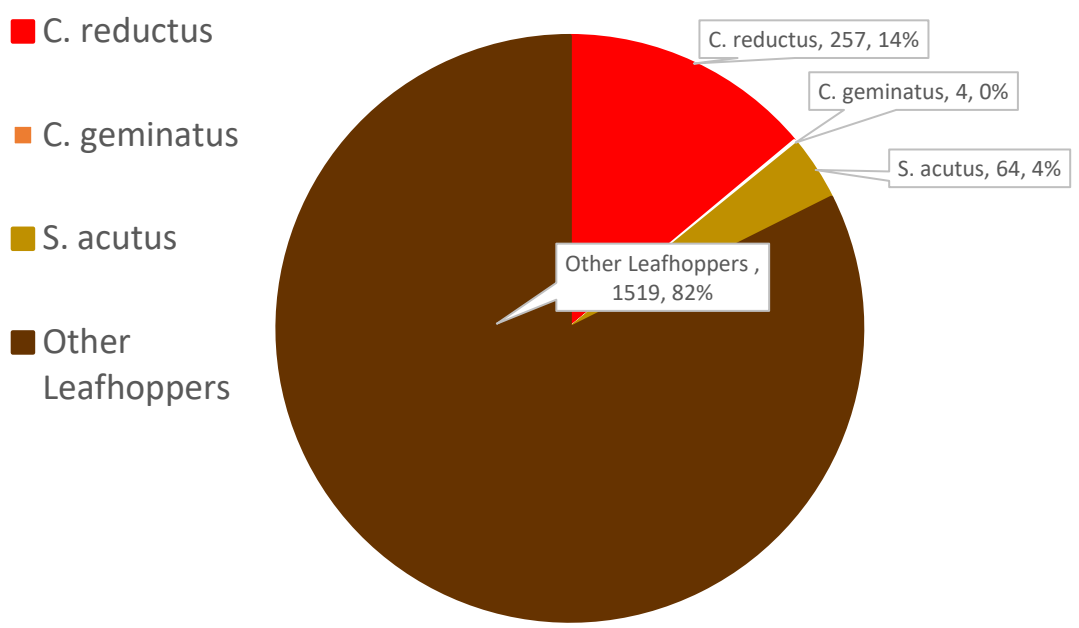

Figure 1. Proportions of leafhopper species collected from the 22 sites in 2021. In addition, we collected 406 nymphs, for which the species is unknown.

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, although we have yet to complete our third generation sampling, which occurs in October. Thus far, we have only tested the bodies of the first generation of *C. m. reductus* (85 total) and *C. geminatus* (50 total), and none have tested positive for X-disease phytoplasma, suggesting they have not acquired phytoplasma. This may be influenced by the cold spring in 2022. However, in the first generation, 2 of the 55 *E. variegatus* bodies tested positive for X-disease phytoplasma with *Ct* scores less than 35. Given the long latency period of *E. variegatus* (approximately 50% longer than the *Colladonus* species), it is unclear if the phytoplasma has reached the mouthparts, allowing the phytoplasma to be transmitted. However, this does suggest that the phytoplasma is reproducing in *E. variegatus* and further studies are required to determine the role of this vector in X-disease

epidemiology. We have continued to evaluate 2 other brown species and 3 green species of leafhopper, but none of these have tested positive for X-disease phytoplasma.

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)

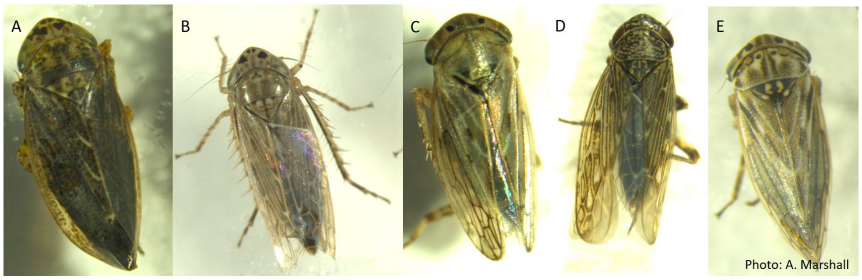

Figure 3. *E. variegatus* (A), and four other types of leafhoppers commonly collected in surveys. Leafhoppers are shown in order of abundance, with A being the highest abundance, and E being the least abundant. Note especially variation in the head shape and patterns, as well as the pronotum (the pirate 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 have proven unsuccessful (all leafhoppers tested negative), and we are retooling to change our approach to evaluating acquisition in nymphs.

3) Photos and website development. We have developed a webpage gallery with high resolution images of known vectors taken from different angles to aid in identification (http://treefruit.wsu.edu/vector-gallery/). In addition to it being available as a webpage, it is also included in the new Little Cherry App. In addition to showing documented X-disease vectors, we now also present high quality images of example leafhoppers that are not vectors, as documented by this project. '**To download the App search for 'Little Cherry Scouting Guide' in your App store. Or for an apple version click here or for Android version click here.'** For more information here http://treefruit.wsu.edu/article/washington-and-oregon-state-extension-announce-a-new-app-for-little-cherry-disease-and-insect-scouting/. We have used the app to train growers and consultants at field days in July and September 2022.

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

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Jensen, D. D. 1969. Comparative transmission of western X-disease virus by *Colladonus montanus*, *C. geminatus*, and a new leafhopper vector, *Euscelidius variegatus*. Journal of Economic Entomology **62**:1147-1150.

Purcell, A. H., and J. S. Elkinton. 1980. A comparison of sampling methods for leafhopper (Homoptera, Cicadellidae) vectors of X-disease in California cherry orchards. Journal of Economic Entomology **73**:854-860.

Zahniser, J. N., and C. H. Dietrich. 2013. A review of the tribes of Deltocephalinae (Hemiptera: Auchenorrhyncha: Cicadellidae). European Journal of Taxonomy **45**:1-211.

Project Title: Insecticidal control of leafhoppers in cherries

Report Type: Continuing Project Report, No-Cost Extension
FINAL REPORT FORTHCOMING BY DECEMBER 2022 (research is still underway)

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Cooperators: Scott Harper, WSU

Project Duration: 2-Year

Total Project Request for Year 1 Funding: $81,166
Total Project Request for Year 2 Funding: $84,185
Total Request: $165,351

Other related/associated funding sources: None

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Item	2020	2021
Salaries[1,2]	$52,827	$54,940
Benefits	$18,373	$19,108
Wages[3]	$3,900	$4,056
Benefits	$366	$381
RCA Room Rental		
Shipping		
Supplies[4]	$4,500	$4,500
Travel		
Plot Fees	$1,200	$1,200
Miscellaneous		
Total	$81,166	$84,185

Footnotes:
[1] Research assistant professor (Nottingham) at 2% FTE of $7,612.5 per month for 12 months.
[2]Postdoc at 100% FTE of $4,250 per month for 12 months
[3]Summer time slip at 20 hours per week for 13 weeks at $15.00 per hour.
[4]Supplies including potted cherries, greenhouse and colony supplies (cages, soil, pots), bioassay supplies (pipette tips, paper cups, lab sprayer supplies), and PCR diagnostic services.

Objectives:

1. **Perform initial screening on a wide range of insecticides (broad spectrum-conventional, soft-conventional, and organic) against leafhoppers for mortality and feeding suppression.**
   Deviations: We were unable to establish a colony in the lab, but were able to continue testing insecticides on field-collected leafhopper adults.
2. **Determine whether X-infected leafhoppers are more susceptible to insecticides than uninfected leafhoppers.**
   Deviations: Concurrent studies in the Northfield and Harper labs indicate that phytoplasma presence in wild-caught leafhoppers too low for this objective to produce useful results.
3. **Determine residual control timelines for the most effective foliar products.**
   Deviations: It is too risky to perform insecticide trial in field plots due to the potential to kill trees, so bioassays were used instead.
4. **Determine the potential for soil applications of systemic insecticides to provide long-term control of leafhoppers and disease transmission.**
   Deviations: This objective was successfully tested in 2020, but with unimpressive control of leafhoppers. A new bioassay method to test systemic insecticides is in progress with results forthcoming.

Significant Findings:

- We are in the process of identifying more conventional insecticides that cause high mortality of *C. reductus* and *E. variegatus* with direct spray bioassays.
- The third leafhopper generation (beginning in Oct) shows signs of being the most abundant this year, indicating control methods must continue throughout the fall.

Methods:

Collection and Transport. *Colladonus reductus* and *Euscelidius variegatus* leafhopper adults were collected from weedy groundcover in organic commercial apple, cherry, and apricot orchards throughout the Columbia River Valley, WA. A modified leaf blower/vacuum with a 5-gallon paint strainer bag affixed to the tube was used to gently vacuum insects from clover, mallow, dandelion, and other weeds (Fig. 1, background). The bag was frequently emptied into a 12" by 24" mesh cage containing fresh vegetation to avoid sublethal injury to the insects (Fig 1, foreground).

Once returned to the lab, leafhoppers were aspirated into vials. They were then promptly divided into replicates and added to assay arenas. Fresh leafhopper collections were made for each experiment.

Fig. 1. Leafhopper collection method

Direct Spray Bioassays. Arenas were constructed using 16 oz plastic deli cups with moist soil and excised cherry leaves kept alive by constant contact with water in floral tubes (Fig. 2A). Leafhoppers were aspirated from collection cages and moved into each arena (5-10 leafhoppers per arena). Each arena was sealed with a plastic lid with a mesh cutout (Fig. 2B). Once leafhoppers were in all arenas and had acclimated for approximately 1 hr, treatments (Table 1) were applied using hand-pump aluminum spray bottles. Insecticide solutions were sprayed through mesh lids to contact the leafhopper, leaf, and soil, as would occur in the field. Containers with sprayed leafhoppers were then stored for 24-72 hr in a greenhouse prior to evaluation. To evaluate efficacy of insecticides, leafhoppers were rated as either alive or dead; "dead" leafhoppers were unable to walk.

Fig. 2. Leafhopper bioassay arenas and collection cages. A) Closeup of one arena without lid to show cherry leaf in floral tube and soil. B) Multiple arenas with lids in foreground; collection cages in background.

Soil Drench Bioassays. Assay arenas were constructed in the same manner as for the Direct Spray Bioassays. Instead of treating insects through the mesh cutout with spray bottles, insecticides (Table 1) were mixed per the label rate and used to fill the floral tubes into which excised cherry leaves were placed. Leaves were allowed to translocate the solution for approximately 1 hr before 5-10 leafhoppers were placed in the assay arenas. They were then exposed to the treatment for 24-48 hours, after which leafhoppers were rated as either alive or dead; "dead" leafhoppers were unable to walk.

Table 1. Insecticides tested against two species of leafhoppers in direct spray and soil drench assays.

Assay	Trade Name	A.I.	Rate	Target
Direct spray	Beleaf SG	Flonicamid	2.8 oz/acre	*C. reductus, E. variegatus*
	Exirel 0.83SE	Cyantraniliprole	20.5 fl oz/acre	*C. reductus, E. variegatus*
	Admire Pro	Imidacloprid	2.8 fl oz/acre	*C. reductus, E. variegatus*
	MBI-306*	Experimental	15 fl oz/acre	*C. reductus*
	MBI-306*	Experimental	20 fl oz/acre	*C. reductus*
Soil drench	Admire Pro	Imidacloprid	2.8 fl oz/acre	*C. reductus, E. variegatus*

*Product expected to be registered for organic use

Results:

Bioassays are currently being conducted with third generation leafhopper adults (population peak was in early October 2022); therefore, results and conclusions for this season will be provided in a separate, Final Report, by December 2022.

2021 Direct Spray Bioassays. Treatment with all conventional insecticides resulted in significantly greater mortality than the control treatment (Fig. 3A). Two treatments, Admire (imidacloprid) and Scorpion (dinotefuran), reached 100% mortality after 24h of exposure. Actara (thiamethoxam), Transform (sulfoxaflor), and Magister (fenazaquin, at high and low rates) resulted in 97%, 90%, 80%, and 72% mortality, respectively. Only one organic treatment, Pyganic (pyrethrins) resulted in high mortality of 98%, which was statistically comparable to the positive control Asana (esfenvalerate) at 96% mortality (Fig. 3B). Treatment with IAP 440 Oil (mineral oil) resulted in only 4% mortality, which was not significantly different from mortality in the control treatment (2%).

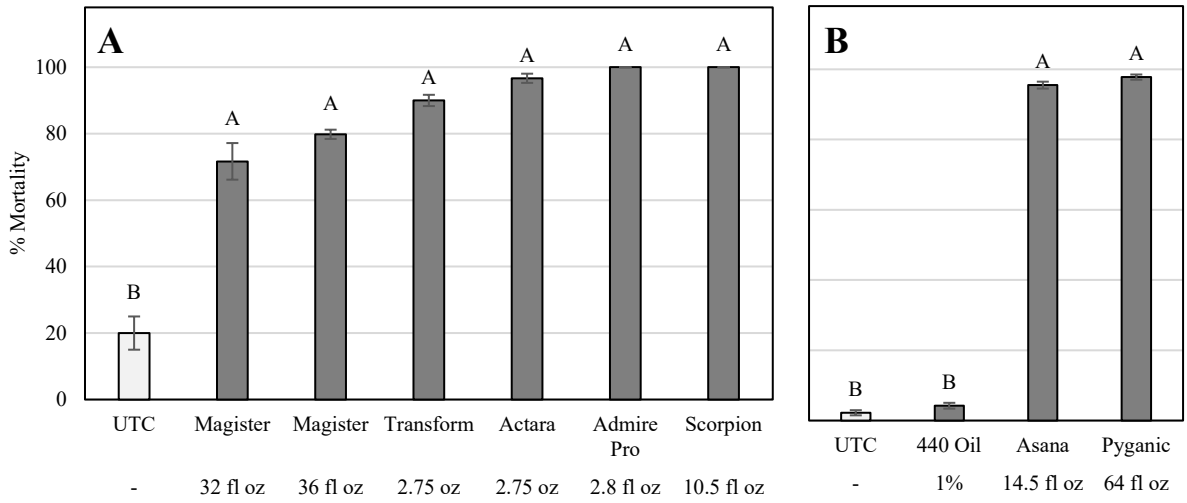

Fig. 3. Conventional (A) and Organic (B) Direct Spray Bioassay. Bars show average leafhopper mortality resulting from each insecticide. Per acre rates shown below each insecticide name. Bars not sharing a letter are significantly different according to Tukey's HSD ($p < 0.05$).

2021 Particle Film Repellency Assays – Petri Dish Bioassay. Treatment had no significant effect on cumulative duration spent in each zone (Fig. 4A). Leafhoppers, surprisingly, spent more time in the zones treated with particle films than in the completely untreated zones. However, those differences were only numerical and not statistically significant.

2021 Particle Film Repellency Assays - Excised Leaf Bioassay. Significantly fewer leafhoppers selected leaves treated with any combination of particle film (Celite [diatomaceous earth] or Surround [kaolin clay]) and oil compared to the untreated control (Fig. 4B). Leafhoppers chose oil-only treated leaves significantly less than untreated leaves, and leaves treated with any particle film significantly less than untreated or oil-only treated leaves. While untreated leaves harbored an average of twelve leafhoppers/leaf, oil-only treated leaves averaged four, and particle film-treated leaves averaged fewer than two.

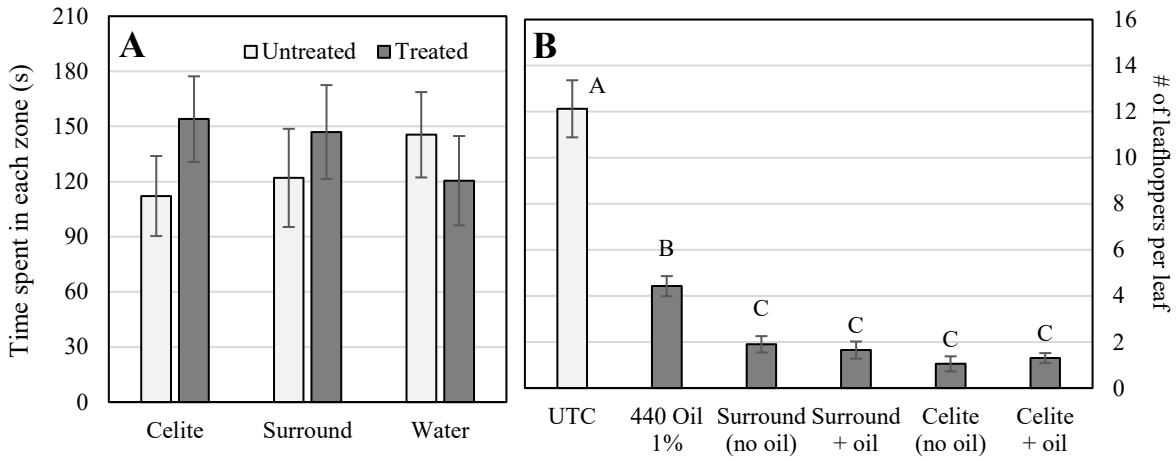

Fig. 4. A) Cumulative duration (s) spent in zones treated with particle films and untreated zones. B) Average leafhopper count on treated and untreated excised cherry leaves. Bars not sharing a letter are significantly different according to Tukey's HSD ($p < 0.05$).

2021 Spray Residue Bioassays. In the first aged residue bioassay, leafhopper mortality was not significantly different among time points for any treatments, indicating that each insecticide performed similarly as 1h, 72h, 168h, and 336h residues (Fig. 5A). However, when experiment-wide mortality was averaged and compared by treatment, Actara resulted in the highest overall mortality (88%), followed by Asana (58%) and Pyganic (32%) (Fig. 5B).

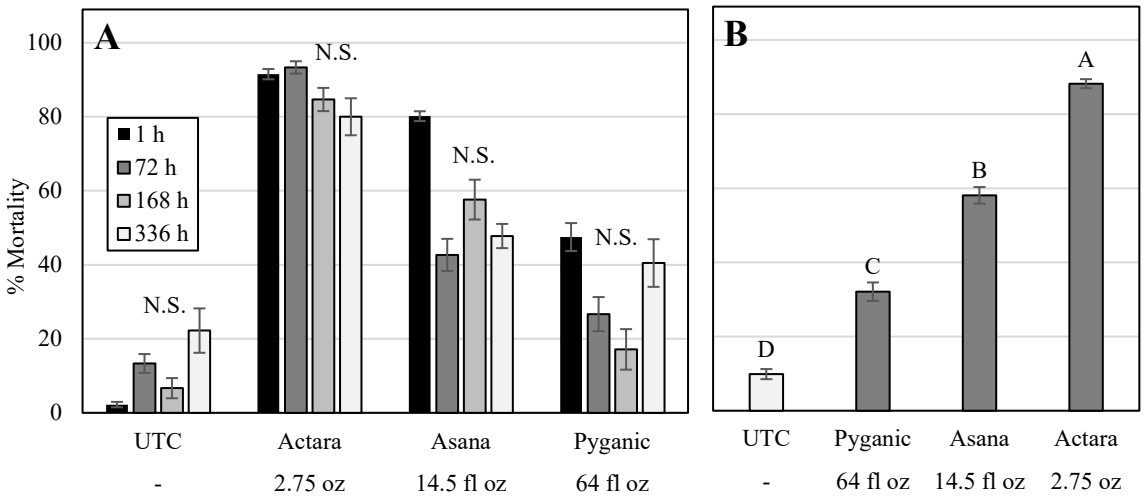

Fig. 5. A) Mortality of leafhoppers exposed to aged insecticide residues. B) Average leafhopper mortality across all residue ages. Per acre rates shown below each insecticide name. N.S. indicates no significant difference, and bars not sharing a letter are significantly different according to Tukey's HSD ($p < 0.05$).

In the second aged residue bioassay, leafhopper mortality was significantly different in the Asana + oil, Pyganic + oil, and control treatments (Fig. 6A). Control mortality was significantly higher in the 24h residue time point, which may indicate the influence of an outside factor that increased mortality in all treatments at that time. However, Asana + oil and Pyganic + oil still performed significantly better as 1h aged residues (90% and 36% leafhopper mortality, respectively) than as 122h aged residues (45% and 0% mortality, respectively). There were no significant differences in leafhopper mortality in the 440 oil treatment, Actara + oil treatment, and Admire + oil

treatment, indicating that these products performed similarly as 1h, 24h, and 122h aged residues. When mortality was averaged across the experiment by treatment, Actara + oil and Asana + oil performed significantly better than all other treatments at 76% and 69% mortality, respectively (Fig. 6B). Experiment-wide mortality in the Pyganic + oil (20%), Admire + oil (14%), and 440 oil (3%) treatments did not differ significantly from control mortality (5%).

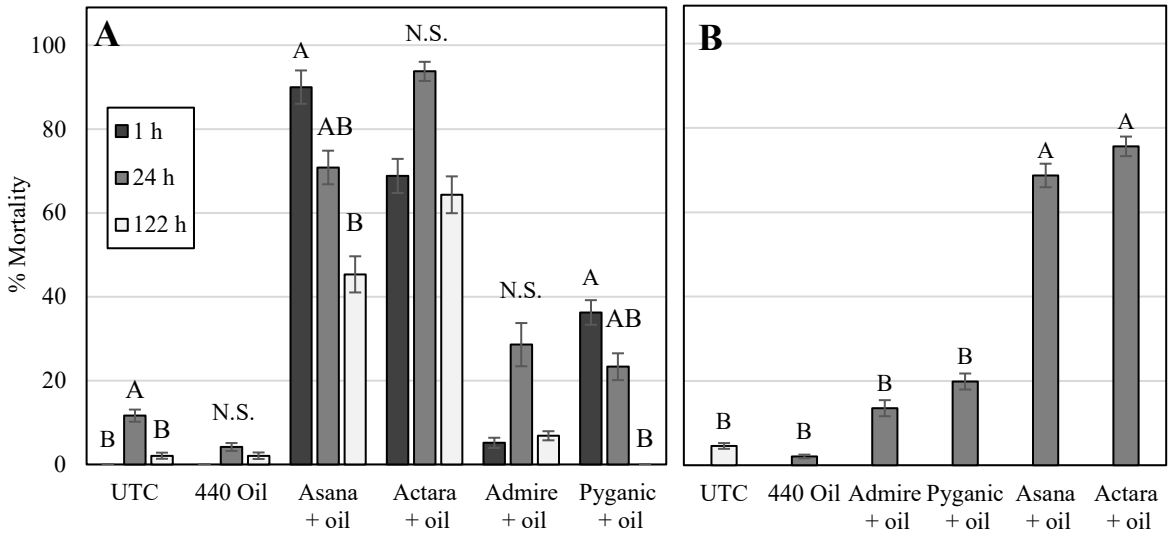

Fig. 6. A) Mortality of leafhoppers exposed to aged insecticide residues. B) Average leafhopper mortality across all residue ages. N.S. indicates no significant difference, and bars not sharing a letter are significantly different according to Tukey's HSD ($p < 0.05$).

Discussion:

Through the 2021 experiments, we identified one organic and five conventional insecticides that are highly toxic to leafhoppers upon direct spray contact. The design of the direct spray bioassays did not necessarily produce perfect contact with all leafhoppers; however, this conservative approach likely produces more field-realistic results because perfect spray coverage is not possible in the field. Future testing of more insecticides, especially organic and soft-selective insecticides, will be crucial for informing management decisions concerning cherry leafhoppers.

Particle film testing using EthoVision video monitoring was unsuccessful due to experimental setup; the smooth surface of the Petri dishes was difficult for the insects to grip, and five minutes of observation was insufficient. However, in the leaf-dip cage choice-test bioassays, both particle films tested greatly reduced the number of leafhoppers harboring on excised cherry leaves. This indicates that both kaolin clay and diatomaceous earth are effective repellents and may have a role in cherry leafhopper management. Evaluation of aged and weathered particle film residues will be necessary to determine long-term effectiveness of these products.

Residues of two conventional insecticides performed well with and without the addition of mineral oil and over the course of several days to weeks of aging. Through these aged residue assays, we were able to establish a protocol to test products and obtain realistic estimates of their actual in-field performance without requiring a full field trial.

We observed that the third and final leafhopper generation of the 2021 season (mid- to late-September) and the 2022 season (beginning in October) were the most abundant of each year. This means that control methods will need to be implemented through the fall to protect trees from X-disease transmission when leafhoppers are at their greatest numbers.

Project Title: Developing a Leafhopper Degree-day Spray Program for Cherry IPM

Report Type: Continuing Project Report

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Cooperators: Teah Smith, Garrett Bishop, Jenna Voelker, Dr. Katlyn Catron, Dr. Rodney Cooper, Dr. Scott Harper, Dr. Adrian Marshall, Dr. David Crowder, Dr. Vince Jones

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 71,536
Total Project Request for Year 2 Funding: $ 74,397
Total Project Request for Year 3 Funding: $ 77,373
Total Request: $223,306

Other related/associated funding sources: Awarded
Funding Duration: 2020-2021 (2022-2024 Pending)
Amount: $26,000
Agency Name: Corteva Agriscience, Gowan Co, Brandt Co., Nichino America, Kemin Industries
Notes: Insecticide efficacy trials funding will cover travel to field sites, 10% balance in postdoc's salary and miscellaneous project costs.

Budget
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Item	2022	2023	2024
Salaries[1,2]	$47,727	$49,636	$51,621
Benefits[1,2]	$17,498	$18,198	$18,926
Wages[3]	$5,760	$5,990	$6,230
Benefits	$551	$573	$596
Equipment	$0	$0	$0
Supplies[4]	$0	$0	$0
Travel[4]	$0	$0	$0
Miscellaneous	$0	$0	$0
Plot Fees	$0	$0	$0
Total	$71,536	$74,397	$77,373

Footnotes:
[1]Nottingham Salary: $7,612 x 12 mo x 2% FTE = $1,827 for year 1 x 1.04 each additional year. Benefits at 29.9%
[2]Postdoc Salary: $4,250 x 12 mo x 90% FTE = $45,900 for year 1 x 1.04 each additional year. Benefits at 36.9%.
[3]Non-student temporary employee: $15/hr x 16 hrs/wk x 24 wks = $5,760 for year 1 x 1.04 each additional year. Benefits at 9.6%
[4]Funded with industry gifts and leveraged matching funds.

Objectives

1. Collect model development and validation data: scout selected WA and OR orchards containing high populations of leafhoppers twice per week while compiling weather data.
Future goals: Finish identifying and counting leafhoppers from 2022 vacuum samples in the off-season, continue data collection in 2023 and 2024.
Deviations: Initial scouting occurred once weekly to determine productive sites.
2. Modeling phenology curves: Use leafhopper abundance data to parameterize and validate the model.
Future goals: Analyze data from 2023 collections and beyond.
Deviations: None.
3. Insecticide trials: Screen conventional and organic insecticides for efficacy against leafhoppers and partner with growers to test spray timings.
Future goals: Find grower partners to include on spray timing tests.

Significant Findings

- We established 13 sample sites throughout central WA and north-central OR and installed remote air temperature monitors for 8 sites.
- We collected leafhoppers weekly from April to September 2022 using sticky cards and vacuum sampling
    - Phenology of *Colladonus reductus, C. geminatus, Euscelidius variegatus*, and other leafhopper adults was plotted using sticky card data

Methods

Site Establishment. Thirteen orchard sites were selected throughout central and southern Washington and north-central Oregon. Sites were chosen based on location, leafhopper catch in previous years (if available), ground cover, crop grown, and management strategy (organic or soft-conventional only). Crops included sweet cherry, apple, pear, apricot, and nectarine (Table 1) and were either organic or minimally managed blocks. At sites not close to established AWN or Agrimet sensors, METER Group ZL6 Advanced Cloud Data Loggers with ECT/RT temperature sensors were installed to record air temperature every 15 minutes.

Table 1. Sample Sites

Site	Fruit	Closest town	Weather station
1	Apricot	Wapato	ZL6
2	Cherry	Zillah	ZL6
3	Cherry	Prosser	AWN
4	Cherry	Prosser	ZL6
5	Nectarine	Pasco	ZL6
6	Cherry	Royal City	ZL6
7	Apple	Rock Island	ZL6
8	Apple	Rock Island	ZL6
9	Pear	Peshastin	ZL6
10	Pear	Wenatchee	AWN
11	Cherry	Hood River, OR	Agrimet
12	Cherry	Mosier, OR	Agrimet
13	Cherry	Mosier, OR	Agrimet

Sampling. Leafhoppers were sampled weekly from all orchards starting April 27, 2022. At each site, two sticky cards were deployed at the lowest canopy level and were replaced each week with fresh cards. The number of leafhopper nymphs, *Colladonus reductus* adults, *C. geminatus* adults, *E. variegatus* adults, and any other leafhopper adults on each card was recorded. This count data was plotted over time to visualize population peaks and lulls through the season.

Additionally, three leafhopper samples were taken from ground cover at each site using a modified leaf blower/vacuum. A plastic hoop (32" inner diameter) was laid on a patch of weedy ground cover and the area within thoroughly vacuumed. A 5-gallon paint strainer bag, held on the vacuum tube with rubber bands, collected the insects and prevented them from being sucked into the motor. After three hoop areas were vacuumed, the contents of the paint strainer bag were emptied into a zip-top bag. This procedure was repeated three times at each site (Fig. 1). Zip-top bags were returned to the lab and frozen to euthanize the insects and preserve them until they could be sorted from debris and counted.

Fig. 1. Vacuum sample area (left), vacuum sampling technique (center), and transfer of paint strainer bag contents to zip-top bag (right). Photos by Garrett Bishop.

Anticipated results. After leafhoppers in the vacuum samples are sorted, they will be identified and counted (Oct-Dec. 2022). That count data will be compared with sticky card counts and plotted over time (Dec. 2022). Leafhopper abundance data and air temperature data will be used to parameterize and validate the phenology model (beginning Jan. 2023). Additional modeling will be done to determine if changes in sample sites, sampling frequency, or sampling type should be made before continuing weekly sticky card and vacuum samples in 2023.

Potential problems or limitations. Year one went relatively smoothly. Other than the logistical challenges of coordinating safe days to sample with so many growers each week, no major problems or limitations are anticipated for the next two years.

Results and Discussion

Establishment of sample sites and consistent sampling frequency were the primary goals of this first field season, and both were accomplished. The METER data loggers that we installed had no issues collecting high-quality temperature data throughout the season. Almost every site was sampled via vacuum and sticky cards every week, for a total of over 400 sticky card counts and 600 vacuum samples as of October, with sampling still ongoing. Adult *C. reductus* activity began in mid-May with a peak in early June, a second peak in early August, and a third peak building as of report writing (Fig. 2). Preliminary examination of sticky card and temperature data at a highly productive sample site outside of Royal City, WA, shows a similar trend with logical variations in temperature throughout the season (Fig. 3).

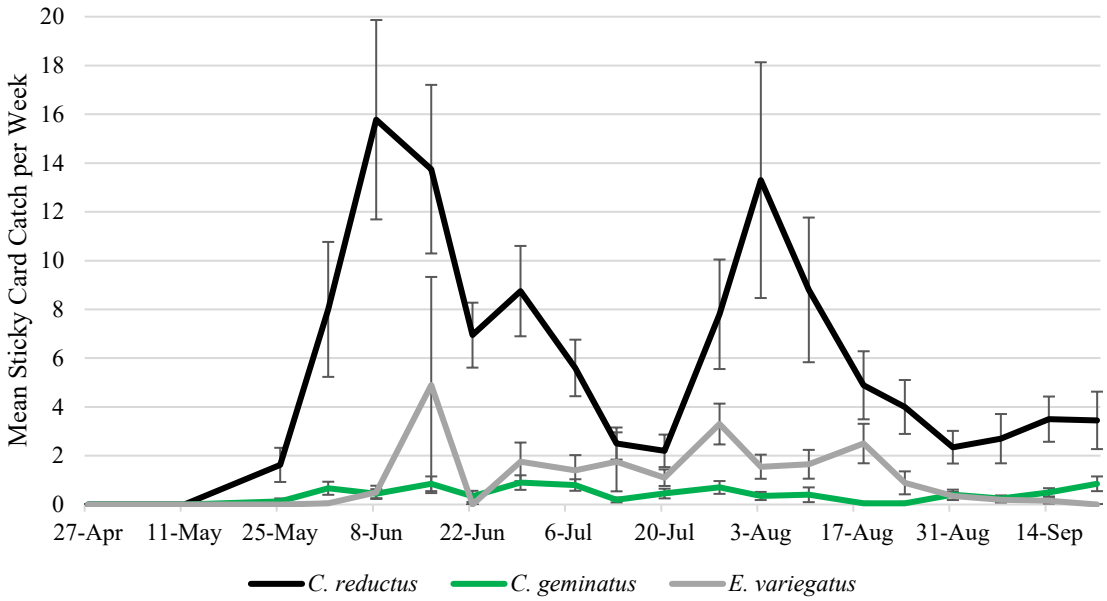

Fig. 2. Mean WA weekly sticky card catch of leafhopper species of interest throughout 2022 growing season. Error bars represent standard error of the mean.

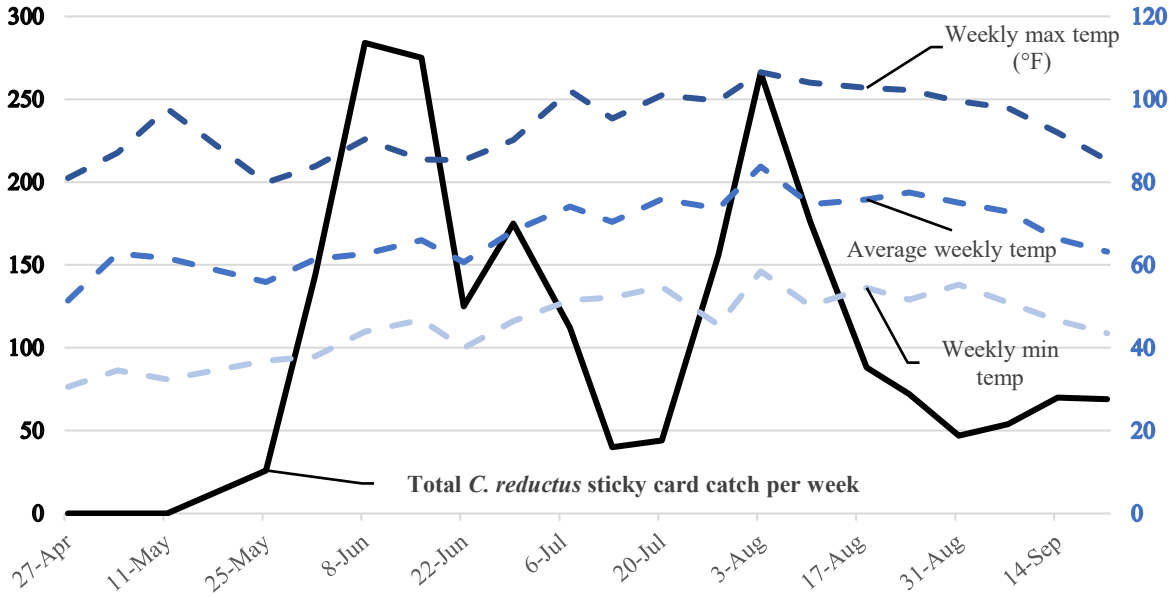

Fig. 3. Total *Colladonus reductus* sticky card catch and weekly temperatures (minimum, average, and maximum) as measured by a METER weather station for a single sample site outside of Royal City, WA.

The vast majority of leafhoppers caught using sticky cards were *C. reductus* or other unidentified (and likely non-vector) species (Fig. 4). Though insects caught using vacuum samples have not been identified, observations during their processing suggest that finer-resolution population curves will be possible for *C. geminatus* and *E. variegatus* using those data, as well.

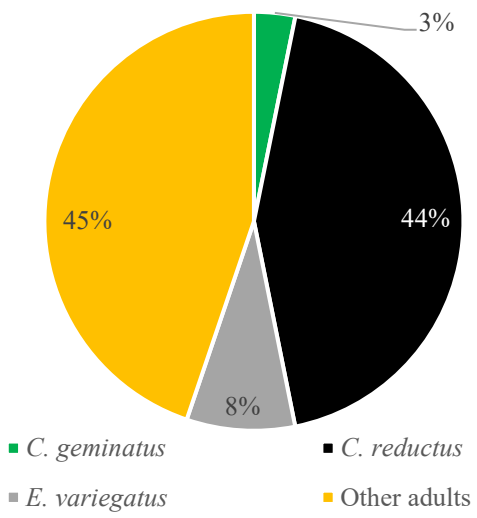

Fig. 4. Proportion of 2022 WA leafhopper sticky card catch composed of each species.

Using data from sticky cards and vacuum samples, we will be able to determine if sticky card monitoring is sufficient to track *C. reductus* populations for eventual phenology-based control. Additionally, we have collected enough data in Year 1 to begin parameterizing and testing phenology models for all three species of interest. While a finished model is still a few years off, this data is foundational for helping growers anticipate the population growth of X-disease vectoring leafhoppers and begin treating for them proactively instead of reactively.

Project Title: Dispersive distance of cherry X-disease vector leafhoppers within managed sweet cherry orchards

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Co-PI (3):
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Co-PI (4):
Organization:
Telephone:
Email:
Address:
City/State/Zip:

Cooperators:

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

Other funding sources: None

Budget 1

Organization Name: Agricultural Research Foundation **Contract Administrator:** Charlene Wilkinson
Telephone: 541-737-3228

Email address: Charlene.wilkinson@oregonstate.edu

Item	2022	2023	2024
Salaries[1]	$7,975	$8,215	$8,461
Benefits	$5,575	$5,742	$5,914
Wages			
Benefits			
Equipment			
Supplies[2]	$4,500	$4,500	$4,500
Travel[3]	$1,000	$1,000	$1,000
Miscellaneous			
Plot Fees			
Total	$19,050	$19,457	$19,875

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

Budget 2

Organization Name: Agricultural Research Foundation **Contract Administrator:** Charlene Wilkinson
Telephone: 541-737-3228
Email address: Charlene.wilkinson@oregonstate.edu

Item	2022	2023	2024
Salaries[1]	$1,000	$1,000	$1,000
Benefits	$	$	$
Wages			
Benefits			
Equipment[2]	$2,127	$2,453	$1,679
Supplies			
Travel[3]	$300	$300	$300
Plot Fees			
Miscellaneous			
Total	$3,427	$3,753	$2,979

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.
2) Describe dispersive distance and rate of movement over time of key leafhopper vector species, within cherry orchards.
3) Describe rate of movement relative to prevailing wind direction and outside orchard habitat.

In year **one** we worked to establish the most viable technique for permanently and effectively marking and capturing leafhoppers with both proteins and dayglo powder. Details to work out in year one include how glue from sticky-traps will impact our ability to detect markers, and

Timeline

Objective	Y1	Y2	Y3
1. Develop methods of marking	X		
2. Describe dispersive distance inside orchard		X	X
3. Describe dispersive distance outside orchard		X	X

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. Do both egg whites and milk work as protein markers for grass and leafhoppers?
2. Does trapping with sticky cards work? i.e. can we get a positive signal when insects are collected this way (on glue)?
3. Because some insect parts might be left behind, can we hole punch and test insect + card?
4. Will samples still test positive after sitting on a sticky card for 1 week?
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?
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

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.

2**. Does trapping on sticky cards work? i.e. can we get a positive 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.

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.

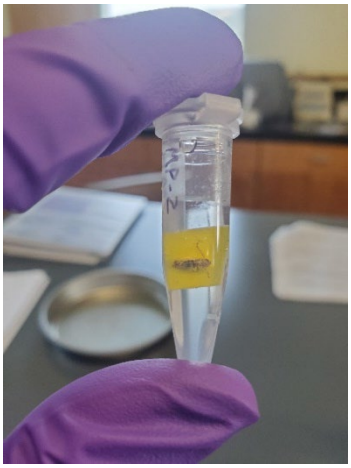

Figure 2. leafhopper + sticky card

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.

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.

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.

Results table

		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

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 1. 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.
-

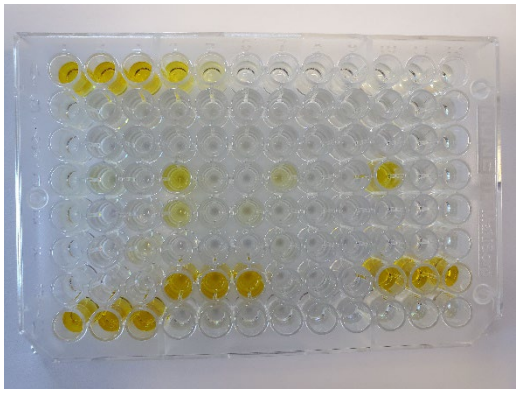

Figure 2. ELISA tray control results

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.

Conclusions and Future Directions

We accomplished a lot in this first season. Although our sample size is small, a 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).

We are now performing additional experiments to address the following questions:

1. Will marked leafhoppers still be positive after a week on a sticky card, exposed to the orchard environment (sun and irrigation water)?
2. Will marked leafhoppers contaminate unmarked leafhoppers on a sticky card in the presence of irrigation (proteins washed onto unmarked insects)?
3. Will being sprayed directly kill or impair leafhoppers?
4. Will live marked leafhoppers be positive a week later or can they clean the protein off?

We have made good progress on objective one and are ready to begin mark release recapture in the field beginning in year 2.

Project Title: Coordinating SWD and X Disease Management
Report Type: Continuing Project Report

Primary PI: Elizabeth H. Beers
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Address 2: 1100 N. Western Ave
City/State/Zip: Wenatchee, WA 98801

Cooperators: Ash Sial, Rufus Isaacs, Kent Daane, Hannah Burrack, Joanna Chui, Frank Zalom

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 24,865
Total Project Request for Year 2 Funding: $25,800
Total Project Request for Year 3 Funding: $26,772

Other related/associated funding sources: Awarded
Funding Duration: 2020- 2024
Amount: Beers: 18,634, 17,751, 16,890; Northfield: 72,197, 73,313, 73,817
Agency Name: USDA ScRI
Notes: This USDA-SCRI project is a national collaboration of SWD researchers covering sweet cherries and berries. Funding is for 4 years (Sept. 1, 2020 through August 31, 2024).

WTFRC Collaborative Costs: none

Budget 1
Primary PI: Elizabeth H. Beers
Organization Name: Washington State University
Contract Administrator: Stacy Mondy
Telephone: 916-897-1960
Contract administrator email address: arcgrants@wsu.edu
Station Manager/Supervisor: Chad Kruger
Station manager/supervisor email address: cekruger@wsu.edu

Item	Year 1: 2021	Year 2: 2022	Year 3: 2023
Salaries[1]	13,752	14,302	14,874
Benefits[2]	4,839	5,033	5,234
Wages[3]	3,900	4,056	4,218
Benefits[4]	874	909	946
RCA Room Rental			
Shipping			
Supplies	$1,500.00	$1,500.00	$1,500.00
Travel			
Plot Fees			
Miscellaneous			
Total	$24,865.00	$25,800.00	$26,772.00

Footnotes: [1]Salaries: 0.25 FTE post-doc; [2]Benefits (salaries): 35.2%; [3]Wages: $15/hr, 20 hr/week, 13 weeks/yr; [4]Benefits (wages): 22.4%.

Objectives:
(Objectives 1-5 are the objectives in the leveraged SCRI proposal covering a broad range of US regions and crops affected by SWD; Objective 6 is an additional objective solely for Washington cherry and is the main focus of this continuing report.)

1. Implementation of best management programs for sustainable management of SWD in collaboration with grower influencers.
2. Develop economics-based decision aid tools to support the identification and implementation of profit-maximizing SWD management strategies.
3. Evaluate sustainable alternatives to insecticides for long-term SWD management.
4. Assess and reduce the risk of insecticide resistance development.
5. Develop and disseminate actionable recommendations that enable producers to optimize pest management decisions, and evaluate their impact.
6. Determine the impact of SWD controls on leafhopper vectors of X-disease.
    a. As the sustainable alternatives to SWD insecticides (Obj. 3) are currently being tested and adapted for the unique climate and growing conditions that defines Eastern Washington cherry production, Obj. 6 was altered to assess the impacts of X-disease leafhopper management on SWD populations in Eastern Washington cherry orchards.
    b. Given the potential horticultural benefits of Extenday groundcover (Extenday, Union Gap, WA) and Surround WP kaolin clay foliar application (NovaSource, Phoenix, AZ) and recent findings on the potential for these products to control leafhopper vectors of X-disease phytoplasma, the aim of the revised objective was to assess these products as part of an integrated approach to SWD management in Eastern Washington cherry production.

Significant Findings:
Objective 6
- There is preliminary evidence that biorational control of leafhoppers and SWD will be complementary

2022:
- At Cashmere 1, Extenday reduced SWD adult counts by 53.7% while herbicide applications reduced SWD counts by 22% in comparison to the Control.
- At Cashmere 2, Extenday applied postharvest reduced SWD adult counts by 55.9% while herbicide applications increased SWD counts by 71% in comparison to the Control.

2021:
- At the Wenatchee site, Extenday applied postharvest reduced SWD adult counts by 65.83% while Surround reduced SWD adult counts by 66.60% in comparison to the Control. Mowed blocks were comparable or hosted more SWD than the Control.
- At the Wapato site, Extenday applied postharvest reduced SWD adult counts by 47.34% while Surround reduced SWD adult counts by 37.32%.

Methods:
Objective 6
2021
The impacts of post-harvest X-disease vector leafhopper management on SWD populations was assessed in 2021 at two cherry orchard sites near Wapato and Wenatchee respectively. The Wapato cherry orchard consisted of 28 acres of 'Sweetheart' cherries and was conventionally managed. The Wenatchee cherry orchard consisted of ~25.9 acres of 'Coral Champagne' cherries and is in the first year of transitioning from conventional to organic management.

At the Wenatchee orchard, four treatments were assessed: Extenday groundcover, Surround kaolin foliar application, weekly mowing, and an untreated control. Each treatment was repeated in a

randomized block design with 4 replicate blocks for each treatment. Each block was 200 ft long and 12 rows wide. Treatments were maintained from mid-July until early November with adult SWD trapped throughout this period.

Each replicate had two traps, one at 50 ft and one at 150 ft from the block 's edge in the middle (seventh) row. The traps consisted of a modified 32 oz plastic jar with a Scentry SWD Lure (Scentry Biologicals Inc., Billings, MT) suspended from the lid in front of screened holes to allow the odor of the lure to diffuse, and the flies to enter the trap. Flies were retained and preserved in 300 mL of drowning solution (5 L water, 50 g sodium benzoate, 50 mL unscented dish soap). Drowning solution was changed and trap contents collected every two weeks. Trap contents were assessed under a dissecting microscope (Leica Microsystems, Wetzlar, Germany) and the number of SWD males and females as well as other *Drosophila* flies (specimens not identified as *Drosophila suzukii*) was noted. The Scentry SWD lures were replaced every 6 weeks.

At Wapato, three treatments were assessed; Extenday groundcover, Surround kaolin foliar application, and an untreated Control. Each treatment was repeated as two replicate blocks. Each block was 200 ft long and 12 rows wide. Treatment blocks were maintained from mid-July until mid-October when the treatments were removed in preparation of the block's removal in November (due to high prevalence of X-disease). Sampling for adult SWD was conducted as described above, except there were four traps per block (at 50 feet in row 5, 100 feet in rows 6 and 8, 150 feet in row 7).

The Wapato site's cherry trees were removed in October 2021 due to the ongoing X-Disease epidemic, preventing this site from being surveyed in 2022. As the Wenatchee site was transitioning to certified Organic management, it could not participate in the 2022 experiments given the addition of the conventional herbicide treatment.

2022

In 2022, we conducted an assessment of two cultural control practices (Extenday and Herbicide) compared to an untreated Control at two conventionally managed orchard sites near Cashmere, WA. The first orchard (Cashmere 1) consisted of 5.37 acres of 'Rainier' sweet cherries. The second orchard (Cashmere 2) consisted of 3.13 acres of 'Rainier' sweet cherries.

At each orchard, the three treatments (Extenday, Herbicide, and untreated Control) were repeated as two replicate blocks. Each block was 130 ft long and 6 rows wide. Treatment blocks were setup in late May and maintained until the end of October. The Herbicide treatments consisted of 1 preharvest groundcover application of SPUR (Clopyralid, Albaugh LLC, Ankenny, IA) on May 20th and 1 postharvest groundcover application of Venue (Pyraflufen ethyl, Nichino America Inc, Wilmington, DE) on 25 July. At Cashmere 1, the Extenday Block was maintained for the duration of the experiment. At Cashmere 2, Extenday was maintained by the grower collaborator across all 3 treatment blocks from 30 May 30-27 June due to concerns of ripening. The Extenday was removed from all Cashmere 2 blocks by June 2 for harvest and was then re-applied solely to the designated Extenday Blocks on 15 July. As such, our Cashmere 2 analyses consist only of post-harvest comparisons.

Surveys to collect and identify SWD adults was conducted as described for 2021, except with two drowning traps per block collected and changed weekly. The traps were hung at 30 ft in row 2 and at 65 ft in row 3 respectively.

Results and Discussion:
2021

SWD counts across all treatments were initially low, potentially due to the extreme heatwave events that the Pacific Northwest experienced during the summer of 2021. At Wapato, SWD counts in traps started to increase in late August while at Wenatchee, SWD counts remained low until late September (Figure 1, Figure 2). At the Wenatchee site, there was a significant effect of treatment ($X^2 = 175.7$, df = 3, $P < 0.0001$) on SWD collected per trap. Extenday applied postharvest reduced SWD adult counts by 72% while Surround reduced SWD adult counts by 71% in comparison to the control (Figure 3, Table 1). Mowed blocks hosted more SWD than control blocks. At the Wapato site, there was a significant effect of treatment ($X^2 = 18.919$, df = 2, $P < 0.0001$) on SWD collected per trap. Extenday applied postharvest reduced SWD adult counts by 47.9% while Surround reduced SWD adult counts by 41.3% (Figure 4, Table 2). At Wenatchee, the effect of treatment on the proportion of female SWD per trap was not significant. At Wapato, the effect of treatment ($X^2 = 11.221$, df = 2, $P < 0.01$) on the proportion of female SWD per trap was significant. Surround and Extenday treatments had a greater proportion of female SWD per trap than the control treatments (Table 2). There was a significant effect of treatment ($X^2 = 12.819$, df = 3, $P < 0.01$) on the proportion of SWD among total *Drosophila* collected per trap. The Surround treatments hosted a smaller proportion of SWD than the mowing or control treatments while the number of SWD per total Drosophilids per trap for Extenday treatments was comparable to all other treatments (Table 1). The effect of treatment on the proportion of SWD among total Drosophilids collected per trap at the Wapato site was not significant.

2022

In 2022, SWD counts in traps at both Cashmere sites remained low until mid-September (Figure 5, 6). At Cashmere 1, there was a significant effect of treatment ($X^2 = 8.4172$, df = 2, $P < 0.05$) on SWD collected per trap. Extenday reduced SWD adult counts by 57% while Herbicide reduced SWD adult counts by 22% in comparison to the control (Figure 7, Table 3). At Cashmere 2, there was a significant effect of treatment ($X^2 = 12.601$, df = 2, $P < 0.01$) on SWD collected per trap. Extenday applied postharvest reduced SWD adult counts by 55.9% while Herbicide treated blocks increased SWD adult counts by 71% (Figure 8, Table 4). At the Cashmere sites, the effect of treatment on the proportion of female SWD was not significant. The effect of treatment on the proportion of SWD among total Drosophilids collected per trap at Cashmere 1 was not significant. There was a significant effect of treatment ($X^2 = 7.6855$, df = 2, $P < 0.05$) on the proportion of SWD among total *Drosophila* collected per trap. The herbicide treated blocks at Cashmere 2 had a slightly greater proportion of SWD among total drosophilids caught than the control. (Table 4).

The preliminary results from 2021 and 2022 suggest that postharvest canopy and groundcover-based management of leafhopper vectors of X-disease phytoplasma may also reduce SWD populations in cherry orchards. The reduced counts of SWD observed for Extenday and Surround treated blocks were observed in orchards representative of high pressure and low pressure situations for both SWD and X-disease leafhoppers. The 2022 trials support the use of Extenday to control SWD although herbicide applications may have a mixed effect. These preliminary results suggest that these integrative management options may be viable under a wide scale of potential pest pressure

Table 1: SWD catch, sex ratio, and proportion of total drosophilids caught by treatment, Wenatchee, 2021

Treatment	n	SWD/trap/2 wk	%Reduction	Female SWD/Total SWD	SWD/Total drosophilids
1.Control	4	50.00 b	-	0.56 a	0.12 b
2. Extenday	4	14.00 a	72.0%	0.57 a	0.10 ab
3. Kaolin	4	14.48 a	71.0%	0.60 a	0.08 a
4. Mowing	4	72.54 c	-45.1%	0.60 a	0.13 b

Table 2: SWD catch, sex ratio, and proportion of total drosophilids caught by treatment, Wapato, 2021

Treatment	n	SWD/trap/2 wk	% Reduction	Female SWD/Total SWD	SWD/Total drosophilids
1. Control	2	311.10 b	-	0.38 b	0.03 a
2. Extenday	2	162.08 a	47.9%	0.44 a	0.02 a
3. Kaolin	2	182.70 a	41.3%	0.47 a	0.02 a

Table 3: SWD catch, sex ratio, and proportion of total drosophilids caught by treatment, Cashmere 1, 2022

Treatment	n	SWD/trap/wk	% Reduction	Female SWD/Total SWD	SWD/Total drosophilids
1. Control	2	9.09 b	-	0.45 a	0.06 a
2. Extenday	2	3.88 a	57.3%	0.54 a	0.08 a
3. Herbicide	2	7.06 ab	22.3%	0.48 a	0.05 a

Data collection still in progress.

Table 4: SWD catch, sex ratio, and proportion of total drosophilids caught by treatment, Cashmere 2, 2022

Treatment	n	SWD/trap/wk	% Reduction	Female SWD/Total SWD	SWD/Total drosophilids
1. Control	2	4.06 ab	-	0.51 a	0.04 a
2. Extenday	2	1.79 a	55.9%	0.55 a	0.07 ab
3. Herbicide	2	6.95 b	-71.2%	0.52 a	0.06 b

Data collection still in progress.

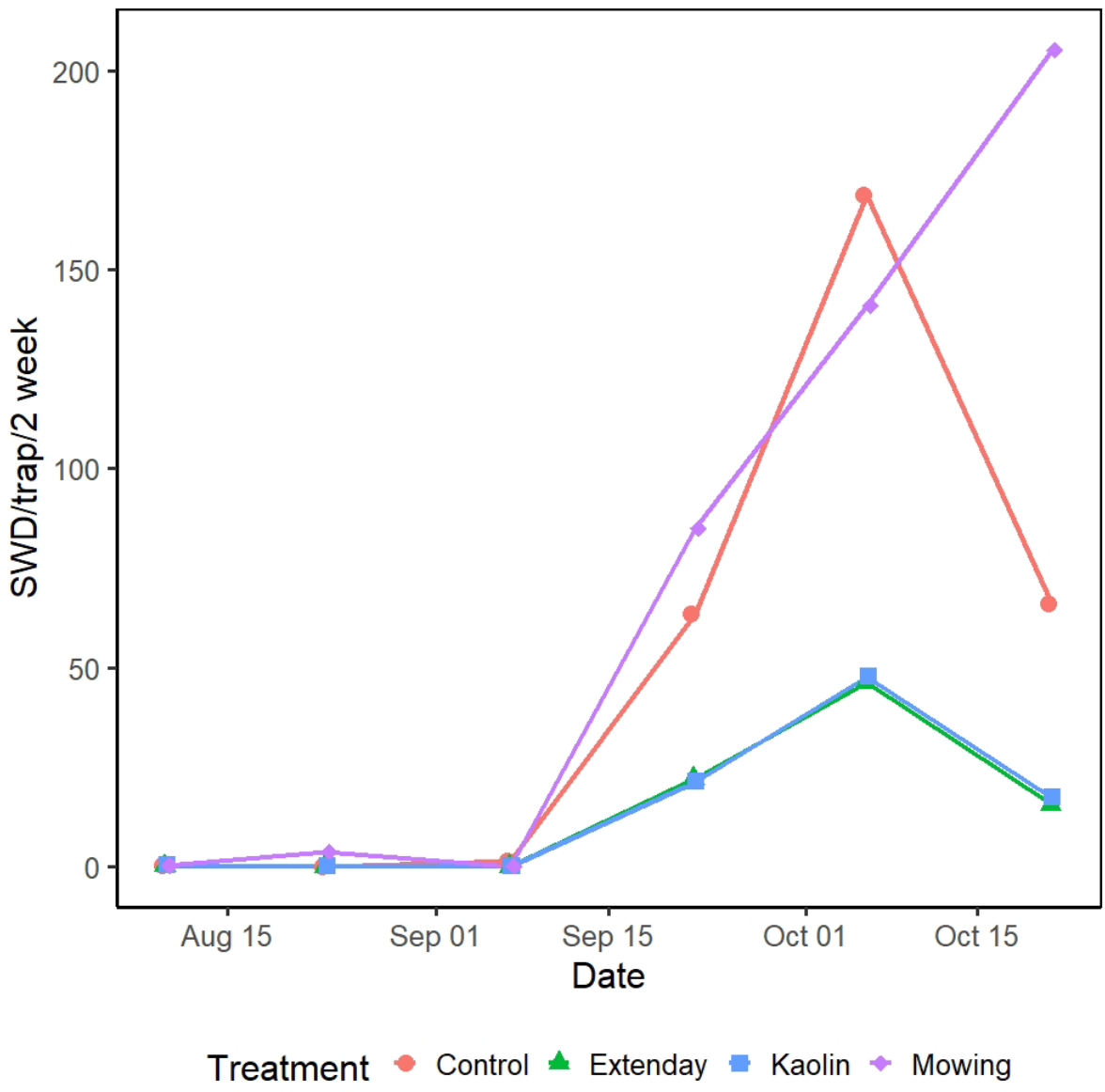

Fig 1. SWD adult counts per trap per sampling date by treatment, Wenatchee, 2021.

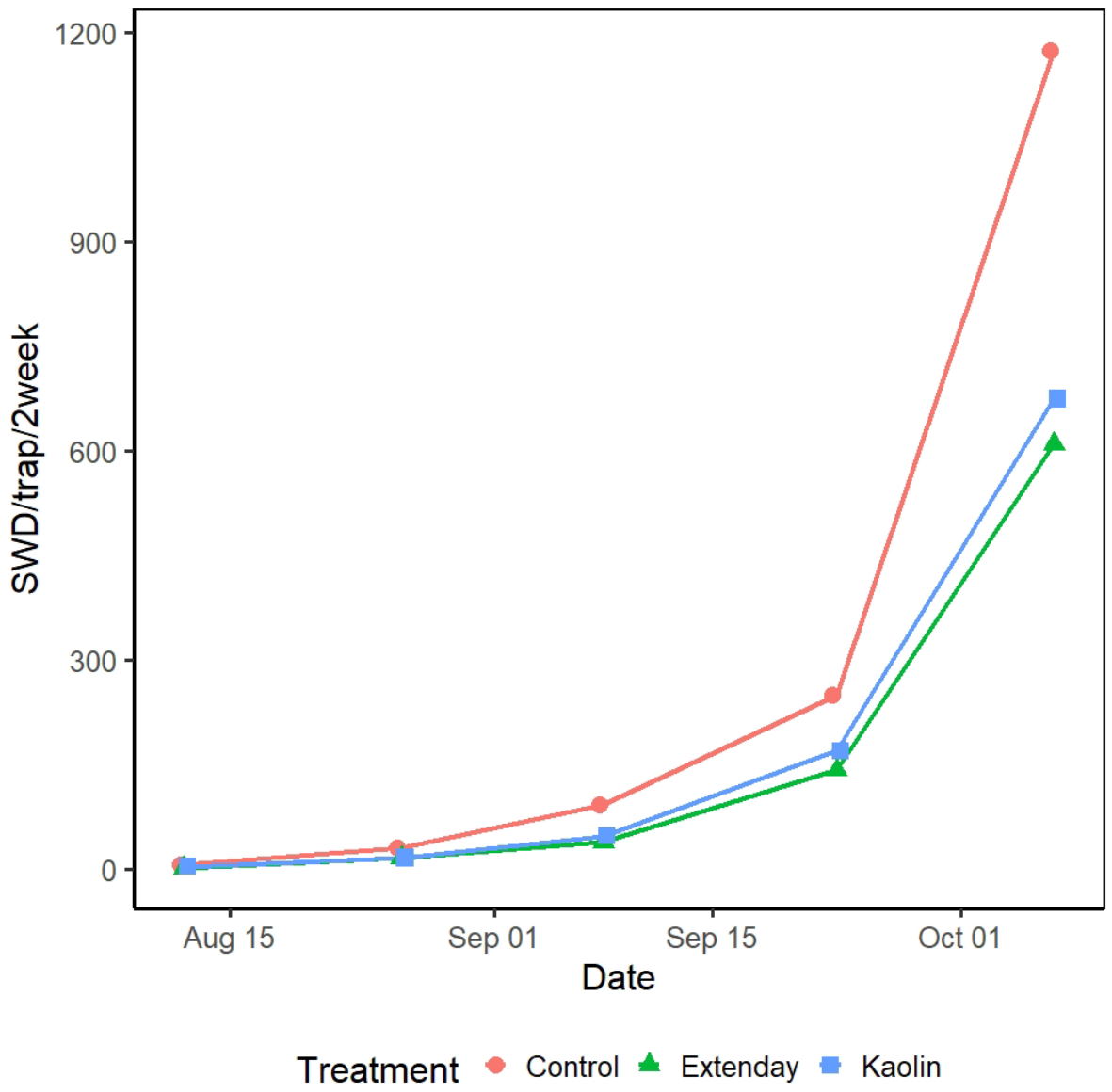

Fig. 2. SWD adult counts per trap per sampling date by treatment, Wapato, 2021.

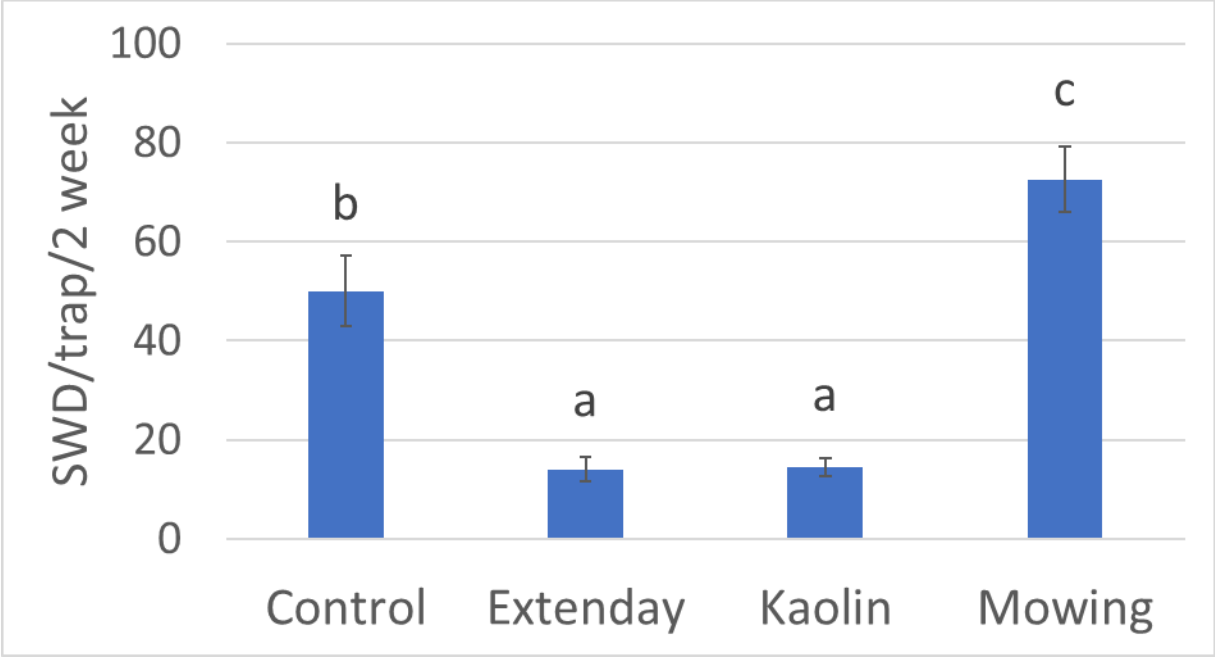

Fig. 3. SWD adult counts by treatment, Wenatchee, 202.1

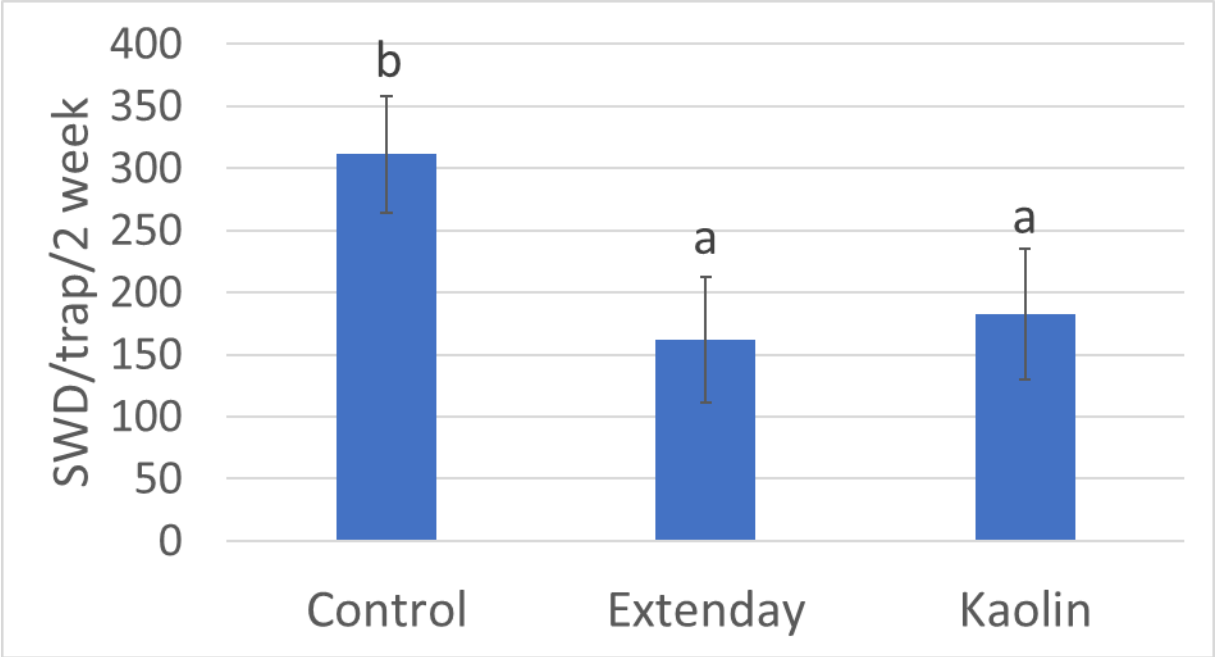

Fig. 4. SWD adult counts by treatment, Wapato, 2021.

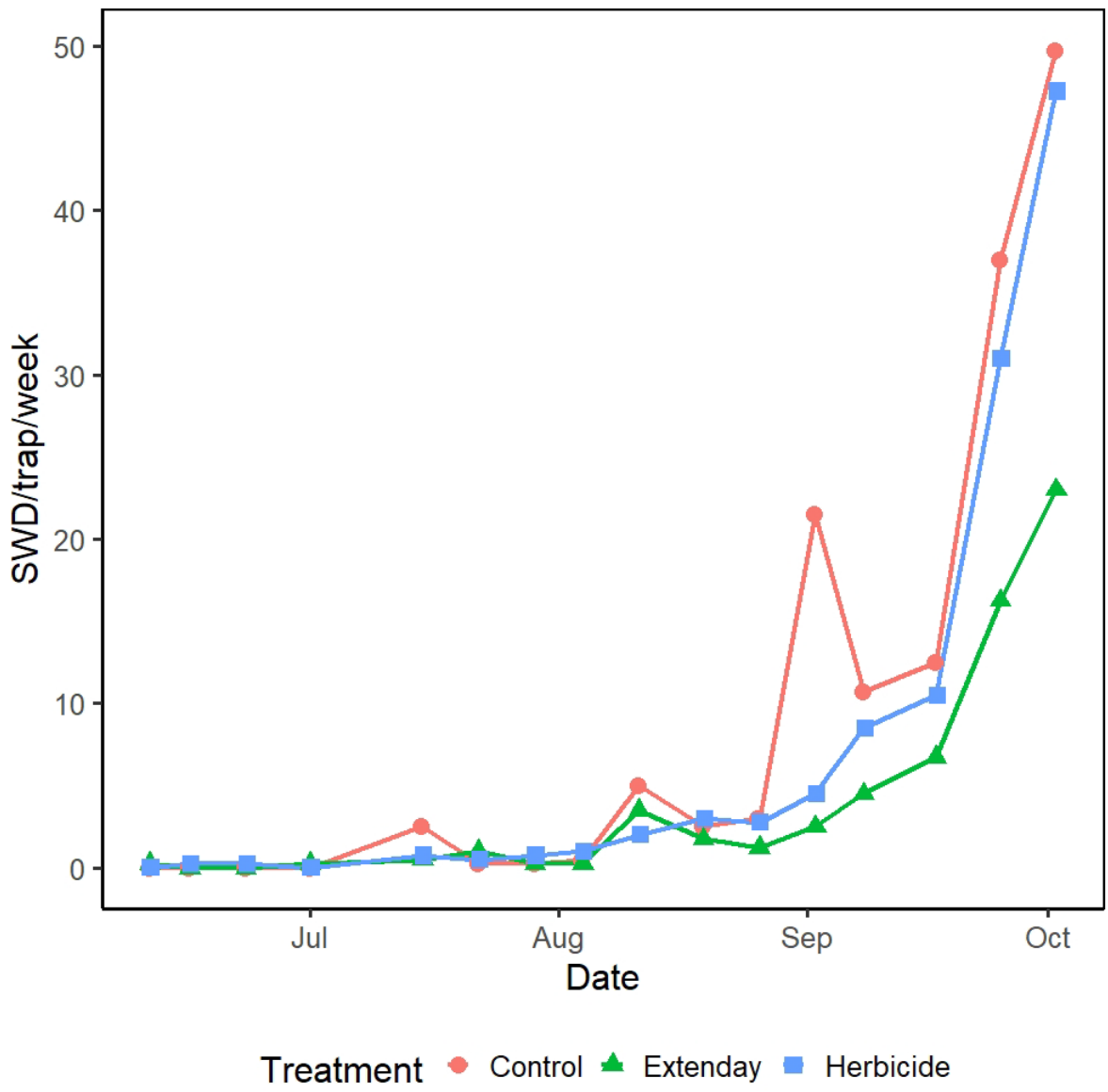

Fig 5. SWD adult counts per trap per sampling date by treatment, Cashmere 1, 2022.

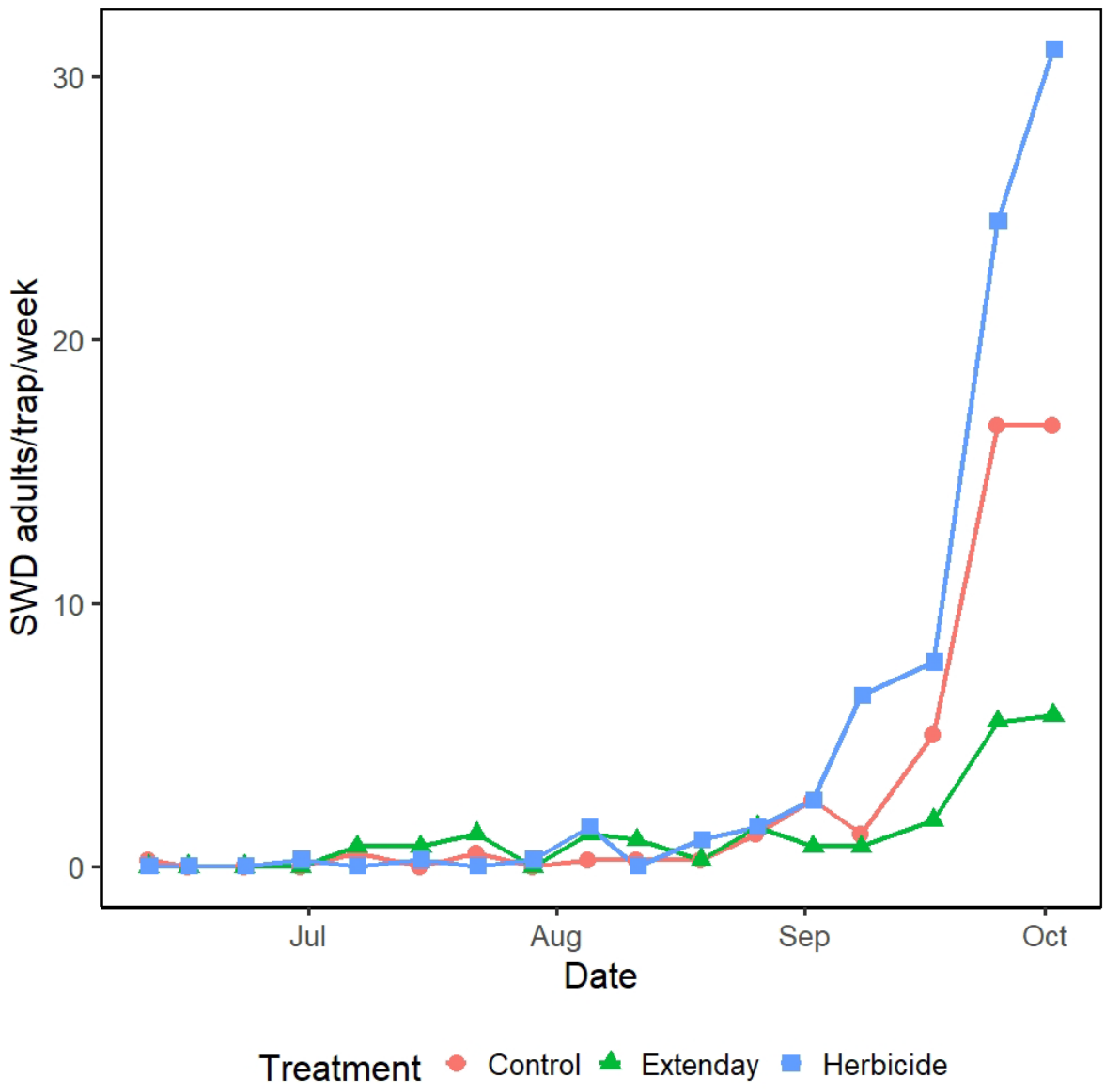

Fig. 6. SWD adult counts per trap per sampling date by treatment, Cashmere 2, 2022.

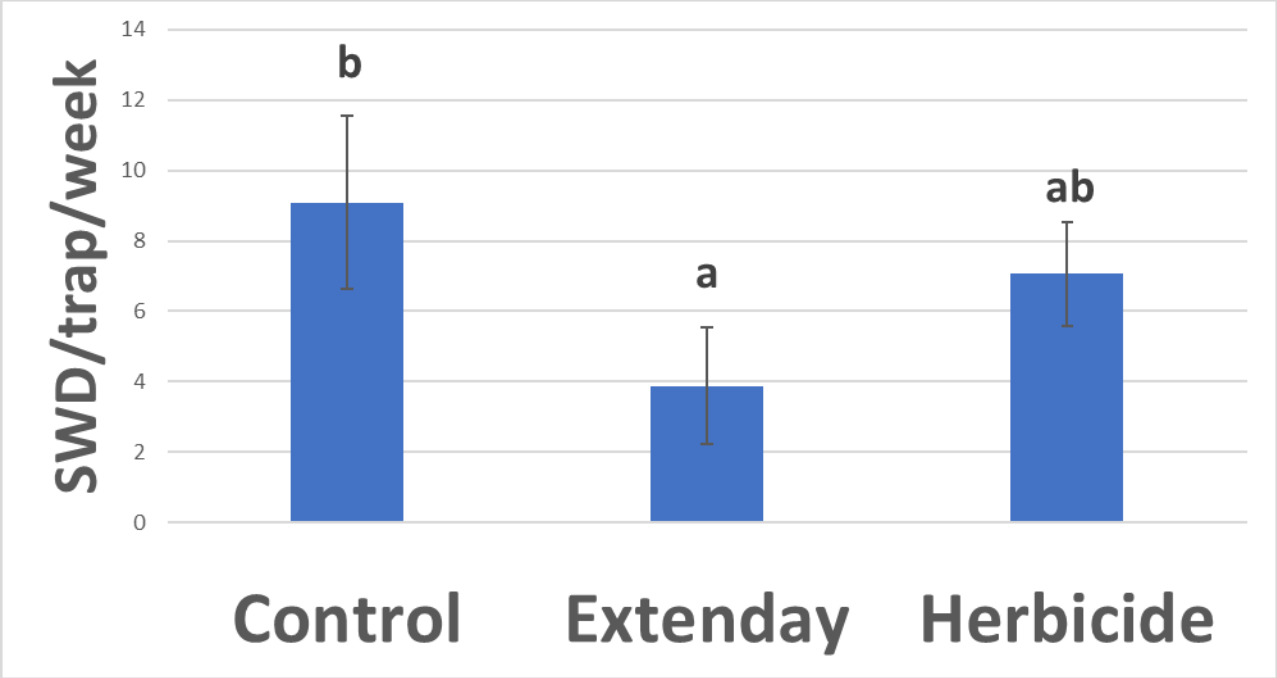

Fig. 7. SWD adult counts by treatment, Cashmere 1, 2021.

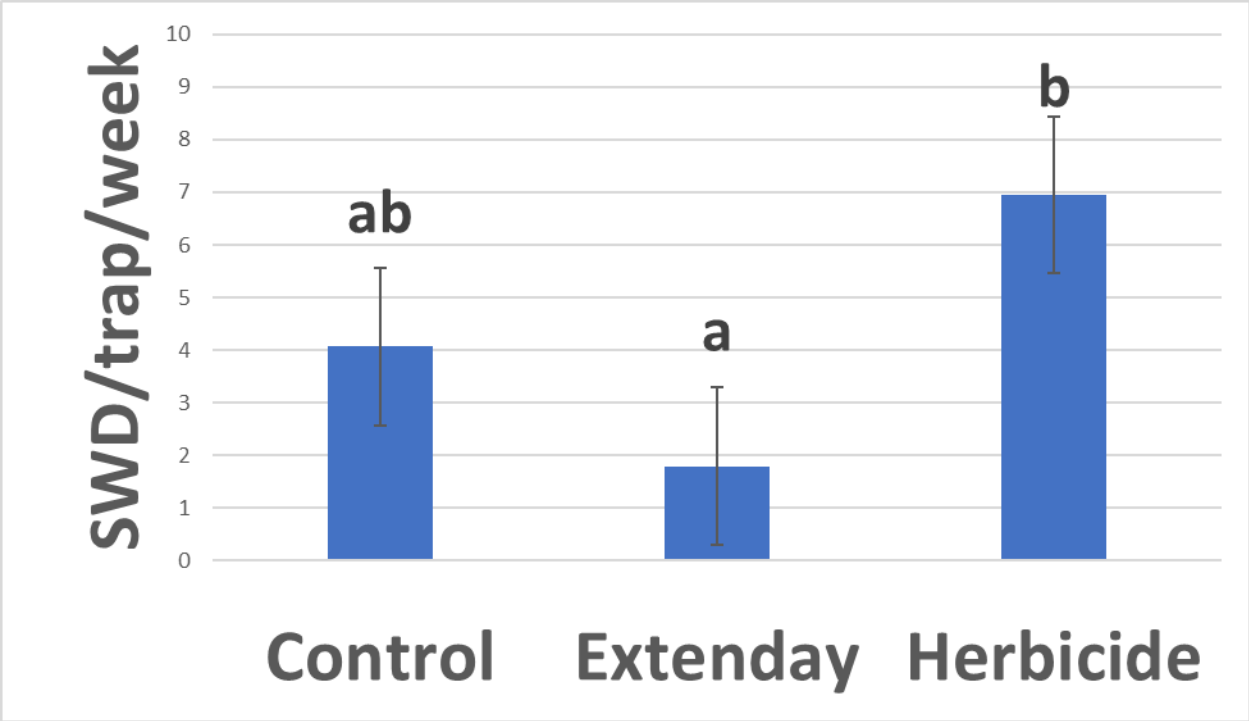

Fig. 8. SWD adult counts by treatment, Cashmere 2, 2022.

FINAL REPORT **PROPOSED DURATION**: 1 Year

Project Title: Towards identification of LCD linked volatile biomarkers

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Collaborator: Sindhuja Sankaran
Cooperators: Garrett Bishop, GS Long

Equipment cost match: $60,000 (FAIMS-Lonestar VOC Analyzer, Owlstone Medical, UK)

Total Project Request: **Year 1: $62,310** **Year 2:** **Year 3:**

Other funding sources: **None**
Amount:
Agency Name:
Notes:

WTFRC Budget: none

Organization Name: WSU-IAREC **Contract Administrator: Katy Roberts**
Telephone: 509-335-2885 **Email address: arcgrants@wsu.edu**

Item	2021	2022
Salaries	37,800	
Benefits	13,212	
Wages		
Benefits		
Equipment		
Supplies	7,750	
Travel	3,548	
Miscellaneous		
Plot Fees		
Total	62,310	0

Footnotes: 9-month salary support ($37,800 plus $13,212 benefits) for a postdoctoral researcher is requested. Postdoc will work closely with the PIs in planning and conducting experiments, data analytics and reporting. $3,500 requested to procure scrubber(s) for existing FAIMS unit along with recalibration. $3,500 engineering plant volatile trapping system, and towards procurement of specialty jars for VOC trapping, Teflon caps and tubing, labels, gloves, zip ties, chem-wipes as well as analytical standards needed to confirm VOC biomarkers. $750 is requested towards procurement of N2 gas ($75/tank). $348 is requested for field sampling related travel (20 miles x 30 trip x $0.58/mile) and $1,000 towards extension outreach activities. $2,200 is requested for Postdoc to participate in regional/international (within US) conference to share project outcomes.

Objectives.
#1. This project aims to screen infected and uninfected cherry plant parts, i.e. limb, leaves, stem and fruit tissues of the highly susceptible 'Bing' cultivar at different growth stages to identify potential volatile biomarkers associated with X-disease and/or LCD infection. Once biomarkers are identified, the platform will be trialled in controlled and field environments.

#2. Pertinent technology and finding will be communicated to the industry by an array of outreach and extension methods; including a technology demonstration or video/webinars, grower meetings, and the tree fruit newsletter "fruit matters".

For this continuation report, we have focused reporting on following two specific aims:

1. To evaluate feasibility of portable FAIMS towards LCD symptoms detection; and
2. To identify the earliest pre-symptomatic growth stage where LCD symptoms detection is possible with FAIMS system.

Significant findings

- A portable FAIMS system could detect LCD symptoms from field samples of 'Bing' Cultivar starting shuck fall to the post-harvest growth stages. System could also detect the symptoms for *cv*. Benton, Tieton and Cristalina at post-harvest growth stage (field as well as greenhouse samples).
- The FAIMS also detected the LCD symptoms from root tissue samples collected at post-harvest stage (*cv*. Benton and Skeena).
- The third ion current peak (see fig. 3; in the CV-DF ranges of -0.72 to 0.51 V & 72 to 98%) was consistent distinguishing feature in the spectra for infected samples but not for the healthy samples.
- The ion current for the infected samples was consistently higher than the healthy samples for identified significant CV-DF combinations.

Industrial and economic significance. Findings of this study suggest that it would be possible to achieve high throughput detection of LCD symptoms using a portable FAIMS system starting pre-symptomatic growth stages. The FAIMS system could thus be useful as a complimentary LCD confirmation tool in the laboratory along with qPCR. Robust evaluation: for additional larger datasets at each of the growth stage for a given cultivar and 2) different susceptible cultivars needs to be performed, before industry considers using such system for high throughput and reliable LCD symptoms detection.

Methods
Sample preparation. **<u>Aim 1</u>**. The shoot limb samples of cherry trees were collected for the post-harvest growth stage from an orchard located in Buena, WA (*cv*. Benton). The samples sized approximately 15 cm in length and comprised of leaves and stems. The samples were collected from six trees of which three were confirmed with LCD infestation, and three with no detection (Healthy). These confirmations were provided by the WSU-Clean Plant Network (WSU-CPN) based on the molecular analysis (qPCR) in the previous growth season (2020).

Each sample contained four limb units collected randomly from the trees and four replicate samples were collected per tree. As a reference to the field samples, samples were collected from two confirmed negative (Healthy) trees of the same cultivar managed in a green house facility.

Aim 2. The shoot limb samples of cherry trees (*cv*. Bing, size: same as above) were collected at the flowering, shuck fall, pit hardening, first straw, and harvest growth stages from an orchard located in Wapato, WA. The limbs included flowers at flowering stage, some flower petals at shuck fall stage, light green fruits at pit hardening stage, yellowish fruits at first straw, and matured fruits at the harvest stage. Leaves and stems were present in all above samples at all the stages. Total nine trees were selected in the orchard; of which, six were confirmed with LCD infestation, and three with no detection. As in objective 1, these confirmations were provided by the WSU-CPN. Three replicate samples were collected randomly from each selected tree. Similar to objective 1, the reference samples were collected from confirmed negative trees of the same cultivar managed in a green house facility of the CPN. The samples considered for Aim#1 were also included in this objective for postharvest growth stage analysis.

The collected samples were kept in sanitized glass jars of 1 gal and sealed with a cling film wrap to allow aerobic respiration. The sealed jars were then stored for a duration of 3 hours for volatile headspace accumulation. Post the storage period, the volatile headspace of the jars was sampled using a portable FAIMS system.

Volatile sampling. Post the storage duration of each sample, the cling wrap was removed, and the jar was immediately covered with a Teflon lid (fig. 1). The lid had two openings of which one was connected to the carrier gas cylinder (inlet) and the other connected to the ionization chamber of the FAIMS (outlet) through Teflon tubes. Nitrogen was used as a carrier gas that streamed at a flow rate of 1.5 L/min and pressure of 50 kPa inside the jar through the inlet to push the accumulated headspace with volatiles through the outlet into the ionization chamber (fig. 1). These volatiles gain charge in the ionization chamber and then move and deflect in proportion to their mass, under the influence of a dispersion field (DF or electric field) and compensation voltage (CV or electric potential). Such movement/deflection creates ion current spectra. Total six ion current spectra were collected for each sample jar and pertinent ion current spectra files were saved in the FAIMS computer. These files comprise of ion currents for a total of 26,112 CV-DF (512×51) combinations.

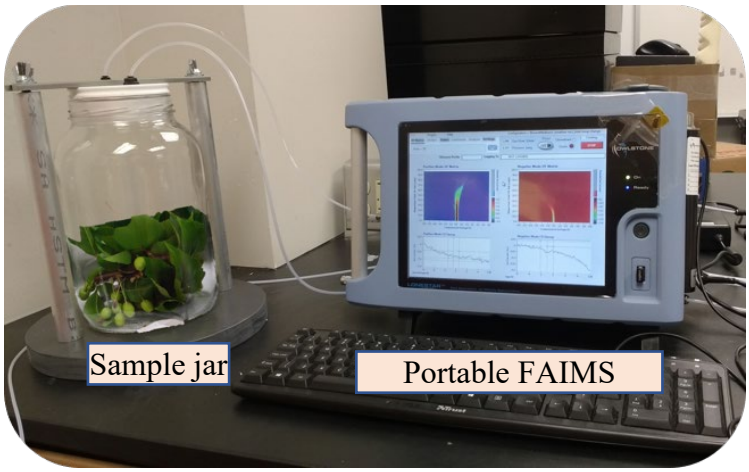


Figure 1. Volatile headspace sampling of cherry samples using a portable FAIMS system.

Data analysis. The data analysis steps are summarized in figure 2. The ion current spectra files were extracted into "*csv" format for further analysis. For each sample, two middle ion current spectra were used during the analysis. The current patterns were initially evaluated to identify the distinctness between the LCD positive (Infected) and non-positive samples (Healthy). Based on initial visual observations, a consistent threshold filter was applied to extract the ion current peaks for the two sample types. A region of interest (ROI) was then fixed for a range of CV and DF for all the samples. The ion current feature in this ROI was extracted for each ion current spectra and statistical difference in their magnitudes was evaluated between the infected and healthy samples. Next, a principal component analysis (PCA) was conducted to recognize the differences between the two sample types. All such analyses were first conducted for the field samples and were then contrasted with the green house samples.

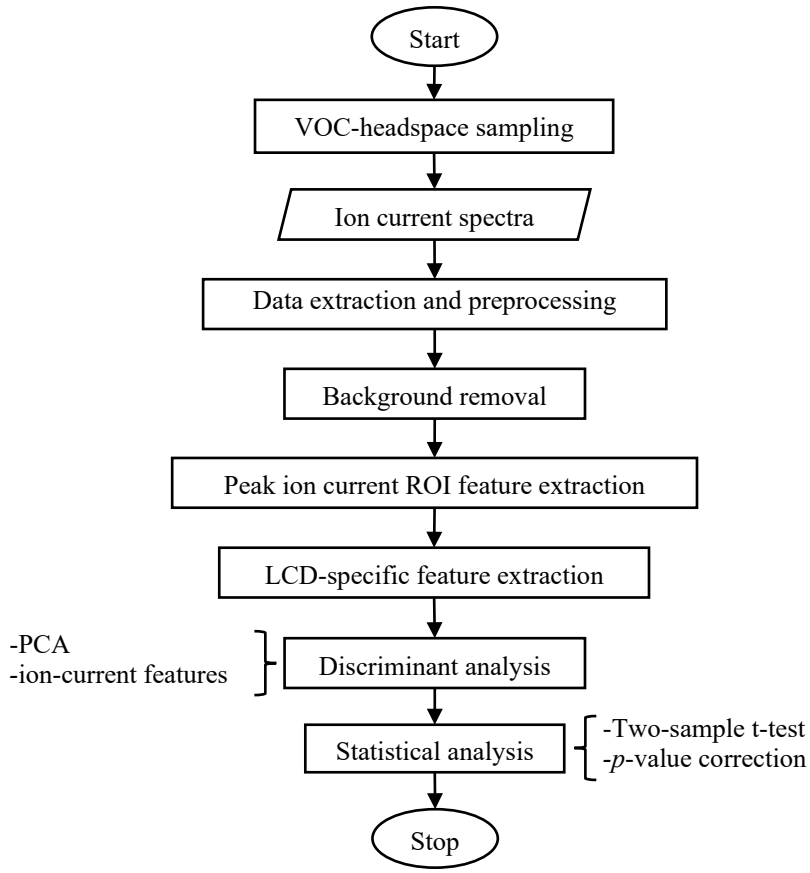


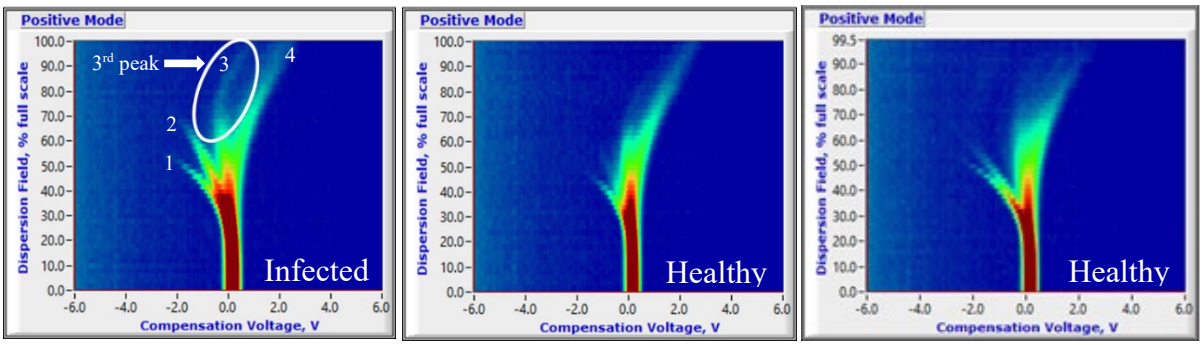
Figure 2. Data analysis pipeline for evaluating FAIMS for LCD detection.

Results and Discussion

<u>Aim 1</u>. To evaluate feasibility of portable FAIMS towards LCD symptoms detection.

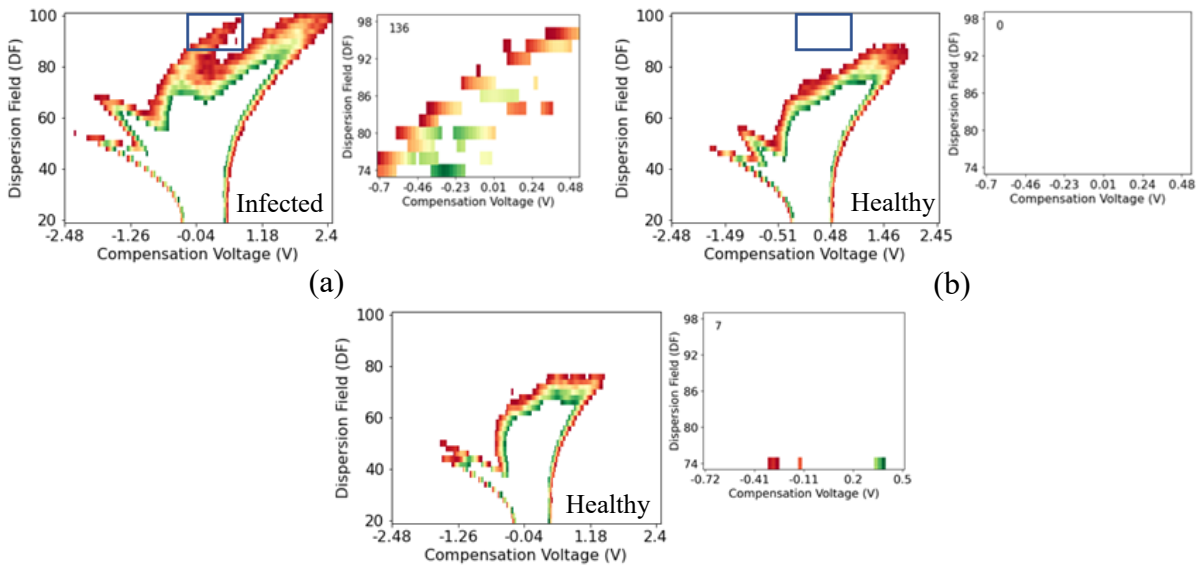
The raw ion current spectra derived as an output of the volatile-headspace sampling by FAIMS system were distinct for infected and healthy samples at postharvest stage. Herein, a third ion current peak was consistently dominant for the infected samples (See fig. 3a for CV (x-axis) range of -0.72–0.51 V, and for DF (y-axis) range of 72–98%). However, such peak was not observed in the ion current spectra for the healthy samples (fig. 3b). This observation suggests that the healthy samples may not display a third peak in the ion current spectra as was also observed by the ion current spectra for healthy reference samples from greenhouse (fig. 3c).

The processed ion current spectra obtained after noise removal from the raw spectra is shown in figure 4. Herein, the presence of ion currents in the fixed ROI (CV-DF ranges of -0.72–0.51, and 72–98%) confirms the above observations for LCD infected samples (fig. 4a). The absence or negligible ion currents in ROI for healthy samples also confirms the above observation for healthy samples (figs. 4b and 4c).



(a) (b) (c)

Figure 3. Ion current spectra for (a) infected and (b) healthy samples from the orchard and (c) healthy samples from green house (Postharvest growth stage).

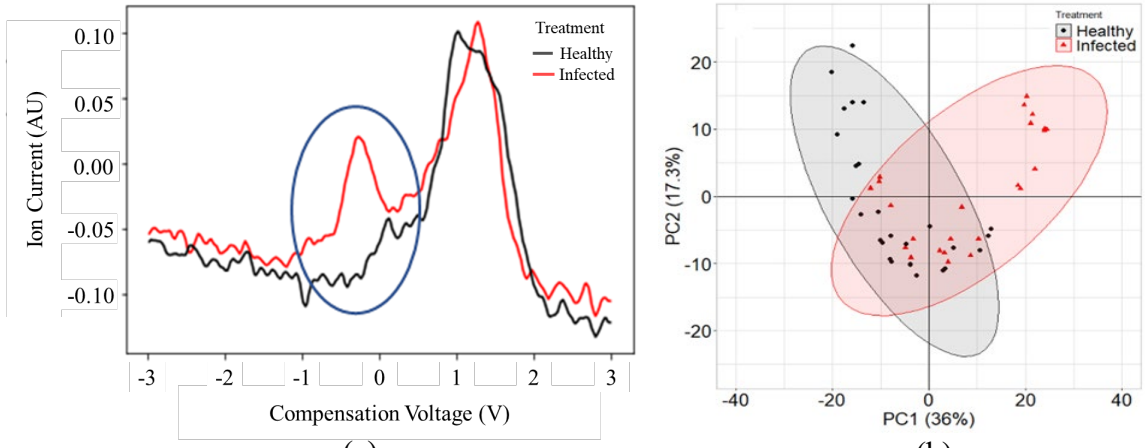


(a) (b)

(c)

Figure 4. Filtered ion current spectra and features in the fixed region of interest for (a) infected and (b) healthy samples from field and (c) healthy samples from the greenhouse.

Post feature extraction, the magnitude of ion current for the infected samples was significantly and consistently higher than the healthy samples (fig. 5a). These ion currents for all the infected and healthy samples, when analyzed with PCA, showed distinct patterns (fig. 5b). Overall, FAIMS could be highly suitable for detection of LCD symptoms at postharvest stage. Also, about 40% of the total 26,112 CV-DF combinations (at 5% level) and 11% combinations (at 1% level) were critical and aided in distinguishing the healthy and infected samples.



(a) (b)

Figure 5. (a) Ion current magnitudes and (b) their pattern distinction for healthy and infected samples using principal component analysis.

<u>Aim 2</u>. To identify the earliest pre-symptomatic growth stage where LCD symptoms detection is possible with FAIMS. Similar to observations in objective 1, the third peak (as a dominant peak) was observed from shuck fall until postharvest growth stages (fig. 6). This peak initiated at shuck fall and strengthened in intensity with the crop growth stage. The peak was however inconsistent at the flowering stage (fig. 6a). With these preliminary observations, it can be inferred that LCD symptoms could be detected as early as at the shuck fall growth stage. Moreover, pertinent to the third peak, the ion current magnitudes were significantly higher for the infected samples compared to the healthy samples at all the growth stages (fig. 7).

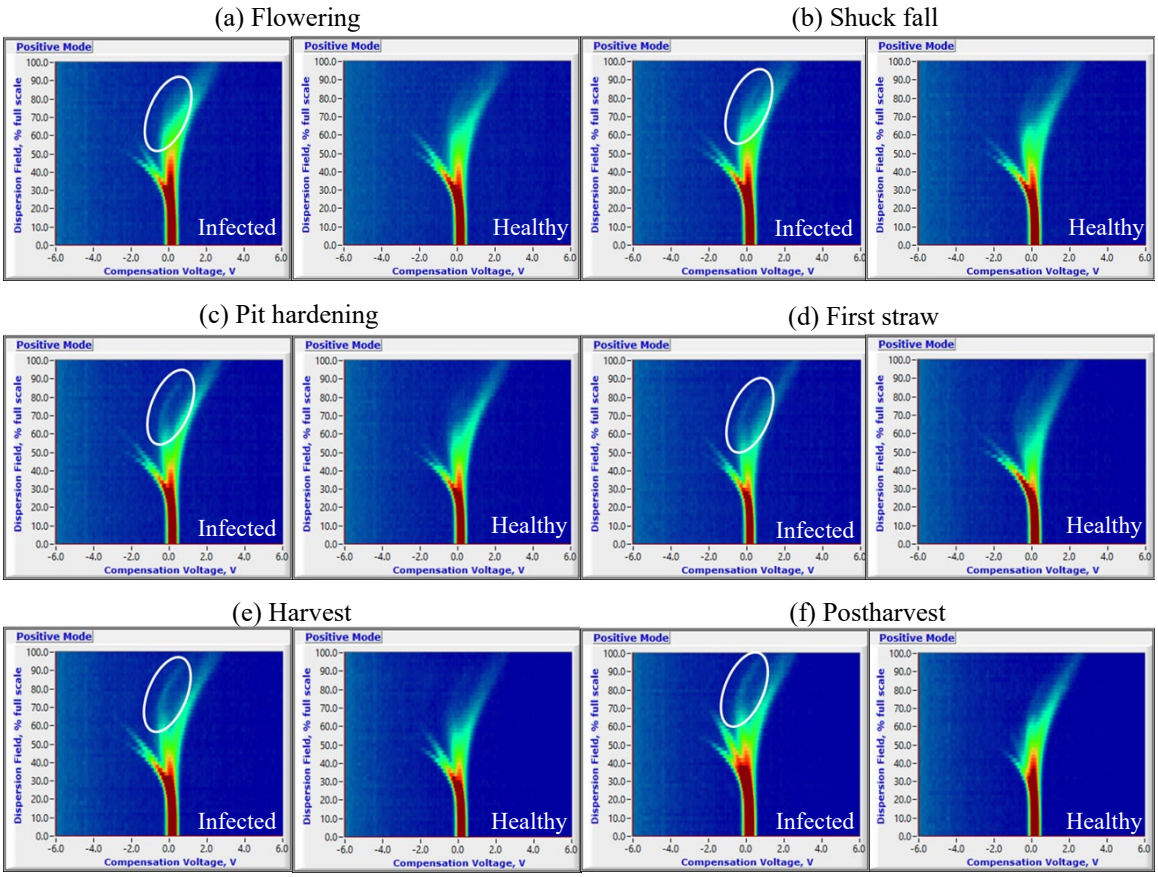


Figure 6. Raw ion current spectra plots for infected and healthy cherry samples collected from the orchard at (a) flowering, (b) shuck fall, (c) pit hardening, (d) first straw, (e) harvest, and (f) postharvest.

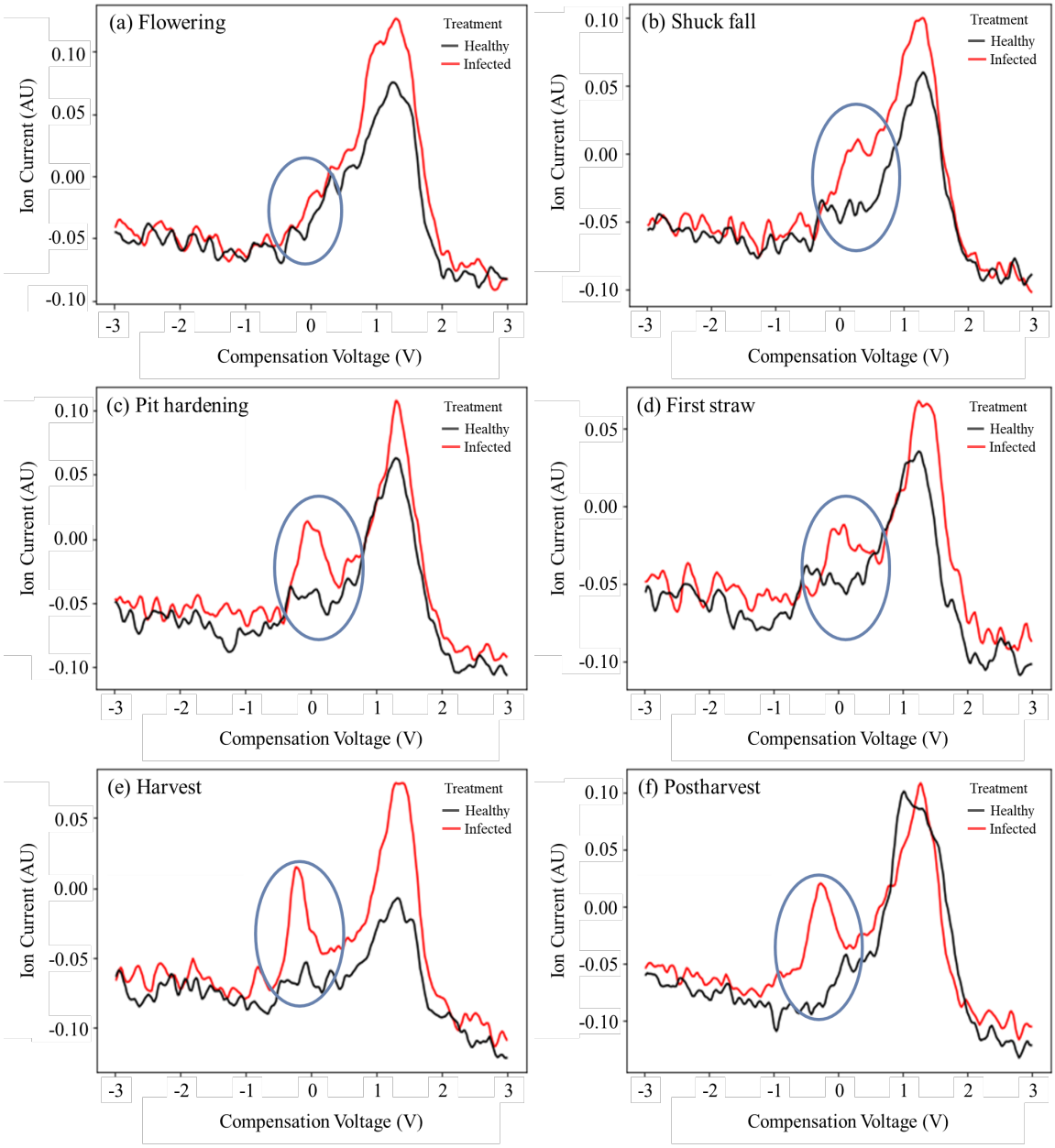


Figure 7. Ion current plots for the infected and healthy samples (differences highlighted in blue ellipse) at (a) flowering, (b) shuck fall, (c) pit hardening, (d) first straw, (e) harvest, and (f) postharvest.

Ongoing and future work:

<u>Robust analysis of existing data, FAIMS system training w/identified CV-DF combinations</u>: The FAIMS ion current data from the season 2020-21 identified the third ion current peak as a feature peak for the infected samples (*cv*. Bing). Additional data was collected to confirm the presence of this feature peak for cultivars: Skeena, Benton, Tieton and Crystalina. The feature peak was observed with different shape and intenisty in all the

tested cultivars. Our team is working on identifying the generalized range of CV-DF cominations that will be potentially used for early and rapid identification.

Collect new datasets: Through new two year project (2022-2024), plant samples including flowers, leaves, fruits and root tissue were collected from the field grown sweet cherry trees for 'Bing' and 'Skeena' cultivars. The FAIMS ion current data for the growing season 2022 confirmed that the signature peak is present in the infected samples.

Linkage with LCD detection dogs: Samples (stem) were collected for infected and healthy trees for the 'Skeena' cultivar at post-harvest stage in 2022 field season. These samples were analyzed using the FAIMS system as reported in the methods section. Samples were also collected from the same trees for LCD detection dogs. Our team is working on the data analysis and these efforts will be contrasted with the LCD detection dogs derived data.

Confirmation of volatile biomarkers release using GC-MS technique: Through new two year project (2022-2024), samples from the healthy and infected trees were collected for in-situ analysis using GC-MS system for the 2022 growing season ('Bing' and 'Skeena'). Results infers that Z-3-Hexenal and Z-2-pentenal are prominantly distiguishable and could be related to the LCD infection in 'Bing' cultivar. Analysis for 'Skeena' cultivar is on going along with additional method of volatile headspace sampling (partially destructive analysis method). These key volatile biomakers linked with the infected samples can be potentially used to 1) identify and develop a customized volatile sensing system, 2) develop FAIMS based detection alert system, and 3) to train the LCD detection dogs.

Executive summary

Little cherry disease (LCD) has been critically affecting the sweet cherry (Prunus avium) industry in the Pacific Northwest. Therefore, this study aimed at evaluating a high throughput field asymmetric ion mobility spectrometry (FAIMS) system towards early detection of the LCD infection of sweet cherry. Total fifteen trees were selected in two cherry orchards at Wapato, WA (*cv*. Bing) and Buena, WA (*cv*. Benton) which were confirmed as infected and healthy by the WSU Clean Plant Network. Shoot samples that included flowers, leaves, fruits, and stems were collected from the selected trees in each of the ***six*** growth stages: flowering, shuck fall, pit hardening, first straw, harvest and postharvest. Collected samples were stored in 1-gallon glass jar for three hours for volatile headspace accumulation. Post-storage period, accumulated headspace was sampled with FAIMS, and ion current spectra were acquired for each sample jar. A consistent presence of third ion current peak was observed in the spectra for infected samples but not for the healthy samples. Such infection-specific peak was observed from as early as shuck fall growth stage. Those peaks were present for compensation voltage (CV) range of −0.72–0.51 V and dispersion field (DF) range of 72–98% for all the growth stages. Pertinent to those peaks, the ion current for infected samples was significantly higher compared to healthy samples (Two-sample t-test, $p < 0.05$). Such observations were also supported by the healthy samples collected from greenhouse grown trees. A Principal component analysis showed the distinctness in the patterns formed by the infected and healthy

cherry samples. Similar findings were observed from the 2022 season data for infected and healthy samples from 'Bing' and 'Skeena' cultivar. The presence of third peak (CV: −0.66–0.55 V and DF: 70–98 %) was observed consistently in the 'Bing' cultivar whereas for 'Skeena' cultivar it was somewhat inconsistent. Overall, a portable FAIMS system was able to detect LCD infection symptoms at a high throughput rate and from pre-symptomatic growth stages. With robust databased investigation, portable FAIMS systems can be trained using the common features (e.g. third peak) for alarm-based alerts which could assist in timely identifying the LCD infestation in sweet cherry orchards.

Keywords: Little cherry disease, FAIMS, High throughput capacity, Ion current features, Alarm-based alerts.

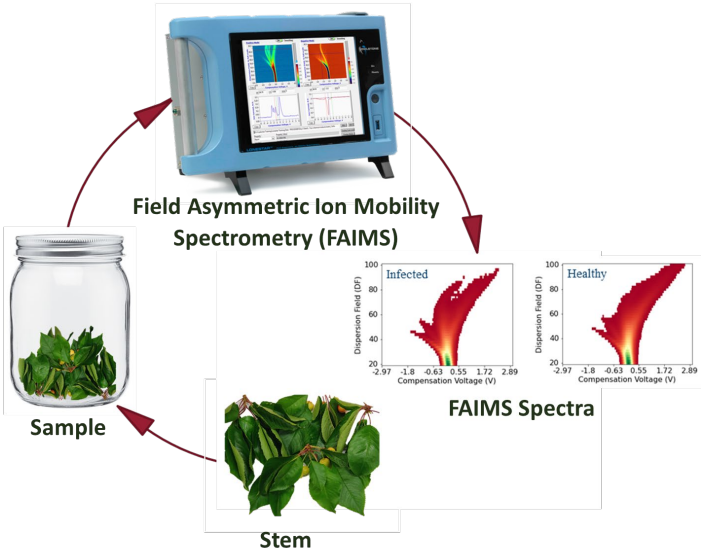


Figure 8. Schematic of little cherry disease detection using a portable FAIMS system.

Towards the identification of Little cherry disease linked volatile biomarkers

Report Type: Continuing Project Report

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Cooperators: Garrett Bishop, GS Long
Collaborator: Lynda Pheasant; Bernardita Sallato; Jacqueline Serrano; Sindhuja Sankaran
Equipment cost match: $60,000 (FAIMS-Lonestar VOC Analyzer, Owlstone Medical, UK)

WTFRC Budget: none

Organization Name: WSU-IAREC **Contract Administrator: Katy Roberts**

Telephone: 509-335-2885 **Email address: arcgrants@wsu.edu**

Item	2022	2023
Salaries	46,200	48,048
Benefits		
Wages		
Benefits	16,014	15,120
Equipment		
Supplies	15,120	16,655
Travel	2,697	2,697
Miscellaneous		
Plot Fees		
Total	80,031	82,520

Footnotes: Year-1: 11-month salary support ($46,200 plus $16,014 benefits) for a postdoctoral researcher is requested. Postdoc will work closely with the PIs in planning and conducting experiments, data analytics and reporting. $1,500 requested to procure FAIMS sampling experiment lab consumables such as PTFE tubing, sampling glass jars, gloves, chem-wipes, headspace trapping cling films and N2 carrier gas. $7,120 requested to procure Tedlar bags ($178/ pack of 10 bags x 40 pack) for plant volatile trapping, and $5,000 towards PI-Ruddell's GC/MS consumables and maintenance. $2,697 is requested for field sampling related travel (150 miles /trip x 20 trips) as well as GC/MS analysis travel from Prosser to USDA-ARS Wenatchee, WA (5 nights per diem + 270 miles/trip). PI Serban requests $1,500 towards extension outreach activities. **Year-2**: request is similar to Year 1 expect salary rate is adjusted by 4% per WSU policies.

Objectives

1. Volatile biomarker-based early X-disease and LCD infection detection for 'Bing' and 'Skeena' cultivars using FAIMS technique,
2. Develop a comprehensive understanding of associated volatile biomarkers release using GC/MS technique, and
3. Conduct pertinent extension education and technology demonstrations.

Significant Findings

Objective 1

- The FAIMS system could detect the disease symptoms from infected 'Bing' trees as early as flowering growth stage. The distinguishing ion current peak was at compensation voltage: -0.66 to 0.55 V & dispersion field: 70 to 98% combination. The ion current peak intensity varied between the growth stages and may be related to titer distribution changes throughout the season.
- For 'Skeena' cultivar, although distinguishing ion current peak was present at flower stage, later stages had confounding results and further investigation is on-going.

Objective 2

- The 'in-situ plant tissue based volatile analysis' using GC/MS suggested that Z-3-Hexenal and Z-2-pentenal were prominently distinguishable and seem to be related to the LCD infection in 'Bing' cultivar. Two additional peaks related to unknown compounds do exist and are also being investigated. Team is also in process of analyzing GC/MS samples for 'Skeena' cultivar and of the 'partially destructive volatile analysis method' for both cultivars.

Objective 3

- The project results were shared with stakeholders during 'LCD Field Day' (June 21, 2022) held at Buena, WA (Attendance: ~70). The project outcomes were also shared to the research community as a session talk at The American Society of Agricultural and Biological Engineers-AIM 2022 held at Huston, TX.

Methods

Sample collection and preparation. Throughout the 2022 growing season, samples, including flowers, leaves, and fruits, were collected from the lower canopy zones of the field-grown trees in Washington State (Wright et al., 2021; 2022). The samples were collected at the vital growth stages: flowering, shuck fall, pit hardening, first straw, harvest, and post-harvest for the cultivars "Bing" and "Skeena" (Table 1; Fig. 1). Trees in the experiment were selected based on the preliminary infection results provided by Clean Plant Network (CPN), Prosser, WA. Trees and branches were labeled with colored tape and labels for consistent

data collection throughout the season. The root tissue analysis was conducted at the post-harvest growth stage for both cultivars.

Table 1. Experimental design

Site	Cultivar	Growth stage	Method*	Samples
Site 1	Bing	Flowering	1,2,3	Infected: 5 Negative: 4
		Shuck fall	1,2	
		Pit hardening	1,2	
		First straw	1,2	
		Harvest	1,2,3	
		Post-harvest	1,2	
Site 2	Skeena	Flowering	1,2	Infected: 4 Negative: 4
		Shuck fall	1,2	
		Pit hardening	1,2	
		First straw	1,2	
		Harvest	1,2,3	
		Post-harvest	1,2	

*1: FAIMS; 2: GC/MS; 3: qPCR technique

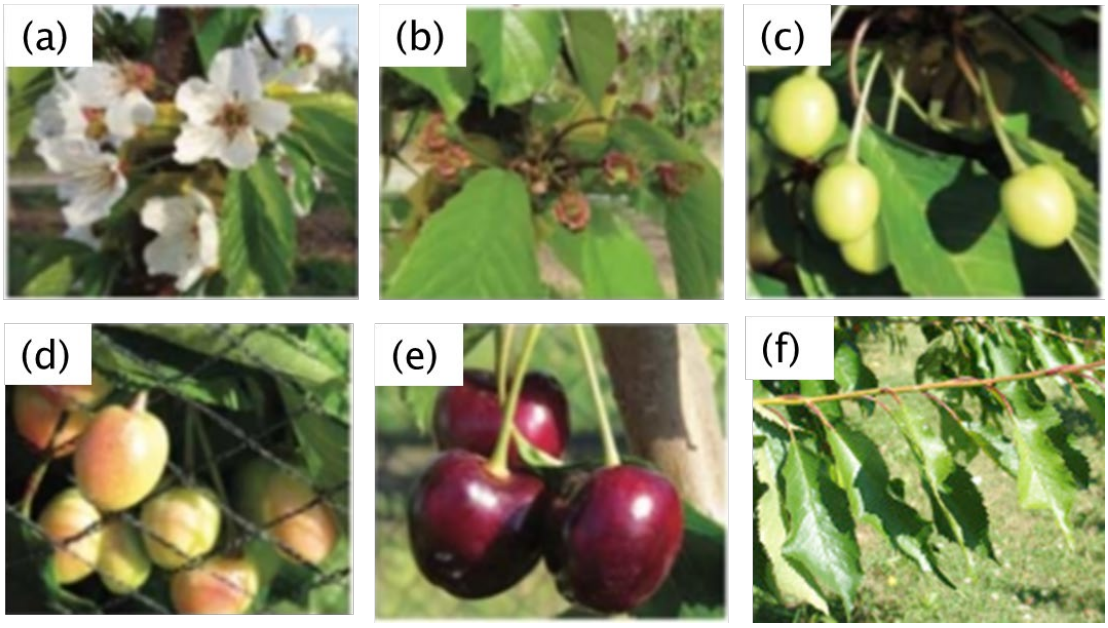


Figure 1. Samples at different growth stages, (a) flowering; (b) shuck fall; (c) pit hardening; (d) first straw; (e) harvest, and (f) post-harvest.

FAIMS Sampling. Collected samples were stored in the 1-gallon glass (sterilized) jars. Each sample jar was covered with a food-grade cling film for aerobic storage conditions (Fig. 2). Samples were then analyzed using a portable FAIMS system using a custom-developed unit (Arasaradnam et al., 2016; Kothawade et al., 2021). The glass jars were covered by a Teflon lid having two stoppers with two holes,

one as an inlet for the carrier gas source (nitrogen air) and another to flush out the VOCs to the FAIMS ionization chamber. A total of six scans were conducted for each sample jar at the optimized operation parameters (flow rate: 1.5 L min^{-1} and pressure: 60 kPa).

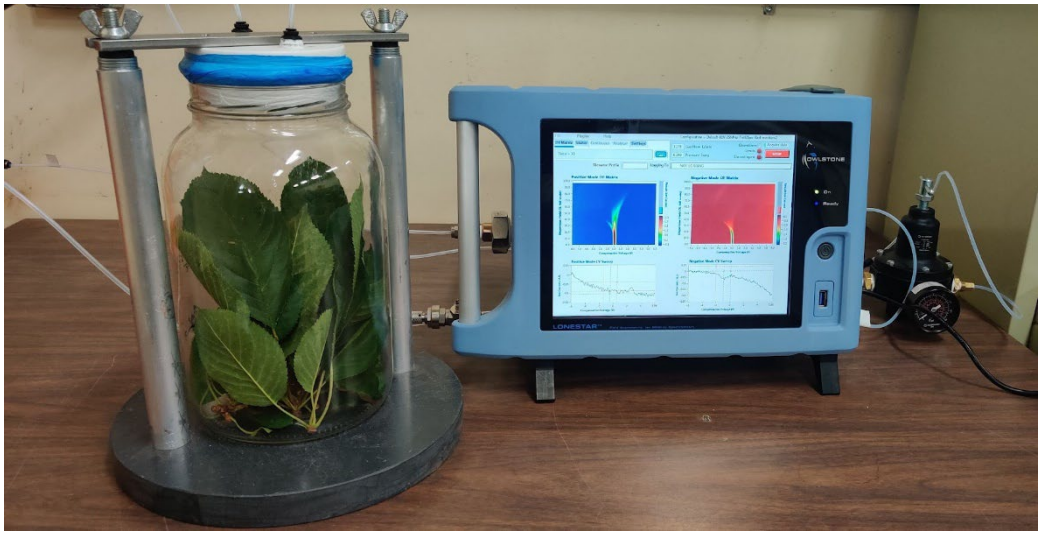


Figure 2. A portable FAIMS system analyzing cherry leaves volatile profile.

The FAIMS scans output is an ion current spectrum that is proportional to the mass of distinct VOCs under a range of dispersion fields (DF: 0 to 100%) and compensation voltages (CV: -6 to 6V). The ion current spectrums from all scans generate three-dimensional data consisting of 51 DFs, 512 CVs, and resultant ion currents (arbitrary units, AU). The system was purged for about 40 minutes using nitrogen air before scanning the next sample jar to remove the residues from the previous sample. A blank jar was also examined as a reference in data analysis for each sampling day.

GC-MS analysis. The in-situ plant tissue based volatile analysis was conducted at the first straw and harvest stage. For the sample collection, 50 ml falcon tubes were used to store the samples (5 leaf/replicate). Liquid nitrogen was used to flash freeze the samples and stored them in dry ice until the samples were moved to the -80 °C facility. The plant tissue was ground using liquid nitrogen and kept at an -80 °C facility. The scaling of ground plant tissue (0.5g) in 20 ml glass vials was performed in the box filled with liquid nitrogen to avoid thawing the samples at room temperature.

HPLC water, 5-Hexen-1-ol, and Isopropyl butyrate was used to prepare an internal standard (ISTD) for further volatile headspace sampling. A polystyrene box was then filled with liquid nitrogen (1" covering the bottom), and an aluminum block with samples was placed in the box. Next, 10 µl ISTD and 1 ml NaCl saturated solution were added to the samples, and all samples were incubated at room temperature, followed by sonication and agitation. Aluminum tray with labeled glass vials was placed on the GC-MS system. Volatile headspace was adsorbed onto glass traps packed with Tenax TA porous polymer (TDU tubes, Gerstel, Linthicum, MD, United States) (Lee et al., 2002; Hewavitharana et al., 2019). Thermally desorbed analyte was injected and analyzed using Agilent (Santa Clara, CA, United States) 6890/5975 GC-MS equipped with a Gerstel (Baltimore, MD, United States) Multipurpose Sampler (MPS), Dynamic

Headspace Sampler (DHS), and Thermal Desorption Unit (TDU) (Rudell et al., 2009; Hewavitharana et al., 2019).

Samples including leaf tissue were collected from the same sites as reported above and stored in the 1-gallon glass jars for the partially destructive volatile analysis method. Volatile headspace from the jars was trapped on the adsorbent 'Tenax TA' packed in glass collectors using a vacuum pump. The glass traps were then analyzed on 6890/5975 GC-MS as reported above.

Figure 3. A GC-MS system used for in-situ volatile headspace analysis.

Chemical standards. In coming months, chemicals associated with the LChV-2 and X-disease phytoplasma will be analyzed using the FAIMS system as standards. The FAIMS operational parameters for the chemical standards will be optimized based on the different concentrations. Every analyte has a unique pattern. Prior to the experiments, FAIMS scans for these specific analytes will be recorded. For a sampling of these chemicals, the same setup will be used for consistency.

Molecular analysis. The samples were collected within a similar time frame and sites. The collected samples were kept in a cooler with ice packs. Post-sample collection, a small section of stem from each branch was used for phloem tissue extraction. Extracted tissue was chopped using a razor blade and stored in a bead-beating tube. For further processing, 0.1 g tissue was scaled in labeled tubes. The Nucleic acids from the samples were then extracted using the CTAB extraction method and stored at -20 °C. The amplified samples were used as the template for the qPCR reaction. The results of the molecular analysis were used for comparative analysis.

Data analysis

The raw FAIMS scans from the volatile's headspace analysis were extracted to the convenient file format ('.txt' and '.csv'). After preprocessing, relevant ion current peaks were used for further research. The third ion current peak was observed as a representative of the ion current response associated with the volatiles released from the infected samples, which was not consistently present in the negative samples. Based on the relevant ion current peak was extracted using a fixed range of CV and DF. Statistical analysis was performed on the extracted data, and the significant CV-DF combinations were used for further

analysis. The key LCD-associated volatile biomarkers were identified by matching mass spectra to the Wiley/NIST [National Institute of Standards and Technology] library in the software MassHunter (Agilent, Santa Clara, CA, United States). The extracted peak responses for the volatile compound were analyzed using different dimensionality reduction method like Principal component analysis (PCA) and Orthogonal partial least squares discriminant analysis (OPLSDA). The key volatile compounds were identified using statistical analysis and OPLSDA based VIP scores assessment. These results will be validated by comparing the GC RTs with the authentic standard chemical compounds. Moreover, some of the important chemicals identified by GC-MS analysis will be analyzed using the FAIMS system for further analysis that will help to quantify the associated variations in the volatile headspace for the LCD-infected samples (Fig. 4).

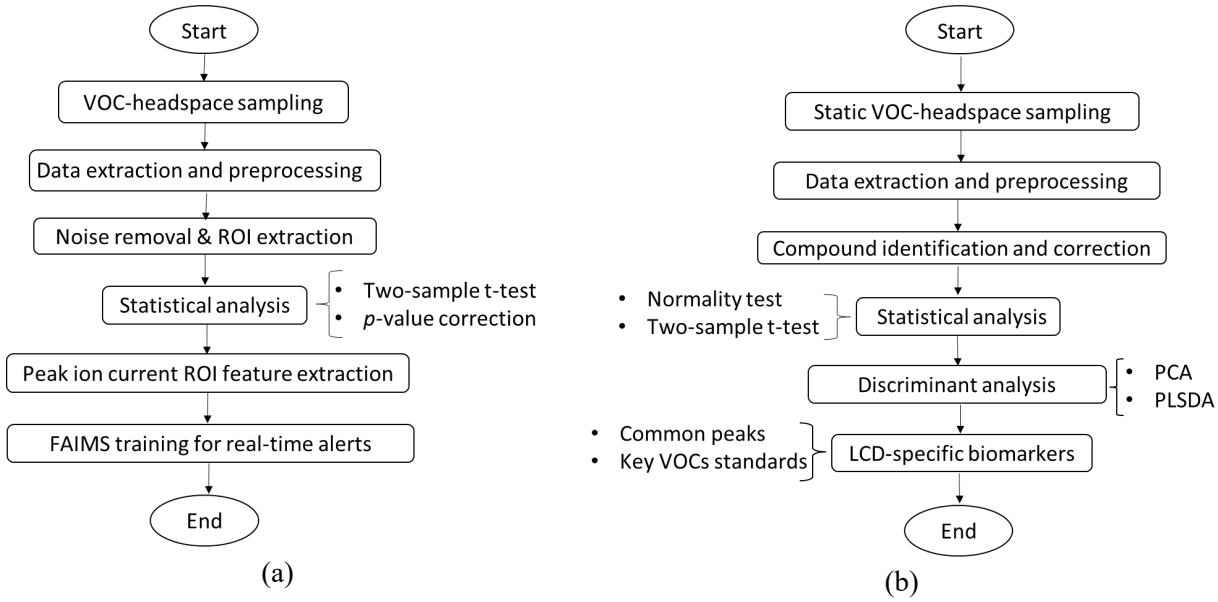


Figure 4. Data analysis flowchart (a) to identify CV-DF ion current features and (b) volatile biomarkers associated with LCD infection.

Results and Discussion

Objective 1. Volatile biomarker-based early X-disease and LCD infection detection for 'Bing' and 'Skeena' cultivars using the FAIMS technique.

The FAIMS data (ion current plots, Figure 5) were different for LCD-positive and healthy (non-detect) samples. The spectra produced four peaks, out of which the third peak (CV: -0.6 to 0.55 V and DF: 70 to 98%) was found to be a distinguishing feature. The observations suggest that third peak was not present in most of the healthy samples. Similar results were found in the 2021 season data for the 'Bing' cultivar. LCD-linked ion current peak was observed in the samples at flowering growth stage and was present throughout all the growth stages with different shape and pattern. The intensity and shape of this third peak varied between the samples and it could be due to the variation in biological composition of sample and the extent of the pathogen titer distribution in the tree.

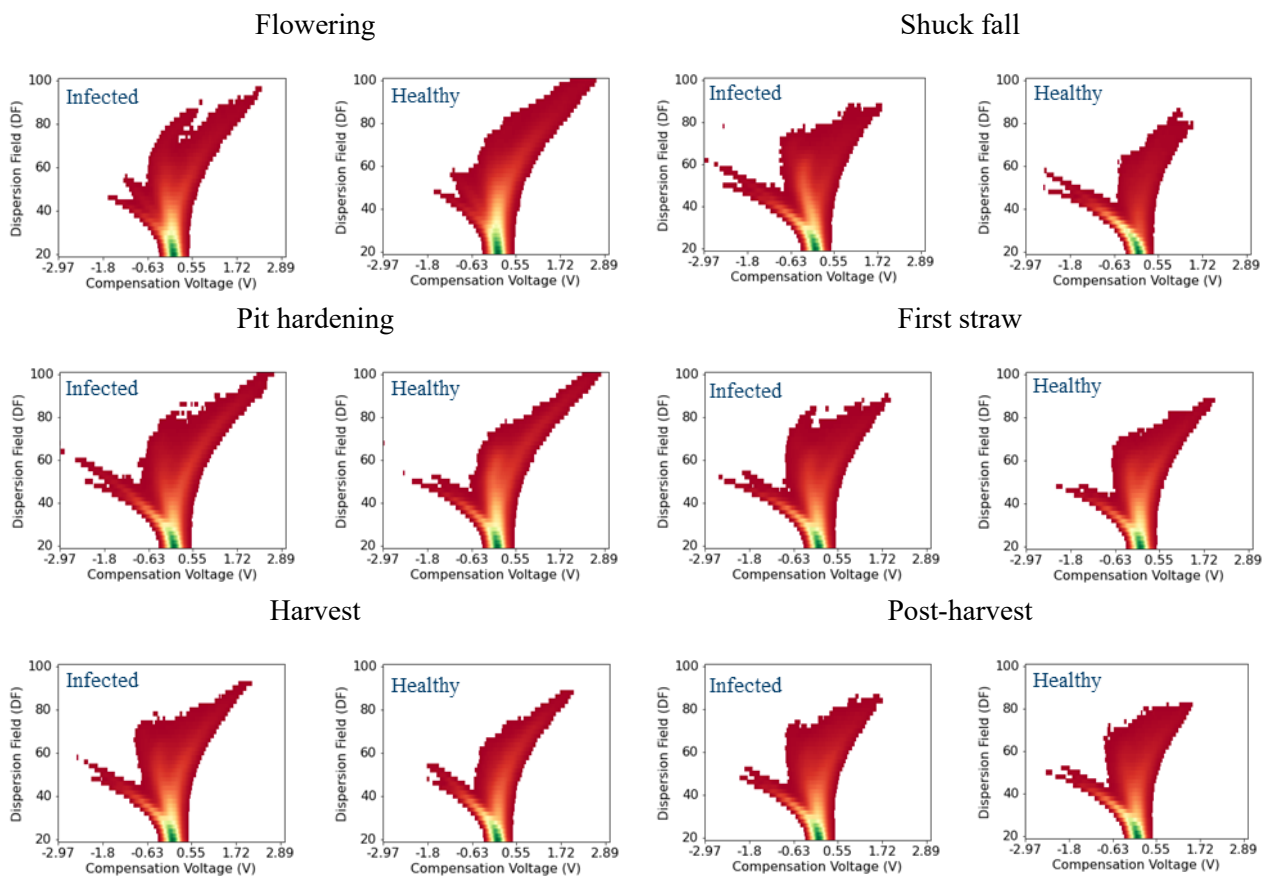


Figure 5. Typical FAIMS ion current spectra for the 'Bing' cultivar at the key growth stages.

Similar to the 'Bing' cultivar, the third peak (Fig. 6) was observed in a different shape and pattern for 'Skeena'. Occurrence of the distinguishing peak was inconsistent for 'Skeena' cultivar. This could be due to the cultivar specific plant volatile release pattern and can also be attributed to the 2022 season, which had a delayed growing cycle due to the late-season snow at flowering stage.

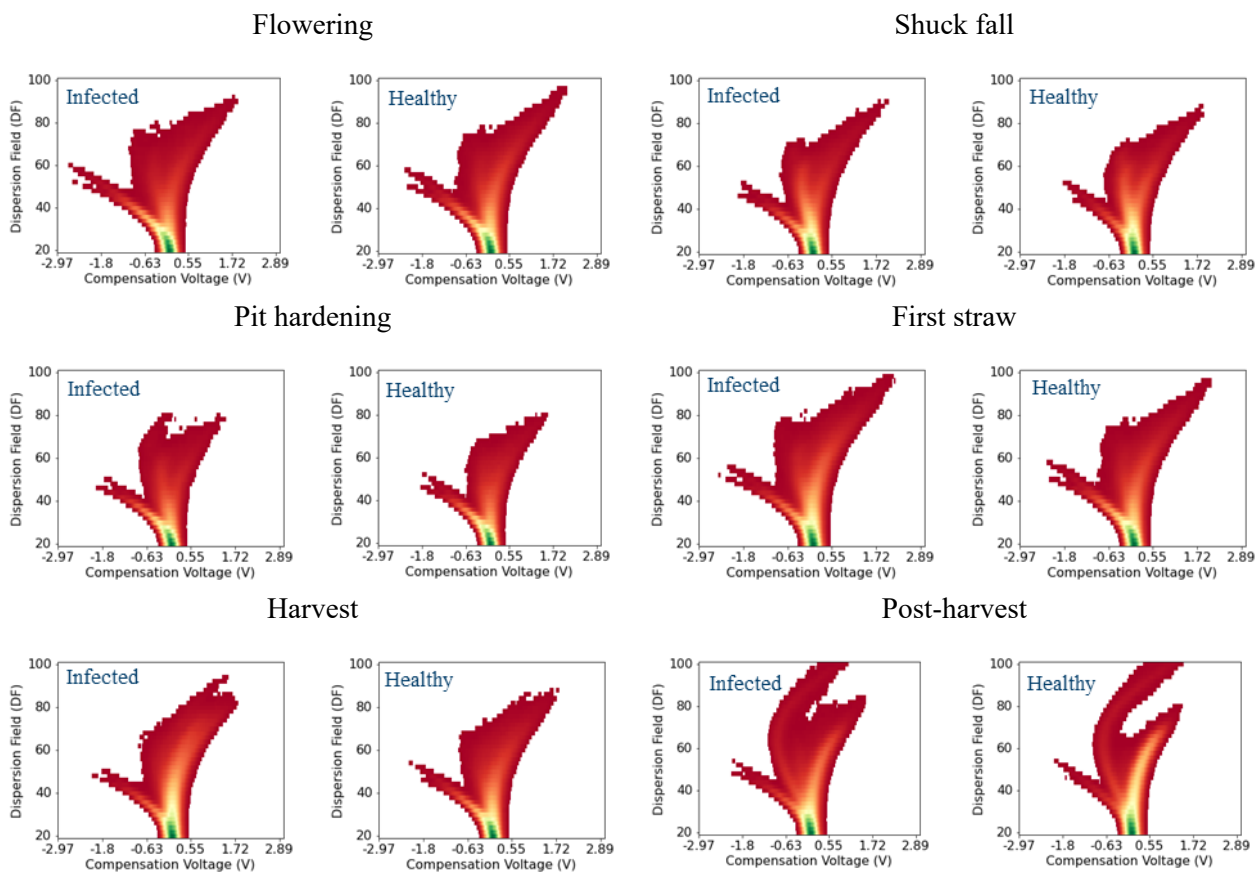
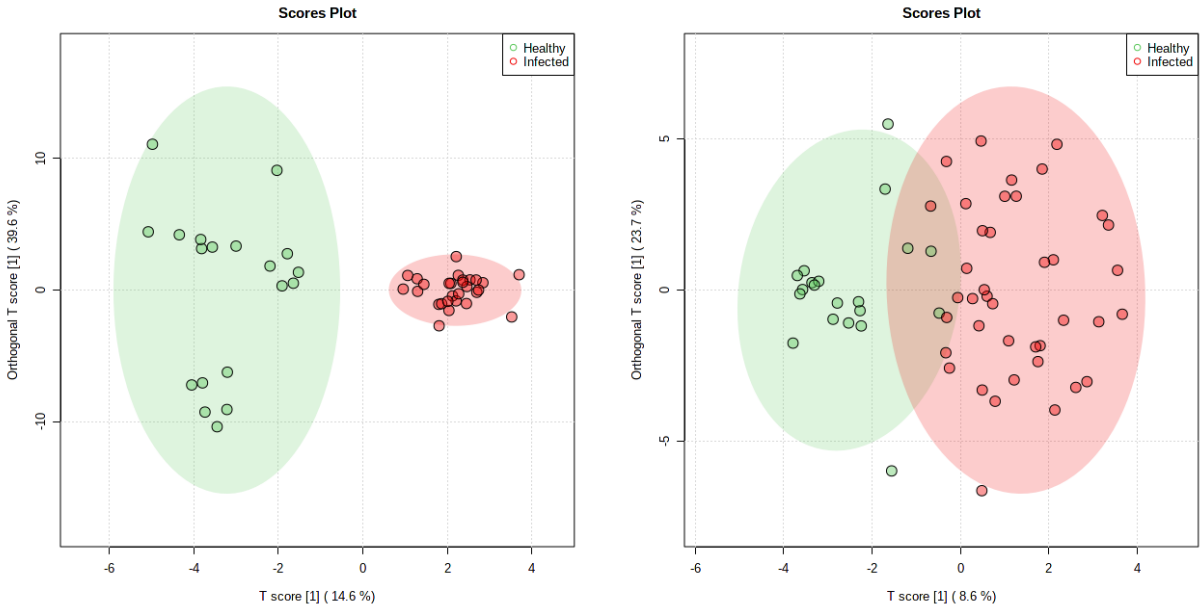


Figure 6. Typical FAIMS ion current spectra for the 'Skeena' cultivar at the key growth stages.

Above results need further statistical analysis and investigation to identify the key CV-DF combinations and ranges that can be used to identify the prominent peaks from an unknown sample. Also, the key volatile compounds identified using the GC-MS analysis will be analyzed through the FAIMS system for validation of the key peaks. Similar experiments for both cultivars with an additional number of samples will be conducted for 2023 field season. Our team has also collected FAIMS spectra of root samples for both cultivars. We are analyzing these spectra's as well.

Objective 2. Develop a comprehensive understanding of the associated volatile biomarkers release using the GC-MS technique.

The GC-MS analysis detected more than 100 compounds in the volatile headspace generated from the leaf tissue. A total of 15 significant features (peaks) were found after the statistical analysis ($p < 0.05$) of the extracted peaks from the gas chromatograph and mass spectra. Orthogonal PLSDA revealed the distinguished patterns between healthy and infected samples at first straw and harvest stage (Fig. 7). The key volatile compounds were filtered using the VIP scores from the OPLSDA analysis. It has been observed that the compounds Z-3-hexenal and Z-2-pentenal are prominent in the infected samples.



(a) (b)

Figure 7. Orthogonal Partial Least Squares-based pattern separation of the GC/MS spectral data for selected peaks at (a) first straw, and (b) harvest growth stage for 'Bing' cultivar.

Further research will be focused on running the identified key volatile compounds through the GC/MS system as 'standards' for confirmation. Also, the data generated from the 'partially destructive volatile headspace sampling' method for the same experimental sites and samples is being evaluated to confirm the key volatile compounds related to the infected samples. Additional metadata for each site and cultivar, such as titer level, other disorders, spray applications and visually observed symptoms will be considered during multivariate statistical analysis to confirm the qualitative and quantitative assessment of volatile compounds. As in 'Bing' cultivar, the analysis on the 'Skeena' cultivar is being performed.

Objective 3. Conduct pertinent extension education and technology demonstrations.

The project results were shared with stakeholders during 'LCD Field Day' (June 21, 2022) held at Buena, WA (Attendance: ~70, Fig. 8 left). The project outcomes were also shared to the research community as a session talk at ASABE-AIM 2022 held at Huston, TX (Fig. 8 right).

Figure 8. (left) demonstration of FAIMS system at LCD field day 2022, Buena, WA; (right) presentation of LCD findings at ASABE-AIM 2022, Houston, TX.

FINAL PROJECT REPORT YEAR: 1

Project Title: Canine LCD Detection Skills Applied to Nursery and Orchard Settings

PI: Lynda Pheasant
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Co-PI: Corina F. Serban
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Address: 2403 S 18th St. Suite 100
City/State/Zip: Union Gap/WA/98903

Cooperators: Michael Barclay and Janice Barclay (WKC instructors); WKC board members; WKC dog handlers (volunteers); Nathan J. Hall and Mallory DeChant (Texas Tech University), Hallie McMullen (Scentsational Detection Dogs); Scott Harper (WSU); Lav Khot and Gajanan Kothawade (WSU); Jeffery Bullock (Wenatchee Valley College); Teah Smith (Zirkle Fruit); Hannah Walters (Stemilt Growers)

Total Project Request: $ 45,500

Other funding sources: None

WTFRC Collaborative Expenses: None

Budget 1
PI: Lynda Pheasant
Organization Name: Wenatchee Kennel Club (WKC)
Contract Administrator: Lynda Pheasant
Telephone: (509) 393-9394
Contract administrator email address: pro-ag@charter.net

Item	2022
Salaries	
Benefits	
Equipment	$8,170
Supplies	$14,830
Travel	$12,000
Miscellaneous	$9,000
Plot Fees	
Total	$44,000

Footnotes: WKC budget consists in the following: Equipment: goes towards training equipment, technology equipment and freezer; Supplies: training aids and nursery stock; Travel: mileage reimbursement to participants and cooperators; Miscellaneous: cost for sample analysis.

Budget 2
Co PI: Corina F. Serban
Organization Name: Washington State University
Contract Administrator: Anastasia (Stacy) Mondy

Telephone: (509) 335-2885
Contract administrator email address: arcgrants@wsu.edu

Item	2022
Salaries	
Benefits	
Equipment	
Supplies	$593
Travel	$907
Miscellaneous	
Plot Fees	
Total	$1,500

Footnotes: Co PI Serban requests $593 towards extension outreach activities and $907 for travel.

OVERVIEW:

Little Cherry Disease (LCD) is an umbrella term used for two different pathogens – Little Cherry Virus-2 (LChV-2) and X-disease phytoplasma (XDP), that cause similar symptoms in cherries. LCD has reached an epidemic level of infection in Washington sweet cherry orchards, causing the removal of ca. 1,000 acres in the past five years according to a survey conducted by WSU/OSU Extension. Once a tree is infected, there is no cure for it, and the only control measure is the tree removal. Several factors are preventing the sweet cherry industry to stop the spread of the LCD infection. Among the most important, is the lack of optimized or new screening methods to quickly identify LCD infections. Canine detection skills project could provide an early detection tool for LCD identification, control, and eradication. The next step toward industry adoption and implementation is to conduct the structured integration of LCD detection dogs into nursery and orchard settings for further "live" training and assessment of skills. This project is designed to increase detection proficiencies and facilitate transition of canine detection skills from controlled settings to field environments.

The WKC is a non-profit (501c3) all-volunteer organization that was established in 1963. The WKC dogs that participated in this project varied in size, age, gender, breed, and training experience. The dogs are scent-sport competitor dogs trained by their owner/handler to perform a variety of canine sports such as obedience, rally, agility, hunting, lure coursing, barn hunt and competitive scent work. WKC trained consistently 5-7 LCD detection dog/handler teams to increase proficiencies to facilitate transition to field environments. Canine participants in this project include pedigreed dogs (some with champion/grand champion titles), dogs of nondescript "All American" breeding, and rescue dogs. The breeds that participated were: Rhodesian Ridgeback, Standard Poodle, German Shepherd, Rescued Mix-breed, Wheaten Terrier, Entlebucher Mountain Dog, German Shorthair, and Labrador Retriever. WKC dogs are not "purpose bred" for detection work, nor are the handlers paid professionals. Participants volunteered their expertise as dogs' handlers along with their dogs to the development and success of this LCD detection dog project. Geographically, participants represent all four counties of North-Central Washington: Chelan, Douglas, Grant, and Okanogan – counties having substantial economic investment in the cherry industry.

In March 2021 WKC initiated an Agricultural Detection Dog program as a pilot project to explore the following objectives: 1) Can companion dogs be trained to detect LCD, and 2) Can WKC develop an appropriate Agricultural detection dog training program. PI Pheasant has served as the WKC Project Coordinator from the onset. Board members Mike and Janice Barclay served as instructors from March – November 2021. Throughout the project, Board members John and Helen Njus have served as

professional videographers for WKC. The Barclays brought in professional detection dog trainer Cooperator Hallie McMullen who offered training consultation and establishment of a control group in Idaho. As a proof of concept, WKC invited Collaborator Nathan Hall (Director of the Canine Olfaction Research Laboratory at Texas Tech University) to evaluate proficiencies of the LCD Detection dog programs, at WKC and in Idaho. Mid-August 2021 the Ag Dog program lost three handlers and had to re-build by adding three other new handlers. On August 23, 2021, PI Pheasant (WKC) met for the first time with Corina F. Serban (WSU Extension) to explore the continuance of the WKC's "proof of concept" pilot project into submitting a proposal project with the WTFRC. Based on that meeting and seeing the Ag Dog class in action, Pheasant and Serban agreed to be Co-PI's on the research project and submitted a new research proposal to the WTFRC in Oct 2021. The loss of the trainers for the Ag Dog class in Nov 2021, Mike and Janice Barclay, left the project with new challenges, creating a delay and reset of the project. In Jan 2022 the Canine LCD Detection research project was formally initiated. Class program was hampered by extreme weather and by lack of root and bark samples. In March, Cooperator Hallie McMullen conducted a workshop with focus on preparation for April 2022 evaluations. Cooperator Mallory DeChant replaced Cooperator Nathan Hall conducting the evaluations at WKC (none performed in Idaho). Two out of the six dogs passed the proficiency test with 90% accuracy. Moving forward, the dogs were given opportunities to work in the "field" environments even though several of them had zero experience in a commercial cherry orchard. Throughout 2022 the Ag Dogs have held nine instructional field experiences at local cherry orchards and research plots. In July 2022, due to numerous events such as extreme heat, and the need to increase the training proficiencies it was decided to hold the trainings and evaluations indoors at WKC Training Center in East Wenatchee. PI Pheasant stepped away from this project at the beginning of August 2022. Afterwards Co-PI Serban lead the trainings for the month of August while coordinating the weekly training plan with Cooperator Hallie McMullen. At the end of this project Cooperator Hallie McMullen held an evaluation on Sept 5, 2022.

OBJECTIVES:

1. *Increase LCD detection proficiencies in controlled indoor/outdoor settings*
   Three double blind tests/evaluations were run during this project. One prior to the start during proof of concept with Collaborator Nathan Hall (August 2021), one at the midpoint with Collaborator Mallory DeChant (April 2022), and one at the end with Collaborator Hallie McMullen (September 2022).

2. *Develop next-step training protocols to facilitate transition into field experience*
   The dogs were given introduction to the plant materials and orchard experiences. This objective was in development including developing of the protocols. As the dogs were reaching proficiency in Objective 1, they would be able to move forward to Objective 2. With the time and training delays created by the loss of Mike and Jan Barclay, the group was not able to achieve these goals in the time available.

3. *Provide introductory field experiences to enhance LCD detection dog confidence and skill*
   Throughout 2022 the Ag Dogs have held nine instructional field experiences at local cherry orchards and research plots. The group decided to not try to make it to the nurseries because of all the unknowns and the time it would take to test nursery stock, get the results back and then get the dogs in the field.

4. *Improve sample management and analysis*
   Critical to this project is the management and analysis of cherry plant material samples provided to LCD detection dog training classes. Cooperator Hannah Walters provided samples in 2021 and

Cooperator Teah Smith provided samples in 2021 and 2022 on-as needed basis. Cooperator Scott Harper provided some of the PCR tested samples in 2022 as well. Additionally, Cooperators Lav Khot and Gajanan Kothawade took samples from some of the same trees that the dogs used in their trainings to evaluate the volatile profile using FAIMS and CG/MS techniques.

5. *Provide education and extension on "Canine LCD Detection Skills"*
   In July 2021 WSU hired a new Little Cherry Disease (LCD), Information Technology Transfer (ITT) Extension, Corina F. Serban. Co-PI Serban provided education and extension opportunities for WKC, collaborators and industry wide outreach.

SIGNIFICANT FINDINGS:

- The desired percentage of proficiency was not reached by all the dogs. However, the performance of the dogs did support positive proof of concept that any dog can be trained to detect LCD. The group would need additional time to meet the levels of proficiency included in the proposal.
- Some of the factors that may have impacted the results include:
    - New dogs entered the program and a couple of veteran dogs left the program due to medical/other issues.
    - The loss of the trainers for the team, Mike and Janice Barclay, creating a delay and reset for the project.
- Environmental conditions contributed to the success or failure of the dogs accurately alerting on positive samples in the orchard. High temperatures physically limited the dog's ability to work in the field during the summer months. Handlers needed to take in the account the direction and force of the wind. There were also concerns about the presence of pesticides, weeds such as goat heads, cherry cannons, and poisons set out for vermin. These variables could only be learned by working in the orchards and not by working only in controlled environments.
- This work done in this project has created the opportunity for next steps in the process moving forward. There was a lot of learning done regarding LCD by the group which will increase performance timelines and improve methodology.
- The next steps for canine LCD work should include a small group of focused dogs that are trained on a regular basis, at least several times a week using a specifically developed training schedule including regular validation testing. The steps would include odor validation, proofing, and experiences in the appropriate field setting.

METHODS:

1. *Increase LCD detection proficiencies in controlled indoor/outdoor settings.*

<u>August 2021 evaluation.</u> All plants materials were provided by Cooperators Hannah Walters and Teah Smith. Tree cuttings were placed in labeled Ziplock bags and held at refrigerated temperatures until use. All samples were coded as either samples obtained from positive trees or negative trees with a unique sample ID. Dogs were tested in a three alternative forced choice test. Unused mailer boxes were used to hold either a glass 8 oz mason jar with a screen/perforated lid (Wenatchee location) or a stainless-steel canister with a perforated lid (Idaho location). A hole in the cardboard box was made to hold the container with the sample upright on the floor, giving the dog access to sniff. All dogs completed 15 three alternative forced choice test experimental trials. For each trial, one diseased cutting and two non-diseased cutting were presented. Individual dogs were tested with the same three samples for all 15 trials, but different clipping samples were given to each dog. Some dogs were tested with the same positive source sample (but different samples within the source).

April 2022 evaluation. Prior to the start of the testing period, the handlers were allowed 5 training trials so they could become familiar with the search pattern and calling out an alert. The test period consisted of each dog searching 6 canisters for a total of 10 trials. The odorants utilized were positive cutting, negative cutting, grass, gravel, and a blank canister. One out of the six cannisters had a positive cutting. The position of each odorant in the canisters was pseudo-randomized so the target odor was not in the same position for more than 3 trials in a row. Handlers were blind to the location of the target odor (positive cutting) and were asked to call out the number of the canister their dog was alerting to, the experimenter then indicated if dog was correct or incorrect. As typical practice for WKC training, handlers searched the cannisters until dog located the correct target cannister or the handler called an all clear.

RESULTS AND DISCUSSION:

1. *Increase LCD detection proficiencies in controlled indoor/outdoor settings.*

   Three double blind tests/evaluations were run to test the LCD detection proficiencies. One prior to the start during proof of concept with Collaborator Nathan Hall (August 2021), one at the midpoint with Collaborator Mallory DeChant (April 2022), and one at the end with Collaborator Hallie McMullen (September 2022). In addition, Co-PI Serban collected data from the training classes held during August 2022 to track the proficiencies of each dog indoors. The dogs performed at different levels during these tests.

   - August 2021 evaluation. The overall results from this project showed that two of the eleven dogs showed proficient detection of the LCD infected samples from the healthy samples by showing immediate detection during warm-ups and detection above change levels. An additional 4 dogs reached above chance levels with reinforced experience with the target material, but 5 dogs did not show detection above chance levels. Together, these results provide a positive proof-of-concept that citizen science trained dogs can successfully discriminate LCD infected cherry samples from healthy cherry samples, but not all dogs meet proficiency to demonstrate above chance performance.
   - April 2022 evaluation. The objective of this evaluation at the WKC was to evaluate the accuracy of six dogs that have previously been trained to detect LCD. The results below show that two dogs out of six passed the proficiency evaluation with 90% accuracy.

Dog ID	Training period Accuracy	Testing period Accuracy	Testing period miss rate	Testing period False Alerts
1	20%	50%	10%	11 times
2	0%	40%	10%	6 times
3	40%	40%	0%	7 times
4	30%	30%	20%	10 times
5	100%	**90%**	0%	1 time
6	30%	**90%**	0%	1 time

   - Classes held indoors in August 2022 to track the proficiencies of each dog over time.

Note: N.A means the dog was not present.

Dog ID	Testing period Accuracy (%)			
	Aug 8	Aug 15	Aug 22	Aug 29
A	71%	60%	**83%**	40%
B	57%	60%	67%	60%
C	57%	60%	67%	40%
D	43%	N.A	33%	60%

E	71%	40%	N.A	40%
F	57%	**80%**	33%	N.A
G	43%	N.A	N.A	N.A

- September 2022 evaluation. The overall results show that all participants have been working on their handling skills. The dogs were working more independently. It also appeared that the dogs were more definitive in their answers. There were still some issues with dogs choosing the negative samples, but there was progress in that area as the dogs made choices sooner and weren't waiting for handler to help. There are several factors that would be next steps for resolving these things, such as changing out canisters, so the dogs aren't influenced by odors left by other dogs and so on. There weren't obvious consistencies in errors, for instance the dogs didn't show a propensity to false alert if the negative came before the positive or vice versa. However, there was an increase in false indications in the last 4 runs. There are several different factors that could play into that result, such as search endurance, odor influence from not replacing canisters, or others. Results are shown in table below:

Dog ID	Testing period Accuracy	Testing period miss rate	Testing period False Alerts
A	50%	0%	5 times
B	70%	0%	3 times
C	40%	0%	6 times
D	60%	0%	4 times
E	**80%**	0%	2 times

2 *Develop next-step training protocols to facilitate transition into field experience.*

The dogs were given introduction to the plant materials and an orchard experience. This objective was in development including developing of the protocols to facilitate transition from controlled indoor/outdoor setting to field experience in nurseries and orchards. As the dogs were reaching proficiency in Objective 1, they would be able to move forward to Objective 2. With the time and training delays created by the loss of Mike and Jan Barclay, the group was not able to achieve these goals in the time available.

3 *Provide introductory field experiences to enhance LCD detection dog confidence and skill.*
The first introductory field experience in a cherry orchard was on May 23, where several of the dog/handler teams had never had exposure inside a cherry orchard. The dogs found little connection between LCD odors in sample cans and LCD odors in trees. WKC developed a program for carefully staging the introduction of LCD detection in orchard settings. Over the next several weeks field classes were held at local orchards where the dogs learned orchard search techniques, trying to find positive samples, and ignoring the negative samples contained in a variety of packaging materials placed at various elevations within the orchard setting. On June 27th, Ag Dogs returned to a different orchard where dogs invested time canvassing the ground cover plants rather than indicating the positive source trees. On July 25 three dog/handlers teams had the opportunity to travel to WSU research orchards in Prosser for additional field training. The group quickly learned that the site raised far more questions than it provided support for LCD detection dog training because of different inconsistent factors such as smaller inoculated "rootstocks" type trees, tall ground cover, cannon noises and extremely hot weather.

4 *Improve sample management and analysis.*

In addition to samples being provided by Cooperator Hannah Walters (2021) and Cooperator Teah Smith (2021 and 2022), the samples also had corresponding information such as titer levels, collection date and orchard location. WKC had a designated secured refrigerator where the samples were stored for dog training use. The WKC handlers developed their own training record sheets that they used in the field introductions to collect observations tracking each dog's progress.

5 *Provide education and extension on "Canine LCD Detection Skills"*

Education and Extension opportunities provided to WKC and collaborators

- Co-PI Serban hosted monthly 1 h virtual meetings 'Canine LCD Detection Collaborators Meeting' in February, March, April, May, July, and August 2022, where the progress of this project was shared with all the collaborators, and everyone was invited to provide feedback (15 participants).
- Dr. Tobin Northfield (WSU Entomology) was invited to give a presentation on Little Cherry Disease topic to the WKC handlers, to learn more about the topic and provide them opportunity to ask questions. August 8, 2022 (12 participants).
- PI Pheasant hosted 'Ag Dogs semi-annual meeting' on July 25, 2022 (10 participants).

Industry wide outreach

Project was mentioned in several oral presentations by Co-PI Serban at the:

- Tree Fruit Endowment Advisory Committee Meeting, March 15, 2022, Prosser WA *('Little Cherry Disease Extension & Outreach Program'; 30 participants)*
- LCD Northwest Horticultural Council -USDA Meeting, Feb 25, 2022 *('Extension Activities in Response to Little Cherry Disease'; 17 participants)*
- Cherry Institute Annual Meeting, Jan 7th, 2022, Yakima WA *('Latest Developments on Little Cherry Disease'; 100 participants)*
- WSDA Tree Fruit Technology Fall Tour, Oct 15, 2021, Prosser WA *('Little Cherry Disease Extension & Outreach'; 20 participants)*

Project details were shared by PI Pheasant (oral presentation) under 'New Experimental Research Projects: Early Detection' session at the Little Cherry Disease Day, on Feb 16, 2022, in Ellensburg WA (178 participants) organized by Co-PI Serban. Preliminary results/project details were shared by PI Pheasant (oral presentation) and a demonstration by Canine LCD detectors-in-training (two handler/dog teams) at the Little Cherry & X-disease Field Day on Jun 21, 2022, in Buena WA (57 participants) organized by Co-PI Serban. Following the Little Cherry & X-disease Field Day on Jun 21, 2022, Co-PI Serban had one interview in YaktriNews.com together with a field day summary (https://www.yaktrinews.com/researchers-use-dogs-to-sniff-out-infected-cherry-trees-in-eastern-washington/). Other Newspapers and Periodicals featuring project details:

- McClain, Sierra Dawn. "Pilot project uses dogs to sniff out little cherry disease". Capital Press, June 2, 2021. https://www.capitalpress.com/ag_sectors/orchards_nuts_vines/pilot-project-uses-dogs-to-sniff-out-little-cherry-disease/article_dd7230e0-c30c-11eb-9145-9baccaec1558.html
- Brown, Trent. "A nose for the orchard – Wenatchee Kennel Club trains 'ag dogs' to detect little cherry disease", June 15, 2022. https://www.wenatcheeworld.com/news/local/a-nose-for-the-orchard/article_63da7a4e-eb6f-11ec-aa9d-7fdcb8c20fb9.html

EXECUTIVE SUMMARY
Project title: Canine LCD Detection Skills Applied to Nursery and Orchard Settings

Keywords: Canine, Dog, Little Cherry Disease, Early LCD detection, Ag Dogs, Little Cherry Virus-2 (LChV-2), X-disease phytoplasma (XDP)

Little Cherry Disease (LCD) is an umbrella term used for two different pathogens – Little Cherry Virus-2 (LChV-2) and X-disease phytoplasma (XDP), that cause similar symptoms in cherries. Once a tree is infected, there is no cure for it, and the only control measure is the tree removal. Several factors are preventing the sweet cherry industry to stop the spread of the LCD infection. Among the most important, is the lack of optimized or new screening methods to quickly identify LCD infections. Canine detection skills project explored the possibility of providing an early detection tool for LCD identification, control, and eradication. This project was designed to increase detection proficiencies and facilitate transition of canine detection skills from controlled settings to field environments. The desired percentage of proficiency was not reached by all the dogs. However, the performance of the dogs did support positive proof of concept that any dog can be trained to detect LCD. The group would need additional time to meet the levels of proficiency included in the proposal. This work done in this project has created the opportunity for next steps in the process moving forward. There was a lot of learning done regarding LCD by the group which will increase performance timelines and improve methodology. The next steps for canine LCD work should include a small group of focused dogs that are trained on a regular basis, at least several times a week using a specifically developed training schedule including regular validation testing. The steps would include odor validation, proofing, and experiences in the appropriate field setting.

Project Title: Physiology-based identification of X-disease infected cherry trees.

Report Type: Continuing Project Report

Primary PI: Kelsey Galimba
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Co-PI 3: Corina Serban
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Cooperators: John Byers (Grower), Tim Hudson (Grower), Ken Newman (Grower), Garret Bishop (G.S. Long)

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $30,657
Total Project Request for Year 2 Funding: $42,419
Total Project Request for Year 3 Funding: $33,596

Budget 1
Primary PI: Kelsey Galimba
Organization Name: Oregon State University
Contract Administrator: Charlene Wilkinson
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Contract administrator email address: charlene.wilkinson@oregonstate.edu
Station Manager/Supervisor: Steve Castagnoli
Station manager/supervisor email address: steve.castagnoli@oregonstate.edu

Item	2022	2023	2024
Salaries[1]	$14,356	$14,787	$15,230
Benefits	$7,928	$8,166	$8,411
Wages			
Benefits			
Equipment[2]		$9,552	
Supplies[3]	$2,000	$2,000	$2,000
Travel	$500	$1,000	$1,000
Miscellaneous			
Plot Fees			
Total	$24,784	$35,505	$26,641

Footnotes:
[1] Estimated salary for one FRA to perform sample collection, testing and data analysis + 2 weeks of PI summer salary.
[2] Field testing equipment for NIR and Ca2+.
[3] Lab supplies and reagents.

Budget 2
Co PI 2: Ashley Thompson
Organization Name: Oregon State University
Contract Administrator: Charlene Wilkinson
Telephone: 541-737-3228
Contract administrator email address: charlene.wilkinson@oregonstate.edu

Item	2022	2023	2024
Salaries	$3,836	$3,836	$3,836
Benefits	$2,037	$2,078	$2,119
Wages			
Benefits			
Equipment			
Supplies[1]			
Travel[2]			
Miscellaneous			
Plot Fees			
Total	$5,873	$5,914	$ 5,955

Budget 3
Co PI 2: Corina Serban
Organization Name: Washington State University
Contract Administrator: Stacy Mondy
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Contract administrator email address: arcgrants@wsu.edu

Item	2022	2023	2024
Salaries			
Benefits			
Wages			

Benefits			
Equipment			
Supplies[1]		$500	$500
Travel[2]		$500	$500
Miscellaneous			
Plot Fees			
Total		$1,000	$1,000

Footnotes:
[1&2] Travel and supplies to sample leaves from newly-infected trees and send them to MCAREC for starch testing.

Objectives

1. Characterize the degree of leaf starch content changes in sweet cherry trees with verified *Candidatus* P. pruni infections (both established and new), using lab-based methods.

2. Identify accurate, efficient procedures to test leaf starch content in a field setting, by comparing methods such as iodine tests and spectroscopy.

3. Explore other potential physiology-based methods for identification to determine if any of them can be developed further as X-disease identification tools.

Significant Findings

Objective 1
- Five collections of leaves from infected and uninfected trees in three locations were made and samples were frozen for lab testing.

Objective 2
- All leaves sampled in Objective 1 were scanned with a hand-held spectrometer to gather spectral reflectance data for >2,000 individual wavelengths.
- Testing method using small plastic bags, sandpaper and iodine solution doesn't show significant visual differences between infected and non-infected leaves.
- Conflicting with previous published research, non-infected sweet cherry leaves appear to contain substantial amounts of starch.
- Starch content of cherry leaves appears to by cyclical, with quantities and spatial distribution varying throughout the day.

Objective 3
- Collections of phloem sap from infected and uninfected trees in Objective 1 were made and frozen for further analysis.

Methods

Objective 1. Characterize the degree of leaf starch content changes in sweet cherry trees with verified *Candidatus* P. pruni infections (both established and new), using lab-based methods.

X-disease infections were identified and verified using qPCR (OSU Plant Clinic, Corvallis OR) at three separate locations in The Dalles, OR. Cultivars included 'Bing', 'Benton', and 'Royal Ann'. Collections of leaves from 3-5 infected and 3-5 non-infected trees were made at five dates throughout July and August. Collections were taken preferentially from limbs that bore symptomatic fruit, and from lower on the tree. For each tree, samples from 10 leaves were weighed and flash frozen for further processing to analyze starch content. A colorimetric Starch Assay Kit was purchased from Cell Biolabs and will be used to process samples before the 2023 field season.

Objective 2. Identify accurate, efficient procedures to test leaf starch content in a field setting, by comparing methods such as iodine tests and spectroscopy.

Simultaneous collections were made from the same trees to use for both iodine testing and for spectral analysis. For the spectrometer, a midpoint between the distal and proximal ends and between the midvein and leaf margin was scanned for each leaf. Data is currently being organized for analysis. Communications with a statistician (Dr. Clark Kogan, statistician on the Cherry Cold Hardiness project) have been initiated to determine whether modeling will be necessary to develop spectral signatures for X-Disease.

For the iodine testing, a number of approaches were evaluated. The first followed the protocol outlined in Takushi *et al.* 2007. The adaxial surface of each leaf was scratched with a one-inch square of fine-grit sandpaper until the surface of the paper was coated. Sandpaper was added to a plastic bag with diluted iodine and color of the solution was observed.

The second method involved clearing leaves before staining with iodine. At first whole leaves were attempted, but the size of the leaves proved difficult to manipulate and limited the number of leaves we could process. To optimize, we switched to using 1x1 inch squares that were cut from the basal portion of each leaf blade, avoiding the midrib if possible (Fig. 1). Leaf squares were boiled for two minutes, then soaked in room temperature (RT) water to cool. They were then boiled in 90% EtOH until bleached, changing out EtOH if needed. Once squares were bleached, they were placed in RT water bath to rehydrate. Pictures were taken of bleached squares and then they were added to a glass dish with iodine solution. They were allowed to remain in contact with iodine for ~1 minute before being rinse in a water bath and photographed.

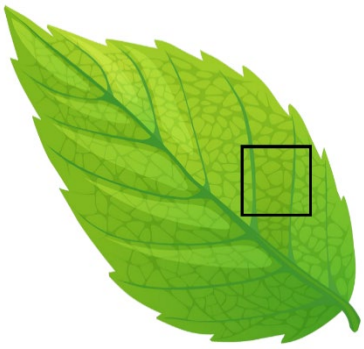

Figure 1. Leaf area that was used to clear and stain with iodine.

Objective 3: Explore other potential physiology-based methods for identification to determine if any of them can be developed further as X-disease identification tools.

Phloem sap was collected from trees used in Objective 1. ¾ inch sections of first- and second-year wood were cut, scored, and centrifuged as in Hijaz & Killiny 2014, then flash frozen for further analysis. Collected liquid should consist of both xylem and phloem contents.

Problems/Limitations Encountered

- Staffing has been a major issue in 2022. We were able to hire a full-time research technician at the beginning of August, 2022 but she unfortunately resigned two months later. Out of five prospective summer students, only one followed through with applying and taking the position. The majority of all research is being performed by one full-time FRA and one part-time technician.
- Obtaining materials and supplies necessary for research has been challenging because of supply chain issues. One example: Potassium Iodide ordered in September didn't ship until January.
- Over 1/3 of the infected trees we used in 2022 from grower's orchards were pushed out, and the others are slated for removal. This highlights the need for infected trees at MCAREC that we can use for experimentation. We are currently designing and planning for a screen house that will be able to house and contain them. In the meantime, we are attempting to infect turnip and other herbaceous species used as model systems for X-Disease in the past, using cherry material from The Dalles and the leafhopper *E. variegatus*.

Results and Discussion

Objective 2. Identify accurate, efficient procedures to test leaf starch content in a field setting, by comparing methods such as iodine tests and spectroscopy.

All leaves that were frozen for starch content analysis were scanned with a hand-held spectrometer to gather spectral reflectance data. Data for each leaf consists of the reflectance value for 2,048 individual wavelengths ranging from 140 – 1334 nm. Data analysis is pending.

Iodine testing was attempted in two different ways. The sandpaper + baggy method that was shown to work in HLB infections in citrus has yet to show significant visual differences between infected and non-infected leaves. When the method is used on potato tubers, black cells can be seen floating in the iodine solution (Fig. 2). While darker leaf material can be seen floating in solution containing sandpaper from both infected and non-infected samples, it is never as dark as the starch-filled cells from the potato. Additionally, the solution doesn't appear to differ between the infected and non-infected leaves. This testing was performed at five different dates and on different cultivars with similar results.

The clearing and iodine staining method using leaves or portions of leaves gave unanticipated results. Previous research done at WSU in the 1980's asserts that normal (i.e. uninfected) sweet cherry leaves contain no, or very little starch. This would mean that the extra starch we hope is being accumulated in X-Disease infected leaves should be easy to distinguish. However, what we found after clearing leaves from X-Disease infected and non-infected trees is that both seem to contain starch, and in some cases (Fig. 3) uninfected leaves contain much more starch. This led us to expand our investigation. Additional testing suggests that starch does occur in cherry, and that its metabolism is dynamic, changing in response to circadian rhythms and possibly to light/solar exposure. Uninfected and infected eaves that had been kept in a dark refrigerator overnight showed no starch in mesophyll cells, but instead obvious starch granules in the guard cells surrounding the abaxial stomata (Fig. 4). Leaves from uninfected (presumably) 'Bing' trees at MCAREC showed that while starch is present in both leaves exposed and shaded

from the sun, it appears more uniform and more concentrated in shaded leaves (Fig. 5). These results will be verified by starch assay. Altogether, the results from iodine testing in 2022 indicate a strong need for additional research into starch metabolism in sweet cherry leaves, with strong evidence that former conclusions need to be re-explored before starch can be used as a marker for X-Disease infection.

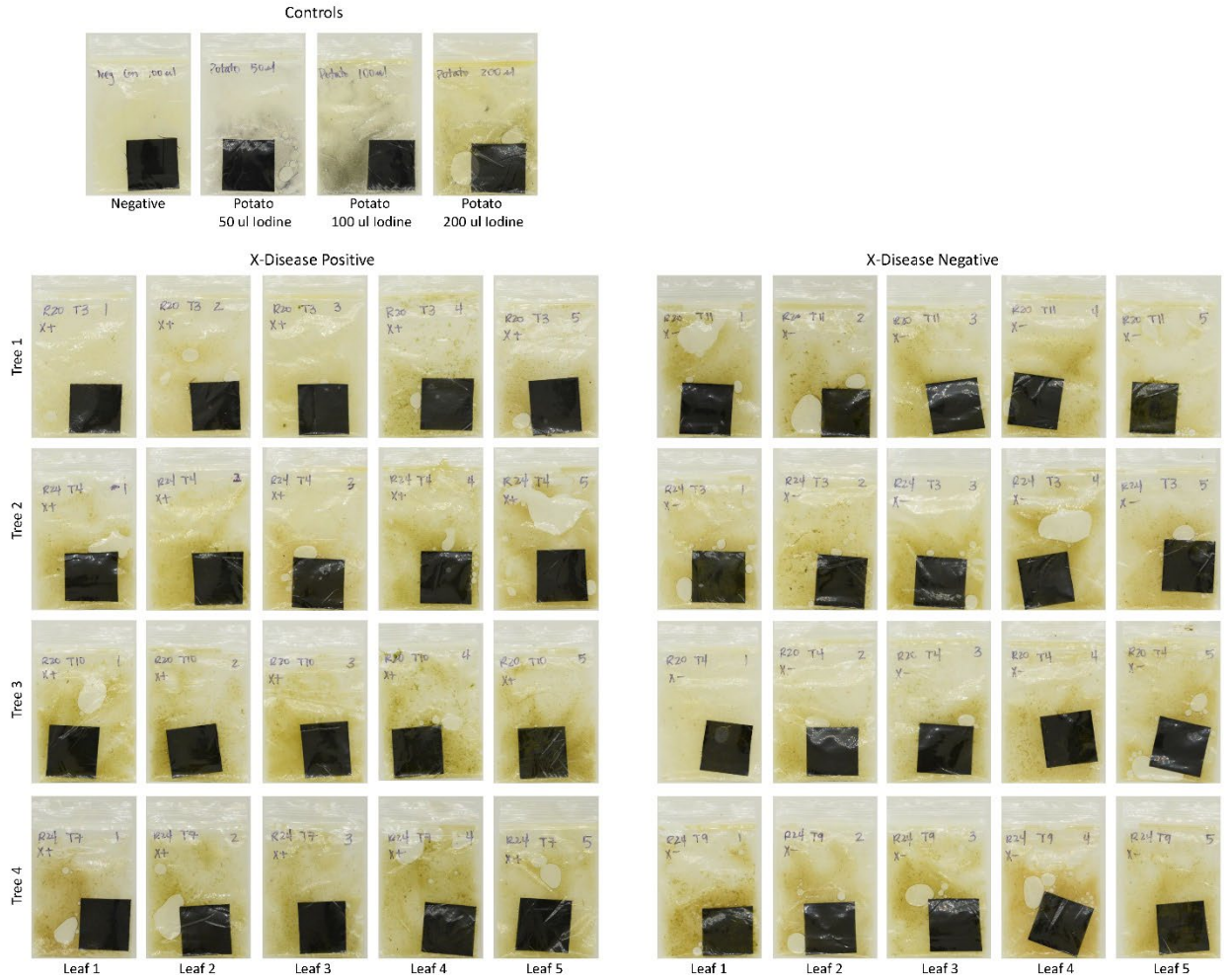

Figure 2. Baggy iodine method to detect starch in infected leaves. Sandpaper squares were used to accumulate leaf tissue from infected (left) and non-infected (right) leaves. 100 ul of iodine and 1 ml of water were added and color change was observed. No apparent difference between infected and non-infected leaves is present.

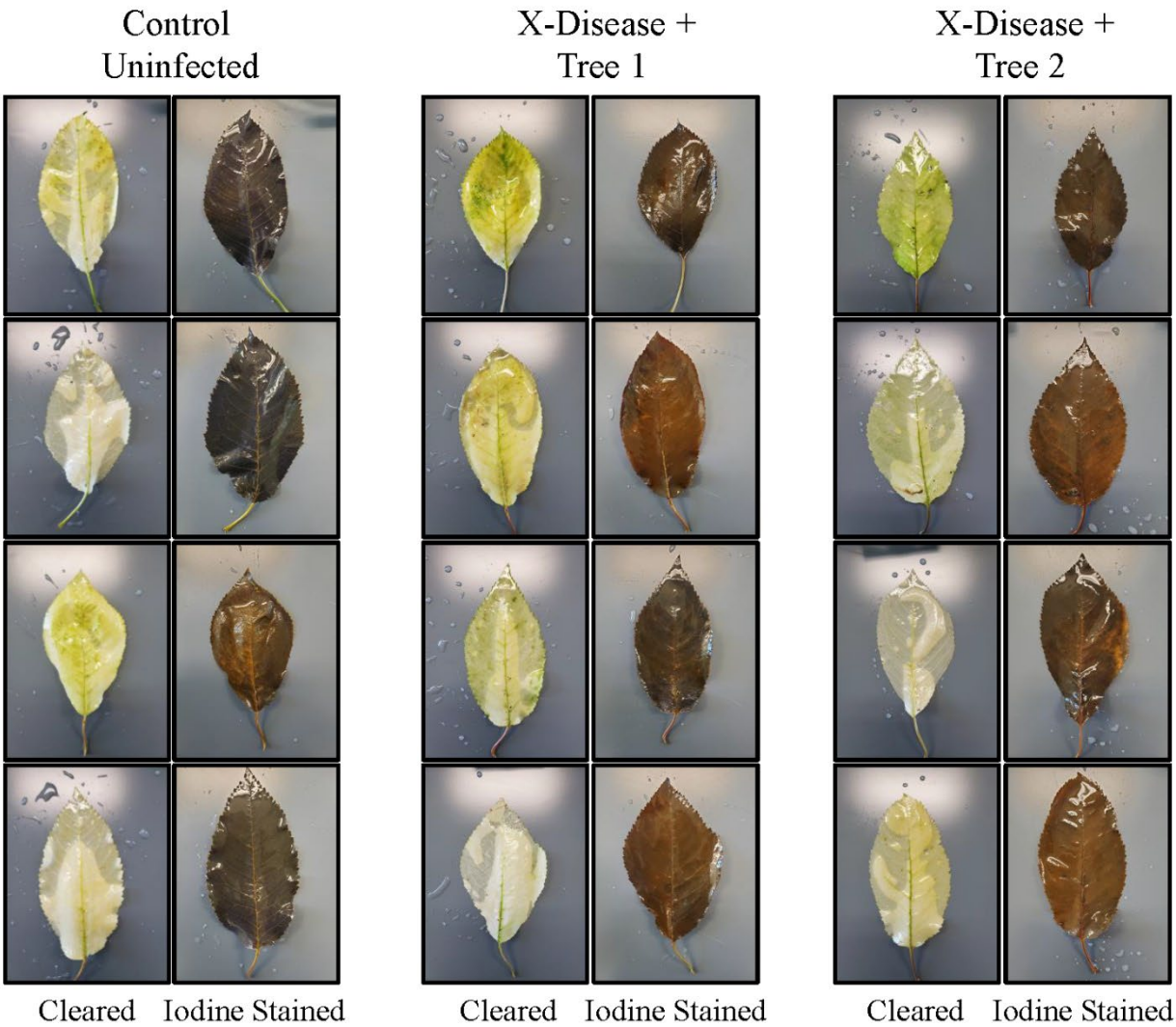

Figure 3. Clearing iodine method to detect starch in infected leaves. Leaves were cleared using boiling ethanol and stained with iodine. Uninfected leaves appear to contain higher levels of starch than infected leaves.

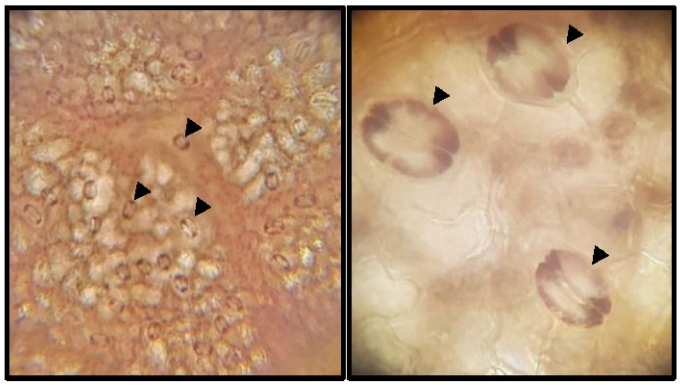

Figure 4. Leaves kept in the dark overnight prior to clearing and staining showed redistribution of starch to the guard cells of the stomata, indicated with black arrows.

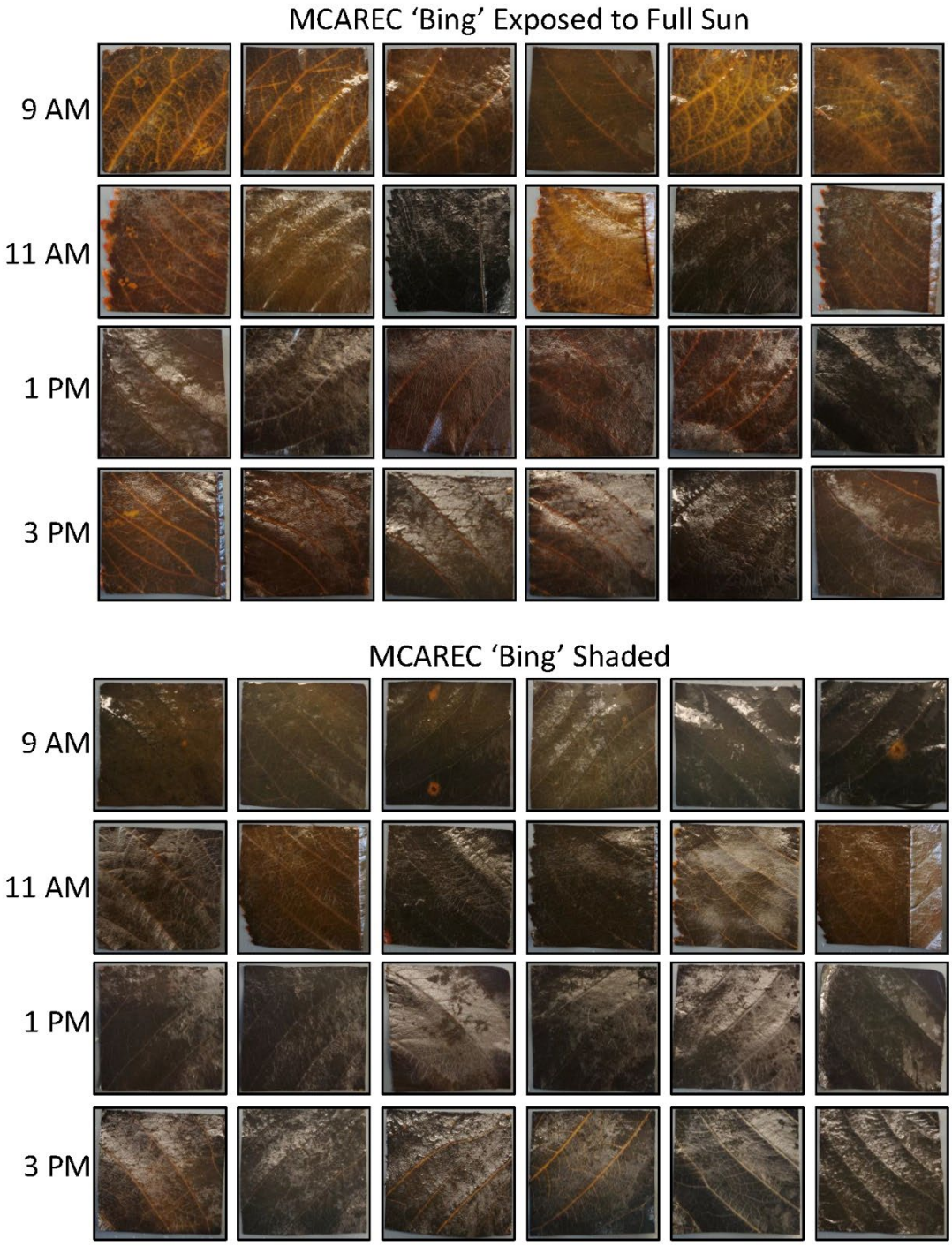

Figure 5. Clearing and staining method used on uninfected leaves, from exposed and shaded portions of the canopy. The presence of high levels of starch in uninfected leaves was unanticipated, as previous published research states that starch is absent in sweet cherry leaves. The higher concentration of starch in shaded leaves supports the new hypothesis, that starch is present in cherry leaves, but is dynamic, changing throughout the day and with different solar exposure.

Project Title: Improved timing for initial SWD sprays in blush and dark sweet cherry

Primary PI: Nik Wiman
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City/State/Zip: Aurora, OR

Cooperators: JD Walker, Les Stephens, Chris Adams
Project Duration: 2-Year

Total Project Request for Year 1 Funding: $21,443
Total Project Request for Year 2 Funding: $18,249

Other funding sources: None

WTFRC Collaborative Costs: None (OSCC Project)

Budget 1
Organization Name: Oregon State University, Agricultural Research Foundation
Contract Administrator: Charlene Wilkinson
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Email address: Charlene.Wilkinson@oregonstate.edu

Item	2021	2022	TOTAL
Salaries[1]	6,750	6,953	13,703
Benefits[2]	4,772	5,014	9,786
Wages (Hourly)[3]	3,360	3,360	6,720
Benefits	0	0	0
Equipment[4]	3,500	0	3,500
Supplies[5] (Insect colonies, cups, cages)	460	320	780
Travel[6]	580	580	1160
Miscellaneous	0	0	0
Plot Fees	0	0	0
Total	21,443	18,249	$39,692

Footnotes: [1]Faculty research assistant Heather Andrews, 0.15 FTE. [2] Benefits, Faculty Research Assistant (0.70). [3]Wages for student assistant at $14.00/hr and 30 hr/wk for 8 wk (no OPE associated or permitted). [4]Approximate cost for colorimeter and firmness tester unit. [5] Approximate cost for insect rearing, supplies such as cages, traps and lures. [6] Travel is based on OR mileage reimbursement rate ($0.58/mile) for 100 miles per week for 10 weeks (1k miles) to travel to and from field sites.

OBJECTIVES

This project was intended to help address the issue of SWD risk in blush and dark sweet cherry as a function of fruit maturation variables including fruit size, color, firmness, pH, brix, and heat unit accumulation as well as by examining potential for traps to predict risk. We worked at two Willamette Valley orchards where we have excellent cooperators and access to multiple cultivars with unsprayed fruit, so that we can examine risk factors in blush compared to dark cherries. Our goal is to improve understanding of SWD fruit infestation risk and to evaluate potential tools that can more effectively inform growers about risk.

Objective 1) Associate cherry ripening factors with attack by SWD. As fruit ripen, we are quantifying fruit ripening data including color, brix, pH, hardness, size along with SWD damage and infestation data to associate ripening factors with natural and induced damage and infestation by SWD.

Objective 2) Associate environmental and trapping data with fruit ripening and SWD attack. SWD will be monitored on-site with traps and data loggers will be used to capture environmental data to associate with ripening factors and SWD infestation data at each site. These data will help make the results more broadly applicable to other cherry production regions.

Objective 3) Analyze all variables to determine relative importance and distill results into digestible, easily implemented grower recommendations.

SIGNIFICANT FINDINGS

- We followed color development in 5 cherry cultivars, both blush and dark, to associate color with SWD fruit infestation data and trap captures.
    - Our goal is to use simple RGB color analysis that could be captured in the field with a cellphone.
    - We used photos of fruit and RGB analysis in the open-source image analysis software ImageJ to demonstrate color trends in fruit as it ripened.
    - For cv. 'Rainier', increasing red and blue and decreasing green occurred as fruit matured (Fig. 1 from 2021 report). More analysis is needed for the 2022 color data.
    - Color data for both years need to be aligned on a degree day scale to determine how color variables relate to environmental variables and probability of attack by SWD

- We examined the capture of SWD on dry and wet traps to evaluate the relationship between captures and SWD fruit infestation data. Two Trece lure formulations were evaluated along with one AlphaScents trap in 2021. In 2022 we dropped the AlphaScents trap because it was not catching SWD. In 2022 we added the Scentry liquid trap as a sensitive standard. We also had to change Trece traps because the specific lure was discontinued. Instead, we evaluated the dry vs. the liquid version of the Trece broad spectrum lure. Three reps of each trap were deployed at each site.
    - From 2021 we reported that SWD were caught in Trece dry traps starting in mid-June. We reported the highest numbers were found at the higher elevation site (potential heat evasion strategy), and some interesting trends were detected:
        - The broad-spectrum lure caught the most SWD
        - While the broad-spectrum lure did have more by-catch, on rare occasions the specific lure had huge by-catch
        - By-catch increases time required to count SWD on the sticky cards and could affect captures of SWD by occupying space on the card.

        - There was a very strong correlation between male and female SWD caught on the broad-spectrum trap, though the trap caught mostly females.
            - The strong correlation between male and female captures suggest that only males could be counted, and the number of females estimated with strong confidence.
            - Counting just males based on presence of wing spots could save time when checking traps.
    - From 2022 trapping data we see that the Scentry trap is far more sensitive than the two Trece trap types (**Fig. 1)**. However, increased sensitivity is only beneficial if it results in detections when other traps are not working and thereby informs about population trends that are difficult to detect. However, that was not the case, when the Scentry trap was catching SWD, so were the Trece traps, just at a lower rate (**Fig. 2)**. This suggests that much labor can be saved by counting flies on panel traps rather than dealing with liquids.
        - The earliest detections in mid-May preceded infestation of the crop (**Fig. 2)**
        - Very few flies were being captured at the time when the first infestions of fruit occurred in mid-June.
        - The Scentry trap and Trece liquid traps had the strongest linear relationship between male and female captures (**Fig 3)**. The dry red panel Trece trap appeared to be more attractive to males, as there were multiple occasions where males were caught but females were not.

- Firmness and size of fruit were captured by the FirmTech machine on a weekly basis for 5 cherry cultivars, both blush and dark.
    - These firmness data followed distinct trajectories that can be easily modeled (**Fig. 4**)
    - 2022 was a much cooler season than 2021, and this was evident by the delayed softening of fruit in 2022 (**Fig. 4**).
    - Individual varieties showed distinct trajectories in softening, indicating that risk of SWD attack differed by variety.

- We also examined pH and Brix trends to associate these variables with fruit infestation data and trap captures. There were major differences between the cultivars depending on the time of sample, we reported on this in 2021.

- First detections of infested fruit in both seasons were in early June (**Fig. 5**). There were far more detections of natural infestation in 2022 vs. 2021. Wild detections coincide with initial softening of fruit.

- Another tactic we used to evaluate fruit susceptibility was to artificially infest fruit harvested fruit with SWD reared in the lab (**Fig. 6**). Fruit first became susceptible in late May in 2021 and in early June 2022.

- Data loggers were deployed in the field sites and these data will ultimately be used to calculate heat units to associate with all of the other variables (not analyzed yet).

- Nonlinear multivariate analysis will ultimately be performed on all the variables to better understand their relative power to predict fruit infestation. This analysis can be used to produce a cherry risk model (not analyzed yet).

METHODS

Objective 1) We sampled untreated fruit from two blush (Rainier and Royal Anne) and four dark cherry varieties (Benton, Skeena, Sweetheart, and Van) every week. We originally planned to sample just two blush and two dark cherry varieties, and to sample every 2-3 days, but logistically we could not achieve this. We determined color of fruit from RGB (Red, Green, Blue) analysis using the open-source software ImageJ, not as determined by colorimeter as originally planned. Firmness of fruit was determined by FirmTech machine. Originally, we had planned to use penetrometer nfor the firmness data and the FirmTech was a big improvement. Dissolved soluble solids of the fruit was measured by refractometer, and pH of fruit as measured by digital pH meter. These fruits were assessed for signs of SWD damage. We then used the saltwater test on a subsample of fruit to evaluate infestation level. SWD emergence was monitored from a random collection of 100 fruit (representing more than 20 cultivar/rootstock combinations). These fruits were brought back to the laboratory and oviposition damage by SWD was estimated under magnification Fruit were placed in cups (we originally planned to use cages) to allow any SWD emerge. After two weeks, the number of adults that had emerged was determined. In addition to rearing wild SWD, a subset of collected fruit with no visible signs of damage were exposed to laboratory colonies of SWD to determine fruit susceptibility. For this experiment, we added a male and female SWD from our laboratory colony to individual fruit in cups and subsequently evaluated success of SWD to attack and reproduce on the fruit.

Objective 2) We evaluated commercial dry SWD traps which were monitored weekly to determine relationships of trap captures to other measured parameters of fruit ripeness and SWD infestation levels of fruit, and b) temperature data loggers will be placed in trees at research sites to calculate growing degree-days as the time scale for all data. Traps will be placed in untreated as well as treated areas of the orchard as well as natural borders at cooperator farms and will be checked at each orchard visit (Obj. 1). Data loggers will be placed in tree canopies as soon as Jan 1 to allow precise calculation of growing degree days at each site.

Objective 3) Ripening parameters will be associated fruit infestation rates by SWD for blush and dark cherry varieties and will be distilled down to make simple grower-oriented rules for initial treatments against SWD for commercially relevant cherry varieties. For example, simple rules for guiding management timing and fruit susceptibility to SWD attack based on the accumulation of growing degree days could be helpful metrics that would not require trapping or frequent monitoring of fruit. It will also be important to examine SWD susceptibility as a function of ripening asynchrony, i.e., SWD risk quantified as a percentage or proportion of all fruit. For example, what proportion or percentage of susceptible fruit should be considered a risk factor. Comparison of different varieties will provide an interesting basis for comparison of results. Results will be summarized in an Extension document targeting PNW commercial cherry growers (regional).

RESULTS

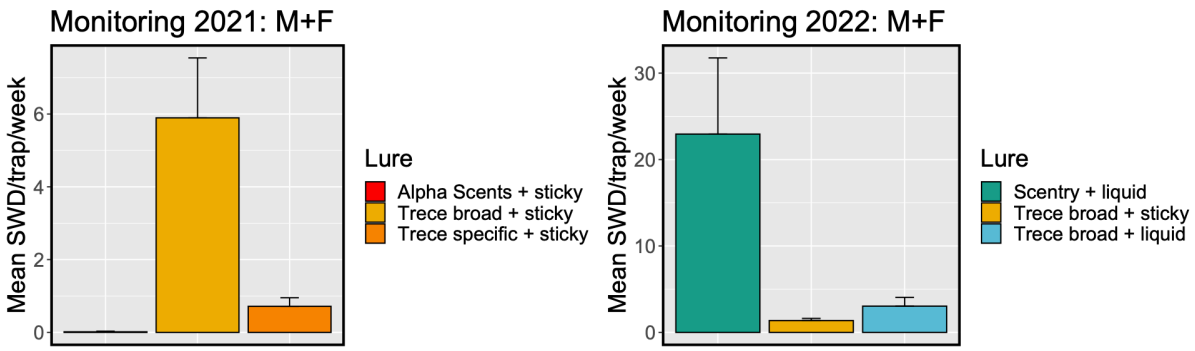

Figure 1. *Captures of SWD on' traps at the two cherry orchards. Alpha Scents was not used in 2022, Trece specific lure not available in 2022.*

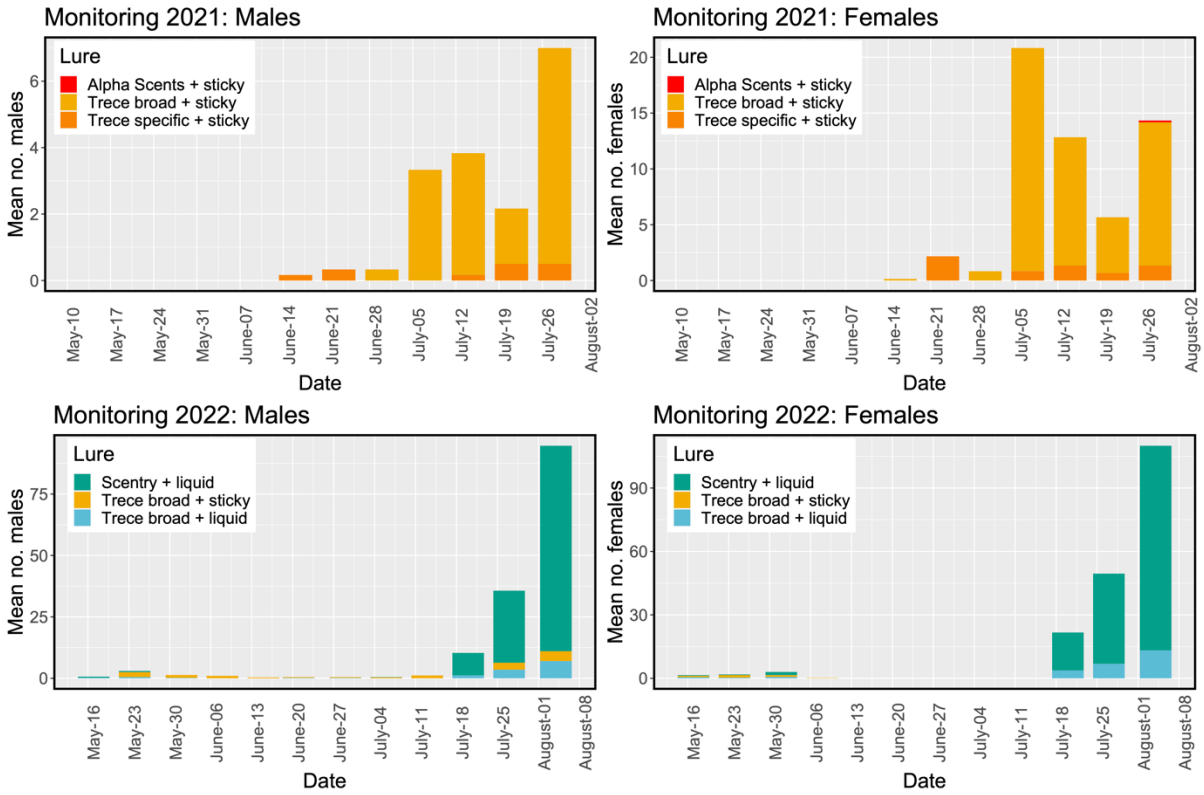

Figure 2. *The monitoring objective was modified in 2022. The AlphaScents trap was not used due to lack of captures in 2021, and the Trece narrow spectrum lure was no longer available. We instead compared the sensitive Scentry trap to the Trece broad lure in both a dry panel and liquid configuration. The Scentry trap was very attractive later in the season but caught comparable SWD numbers to the Trece traps early in the season. Trap captures were very low at the time of first fruit infestation.*

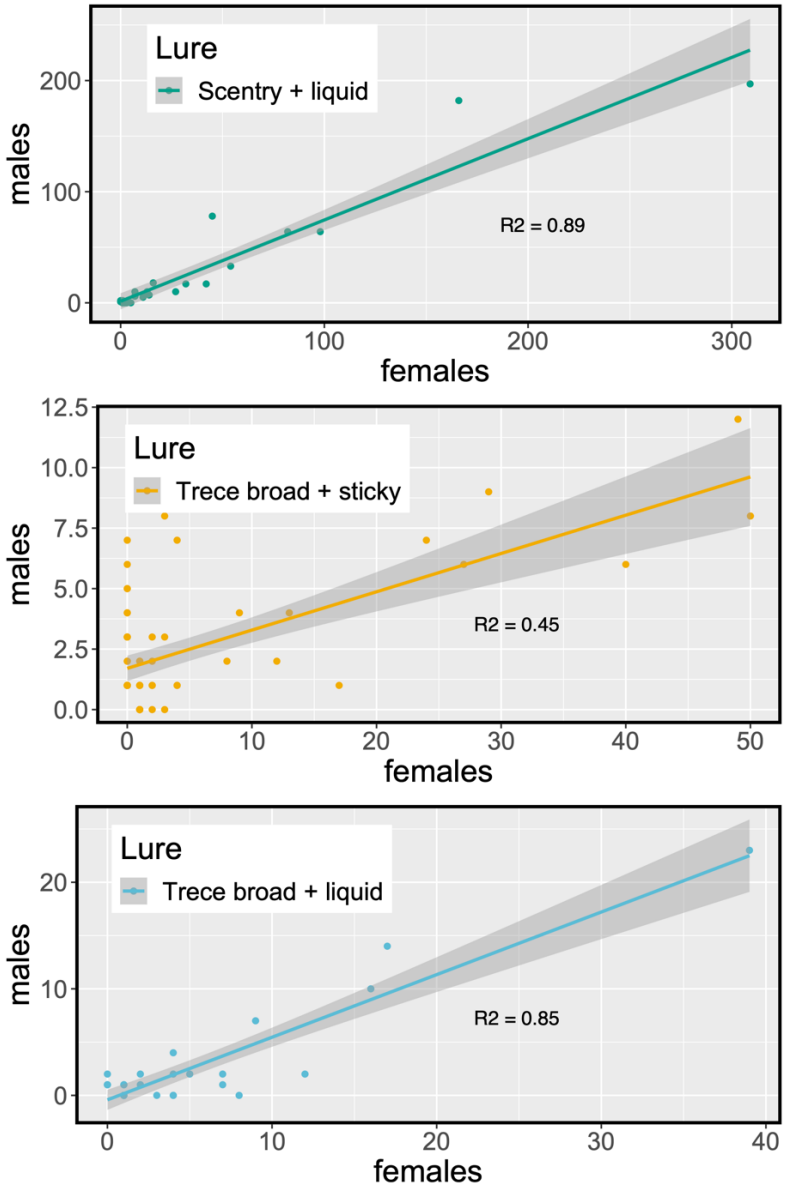

Figure 3. *The relationship between male and female captures was not the same for all traps. The Scentry trap and Trece liquid traps had the strongest linear relationship between male and female captures. The dry red panel Trece trap appeared to be more attractive to males, as there were multiple occasions where males were caught but females were not.*

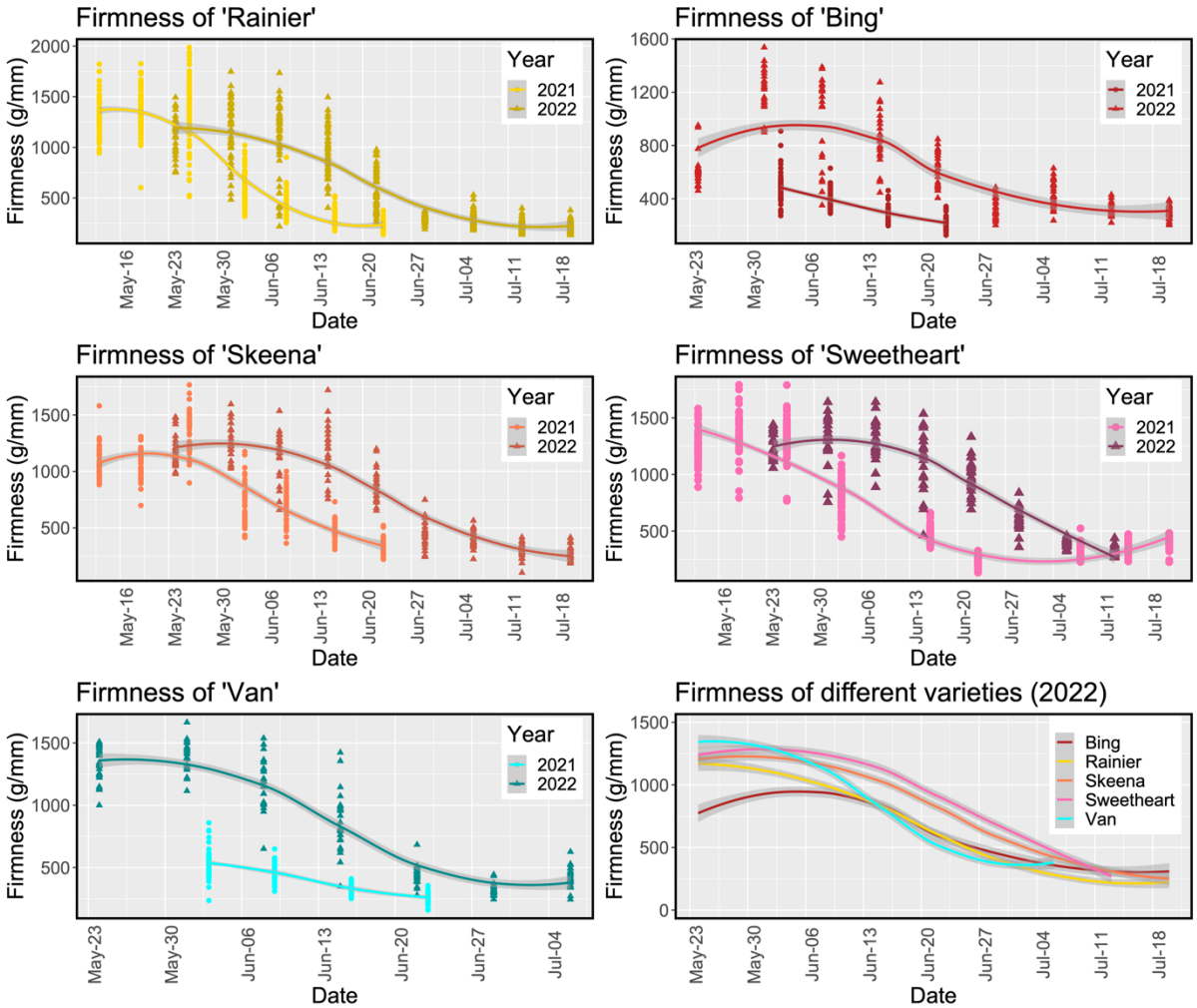

Figure 1. *Firmness of all varieties in 2021 and 2022 as determined by the FirmTech. Firmness followed a very well-defined pattern that can be modeled with high confidence. There was a clear difference in firmness from 2021-2022. The cooler 2022 season delayed fruit maturation by about 3 weeks at the beginning of the season. Environmental effects can have a strong influence on optimal spray timing. Differences in maturation between the varieties also indicates fruit susceptibility to attack by SWD varies by variety. 'Skeena' and 'Sweetheart' are predicted to have delayed susceptibility compared to the other varieties examined.*

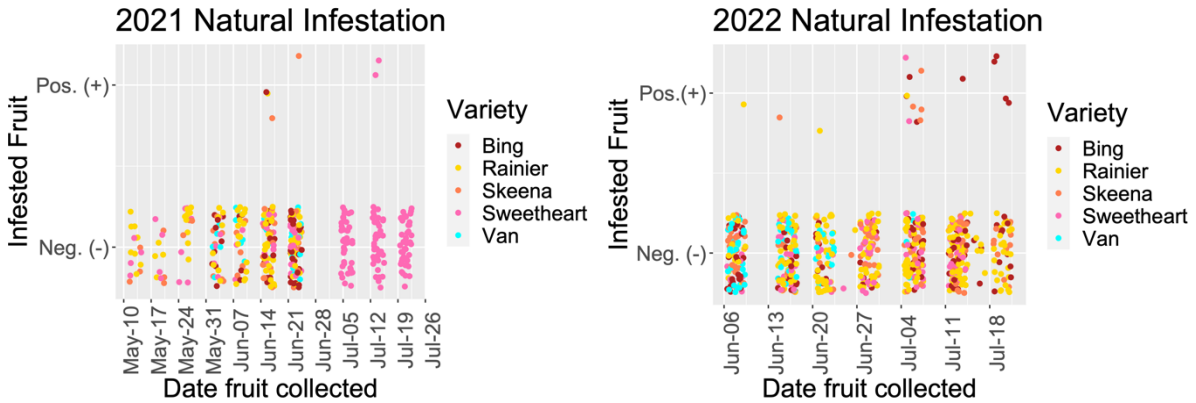

Figure 5. *Natural infestation of all varieties in 2021 and 2022. First detections of infested fruit in both seasons were in early June. There were far more detections of natural infestation in 2022 vs. 2021. Wild detections coincide with initial softening of fruit.*

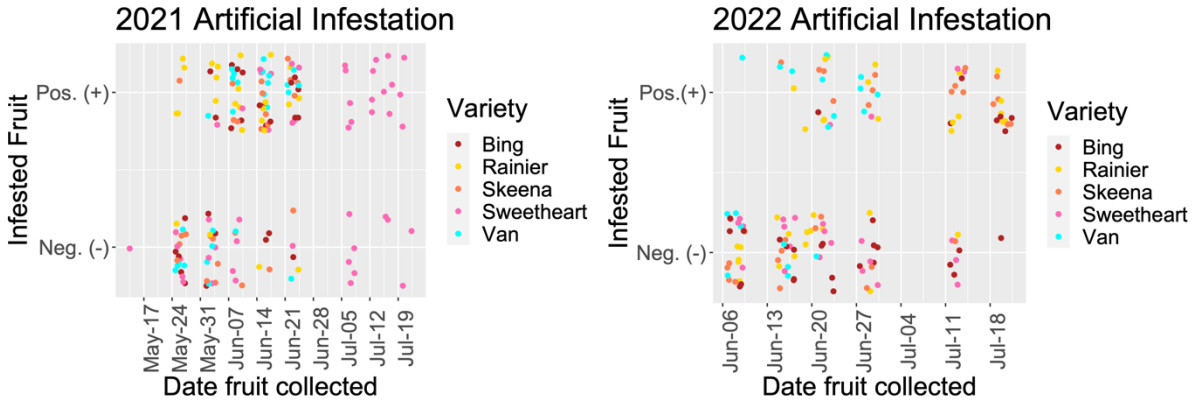

Figure 6. *Artificial infestation of all varieties in 2021 and 2022. These fruit were collected from the field and then SWD were added to determine if fruit were susceptible to attack. These data indicated more of the fruit were susceptible than suggested by the naturally infested data.*

Project Title: SWD in-orchard movement and overwintering population dynamics

Report Type: Continuing Project Report

PI: Dr. Christopher Adams
Organization: OSU MCAREC
Telephone: 248-850-0648
Email: chris.adams@oregonstate.edu
Address: 3005 Experiment Station Drive
City/State/Zip: Hood River, OR 97031
PROPOSED DURATION: 3 Years

Cooperators: Stacey Cooper, Mike Omeg, Brian Nix

Project duration: 3 years

Total Project Request for Year 1 Funding: $43,952
Total Project Request for Year 2 Funding: $41,137
Total Project Request for Year 3 Funding: $42,254

Other related/associated funding sources
Agency name: Helium Foundation, IoT grant
Amount: $100,000
Funding duration: 2023
Status: Preproposal approved and highly ranked. Full proposal submitted, decision pending.

Budget
Primary PI: Dr. Chris Adams
Organization Name: OSU Agricultural Research Foundation
Contract Administrator: Charlene Wilkinson
Telephone: 541-737-3228
Email address: Charlene.wilkinson@oregonstate.edu
Station Manager/Supervisor: Stuart Reitz
Station Manager/Supervisor email: Stuart.rietz@oregonstate.edu

Item	2021	2022	2023
Salaries[1]	$21,266	$21,904	$22,561
Benefits	$14,886	$15,333	$15,793
Wages			
Benefits			
Equipment[2]	$3,900	$0	$0
Supplies[2]	$2,400	$2,400	$2,400
Travel[4]	$1,000	$1,000	$1,000
Miscellaneous[5]	$500	$500	$500
Plot Fees			
Total	$43,952	$41,137	$42,254

Footnotes:
[1]Adams lab Faculty Research Assistant at 0.40 FTE (2 days /wk), with 3% increase in years 2 and 3; OPE 70%
[2]Dragino LHT65 temp & humid sensor ($50 each) x 60, Long Range Transceiver ($450 each) x2
[3] Research consumables, SWD bait and traps (40 traps per rep x 3reps = 120 traps) bait replaced weekly for 24 weeks (Jul-Dec) aprox. $100 x 24 wk = 2400
[4] Travel to field plots
[5] supplies:cups, wire, flagging, misc.

Objectives

1) Collect data on overwintering flies and temperature data.. (Year 1 beginning fall/winter 2022)

Progress: Orchard sites identified for this first season of winter data collection. Gateway modems & Temperature and humidity sensors purchased.

2) Correlate terrain, vegetation and microclimate temperature data with trap counts to determine if specific in-orchard habitats are more favorable to overwintering SWD. (year 2 & 3)

Progress: data analysis will occur in year 2 & 3.

3) Determine if overwintering SWD can be targeted with off season sprays or attractive baits. (yr 3)

Progress: data analysis will occur in year 2 & 3.

Significant findings

- We established 4 orchard blocks across The Dalles OR for the installation of remote temperature and humidity sensors, wireless network model, and insect traps.
- Hardware ordered and received. Installation of sensors and insect traps will occur in the coming weeks.

Timeline

Objective	2022/2023	2023/2024	2024/2025
1. **Winter** Trapping	X	X	X
2. Correlate temperature and terrain		X	X
3. Winter sprays		X	X

Methods

1) Expand preliminary overwintering trapping data to include more orchards with variable border habitats. (year 1)

The inspiration for this research came from visiting an orchard in The Dalles (Figure 1) and observing that there were no wild resources outside of the managed cherry orchard. The conventional wisdom is that SWD leave cherry orchards after harvest to find suitable overwintering sites. In addition, this area experiences high winds most of the year, so flies should need to seek shelter. At the center of the orchard is a valley with some wild blackberry habitat. Our hypothesis was that overwintering SWD would move to the valley at the center of the orchard to seek shelter for the winter. In an effort to capture the microclimate across the orchard we equipped the orchard with 20 temperature and humidity readers and connected them to a central hub modem with cell phone connectivity for remote data access (Figure 2). We then placed around 30 SWD traps across the orchard in an effort to capture this theoretical movement. While catch data from the traps in the valley was the highest (>4000), other traps still caught SWD at very high levels (1000s) (Figure 3). This would suggest that flies are not moving out of the orchard and not seeking shelter within the orchard. Many questions remain to be answered. We propose to expand this research project to two other orchards and try to replicate these results.

2) Correlate terrain, vegetation and microclimate temperature data with trap counts to determine if specific in-orchard habitats are more favorable to overwintering SWD. (year 2 & 3)

A key question of this research is: Are temperature, wind, or habitat (or all of the above) correlated with successful SWD overwintering here in the PNW, or are flies just remaining in orchards regardless of these factors. In selecting additional orchards, we will look for location with variable off-site habitat and different topography. Catch data will be tested with multi-variant statistical analysis to look for correlation with factors such as elevation, temp, humidity, habitat, etc.

3) Determine if overwintering SWD can be targeted with off season sprays or attractive baits. (yr 3)

Depending on what we learn from objective 2 we will develop a management plan to attack SWD where they are. We could spray them only in targeted locations, i.e. where they have hunkered down in protected valleys or blackberry patches, or we will plan on full orchard sprays. We have plans for two approaches. One approach would be a conventional insecticide spray such as pyrethroids and organophosphates. A second approach is to spray a non-caloric sweetener (Erythritol) that has been shown to be lethal to SWD. We will set up randomized complete block design for each and try to standardize block size and planting.

Figure 1. Orchard in The Dalles were preliminary research was conducted, showing extreme topography within orchard. There is 80 m (262 ft) of elevation difference at this orchard, with a valley in the center. Note lack of habitat outside orchard.

Results and Discussion

This research is scheduled to begin this fall. Supplies have been ordered and research plots have been identified.

FINAL PROJECT REPORT **YEAR**: 3 of 3

Project Title: Pesticide residues on WA cherries

PI: Tory Schmidt
Organization: WTFRC
Telephone: (509) 665-8271 x4
Email: tory@treefruitresearch.com
Address: 1719 Springwater Ave.
City/State/Zip: Wenatchee, WA 98801

Cooperators: Gerardo Garcia, Sandy Stone, Pacific Agricultural Labs, Northwest Hort Council, Doug Stockwell, Doyle Smith, various ag chemical companies

Total Project Request: **Year 1:** $4349 **Year 2:** $5450 **Year 3:** $5620

Other funding sources: **Awarded**
Amount: **Chemical supplies**
Agency Name: **Various ag chemical companies**
Notes: **Registrants typically donate chemicals to be tested**

WTFRC Budget

Item	2020	2021	2022
Salaries			
Benefits			
Wages[1]	1269	1350	1400
Benefits[1]	680	700	720
RCA Room Rental			
Shipping[2]	300	300	300
Supplies/Chemicals	300	300	300
Travel[3]	800	800	900
Plot Fees			
Analytical lab fees	~~4000~~ 1000*	2000	2000
Total gross costs	~~7349~~ 4,349*	5,450	5,620
Anticipated Income (contracts and gift grants)	0	0	0
Total net costs	~~7349~~ 4,349*	5,450	5,620

Footnotes: Schmidt estimates 8% of his time is dedicated to this project on an annual basis
Most pesticides tested are donated by their registrants or an ag chemical supply company
1 Wages & benefits primarily for Garcia (spray applications), crew help for Garcia, and Stone (data entry & review)
2 Est. costs to ship cherries overnight to Sherwood, OR
3 Travel costs include hauling equipment to & from plots
*Note: actual lab fees were less than projected in the original budget ($4000) due to simplified trial protocol

2022 SUMMARY

In 2022, sixteen different pesticides were applied successfully by WTFRC staff to commercial Skeena block near Orondo, WA for analysis of residue levels at harvest. Fruit samples were collected on June 30, packaged, and shipped to Pacific Agricultural Labs (PAL) in Sherwood, OR; unfortunately, those shipments which were packed in blue ice in insulated containers and designated for overnight delivery to the lab did not arrive at their destination until 6 days later at which point the temperature inside the container had risen to ambient levels (~75F) for an undeterminable time. This disruption of the cold chain triggered the breakdown of chemical residues on the fruit surface and fatally compromised the results of the subsequent analysis.

In an attempt to potentially salvage some value from the situation, we shipped a redundant set of fruit samples that were collected at the same harvest date from the same trees to PAL for analysis. Unfortunately, by the time the problem was discovered, and the second fruit sample was prepared and packaged, the shipment was delayed several days by staffing and technical issues at the shipping company. As a result, even though the cold chain was preserved for the second shipment, it did not arrive at PAL until 12 days after they had been harvested, meaning that the results of that residue analysis would also be compromised.

PAL did ultimately analyze both sets of samples but due to the shipping issues described above, interpretation of the results is highly problematic. In general terms, we found that residue levels tended to be higher in the first sample group indicating that despite loss of the cold chain, most residues were degraded more significantly by the additional 7 days of delay in the timing of extractions made during sample processing. One notable exception was carbaryl which showed higher residues in the second set of samples, suggesting that this particular compound may be more likely to break down in warm temperatures than other chemistries.

After consultation with several colleagues, we decided to withhold our 2022 results due to concerns about potential misinterpretation of the data by people who might not fully understand or be aware of the complicating circumstances that have confounded our results. Individuals who would like access to the actual results are welcome to contact the PI (tory@treefruitresearch.com) who can provide proper context before potentially sharing the results.

As always, previous project reports and long-term summaries of our cherry residue studies are available at www.treefruitresearch.org.

Project Title: A robust PNW sweet cherry breeding and genetics program, 2022-2024

Report Type: Continuing Project Report

Primary PI: Per McCord
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City/State/Zip: Hood River, OR 97031

CO-PI 3: Cameron Peace
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Address 2: PO Box 646414
City/State/Zip: Pullman, WA 99164

Cooperators: Allan Bros. Fruit, Cherry River Farms, Custom Orchards, Inc. Orchardview Farms, Stemilt Growers, Breeding Program Advisory Committee (BPAC) members

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 183,524
Total Project Request for Year 2 Funding: $ 182,948
Total Project Request for Year 3 Funding: $ 201,863

Other related/associated funding sources:

Awarded
Funding Duration: 2019-2022
Amount: $188,165
Agency Name: WSDA Specialty Crop Block Grant
Notes: "Reducing Cold Damage in Tree Fruit". Co-PI: Matt Whiting

Awarded
Amount: $458,022
Funding Duration: 2020-2023 (1-year no-cost extension)
Agency Name: WTFRC/OSCC
Notes: "Understanding little cherry disease pathogenicity". PI. Scott Harper. Co-PIs: Alice Wright, Per McCord

Awarded
Amount: $599,807
Funding Duration: 2022-2025
Agency Name: USDA NIFA—AFRI
Notes: "Improving grading methods to infer eating quality in sweet cherries under different cold chain scenarios". PI: Carolina Torres. Co-PI's: Rene Mogollon, Per McCord

WTFRC Collaborative Costs: None

Budget 1
Primary PI: Per McCord
Organization Name: Washington State University
Contract Administrator: Stacy Mondy
Telephone: 509-335-6881
Contract administrator email address: arcgrants@wsu.edu
Station Manager/Supervisor: Naidu Rayapati
Station manager/supervisor email address: naidu.rayapati@wsu.edu

Item	2022	2023	2024
Salaries	$52,744.00	$54,854.00	$57,048.00
Benefits	$17,375.00	$18,070.00	$18,793.00
Wages	$39,426.00	$41,003.00	$42,643.00
Benefits	$9,514.00	$9,894.00	$10,290.00
RCA Room Rental			
Shipping			
Supplies	$29,561.00	$31,605.00	$33,181.00
Travel	$6,100.00	$6,100.00	$6,100.00
Plot Fees	$8,700.00	$10,656.00	$12,080.00
Miscellaneous	$2,500.00	$2,500.00	$2,500.00
Total	$165,920.00	$174,682.00	$182,635.00

Footnotes: Salaries includes 1.0 FTE research technician. Wages includes temporary labor for crossing, harvesting, seed extraction/transplanting, plus farm crew wages. Supplies includes costs for fruit evaluation, DNA extraction/genotyping, embryo rescue, propagation supplies/services, orchard maintenance, and equipment maintenance. Travel includes fuel, insurance, vehicle maintenance, and lodging/per diem costs (the latter during pollination season).

If project duration is only 1 year, delete Year 2 and Year 3 columns.

(*Complete the following budget tables if funding is split between organizations,* ***otherwise delete*** *extra tables.)*

Budget 2
Co PI 2:
Organization Name: Kelsey Galimba
Contract Administrator: Charlene Wilkinson
Telephone: 541-737-3228
Contract administrator email address: Charlene.wilkinson@oregonstate.edu
Station Manager/Supervisor: Stuart Reitz
Station manager/supervisor email address: stuart.reitz@oregonstate.edu

Item	2022	2023	2024
Salaries	$3,655.00	$2,718.00	$5,198.00
Benefits	$2,637.00	$1,946.00	$3,723.00
Wages	$3,439.00	$865.00	$4,034.00
Benefits	$625.00	$87.00	$690.00
RCA Room Rental			
Shipping			
Supplies	$4,599.00	$1,000.00	$3,057.00
Travel			
Plot Fees	$2,649.00	$1,650.00	$2,526.00
Miscellaneous			
Total	$17,604.00	$8,266.00	$19,228.00

Footnotes:
1. Salary: for one FRA to perform PGR applications, training, thinning, netting and data collection.
2. Wages: for hourly employees and students to assist with orchard activities and quality tests.
3. Supplies: include irrigation, trellising, block maintenance, and training supplies.
4. Research plot fees ($3,500/acre).

Objectives

1) Continue to generate seedlings, and rigorously evaluate seedlings and selections at all phases of the breeding program, including those now in Phase 3.
   a) Develop protocols for fruit evaluation via a small-scale commercial grade optical sorter (externally funded). –**Delayed until 2023 (Year 2) when sorter is installed.**
2) Test the effects of plant growth regulators on selections that have been advanced to Phase 3.
3) Increase the number of targeted cross made, seeds germinated, and seedlings transplanted
   a) Continue to utilize DNA information for superior and complementary parent selection and seedling screening.
   b) Deploy newly developed DNA tests for cracking susceptibility and fruit firmness.
4) Continue to implement timely and proper practices for orchard management (training/pruning, pest and disease monitoring and control, nutrient management).

Significant Findings

- 121 P1 seedlings were evaluated in the fruit quality laboratory. No seedlings were advanced to P2 from this cohort.
- Limited data was collected from selections in the old P2 trial. R25, an early-ripening self-fertile selection, was selected for re-evaluation in the new multi-location P2 trials.
- Cherry fruit set and fruit quality were severely impacted by adverse weather during the spring of 2022, likely impacting results of PGR trials in Hood River. No significant effects observed.
- For the 2022 crossing season, over 13,600 seed were produced, the highest since 2006.
- Over 2,300 seeds from crosses targeting early ripening were processed through embryo rescue (a program record)

- 764 seedlings from 2021 crosses were planted in 2022. DNA tests for self-fertility, powdery mildew resistance, low cracking, and high firmness were used to eliminate 113 seedlings prior to planting
- PCR testing for Little Cherry Disease identified 21 infected trees, which are in the process of being removed.

Methods

Fruit evaluation

As in prior years, breeding program advisory committee (BPAC) members were invited to inspect P1 seedlings during the harvest. Walkthroughs were conducted generally twice per week (once with BPAC members). Selection criteria in the field were based on fruit size, firmness, and flavor. The new P2 trials (planted 2021) are not yet fruiting, but standard cultivars and several selections from the old P2 trial were harvested at Prosser only. Fruit from field-selected P1 seedlings and P2 standards and selections were evaluated in the laboratory for defects (harvest and post-harvest), weight, diameter, firmness (via Firmtech), stem pull force, color, sweetness (°Brix), and titratable acidity. Total harvest weight was measured for P2 selections and standards on a per-tree basis. P1 selections with an average fruit weight less than 9 g and firmness less than 270 g/mm were generally not evaluated for downstream traits.

An induced cracking test was performed (4-hour soak in deionized water) for P2 selections and P1 seedlings with enough fruit. Prior to storage, P2 samples were treated with a fungicide soak (Shield-Brite® FDL-230SC). Fruit sampled for post-harvest analysis was placed in modified-atmosphere packaging and stored in a walk-in cooler for 4 weeks at approximately 35°F.

Plant Growth Regulators

'R19', a large, firm, early-ripening selection, has been identified as having light crop loads and being prone to cracking. Two branches on each tree were treated with ReTain®, two with Parka®, and two left untreated. ReTain® was applied once, at 50% bloom, at the equivalent of a rate of one pouch (333g)/100 gallons/acre. Flowers were counted prior to the application and fruit were counted at harvest. In addition, color, diameter, weight, firmness, stem retention force, SSC and TA were tested. Parka® was applied twice, at shuck fall and at straw color, at the equivalent of a rate of 1 gallon per acre. Soaking tests were performed with Parka®-treated and untreated controls at harvest to assess cracking. Color, diameter, weight, and firmness were also tested.

Because 'R3' (early season) and 'R29' (mid-season) do not seem to exhibit any specific yield or quality issues, GA_3 was tested on these selections, as it is used as a general quality-enhancer that improves multiple characteristics. ProGibb® was applied with a similar design as above, with three branches from each of five trees treated and three remained untreated. Applications were made between translucent green and straw color, at a concentration of 20 ppm.

Crossing and DNA tests

Crosses were made (a) by hand with emasculated blossoms and a small repurposed makeup applicator for pollination, and (b) by caging entire trees and utilizing bees (mason bees and captured honeybees) for pollination. Aside from a single caged poly-cross with two chosen male parents no open-pollinated seed was collected in 2022. The major criteria for crossing included early/late ripening, large fruit size, self-fertility, and powdery mildew resistance. Other criteria included resistance to LCD, freeze tolerance, and enhancing genetic diversity, the latter including use of several interspecific crosses with sour cherry (*Prunus cerasus*) and Western sand cherry (*P. besseyi*). Crosses were made in the field at the Roza orchard, and in the hoop house at IAREC Headquarters.

Seedlings from 2021 crosses were germinated in cold storage and transplanted to the greenhouse in late fall 2021-early winter 2022. Prior to being transplanted in the field, the seedlings were sampled for

DNA extraction. Based on the traits expected to segregate in particular crosses, seedlings were DNA-tested via PCR for powdery mildew resistance, self-fertility, and a newly developed test for cracking resistance and fruit firmness.

Embryo Rescue

For crosses targeting early ripening and interspecific hybrids, embryo rescue was used as in prior years (since 2019). Several crosses were used to test the effects of two growth media (Quorin-Lepoivre (QL) and Woody Plant Medium (WPM) and two developmental stages (pit hardening and 'straw' phase). Fruit were sterilized in 70% ethanol with a few drops of surfactant, and cut open with a pair of anvil pruners to extract the seed. For nearly all families (including all non-interspecific families), the seed coat was removed prior to placing the seed on the growth medium. Embryos were then stored in a walk-in cooler (39-46 °F) until germination. Embryos harvested early (at or just prior to pit hardening) were incubated at room temperature under LED growth lights (16-hour photoperiod) for 2 weeks prior to being transferred to the cooler. Once germination occurred, embryos were transferred to the growth cart under LED lights until true leaves and roots had formed (approximately 3 weeks). At this point, the embryos were transplanted into potting mix and placed in a clear plastic storage box in a greenhouse for 2 weeks' acclimation. The storage box lid was kept closed for the first week, and opened for short periods each day for the second week, after which acclimation was considered complete.

Disease Screening and Orchard maintenance

New parental trees (potted), and all trees used as parents in field crosses, were screened via quantitative reverse transcription PCR (qRT-PCR) for *prune dwarf virus* (PDV) and *Prunus necrotic ringspot virus* (PNRSV), which are both pollen- and seed-transmissible. In addition, seedling and parental trees in the field that were symptomatic for Little Cherry Disease were screened via qPCR or qRT-PCR for X-disease phytoplasma and Little Cherry Virus 2, respectively. Orchards were sprayed, on average, every 2 weeks from 14 May to 8 October. Pre-harvest target insects were cherry fruit fly and spotted-wing *Drosophila*. Post-harvest (July 30th onwards), the target pests were leafhoppers and two-spotted spider mites. With the chemicals used, San Jose scale, black cherry aphid, and leafrollers were also controlled. Orchard irrigation and most weed control and pruning were undertaken by the IAREC farm crew. Soil samples taken from each block in late spring were used to guide fertilizer applications.

Results and Discussion

Fruit Evaluation

A total of 121 P1 seedlings passed field selection criteria and were evaluated in the lab. None performed sufficiently superior to the standards in multiple years to warrant being advanced to P2 (results not shown), but at least two promising P1 seedlings were identified that will be evaluated for at least one more season.

In addition, four P2 selections (in ripening order: R19, R25, R3, and R47) were evaluated using trees from the old P2 trial. R19 and R3 are currently also under evaluation in P3 trials, with first harvest expected in 2023. R47 was originally only at Prosser, but has been advanced to a multi-location P2 trial (planted 2021). R25 is an interesting case, and is indicative of the value of BPAC involvement in the breeding program. It had been dropped from a multi-location P2 trial (at Prosser, Sagemoor, and Hood River) prior to 2018, likely due to insufficient fruit size. However, it is self-fertile, ripens essentially the same time as 'Chelan', and has superior flavor likely due to a much higher ° Brix (Table 1). Its superior taste vs. 'Chelan' was documented by the BPAC, and is being re-evaluated in a new P2 trial.

Table 1. Characteristics of R25, a selection being re-evaluated in a new P2 trial, compared with standard early-season cultivar 'Chelan'. Results are from 2022, at Prosser.

ID	Color (CTIFL)	Timing	Fruit weight (g)	Row size/Diameter (mm)	Firmness (g/mm)	°Brix/TA	Notes
R25	6.8	Chelan +1	7.7	10.8/24.7	254	19.7/0.51	Self-fertile
Chelan	5.75	--	7	11.1/23.7	223	16.5/0.51	

In 2022, R19, R3, and R47 displayed characteristics typical for these selections from previous years' evaluations (Table 2). Cracking is known to be a problem with R19, but it was especially severe in 2022. Despite such flaws, there is still interest in R19 because of its early market timing and superior fruit quality vs. 'Chelan', the early standard. For each of these selections, additional data from larger-scale P3 trials (Phase 3 for R19 and R3, multi-location P2 for R47) are expected beginning in 2023.

Table 2. Characteristics of R19, R3, and R47. R19 and R3 are currently in P3 trials; R47 was planted in full multi-location P2 trials in 2021. Results reported are averages from 2018-2022 (where available).

ID	Color (CTIFL)	Timing	Fruit weight (g)	Row size/Diameter (mm)	Firmness (g/mm)	°Brix/TA	Notes
Chelan	5.00	(standard)	7.2	10.9/24.2	249	17.2/0.55	
R19	4.92	Chelan + 0	8.9	10/26.9	324	22.4/0.55	Early, sweet and firm, nose cracking, self-fertile but light crops, storage challenges
R3	4.89	Chelan +3-4	9.6	9.9/27.3	311	20.1/0.51	Meaty texture, ripen fully for best flavor
R47	5.59	Chelan +7	10.7	9.6/28.1	376	22.6/0.75	Very firm most years

Plant Growth Regulators

The Mid-Columbia Gorge experienced abnormal spring weather conditions in 2022, with the majority of bloom taking place in cold, overcast conditions. These poor pollination conditions, combined with a substantial late snow event in the beginning of April, caused poor and staggered fruit set in the majority of cherries in the region, including those in the Phase II trial at MCAREC. Additionally, a

prolonged rainy season created issues with quality, causing significantly more cracking than is normally observed in Hood River. All of these factors have the potential to skew the results of the PGR testing performed in 2022. No significant effects were observed for any of the quality parameters measured.

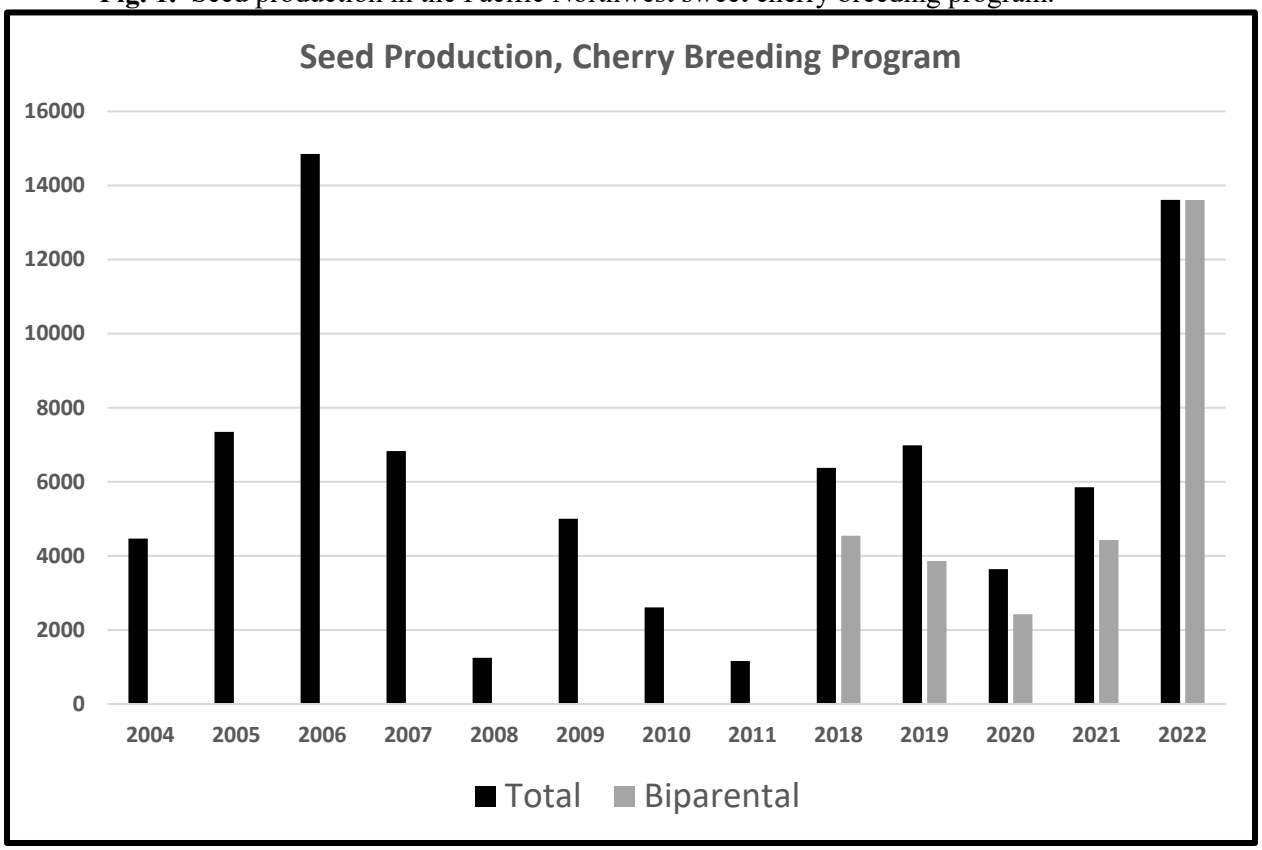
Crossing & Seedling generation

In 2022, the breeding program made 63 crosses, producing an estimated 13,610 seed in total. This is the highest amount of seed produced by the breeding program in a single year since 2006 (Figure 1). This is especially remarkable considering the very unfavorable weather that occurred during bloom and no use of seeds resulting from open-pollination. In fact, essentially all seed (>99%) was collected from bi-parental crosses, which are those with the highest expectation for future outcomes because the breeder has control over choosing both parents (each with desirable characteristics) as well as the particular pairwise combinations (parents that complement each other). For the second year in a row, the WTFRC/OSCC-funded hoop house was successfully used to make crosses indoors, resulting in 798 seed. Attempts to utilize mason bees in the hoop house were unsuccessful, so virtually all of the hoop house seed (98%) came from hand-pollinated crosses. In the future, bee-pollinated crosses in the hoop house will employ bumblebees, which are known to perform well in greenhouse settings. In the field, the opposite situation occurred. The cold spring weather ruined the majority of hand-pollinated field crosses, which were made with emasculated flowers that are more exposed to the elements. However, bee-pollinated crosses performed very well, resulting in 12,375 seed (over 90% of the total). A likely explanation for the remarkable success of bee pollination was the change in the placement of the bucket of male flowers in each cage. In prior years, this bucket was placed on the ground. In 2022, on the advice of a visiting breeder (Lubor Zeleny from Holovousy, Czech Republic), the bucket was secured to branches in the tree canopy. This placed the male blossoms in closer proximity to the female blossoms, as well as the bees (who spend the majority of their time near the ceiling of the cage). It was a simple solution that will certainly be utilized in the future.

Embryo rescue has been used by the program since 2019, and its use has expanded. In 2022, a record number of more than 2,300 embryos were rescued. Current germination results from the experiment involving nutrient media and harvest stage (Table 3) suggests that while certain treatments have an effect in certain families, there is no overall effect of nutrient medium or harvest stage. The number of embryos rescued this year, although significantly more than in prior years, is sustainable, and represents the program's commitment to breeding early-ripening cherries.

In 2022, 764 seedlings (from 2021 crosses) were transplanted to the field. An additional 113 seedlings were eliminated prior to transplanting based on DNA test results. Germination was challenging for this crop, most likely due to seeds being too moist after cleaning, or too much moisture in the stratification medium. Seed cleaning and stratification protocols were carefully reviewed during the 2022 season, and we expect germination for the 2022 crosses (currently ongoing) to be higher than 2021.

Fig. 1. Seed production in the Pacific Northwest sweet cherry breeding program.



Footnotes: The proportion of bi-parental seed is not known with certainty prior to 2018 (no data available 2012-2014 and no crosses were made 2015-2017).

Table 3. Results of the 2022 embryo rescue experiment of testing the effects of nutrient media and harvest stage.

FAMILY	MEDIUM	STAGE	SURVIVED	TOTAL	PERCENTAGE
24	Q	F	3	93	3
24	W	F	40	101	40
24	Q	S	49	110	45
24	W	S	61	157	39
27	Q	F	40	60	67
27	W	F	27	59	46
27	Q	S	N/A		
27	W	S	N/A		
61	Q	F	48	83	58
61	W	F	55	93	59
61	Q	S	9	88	10
61	W	S	8	83	10
62	Q	F	15	90	17
62	W	F	13	108	12
62	Q	S	33	94	35
62	W	S	45	106	42
TOTAL			446	1325	34

Footnotes:
Medium: Q = Quorin-Lepoivre; W = Woody Plant Medium
Harvest stage: F = first harvest (pit-hardening); S = second harvest (straw)

Disease Screening & Orchard Maintenance

The incidence of LCD increased dramatically in breeding program seedling blocks. Whereas 0 out of 28 trees in 2021 tested positive, 17 out of 41 samples tested positive in 2022. Of the positive samples, 12 were infected with XDP, 7 with LChV-2, and two samples were infected with both pathogens. In addition, the Clean Plant Center Northwest (CPCNW) tested symptomatic trees in one of the parental blocks (B53) and found 4 additional trees (out of 6 tested) infected with XDP. These trees have been marked for removal by the IAREC farm crew. Although the cherry blocks at the Roza (and IAREC Headquarters) are being sprayed regularly for leafhoppers, control of broadleaf weed hosts (of XDP) throughout the farm needs to be improved. The breeding program regularly tests for the presence of PDV and PNRSV in breeding materials. Although PDV in particular is prevalent at the Roza farm, only a single mother tree tested positive for PDV, and a second mother tree tested positive for PNRSV (both will be re-tested), out of 29 parental trees tested. Parental trees in the hoop house had a higher incidence of infection, with 9 out of 20 infected with PDV and one sample infected with PNRSV. Infected trees have been removed, or in a few cases, are being retested.

CONTINUING PROJECT REPORT

Project Title: Sweet Cherry Bud Cold Hardiness Model

PI: Kelsey Galimba

Report is forthcoming.

Project/Proposal Title: Nutrient management for high quality sweet cherries

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Cooperators: Denny Hayden, Craig Harris, Luke Anderson (Allan Brothers), Rob Blakey (Stemilt), Aylin Moreno (Washington Fruit and Produce)

Report Type: Continuing Project Report

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 14,716
Total Project Request for Year 2 Funding: $ 15,138
Total Project Request for Year 3 Funding: $ 15,576

Other related/associated funding sources: Root Growth Management to Reduce Ca Deficiency Disorders in Apples and Cherries. P.I. B. Sallato. Awarded.
Funding Duration: 2019 - 2022
Amount: $152,938
Agency Name: Washington State USDA- Specialty Crop Block Grant
Notes:

Budget 1

Organization Name: Washington State University **Contract Administrator:** Katy Roberts
Telephone: (509) 335-2885 **Email address:** arcgrants@wsu.edu
Station Manager: Naidu Rayapati **Email address:** naidu@wsu.edu

Item	2021	2022	2023
Salaries			
Benefits			
Wages[1]	9,600	9,984	10,384
Benefits	928	966	1,004
Equipment			
Supplies[2]	3,888	3,888	3,888
Travel	300	300	300
Miscellaneous			
Plot Fees			
Total	14,716	15,138	15,576

Footnotes: [1] Wages for two temporary support at 15 USD/hour for Sallato's and Torre's lab for 310 hours each (9.4% benefits), plus 600 hours of technician at Sallato's lab at 15 USD/hour and 68.3% benefit. [2] Supplies include laboratory supplies and nutrient samples at 18 USD/sample.

OBJECTIVES

The goal of this project is to improve nutrient management strategies from an understanding of the nutritional composition of good and poor-quality fruit. We proposed to undertake a prospective analysis of orchard growing conditions and fruit nutrient levels and their relationship with key quality parameters: size, firmness, and storability. This research approach permits an in-depth analysis of fruit nutritional content and fruit quality, identifies predictors, determines nutrient extraction/demand, and begins to develop fruit-specific nutritional management strategies for sweet cherry.

1) Identify adequate nutrient conditions for fruit quality in sweet cherry.

2) Determine nutrient demand on different sweet cherry varieties.

3) Identify key conditions leading to better fruit quality and storability in sweet cherry.

4) Develop outreach and educational materials and workshops.

Cultivars were selected by a grower advisory group (collaborators) and include Chelan, Coral Champagne, and Skeena. Three different growing locations were selected for Chelan and Coral Champagne. For Skeena we included five sites.

SIGNIFICANT FINDINGS

- Fruit firmness overall was greater in 2022 compared with 2021, with mean value of 316 $g \cdot mm^{-1}$ (versus 273 $g \cdot mm^{-1}$ in 2021) ($p<0.001$). Consistent with 2021, this analysis reveals the high variability in firmness among cultivars and years.
- Fruit size was overall larger in 2022, varying between 19.4 and 34.9 mm, equivalent 14 and 8.0 row size.
- There were no consistent relationships between fruit firmness and nutritional content
- Fruit Ca^{2+} content across cultivars and sites varied between 0.07 and 0.15% with no relationship to fruit firmness

METHODS

This project takes an observational approach to better understanding the relationships between cherry fruit quality/storability, and fruit nutrient content. There are no imposed treatments, instead, we collected fruit from four commercial warehouses around the state and worked with the natural variability in quality that exists.

The relationship between fruit quality and storability was analyzed for Chelan, Coral Champagne from three commercial orchards and five commercial orchards of Skeena. For each cultivar and orchard, we obtained four replicate bulk fruit samples of at least 5 lbs of the largest and smallest fruit size from the packing house (typically 12-row and 9-row+), in order to have sufficient fruit for storage and nutrient testing from each size category. Each replicated sample from each size category was divided in half (ca. 2.5 lbs). One set of samples were sent to Torre's laboratory at TFREC for storage evaluation test, and the other half were taken to Whiting's laboratory for harvest analysis at IAREC. In Whiting's laboratory, fruit were analyzed individually for weight, size (mm) and firmness (Firmtech II). Further, for each sample unit (ca. 100 fruit each), the 10th and 90th percentile ranking of firmness testing were selected for nutritional analysis (minimum 15 fruit per category) (Figure 1). To determine fruit nutrient content, each fruit sample were separated into pulp, stems and pits to determine fresh and dry weight ratios. Dried tissue samples were homogenized and sent for chemical analysis of nitrogen (N), phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), iron (Fe), manganese (Mn), cupper (Cu), zinc (Zn) and boron (B). To ensure representative and consistent nutrient analyses, samples were sent to Soil Test laboratory (Moses Lake) for total nutrient. Soil Test laboratory is a certified laboratory by the Soil Science Society of America and the North American Proficiency Test Program (NAPT) for plant program assessment (visit https://www.naptprogram.org/about/participants?ssoContinue=1). The laboratory incorporates blind certified sample to monitor nutrient accuracy by utilizing certified material from NAPT program.

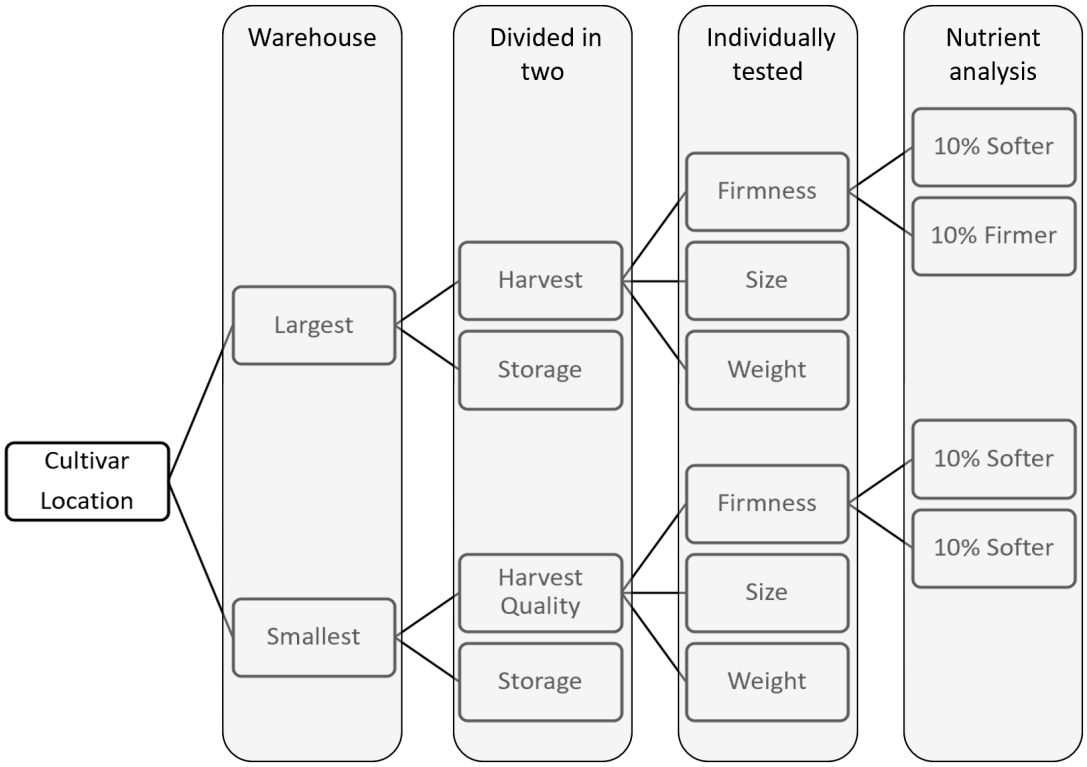

Figure 1. Fruit sampling scheme for nutrient and storability analyses.

In Co-PI Torres's laboratory in Wenatchee, fruit were stored for four weeks in cold storage, and analyzed fruit weight, color, size and firmness, plus storage disorders including decay, stem browning, or pitting.

This experiment will reveal differences between cultivars and growing sites, the relationships between fruit quality (size and firmness) with nutrient content and storability. In the scenario where there is a relation between fruit quality and nutrient content, this research will allow us to establish standards and predictors of nutrient levels for firmer and larger fruit in sweet cherry.

RESULTS AND DISCUSSION

Fruit Quality by size category

In 2022, overall fruit firmness, diameter and weight were greater than in 2021. Firmness of fruit samples exhibited about a 5-fold range, from 130 to 636 $g \cdot mm^{-1}$ and a mean value of 316 $g \cdot mm^{-1}$, versus 123 $g \cdot mm^{-1}$ and 474 $g \cdot mm^{-1}$, and mean value of 273 $g \cdot mm^{-1}$ in 2021 ($p<0.001$). Similar to our results in 2021, our analyses reveal a very high variability in firmness among cultivars and years.
Fruit size was larger overall in 2022, when considering the largest or smallest fruit obtained from the packing house, varying between 19.4 and 34.9 mm, equivalent 14 and 8.0 row size (Figure 1.b). The double bell-shaped distribution reflects both populations of fruit; big and small, obtained from the packing house. And overall fruit weight was 40% larger than in 2021 (data not shown)

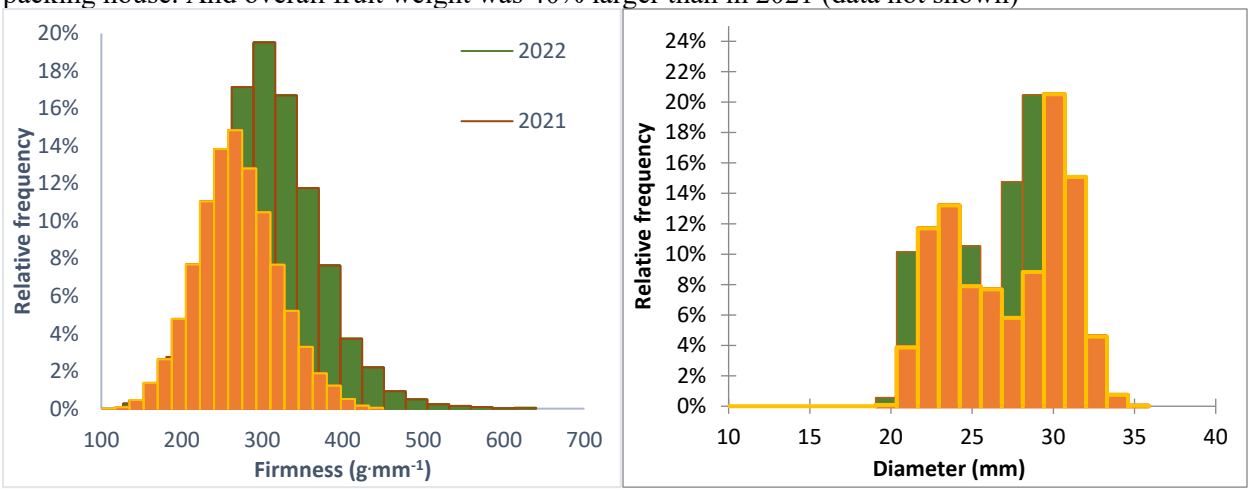

Figure 1. Fruit firmness (left) and diameter (right) distribution in 2021 and 2022.

In 2022 fruit quality parameters for the big size category (Table 1) and small size category (data not shown) demonstrate quality differences between sites.

<u>Chelan</u>: considering only the fruit from the larger size class, fruit firmness and diameter were different in all three sites, with site 2 having the firmest fruit overall (365 $g \cdot mm^{-1}$) and site 1 having the softest (247 $g \cdot mm^{-1}$). Site 3 had the largest fruit (32.4 mm equivalent to row 8.5) and site 1 the smallest (28.8 mm equivalent to row 9.5) (Table 1). Sites 2 and 3 were 37% and 34% firmer in 2022 compared with 2021, while site 1 was 6% softer (data not shown). Chelan sites 1 and 2 are in the same growing area near Roosevelt, thus under similar environmental conditions, however, yield from site 1 was almost three times greater than yield at site 2. In 2021 and 2022, site 1 yielded 9.3 and 7.6 tons/acre respectively, while site 2 yielded 3.6 and 4.6 tons/acre in 2021 and 2022, respectively. Site 3, located north of Pasco, produced 8.3 and 7.3 tons/acre in 2021 and 2022, respectively.

Table 1. 2022 Mean and standard deviation of fruit firmness, diameter, weight, color and soluble solids content of the large category fruit, by cultivar and site. Different letters indicate statistical differences ($p < 0.001$) between sites within cultivars.

Cultivar	Site	Firmness (g/mm ± St.Dev)			Diameter (mm ± St.Dev)			Weight (g ± St.Dev)			Color (CTFL ± St.Dev)			Soluble Solids (Brix ± St.Dev)		
Chelan	1	247	5.6	c	28.8	0.1	c	11.2	0.1	b	5.7	0.4	a	16.6	0.9	a
Chelan	2	365	8.5	a	31.1	0.1	b	11.1	0.2	b	4.9	0.2	b	16.8	0.7	a
Chelan	3	308	6.1	b	32.4	0.0	a	13.1	0.5	a	4.5	0.1	b	14.7	0.6	b
Chelan mean		**307**	**6.7**		**30.8**	**0.1**		**11.8**	**0.3**		**5.1**	**0.2**		**16.0**	**0.7**	
p value			**<0.0001**			**<0.0001**			**<0.0001**			**0.001**			**0.007**	
Coral C	1	302	3.8		29.8	0.1	b	13.2	0.2		5.5	0.1	a	15.1	1.3	b
Coral C	2	296	9.9		31.4	0.1	a	13.6	0.5		4.9	0.0	b	18.1	0.5	a
Coral C	3	294	6.1		30.9	0.6	a	13.0	0.9		4.9	0.3	b	17.5	0.8	a
Coral mean		**297**	**6.6**		**30.7**	**0.3**		**13.3**	**0.5**		**5.1**	**0.1**		**16.9**	**0.9**	
p value			**0.326**			**0.000**			**0.380**			**0.001**			**0.003**	
Skeena	1	315	5.2	cd	30.5	0.0		12.8	0.2	b	5.2	0.3	a	19.2	0.5	bc
Skeena	2	389	6.7	a	30.1	0.1		11.2	0.1	c	4.8	0.2	b	23.1	0.9	a
Skeena	3	330	7.7	bc	30.4	0.1		12.8	0.3	b	5.1	0.2	ab	21.0	1.1	ab
Skeena	4	312	8.0	d	29.9	0.1		12.6	1.2	b	5.2	0.3	a	21.7	0.4	a
Skeena	5	333	12.2	b	29.8	0.8		15.0	0.2	a	5.0	0.1	ab	17.4	2.3	c
Skeena mean		**336**	**8.0**		**30.2**	**0.2**		**12.9**	**0.4**		**5.1**	**0.2**		**20.5**	**1.0**	
p value			**<0.0001**			**0.093**			**<0.0001**			**0.050**			**<0.0001**	

Coral Champagne: firmness was not different between sites, similar to our results in 2021, with values ranging between 294 and 302 $g \cdot mm^{-1}$. All sites were firmer than in 2021, by 16%, 31% and 23%, for sites 1, 2 and 3 respectively (data not shown). Fruit diameter was statistically larger in site 1 and 2, however, with small agronomical differences between all three sites (Table 1). Coral Champagne site 1 yielded 6.05 tons/acre in 2021 and 5.57 tons/acre in 2022. Site 2, produced 2.8 ton/acre in 2021, and 3.2 ton/acre in 2022. Site 3 (data not reported yet)

Skeena; firmness was on average 14% higher than in 2021 (data not shown). Site 1 firmness did not vary between 2021 and 2021, with values above 300 $g \cdot mm^{-1}$ on both years. All other sites were firmer in 2021, by 41%, 10%, 16% and 22% for sites 2, 3, 4 and 5 respectively. Interestingly, in 2022, the firmest site was site 2, consistent with 2021, and the softest was site 4, also consistent with 2021. However, in 2022 all values were above 300 $g \cdot mm^{-1}$. Size was algo higher in 2022, except in site 4 with no differences between years (data not shown). All other sites were between 13% and 18% larger when comparing by years. However in 2022, there were no differences between sites, averaging 30.2 mm (equivalent to row size 8.5).

Firmness, size and nutrient concentration by cultivar and site

Within each size category, fruit from the 10th and 90th percentile ranking of firmness were selected for individual fruit quality analyses and then were combined for nutrient analyses. Each cultivar and site therefore, had four quality categories; big and firm (BF), big and soft (BS), small and firm (SF), small and soft (SS). In this report we focus on nutrient differences between firmness categories, for simplification.

Table 2. Fruit firmness, nutrients and dry matter between soft and firm fruit, for the large size category, by cultivar and site. Different letters indicate statistical differences ($p < 0.05$) between fruit firmness categories, within site and cultivar.

ID		Firmness Cat	Firmness ($g*mm^{-1}$)	N %	P %	K %	Ca %	Mg %	S %	N/Ca	K/Ca	(Mg+K)/Ca	Dry M.	N mg/100g	P mg/100g	K mg/100g	Ca mg/100g	Mg mg/100g	S mg/100g
Chelan	1	FIRM	329 a	0.88	0.16	1.23	0.10	0.07	0.05	**8.83 a**	**12.4 a**	**13.1 a**	**0.20 a**	178	31.4	250	20.3	14.7	10.1
		SOFT	178 b	0.85	0.16	1.27	0.12	0.08	0.05	**7.23 b**	**10.7 b**	**11.4 b**	**0.19 b**	160	30.1	239	22.6	14.1	9.9
		p value	<0.0001	ns	ns	ns	ns	ns	ns	**0.088**	**0.071**	**0.070**	**0.008**	ns	ns	ns	ns	ns	ns
	2	FIRM	471 a	1.11	0.18	1.55	0.15	0.09	0.07	8.22	11.5	12.12	0.23	254	41.1	354	33.6	20.0	**15.4 a**
		SOFT	270 b	1.08	0.17	1.47	0.11	0.09	0.06	9.99	13.7	14.46	0.22	237	37.0	325	23.8	19.3	**13.8 b**
		p value	<0.0001	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.061
	3	FIRM	400 a	0.98	0.13	1.20	0.10	0.08	0.08	9.84	12.2	12.97	0.21	203	27.6	249	20.7	16.2	16.2
		SOFT	218 b	1.04	0.14	1.29	0.10	0.08	0.08	10.34	12.8	13.59	0.20	211	27.8	261	20.5	16.5	15.2
		p value	<0.0001	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Coral Champagne	1	FIRM	396 a	**1.17 a**	0.14	1.16	0.10	0.07	**0.07 a**	**12.0 a**	11.9	12.64	**0.23 a**	**263 a**	**32.2 a**	**262 a**	22.0	**16.4 a**	**15.8 a**
		SOFT	205 b	**1.03 b**	0.13	1.15	0.10	0.07	**0.06 b**	**10.1 b**	11.3	12.06	**0.20 b**	**206 b**	**26.6 b**	**231 b**	20.7	**14.6 b**	**11.5 b**
		p value	<0.0001	**0.091**	ns	ns	ns	ns	**0.040**	**0.080**	ns	ns	**0.070**	**0.006**	**0.008**	**0.042**	ns	**0.086**	**0.006**
	2	FIRM	357 a	1.22	0.18	1.39	0.08	0.07	0.10	15.4	17.4	18.32	0.20	247	36.6	282	16.3	14.8	19.4
		SOFT	248 b	1.13	0.16	1.33	0.08	0.07	0.08	13.8	16.5	17.35	0.20	226	32.4	265	16.5	14.5	15.4
		p value	<0.0001	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	3	FIRM	375 a	0.86	0.13	1.08	0.10	0.07	0.06	8.9	11.3	12.07	0.22	187	28.9	236	21.8	15.8	12.6
		SOFT	218 b	0.86	0.13	1.18	0.10	0.08	0.06	9.0	12.4	13.15	0.21	181	27.5	250	20.6	15.8	12.2
		p value	<0.0001	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Skeena	1	FIRM	385 a	1.04	0.18	0.97	0.09	0.08	0.07	11.8	11.1	12.02	0.20	199	34.0	189	17.2	16.1	14.0
		SOFT	243 b	1.00	0.17	0.98	0.08	0.08	0.07	12.4	12.2	13.22	0.17	169	28.2	164	13.5	13.0	11.8
		p value	<0.0001	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	2	FIRM	510 a	1.17	0.17	0.96	0.08	0.08	0.08	15.3	12.5	13.59	**0.24 a**	**281 a**	**41.8 a**	**234 a**	**19.4 a**	**20.0 a**	**18.7 a**
		SOFT	290 b	1.19	0.18	1.01	0.07	0.08	0.08	17.2	14.5	15.58	**0.18 b**	**212 b**	**32.2 b**	**181 b**	**12.5 b**	**13.8 b**	**13.8 b**
		p value	<0.0001	ns	ns	ns	ns	ns	ns	ns	ns	ns	**0.001**	**0.013**	**0.028**	**0.087**	**0.063**	**0.005**	**0.021**
	3	FIRM	395 a	0.93	0.15	1.02	0.08	0.06	0.06	11.8	13.1	13.87	0.23	212	34.2	235	18.0	14.6	13.3
		SOFT	265 b	0.98	0.15	1.02	0.08	0.07	0.06	12.8	13.0	13.86	0.22	215	32.2	227	17.3	14.4	12.8
		p value	<0.0001	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	4	FIRM	361 a	0.98	0.18	1.11	0.08	0.08	0.08	12.2	13.8	14.85	0.17	168	30.0	190	13.7	13.8	13.1
		SOFT	242 b	1.06	0.17	1.10	0.09	0.09	0.08	12.5	12.9	13.95	0.16	168	27.4	175	13.5	13.5	11.8
		p value	<0.0001	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	5	FIRM	411 a	**1.26 a**	**0.18 a**	1.21	**0.08 b**	**0.09 a**	0.09	**16.9 a**	**16.2 a**	**17.3 a**	0.17	214	31.2	206	12.7	14.9	15.4
		SOFT	259 b	**1.03 b**	**0.16 b**	1.07	**0.09 a**	**0.08 b**	0.07	**12.2 b**	**12.6 b**	**13.5 b**	0.17	171	26.1	176	14.2	13.3	11.9
		p value	<0.0001	**0.056**	**0.084**	ns	**0.050**	**0.024**	ns	**0.041**	**0.022**	**0.019**	ns	ns	ns	ns	ns	ns	ns

Nutrient differences between firm and soft fruit within cultivar and site were inconsistent. For example, for Chelan sites, only in site 1, firmer fruit had higher N:Ca, K:Ca, (K+Mg):Ca and dry matter content, however these differences were not observed in sites 2 and 3. Similarly, for Coral Champagne, only in site 1 we found firmer fruit had higher N and S concentration (dry basis), higher N:Ca ratio, dry matter content and N, P, K, Mg and S concentration (fresh basis). Of all the Coral Champagne sites, site 1 had also the greatest difference between soft and firm fruit (190 $g.mm^{-1}$ difference). In Skeena sites, site 2 and site 5 reported differences between firm and soft fruit, however in different parameters; site 2 firm fruit had higher dry matter and N, P, K, Ca, Mg and S (fresh basis), while site 5 firm fruit had higher N, K, Mg and N:Ca, K:Ca and (K+Mg):Ca, while lower Ca.

Firmness and nutrients concentration differences between years by cultivar

To better understand the relation between firmness and fruit nutrient content, we compared all sites and years, within each cultivar (data not shown), to have greater range of firmness levels. In summary, for Chelan, N, P, K, Mg, S concentration were not associated to firmness nor size categories. However, Ca concentration (dry basis) and N, P, K, Ca and Mg concentration (fresh basis) were 96.6%, 42.6%, 50.4%, 44%, 134% and 22.6% higher in the firmest fruit compared to the softest fruit. Similarly, for Coral Champagne, N, P, K, Ca and Mg concentration (dry basis) were not related to fruit firmness and size categories. However, N, P, K, Ca, Mg and S, were 69.6%, 41.1%, 43.4%, 54.7%, 41.6% and 64.1% higher in the firmest fruit when compared to the softest fruit respectively. In Coral Champagne, the biggest fruit also had 59%, 60.1%, 54.4% and 100% higher N, P, K and S concentration, respectively. For Skeena, N, K, Ca, Mg, S concentration (dry basis) were statistically different but of no agronomical relevance, and similar to Chelan and Coral Champagne, N, P, Mg and S concentration (fresh basis) were 66.4%, 48.8%, 44.6% and 53% higher in N, P, Mg and S, respectively.

Firmness and nutrients concentration differences among years and cultivar

To further assess the relationship between firmness and nutrient levels, we combined all data; cultivars, sites and years, and categorized them in four firmness levels, as we noted that within the soft category there was high variability between orchard location. We analyzed three categories; firm (> 300 $g \cdot mm^{-1}$), soft (200 – 300 $g \cdot mm^{-1}$) and very soft (< 200 $g \cdot mm^{-1}$). Differences were significant only when firmness was very low (Table 3), and only for N, S concentration (in both dry and fresh basis) and N:Ca ratio. No other nutrients measured were significantly different (data not shown).

Table 3. Nutrient and dry matter differences between firmness categories for all sites, cultivars and years. Different letters indicate statistical differences between columns ($p<0.05$)

Category	Firmness range (mean)	N %	S %	N/Ca	N mg/100g	S mg/100g
FIRM	> 300 (358)	1.04 a	0.07 a	11.0 a	200.3 a	13.3 a
SOFT	200 - 300 (231)	1.03 a	0.07 a	10.5 a	193.8 a	13.1 a
VERY SOFT	< 200 (183)	0.89 b	0.06 b	8.8 b	169.9 b	10.9 b
Pr > F(Model)	<0.0001	0.005	<0.0001	0.002	0.021	0.001

4) Develop outreach and educational materials and workshops.

We shared the preliminary results of this project in Jan 7th, 2022 at the Cherry Institute annual meeting (by Sallato) and Columbia Basin Tree Fruit Club 2022.