Project/Proposal Title: Understanding little cherry disease pathogenicity

PI: Scott Harper
Organization: Washington State University
Telephone: 509-786-9230
Email: scott.harper@wsu.edu
Address: 24106 N Bunn Rd
City/State/Zip: Prosser, WA 99350

Co-PI 2: Alice Wright
Organization: Washington State University
Telephone: 509-786-9210
Email: alice.wright@wsu.edu
Address: 24106 N Bunn Rd
City/State/Zip: Prosser, WA 99350

Co-PI 3: Per McCord
Organization: Washington State University
Telephone: 509-786-9254
Email: phmccord@wsu.edu
Address: 24106 N Bunn Rd
City/State/Zip: Prosser, WA 99350

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: Scott Harper
Organization Name: Washington State University
Contract Administrator: Anastasia Mondy
Telephone: 916-897-1960
Contract administrator email address: arcgrants@wsu.edu
Station Manager: Naidu Rayapati
Station Manger email: naidu.rayapati@wsu.edu

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:

- Little cherry and X-disease expression symptom severity correlates with pathogen titer, the more virus or phytoplasma present, the worse the symptoms.
- Beyond fruit size and color reduction, infection by both pathogens also reduces the concentration of most sugars in fruit. Little cherry virus-induced disease differs in that citric acid concentration increases, accounting for the bitter taste.
- Disease outcomes are significantly influenced by both cultivar and orchard location.
- Both pathogens move to and accumulate in developing fruit from bloom through to harvest.
- Preliminary transcriptome analysis indicates X-disease changes the expression of cherry genes associated with secondary metabolism, fruit flavor, ethylene biosynthesis, abscisic acid signaling, and auxin signaling
- A near-complete genome of a Washington isolate of X-disease phytoplasma has been sequenced and used to identify 26 secreted proteins that may be putative effects of disease.

Methods:

Objective 1. We will establish a 1-acre test block at WSU-IAREC consisting of 30 different cherry varieties (Table 1). 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.

Table 1. List of cherry cultivars to be screened for tolerance or resistance to LChV-2 and X-disease phytoplasma in this study.

Group	Cultivars	Group	Cultivars
Commercial	Benton	Genetic diversity	Black Spanish
	Black Pearl		Burgundy Pearl
	Chelan		Hudson
	Coral Champagne		Kristen
	Early Robin		Lambert
	Penny		Moreau
	Rainier		Schmidt
	Santina		Vega
	Skeena		Walpurgis
	Skylar Rae		Windsor
	Sweetheart		Yellow Glass
Potential Resistance/Tolerance	Bing		Yellow Spanish
	Black Tartarian		Attika
	Sweet Ann		Regina
	Utah Giant		Van

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 propose to collect symptom development observations and physiological data from both the controlled field experiments and grower fields throughout the state.

To do so we will focus on two areas. First, recording of symptoms present on known infected trees under controlled conditions 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 LCD 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 or LChV-2, 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. The test plot was established in May 2021 at the WSU Pear Acres field site. Scions (Table 1, underlined) were grafted onto Gisela-6 rootstock in the greenhouse during 2020 and early 2021, with failures re-grafted in the field after planting. Trees were planted in a semi-randomized pattern, and graft-inoculated using PCR-confirmed high-titer LChV-2 genotype LC5 and the prevalent X-disease phytoplasma genotype in late summer of 2021. Peach cv. Lovell was included in the trial as an indicator. Trees will be screened next season (Spring 2022) to determine if inoculation was successful. Additional grafting will be performed in Spring 2022 for the remaining selections in Table 1.

Objective 2. Given the high incidence of X-disease phytoplasma infected trees, efforts have focused on this pathogen, although limited observations were made on LChV-2 infected trees. Trees infected with either pathogen could be grouped into three classes: 1. Asymptomatic, 2. Early infection, which correlated with mild symptoms scattered around the plant, and 3. Established, which correlated with severe symptoms across the entire tree. As can be seen in Table 2, fruit size and color reduction correlated with infection stage, with other developmental abnormalities such as fruit shape changes or flowering at harvest occurring as the infection became established.

The infection stages were also found to correlate with the amount of phytoplasma present in the tree (Figure 1), with early stage, mild symptoms correlating with low titer (~1-100 phytoplasma cells per sample), and established, severe symptoms with high concentrations (~10,000-100,000 phytoplasma cells per sample). Interestingly, plants with asymptomatic infections had similar levels to the early stage, suggesting that the initial disease expression may be triggered by an environmental factor.

Table 2. The effect of different stages of X-disease phytoplasma infection on fruit color and size for each cultivar examined. A more detailed table can be found in Wright et al. (2021).

Cultivar	Infection Stage	Fruit Size (Avg.)	Fruit Color Range	Abnormalities Observed
Benton	Asym.	Normal	Dark Red	-
	Early	>75%	Bright Red to Dark Red	Flattened fruit
	Established	<75%	Yellow-pink to Dark Red	Flattened, pointed fruit. Flower development at harvest
Bing	Asym.	Normal	Dark Red	-
	Early	>75%	Medium to Dark Red	-
	Established	>50%	Yellow to Pink	-
Cristalina	Asym.	Normal	Bright Red to Dark Red	-
	Early	>75%	Medium Red	-
	Established	>50%	Yellow-pink to Bright Red	Pointed fruit
Santina	Asym.	Normal		-
	Early	>75%	Medium Red to Dark Red	-
	Established	>50%	White-pink to Medium Red	Pointed fruit, Flower development at harvest
Rainier	Asym.	Normal	Blush	-
	Early	>75%	Medium Blush	-
	Established	>50%	Yellow	-

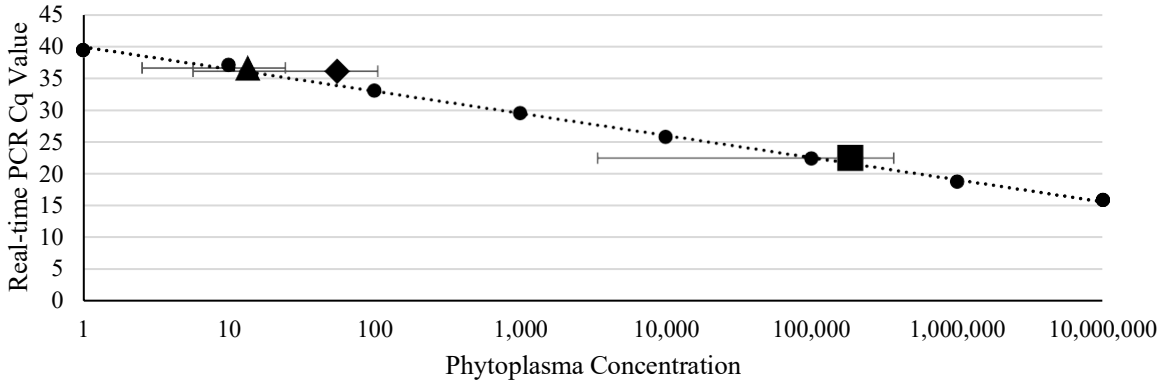

Figure 1. Concentration of X-disease phytoplasma present in asymptomatic (diamond), mild (triangle), and severely (square) symptomatic plants.

As X-disease infected cherries are noted for their bland taste, sugar content (fructose, glucose, and sorbitol) and secondary metabolite content (citric acid, malic acid, and total phenolics) were determined for healthy, early, and established infections for each of the studied cultivars and locations. Our results indicated that there was little difference in sugar content between healthy trees and trees in the early stage of infection. However, for trees with established X-disease infections, all three sugars showed a decrease in concentration across most cultivars and locations. There was no observed change in total phenolics content or malic acid content between healthy trees, early infections, and established

infections. However, a decrease in citric acid content was observed between early and established infections in 'Cristalina' and 'Santina' cherries, indicating potential cultivar-specific effects.

While fewer LChV-2 infected samples were collected, the results for Rainier and Bing were largely comparable to those from their X-disease infected equivalents in terms of color reduction, decrease in size, and sugar content changes. The only significant difference between X-disease infected and LChV-2 infected cherries was fruit citric acid content concentration was lower in trees with high LchV-2 concentrations which correlates with the bitter taste of LCD-afflicted cherries.

Finally, PCA analysis revealed that disease outcomes were the result of three factors, 1. Early vs. established X-disease infections, 2. Cultivar infected, and 3. Location of the orchard (Figure 2). Location effects can significantly affect fruit quality, and can include climate effects such as elevation, rainfall, amount of sunlight, and spring temperatures, or orchard practices such as planting density, pruning, irrigation, nutrient management, and application of growth regulators. Which of these factors are responsible is unknown.

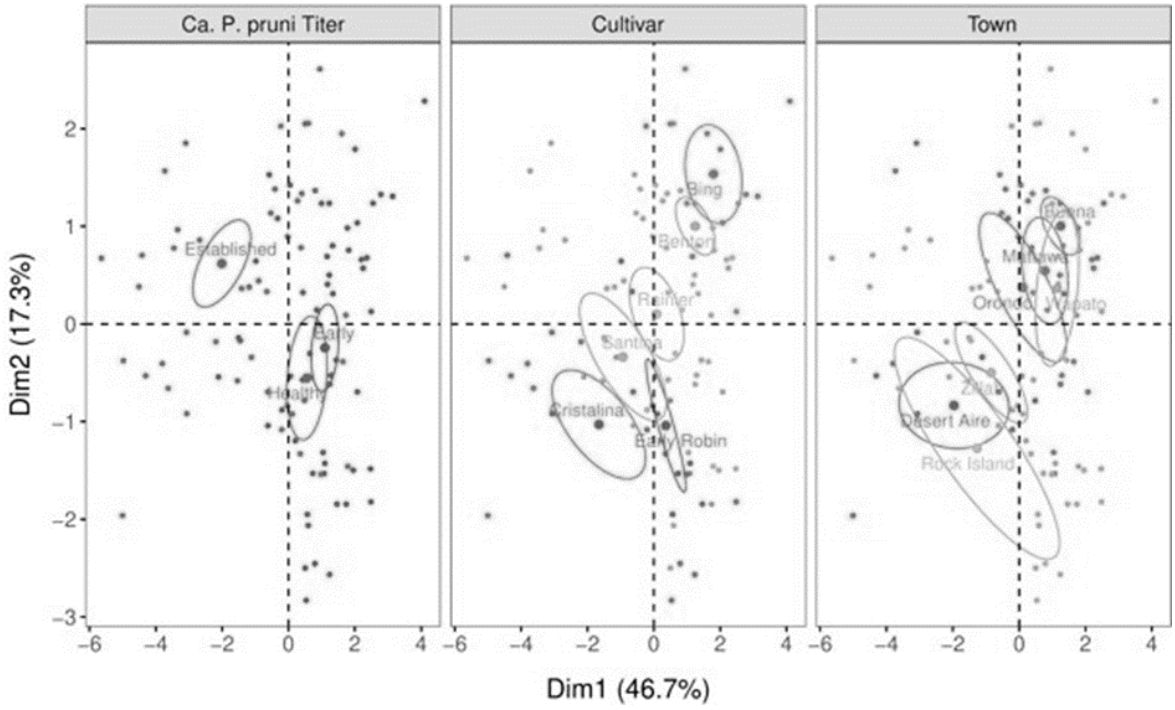

Figure 2. Principal components analysis (PCA) analysis of individual plots comparing pathogen titer, cultivar and location on disease severity.

Objective 3. Before investigating the effect of these pathogens on host gene expression, it was necessary to determine where in the plant they accumulate. LChV-2 distribution had been determined previously in the WTFRC-funded project "*Orchard management practices for little cherry virus 2*" therefore we focused on the X-disease phytoplasma. Following a set of symptomatic Bing trees across two full growing seasons (Figure 3) we found that the phytoplasma would begin accumulating in developing flower buds at bloom, and from there the titer would increase steadily as the resulting fruit developed and matured. Comparable titers were found in both the fruit and attached pedicel.

As both X-disease and little cherry disease symptoms primarily affect the fruit in sweet cherries, and as both are developmental disorders, we decided for focus the gene expression experiments on the fruit and pedicel tissues, with leaf tissues also included to capture pathogen infection-specific changes, or

those caused by other stressors that the experimental plants were responding to. Samples at shuckfall and pit-hardening stages were collected from two Bing cherry blocks, one in Wapato from 2019, 2020, and 2021, and one in Quincy, in 2020 only.

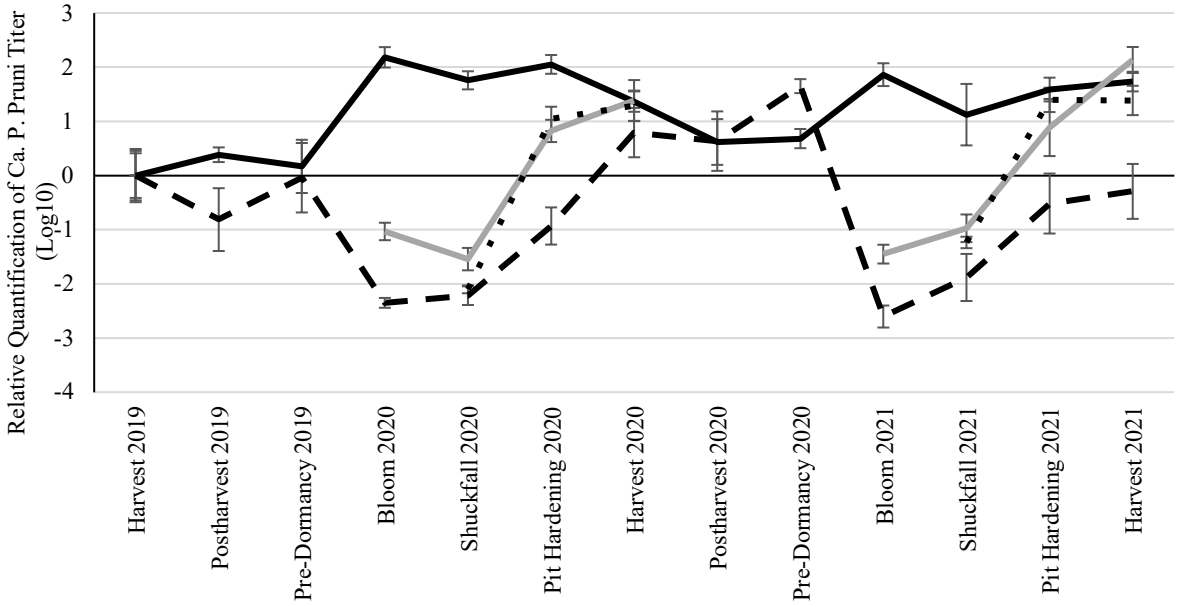

Figure 3. A time course of X-disease phytoplasma titer across two growing seasons in systemically infected Bing trees, as determined by real-time qPCR, in stem (solid line), leaves (dashed line), and fruit (dotted line), and fruit pedicel (grey line).

Data analysis for samples collected during the 2019 and 2020 field seasons is ongoing, while those from the 2021 season will be analyzed this winter. We found that there is significant inter-site, and inter-year variation in gene expression at both timepoints, with relatively few gene expression changes (Log2, P >0.05), in common between timepoints and/or sites (Figure 4).

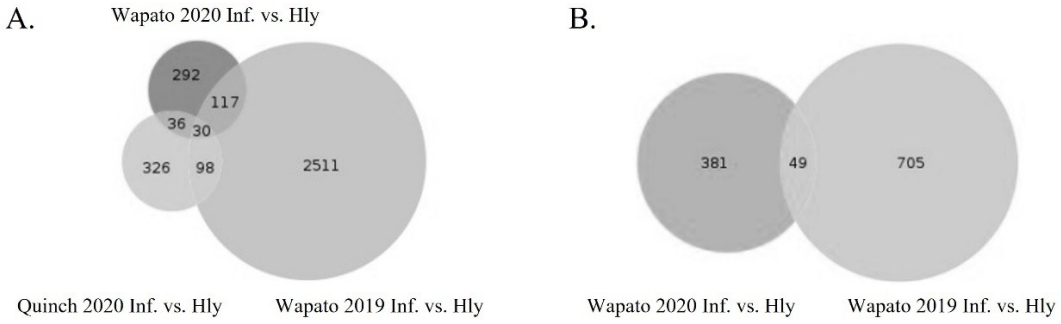

Figure 4. Number of genes showing two-fold or greater expression change between X-disease infected and healthy Bing cherry trees at shuckfall and pit-hardening timepoints.

This does however mean that the genes in common are likely related to X-disease infection, and potentially gene expression. Many were hypothetical proteins whose function is unknown, but among those that could be identified were genes involved in secondary metabolism, hormone metabolism and signaling, and plant growth and developmental regulation (Table 3). Of particular interest are genes

affecting ABA metabolism and signaling as these pathways are important in cherry fruit development., as are genes associated with the phytohormones ethylene and auxin. Also of interest is an (E,E)-alpha-farnesene synthase, as the product of this gene synthesizes an insect chemoattractant which may play a role in facilitating the spread of the phytoplasma. Interestingly this is upregulated in the leaves, which is where the vector leafhoppers feed (T. Northfield, pers. comm).

Table 2. Differentially expressed genes of interest in X-disease infected 'Bing' cherry fruit, fruit pedicels, or leaves, at shuckfall (SF) and pit-hardening (PH) timepoints across the 2019-2020 field seasons.

Tissue	Timepoint	Up/Down	Gene	Function
Fruit	PH	Up	Cytochrome P450 71A1-like	Metabolism associated with fruit flavor
	PH	Down	Fructose-1,6-bisphosphatase, chloroplastic	Gluconeogenesis
	PH	Up	Geraniol 8-hydroxylase-like	Monoterpenoid biosynthesis
	PH	Down	indole-3-pyruvate monooxygenase YUCCA10	Auxin biosynthesis
Pedicel	SF	Down	Plyphenol oxidase, chloroplastic-like	Pigment biosynthesis
	PH	Up	Abscisic acid 8'-hydroxylase 4-like	ABA degradation
	PH	Up	Mitogen-activated protein kinase 18-like	ABA signaling
	PH	Up	Polyphenol oxidase, chloroplastic	Pigment biosynthesis
	PH	Up	Protein PIN-LIKES 3-like	Regulates auxin metabolism
	PH	Up	Transcription factor MYB114-like	Anthocyanin biosynthesis
Leaves	PH	Up	(E,E)-alpha-farnesene synthase-like	Insect chemoattractant
	PH	Down	1-aminocyclopropane-1-carboxylate oxidase homolog 1-like	Ethylene biosynthesis
	PH	Up	Aminopeptidase M1-like	Auxin transport
	PH	Up	Ethylene-responsive transcription factor ABR1-like	ABA signaling
	PH	Down	Auxin efflux carrier component 8	Auxin homeostasis
	PH	Up	Protein NRT1/ PTR FAMILY 4.5-like	ABA transport

Additional RNA-seq is planned for samples collected in 2021. Select genes, or known regulators of these genes, will be included in yeast-two-hybrid or bimolecular fluorescence assays to identify potential protein-protein interactions between phytoplasma effectors and host proteins.

In preparation this process, and to identify putative effectors, we have begun sequencing of the X-disease phytoplasma (*Candidatus* Phytoplasma pruni) genome from a Washington field isolate. To date we have approximately 580 kb of sequence in eight large fragments, from an estimated 680 kb. The low GC-content and high level of sequence repeats has made completing the circular genome difficult. Nevertheless, we now have nearly 200 kb more sequence than the earlier X-disease phytoplasma genomes from other parts of the country and have annotated a total of 484 genes.

Interestingly, the isolate of the X-disease phytoplasma present in Washington state and Oregon is noticeable different from the partial sequences of Californian and Canadian isolates, as well as smaller fragments from Utah and Pennsylvania. These differences might account for the more transmissible, and more virulent phenotype we are observing relative to the older Californian reports from the 1970s and 80s.

Using SignalP 5.0 we have predicted a total of 26 potential secreted peptides that could be putative X-disease effectors, and as the sequence is completed, more will be identified. Curiously, only four of

these proteins have functions identified, the majority being transporter proteins. In contrast, little is known about the functions of the genes in the LchV-2 genome, excepting replication-associated and structural proteins. However, unlike the X-disease phytoplasma, there are only 9 open reading frames in total, and excluding the ORF1a/1b replication proteins, that leaves 7 to be tested by yeast-two-hybrid or bimolecular fluorescence assays.

Our task to meet this objective next year will be: First, complete the set of host genes to be examined by completing analysis of the 2021 season X-disease data, and expand our collection of LChV-2 RNAseq data, with confirmation by real-time RT-qPCR. Second, to conduct the yeast-two-hybrid or bimolecular fluorescence assays using the identified secreted proteins, and/or viral expressed proteins. Cumulatively this will be the first look at how the X-disease phytoplasma, and how Little cherry virus-2 cause similar diseases despite being very, very different organisms.

Publications:

Wright AA, Shires M, Beaver C, Bishop G, DuPont ST, Naranjo R, Harper SJ. 2021. The effect of *Candidatus* Phytoplasma pruni infection on sweet cherry fruit. Phytopathology. DOI: 10.1094/PHYTo-03-21-0106-R.

Project/Proposal Title: Field evaluation of leafhopper controls for X-disease management

Primary PI: Tobin Northfield
Organization: WSU-TFREC
Telephone: 509-293-8789
Email: tnorthfield@wsu.edu
Address: 1100 N Western Ave.
Address 2:
City/State/Zip: Wenatchee, WA 98801

Co-PI 2: Louis Nottingham
Organization: WSU-TFREC
Telephone:509-293-8756
Email: louis.nottingham@wsu.edu
Address:
Address 2:
City/State/Zip:

Cooperators: Garrett Bishop, Jenna Voelker, Teah Smith, Scott Harper, Tianna DuPont

Report Type: Final Project Report

Project Duration: 2-Year

Total Project Request for Year 1 Funding: $ 79,864
Total Project Request for Year 2 Funding: $ 82,558

Other related/associated funding sources: Awarded
Funding Duration: 2021 - 2024
Amount: $244,750
Agency Name: USDA/WSDA Specialty Crop Block Grant
Notes: Primarily funds a 3-year evaluation of Extenday ground cover, with comparisons to a selective herbicide-mowing program, and a control. Also, includes roles of groundcover weeds over the season.

Budget 1
Primary PI: Tobin Northfield
Organization Name: WSU-TFREC
Contract Administrator: Anastasia Mondy
Telephone: 916-897-1960
Contract administrator email address: Anastasia.Mondy@wsu.edu
Station Manager/Supervisor: Chad Kruger
Station manager/supervisor email address: cekruger@wsu.edu

Item	2020	2021
Salaries[1]	50,039	52,040

Benefits[2]	17,325	18,018
Wages		
Benefits		
Equipment		
Supplies[3]	5,000	5,000
Travel[4]	7,500	7,500
Miscellaneous		
Plot Fees		
Total	79,864	82,558

Footnotes:

[1] New postdoctoral researcher position (100% FTE), Louis Nottingham (2%)

[2] 35% (postdoctoral researcher), 25.9% (Nottingham)

[3] Fieldwork consumables and X-disease tests

[4] Domestic travel for research

Objective Recap, Goals, and Anticipated Accomplishments:

1. *Evaluate effects of kaolin clay applied post-harvest on X disease prevalence and density of leafhoppers and predators.*

   As planned, we have finished the two-year trial evaluating the efficacy of kaolin clay to suppress the densities of leafhopper vectors of X-disease (*Colladonus reductus and C. geminatus*) in Yakima and Wenatchee region cherries blocks, as well as Yakima region nectarines.

2. *Evaluate effects of UV-reflective mulch on X disease prevalence and density of leafhoppers and predators.*

   Our shipment of Extenday was delayed by 4 months due to COVID19 preventing us from deploying it in our experimental plots. Thankfully, the growers in Wenatchee region had Extenday in the cherry plots prior to harvest and graciously left it throughout the season for our experimental study. Therefore, in half of our cherry plots (Wenatchee region only) we were able to conduct the first year of evaluating Extenday for suppression and control of the X-disease leafhopper vectors. In 2021 we applied the Extenday to our Yakima region plots as well. We do not present data from two Wenatchee region blocks on Stemilt hill, because we only observed one leafhopper all season in 2020 and did not set up treatments there in 2021. Sampling in 2021 again showed very low leafhopper abundance.

3. *Describe seasonal patterns of leafhopper abundance and map disease incidence in commercial cherry orchards.*
   We tracked the seasonal abundance of leafhoppers over the course of 2020 and 2021. In addition, we mapped disease incidence at harvest in our trial orchards, and we evaluated the spatial distribution of leafhoppers in the different treatments. We found little disease spread over the two funded years of the project. This is partly due to the length of the project covering only 2 years, rather than the proposed 3, such that transmission occurring in 2020 would typically not appear until 2022 or later after a lag in symptoms. Therefore, we instead present differences in the spatial distribution of leafhoppers for the different treatments.

Significant Findings:

- In 2020 Extenday applied postharvest reduced postharvest leafhopper numbers by 81% compared to controls with little to no negative effect on predators in Wenatchee area cherries.
- In 2021 Extenday applied postharvest reduced postharvest leafhopper numbers by 88% and 91% compared to controls in Wenatchee and Yakima region cherries, respectively.
- Extenday provided consistent control despite 50-fold variation in leafhopper abundance in control blocks across Wenatchee and Yakima regions, suggesting it works in both, high-pressure and low-pressure blocks.
- In high-pressure cherry blocks in the Wenatchee and Yakima regions Surround applied postharvest reduced leafhopper numbers by 47-48%, with effects strengthening after the second application.
- Postharvest Surround did not reduce leafhopper numbers in very low-pressure cherry blocks (< 2/trap), or in nectarines, where harvest occurs very late compared to cherries.
- Leafhoppers in Surround-applied blocks appear to forage more in the groundcover than trees compared to control blocks, based on trap capture at 2ft, 4ft and 6ft.

- In control replicates, leafhopper numbers were highest in the middle of the replicate, and in Surround-treated replicates, the numbers were highest at the edge of the replicate adjacent to the untreated buffer trees. Extenday numbers were low throughout the block.
- The project served as preliminary data for a funded WSDA/USDA Specialty Crop Block Grant to compare Extenday to weed management for leafhopper control.

Methods:

We evaluated two control methods (kaolin clay and Extenday groundcover) as additions to the spray rotation currently used on commercial cherry plots in the Wenatchee region (4 plots in one location, and 2 in another) and the Yakima region (2 plots in Wapato) and evaluated kaolin clay in 2 Yakima region nectarine blocks (in Wapato). In 2021 we dropped two of the Wenatchee blocks due to very low leafhopper numbers in 2020 (only one collected all year). We targeted blocks with 1-10% disease prevalence to ensure that the block has disease to control, but that the disease prevalence is not high enough to risk block removal prior to the end of the experiment. However, at the start of the experiment in 2020 we realized that there was very high disease prevalence in the Yakima region cherry blocks, which were removed after the 2021 trial.

Each replicate includes 12 rows with 200 feet of row, with three treatment locations randomized in a split-plot design (Figure 1). Thus, each plot included 36 rows, split in thirds for the three treatments. We evaluated leafhopper abundances and disease prevalence in the middle four rows and used the other rows as buffer rows to reduce spillover effects of the other treatments.

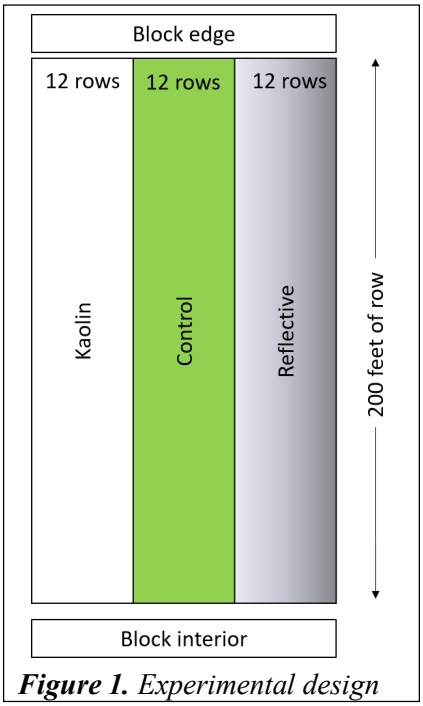

Figure 1. *Experimental design*

Prior to harvest, disease incidence and location within the block was surveyed and recorded for the Yakima region plots. After harvest completion, treatments were applied to assigned plots. In cherry blocks kaolin plots received four kaolin (Surround WP) sprays, one in July, August, September, and October (Table 1) on top of the grower's baseline insecticide program. Kaolin was sprayed at 50 lb/acre and 200 gal/acre. In nectarines, the first application occurred in August, given the later harvest relative to cherries. The postharvest Surround treatment aligns with a typical spray to reduce doubling, and doubling will be recorded in each plot next year. Our order of Extenday was delayed 4 months due to COVID19. Thankfully, our cooperator in the Wenatchee region had Extenday which was deployed in our trial plots from May 27 – October 30. This gave us four replicates of Extenday for the 2020 season. In 2021, we expanded the Extenday treatments to include the 2 Yakima region cherry blocks as well. The nectarine blocks were too small to include Extenday treatments. We did not return to the Wenatchee region blocks that only collected 1 leafhopper in 2020, although collection as part of another project demonstrated there are still extremely low leafhopper numbers there. In our 2021 Wenatchee region cherry blocks we also included a mowing treatment, where the treated area was mowed 5 times instead of 3 for the others. However, this had little effect on the ground cover composition or leafhopper numbers, so for the sake of space we do not present those results.

Figure 2 *5x7 Yellow sticky cards suspended at 2, 4, and 6 ft from the ground.*

After initial treatment application, leafhopper abundance in each treatment (Kaolin clay, Extenday, Control) replicate was monitored using 10 yellow sticky cards (5 × 7 inch) (Fig. 1) in the middle four rows. A yellow sticky card was tied to a cherry tree branch 4 ft from the ground and 25 ft in from each corner of the plot, and two sets of three yellow sticky cards were hung in the middle rows at 2, 4, and 6 ft from the ground using a bamboo pole and braided fishing line (Fig. 2-3). Sticky cards were deployed July 23rd in the Wenatchee region plots and July 31st in the Yakima region plots in 2020 and in 14 and 23 July 2021 in Wenatchee region and Yakima region, respectively. Cards were collected and replaced every two weeks through October, and collected cards were returned to the lab to record leafhopper abundance by species (*Colladonus montanus reductus* and *C. geminatus*). More than 99% of leafhoppers were *C. m. reductus*, so we do not present *C. geminatus* data, apart from 2021 Yakima region cherries that had very few leafhoppers and we then present all known vectors, where we present total vector numbers. Periodical beat sheet sampling was conducted to observe population densities within the tree canopy, but the very low numbers relative to sticky cards suggested it was not an effective method of sampling. During winter months, sticky cards at the Wenatchee region site with all treatments were examined for natural enemy abundance to evaluate non-target effects. To analyze the data we used a generalized linear mixed model, where we assumed a negative binomial error distribution (typical for count data), random effects of replicate block, date, and trap location, a treatment effect, a trap height effect, and a trap by treatment interaction. The random effect for date models variation in leafhoppers over time and accounts for the fact that data collected on the same date will be more similar than those collected on different dates. The random effect for replicate block accounts for the spatial design, that multiple traps occur in the same block and therefore counts within the same block will be correlated, and similarly the random effect for trap location accounts for the fact that the same trap location was sampled multiple times throughout the season. We assumed the control was the baseline

treatment, such that treatment effects model differences between the control and each treatment (Extenday and Surround), the height effect evaluates differences between the heights traps were placed at, and the interactions between trap and height evaluate whether the distribution of leafhoppers across the three heights was different for Surround or Extenday, than in the control blocks.

Table 1. Kaolin clay application timing for cherries and rate by county in 2020. Similar dates and rates were used in 2021.

	KC 1st app	KC 2nd app	KC 3rd app	KC 4th app	Rate
Wenatchee region	Jul 21, 2020	Aug 6, 2020	Sep 4, 2020	Oct 7, 2020	50 lbs/acre 200 gal/acre
Yakima region	Jul 29, 2020	Aug 10, 2020	Sep 9, 2020	Oct 15, 2020	50 lbs/acre 200 gal/acre

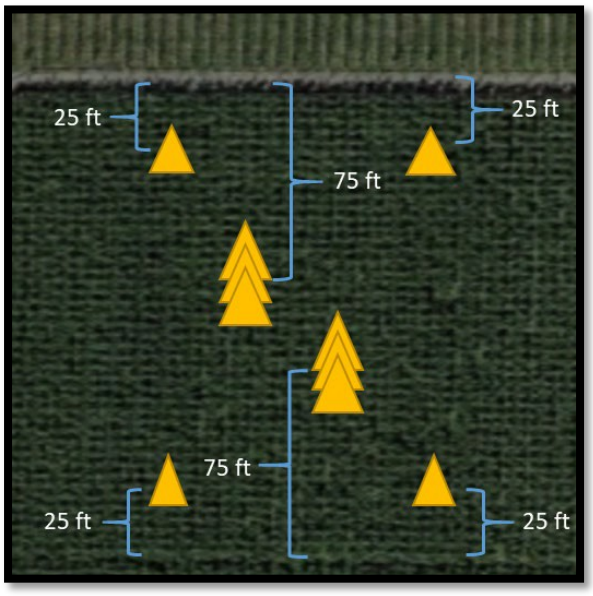

Figure 3 *Trap deployment layout for each Surround, Extenday, and Control experimental plot.*

No-Choice Surround Feeding Study

Kaolin clay (i.e. Surround) covered trees have been shown to reduce feeding and survivorship of other leafhoppers. However, while collecting traps in the Surround sprayed cherry plots, we observed leafhopper presence on leaves frequently. To empirically test if *C. m. reductus* leafhoppers will locate and feed on Surround covered cherry trees we conducted a no-choice feeding study. On

Sep 29 2020, we placed four field collected adult *C. m. reductus* in each of five cages with only Surround covered cherry tree leaves (collected from a sprayed experimental plot (Fig. 7)) and five cages with only non-sprayed cherry trees. We then observed leafhopper feeding behavior at 24, 28, and 46 hrs after initial set-up, recording the number alive, dead, on-plant, off-plant, and actively feeding. Due to space limitations we do not present these data graphically, but the leafhoppers readily feed on both treated and untreated plants.

Results & Discussion:

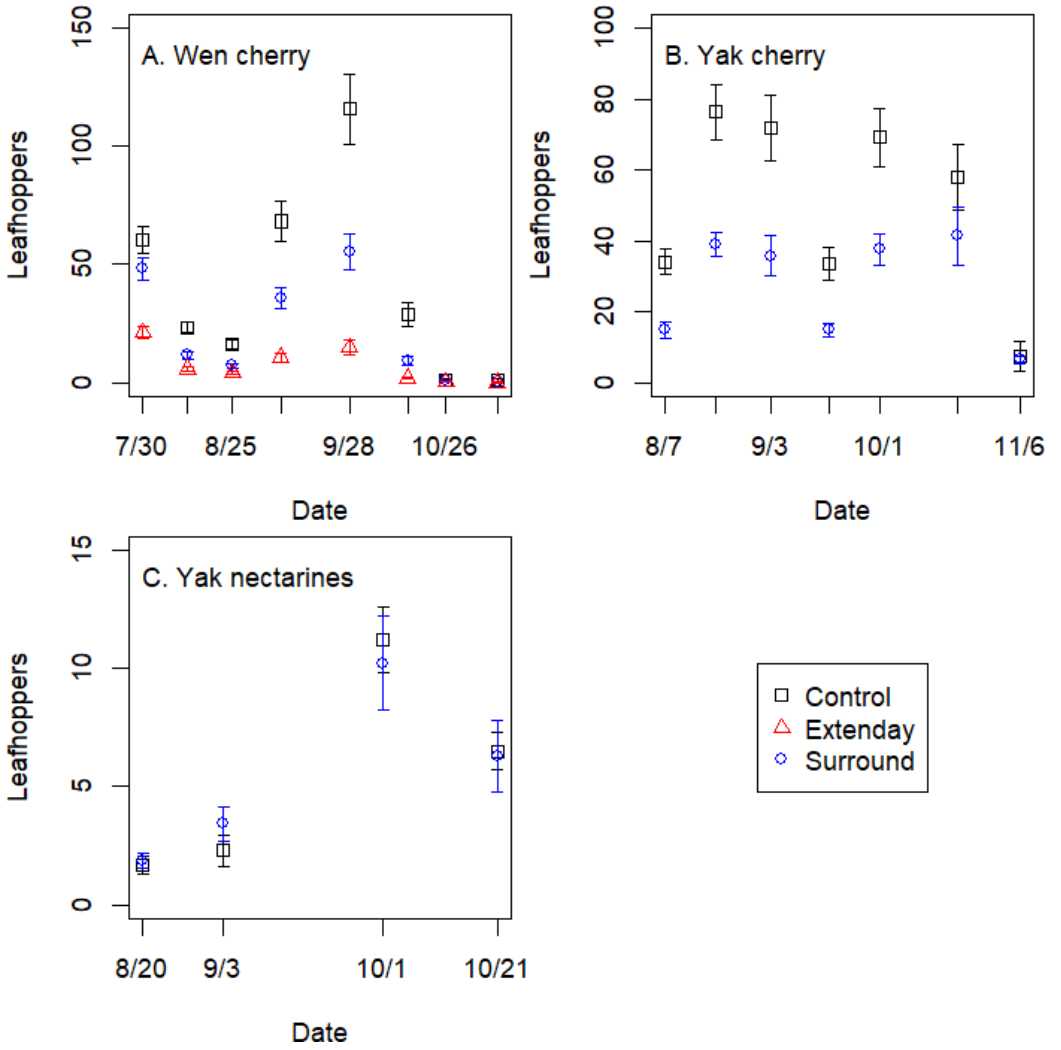

Figure 4. 2020 Leafhopper counts *Mean* C. m. reductus *leafhoppers per trap in 2020 averaged across four plots in control (black squares), Extenday (red circles), or Surround (blue squares) subplots in Wenatchee region cherry blocks (A), Yakima region cherries (B), or Yakima region (C). Dates represent midpoints of 2-week sample periods, except for the September 10th date, which was a 3-week period.*

Objective 1. Surround-kaolin clay.

In 2020, kaolin clay reduced *C. m. reductus* by 47% averaged across the season in each our Wenatchee region (lsmeans comparison: $t = 6.827$, $P < 0.0001$) cherry blocks and our Yakima region

(lsmeans comparison: $t = 8.820$, $P < 0.0001$) cherry blocks, with effects strengthening after the first sample date when only one application had been applied (Figure 4). In addition, leafhoppers were collected at lower heights in treated cherry blocks in Wenatchee (height by Surround interaction: z = -6.080, P < 0.0001), and Yakima (height by Surround interaction: z = -6.502, P < 0.0001) regions.

2021 leafhoppers. As in 2020, in 2021 Surround reduced leafhopper numbers by 48% in Wenatchee region cherries (lsmeans comparions: $t = 23.805$, $P < 0.0001$). This difference is strongest in higher traps (height by Surround interaction: z = -3.879, P = 0.0001; Figure 6), suggesting that the Surround is working to reduce leafhopper movement in and around the tree canopy. In the Yakima region block we observed a 99% drop in leafhopper numbers in control blocks from 2020 to 2021, and in 2021, when leafhopper numbers (all vectors, due to low numbers of *C. m. reductus*) averaged 0.55 per trap we found no significant additional effect of kaolin clay (lsmeans comparion: $t = 2.175$, $P = 0.0777$). However, as in the Wenatchee region it did reduce the height at which leafhoppers were typically caught (height by Surround interaction: $z = -2.179$, $P = 0.0293$). In the Yakima region nectarines, where the harvest date was much later we found no effect on leafhopper numbers within the block in 2020 when leafhopper means were approximately 5, and had only a slight reduction in 2021 when the means were much higher (Figures 4, 5). The effects of Surround reducing the height at which they were captured was not quite significant in 2020 (height by Surround interaction: $z = -1.739$, $P = 0.0820$), but was significant in 2021 (height by Surround interaction: $z = -2.135$, $P = 0.0328$).

Surround effects on predators. In our evaluation of Wenatchee region blocks, the kaolin clay treatment had very little effects on natural enemies, except for lacewings, which had lower densities in the first two weeks of the trial in kaolin-treated blocks (Figure 7).

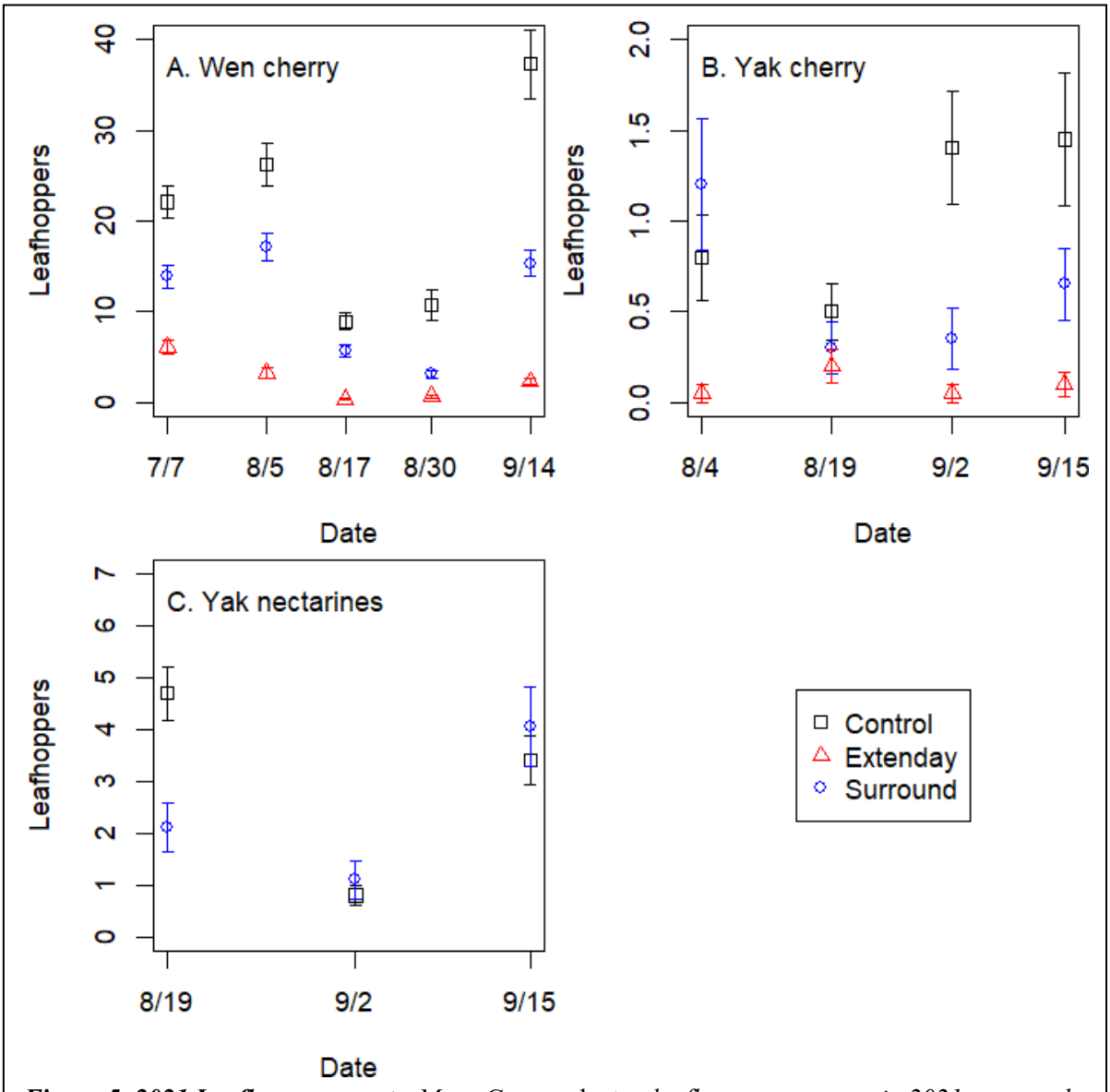

Figure 5. 2021 Leafhopper counts. *Mean* C. m. reductus *leafhoppers per trap in 2021 averaged across four plots in control (black squares), Extenday (red triangles), or Surround (blue circles) subplots in Wenatchee region cherry blocks (A), Yakima region cherries (B), or Yakima region (C). Dates represent midpoints of 2-week sample periods. The Yakima cherry block includes all known vectors combined (*C. m. reductus*,* C. geminatus*,* S. acutus*, and* E. variegatus*), due to a relatively high abundance of all species.*

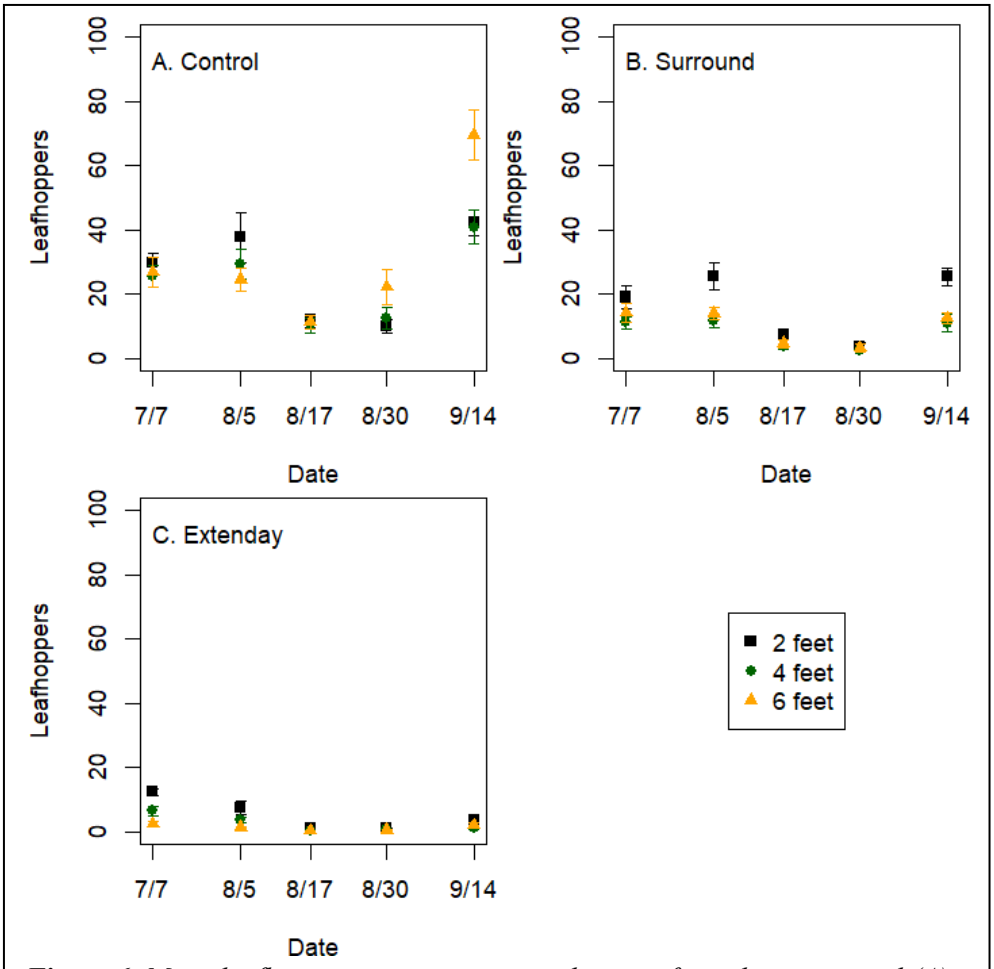

Figure 6. *Mean leafhoppers per trap averaged across four plots in control (A), Surround (B), or Extenday (C) subplots in Wenatchee region cherry blocks, on traps placed at 2 feet (black squares), 4 feet (green circles) or 6 feet (orange triangles). Dates represent midpoints of 2-week sample periods. Data presented are for 2021, and 2020 had similar trends, as do data for Yakima region cherries in 2020 and 2021.*

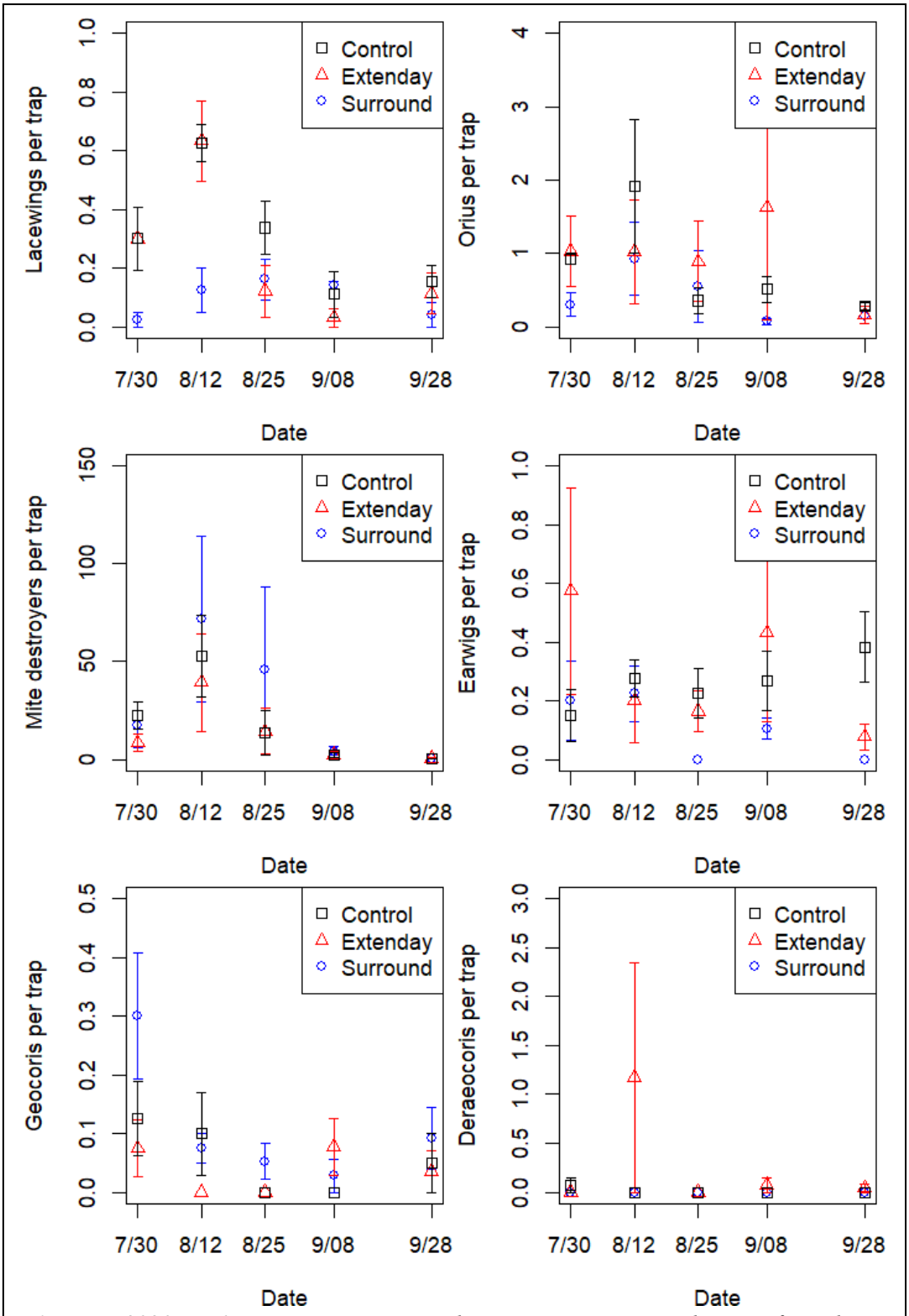

Figure 7. 2020 Predator counts. *Mean predators per trap averaged across four plots in control (black squares), Extenday (red triangles), or Surround (blue circles) subplots in Wenatchee region cherry blocks. Dates represent midpoints of 2-week sample periods, except for the September 10th date, which was a 3-week period.*

Objective 2. Extenday ground cover.

In 2020, Extenday reduced leafhopper numbers by 81% (lsmeans comparison: $t = 13.907$, $P < 0.0001$) and reduced the height at which they were captured (height by Extenday interaction: $z = -2.402$, $P = 0.0163$) in the 4 Wenatchee region cherry plots, providing the best control (Figure 4). Similarly, in 2021, Extenday provided 88% and 91% reductions in Wenatchee (lsmeans comparison: $t = 23.805$, $P < 0.0001$) and Yakima (lsmeans comparison: $t = 5.472$, $P < 0.0001$) region cherries, respectively (Figures 4, 5). The consistency in this control is impressive, given the roughly 50-fold difference in peak leafhopper abundance in the control blocks in the two regions (Figure 5). Extenday also reduced the height at which leafhoppers were caught in Wenatchee region blocks (height by Extenday interaction: $z = -4.907$, $P < 0.0001$), but not in Yakima region blocks (height by Extenday interaction: $z = -0.355$, $P = 0.7229$). These findings suggest that using a ground cover such as Extenday can work to decimate leafhopper populations for high-pressure blocks (e.g., peaks of more than 100 leafhoppers/ trap in Wenatchee 2020 cherry controls) or low-pressure blocks (e.g., peaks of less than 2 leafhoppers per trap in Yakima cherries in 2021). Furthermore, Extenday did not impact the predator community (Figure 5), apart from ground-foraging *Geocoris* bugs, which were rarely collected on sticky traps in any of the treatments.

Given that leafhoppers were collected most in groundcover, the Extenday appears to provide control by simply covering up the weedy hosts that leafhoppers commonly feed on (see continuing report on "Identifying sources of X disease in cherry orchards"). Interestingly, in 2021 detailed analysis of the Wenatchee blocks using Extenday to improve cherry color showed that when Extenday was applied over developing nymphs, the adults appeared shortly after, showing that nymphs can persist under the Extenday. However, once the adults emerged, the populations never recovered, suggesting that the adults did not stay to oviposit under the Extenday cover. Thus, ideal control is likely a pre-harvest nymph treatment paired with Extenday applied after harvest. Furthermore, weed management within tree rows may help with control, as particular traps with higher leafhopper counts were associated with weeds growing over the Extenday from the weed strip. It is likely that it is the physical barrier, rather than the reflective nature of the Extenday that provides the control, but this has yet to be tested.

Objective 3. Phenology and spatial distribution

Phenology. In general, we found three periods of peak abundance of *C. m. reductus* (the most abundant species), in June, August, and September/October (Figure 8). In 2020 Wenatchee region blocks the initial peak was very small, potentially due to control measures in the block and vertical netting surrounding the block. During the first generation we found much higher adult numbers outside the block, indicating low abundance was due to control, rather than phenology. The generation time of *C. m. reductus* is approximately 56 days (Severin & Klostermeyer 1950), so it was feasible that the three peaks of abundance were different generations. To be sure, we collected adult leafhoppers in August from Wenatchee cherries in 2020 and reared them in cages outside the lab. Those adult leafhoppers laid eggs that emerged as adults in October, suggesting that the August and October peaks represent separate generations. Sampling efforts in these plots are ongoing in 2021, as we anticipate leafhopper capture through the end of October.

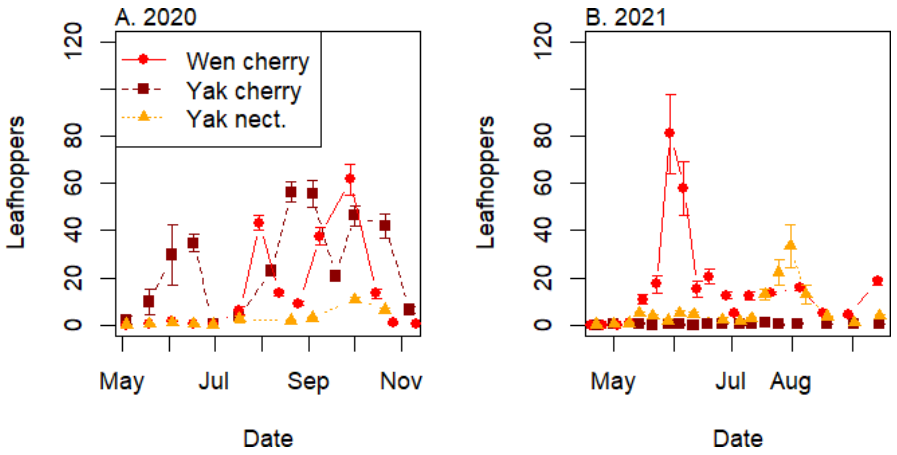

***Figure 8. Phenology**. Patterns of abundance in untreated blocks in 2020 (A), and 2021, in Wenatchee region cherry blocks (red circles), Yakima region cherry blocks (dark red squares), or Yakima region nectarine blocks (orange triangles).*

Spatial distribution. We evaluated the spatial distribution of the different treatments to measure the likelihood of edge effects in controlled blocks. Consistently, the mean number of leafhoppers per trap were highest in the center of control blocks, and highest along the edge of Surround-treated blocks, where the edge of the block is adjacent to untreated border rows (Figure 9). This suggests spillover effects along the row where leafhoppers were moving into the Surround-treated blocks. In Extenday-treated blocks the leafhopper numbers were low throughout the block (Figure 9). Given the shortened timeframe of the funded project relative to the lag between transmission and symptoms as well as the initial degree of infection in the blocks (high disease incidence in Yakima region cherries and very low in Wenatchee region blocks), we were unable to evaluate the distribution of disease prevalence.

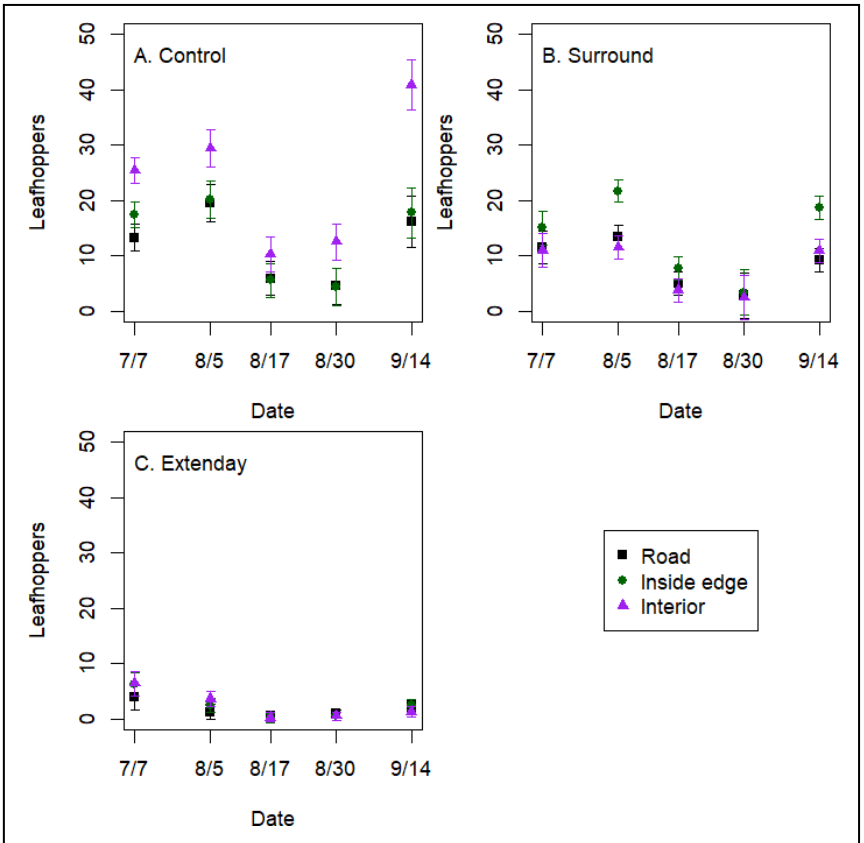

Figure 9. Spatial distribution of leafhoppers. *Abundance of* C. m. reductus *leafhoppers in Wenatchee region cherry blocks in untreated control blocks (A), blocks treated with Surround (B), or treated with Extenday (C). Counts include only traps placed at 4 feet high, either 25 feet from the road (black squares), 25 feet from the edge adjacent untreated buffer trees (green circles), or in the center of the block (purple triangles).While data represent Wenatchee region cherries in 2021, we found similar results in 2020, and Yakima cherries in 2020, and 2021.*

Future plans: We are still collecting leafhoppers from traps in the 2021 season, which extends through October. Given the successful control provided by Extenday, we will use funds provided by a WSDA/USDA Specialty Crop Block Grant to continue Extenday trials and add in a more conventional weed management program to see if similar control can be attained by reducing broadleaf abundance.

References

Severin, H. P., & Klostermeyer, E. C. (1950). *Colladonus geminatus* and *C. Montanus*: Life Histories on Virus-Infected and on Healthy Plants. *Hilgardia*, *19*(18), 553–560.

Executive summary

Project Title: Field evaluation of leafhopper controls for X-disease management

Key words: X-disease, Little cherry disease, *Colladonus* leafhoppers

Abstract:

X-disease is currently at epidemic levels in Pacific Northwest stone fruit orchards. A recent survey of 26% of the PNW cherry industry acreage revealed losses of $120 million due to a combination of X-disease and Little cherry virus 2. While the symptoms of these pathogens are indistinguishable, recent test results suggest the vast majority (93%) of diseased trees are infected with X-disease phytoplasma rather than Little cherry virus 2. The leafhoppers that vector X-disease are abundant after harvest, when phytoplasma titers are high, suggesting this is a key time for phytoplasma transmission. Here, we evaluated two postharvest cultural controls, Surround-kaolin clay and Extenday ground cover, that deter leafhoppers from feeding on trees at the block (Extenday) or plant (Surround) level and potentially prevent transmission. Extenday was effective, reducing leafhoppers by 81% in Wenatchee region cherries in 2020 and 88-91% in Wenatchee and Yakima region cherries in 2021, compared to controls. In year two there was a 50-fold difference in peak densities across the control blocks in Wenatchee and Yakima regions, but these effects were consistent. In 2020 and 2021 high-pressure cherry blocks in the Wenatchee and Yakima region, Surround reduced season-wide leafhopper numbers by 47-48%. Furthermore, the Surround reduced the mean height at which leafhoppers were caught compared to the control plots, suggesting they may not be moving into the trees as much. In Yakima region cherries the clay did not significantly reduce leafhopper numbers, but mean counts were very low, peaking at less than 2 leafhoppers per trap (2-week trapping period). Surround also did not reduce leafhopper numbers in nectarine blocks, potentially due to the late harvest date. In summary, we have identified two cultural controls, with one (Extenday) effective at reducing leafhopper numbers in either high pressure or low pressure blocks, and another (Surround) that reduced leafhopper numbers in high pressure blocks, although not to the same effect as Extenday and also appeared to reduce the movement up into trees from the groundcover.

Project/Proposal Title: Identifying sources of X disease in cherry orchards

Primary PI: Tobin Northfield
Organization: WSU-TFREC
Telephone: 509-293-8789
Email: tnorthfield@wsu.edu
Address: 1100 N Western Ave.
City/State/Zip: Wenatchee, WA 98801

Co-PI 2: W. Rodney Cooper
Organization: USDA-YARL
Telephone: 509-454-4463
Email: Rodney.cooper@ars.usda.gov
Address: 5230 Konnowac Pass Rd.
City/State/Zip: Wapato, WA 98951

Cooperators: Garrett Bishop, Scott Harper, Tianna DuPont

Report Type: Continuing Project Report

Project Duration: 1-Year, 2-Year, 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: $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.

Budget 1
Primary PI: Tobin Northfield
Organization Name: WSU-TFREC
Contract Administrator: Anastasia 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	2020	2021	2022
Salaries[1]	39,629	41,214	42,863
Benefits[2]	4,478	4,657	4,844
Wages			
Benefits			
Equipment			
Supplies[3]	7,000	4,000	4,000
Travel[4]	2,000	2,000	2,000
Miscellaneous			
Plot Fees			
Total	53,107	51,871	53,707

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

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

Budget 2
Co PI 2: W. Rodney Cooper
Organization Name: USDA-YARL
Contract Administrator: Chuck Myers
Telephone: 509-454-4463
Contract administrator email address: Chuck.Myers@ars.usda.gov
Station Manager/Supervisor: Rodney Cooper
Station manager/supervisor email address: Rodney.Cooper@usda.gov

Item	2020	2021	2022
Salaries			
Benefits			
Wages			
Benefits			
Equipment			
Supplies	5,293	3,978	
Travel			
Miscellaneous			
Plot Fees			
Total	5,293	3,978	0

Footnotes:

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:

- 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

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.

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.

Additional Research: Leafhopper Location and Activity

Studies on the behavior of *Colladonus reductus* within orchards is lacking, leaving unknowns such as when they are most active throughout the day and where they are most abundant within a block. We did not observe activity during daylight hours in the feeding trials, but we were unable to replicate dawn or dusk in the growth rooms (due to non-dimming lights), so we sought to identify whether leafhoppers regularly move vertically from ground cover to canopies in four time periods: morning, mid-day, evening, and overnight. To begin addressing these unknowns we used yellow sticky cards (5 × 7 in) to examine leafhopper abundance at two heights, varying distances from the orchard border, and activity throughout a 24 hr period. In two cherry blocks at 6:00am Aug 5th, we deployed 32 sticky cards, one at each height at four distances from the orchard border (40, 80, 120, and 160 ft), and 16 sticky cards, one at each height. At each location, one trap was tied to a branch at 6 ft and another to a wooden stake at 2 ft from the ground. Traps were collected and replaced at 10:00am, 6:00pm, 10:00pm, and

Figure 2 *C. m. reductus* feeding on mallow

6:00am the following morning, and *C. m. reductus* abundance was recorded by height, time, and distance from orchard border.

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.

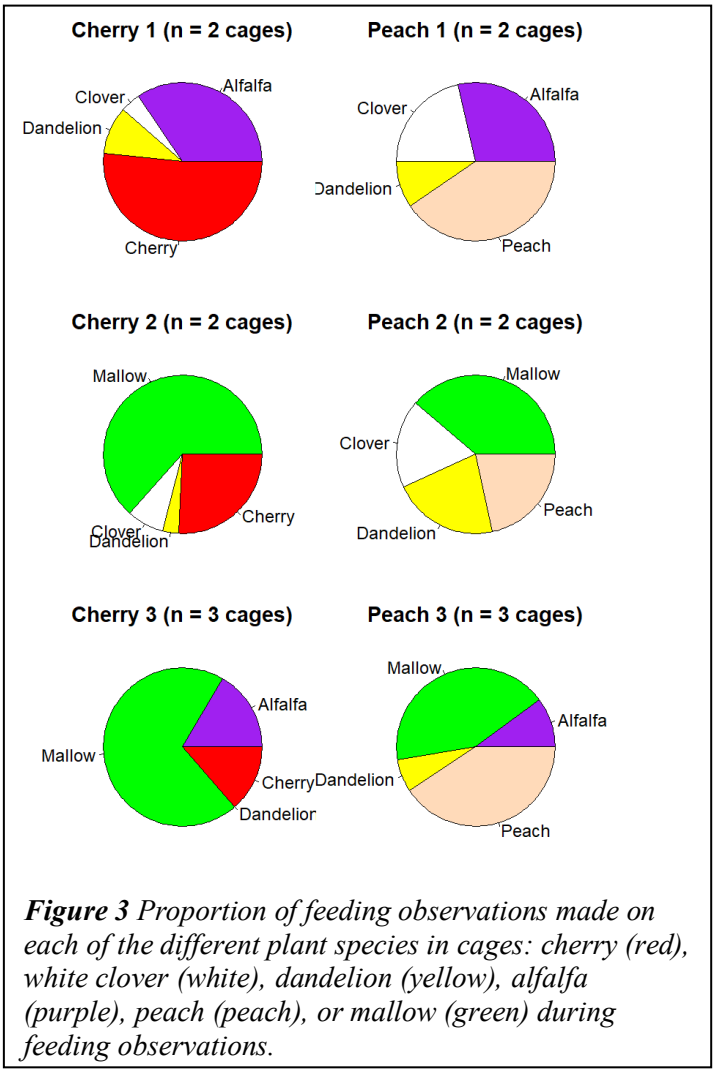

Figure 3 *Proportion of feeding observations made on each of the different plant species in cages: cherry (red), white clover (white), dandelion (yellow), alfalfa (purple), peach (peach), or mallow (green) during feeding observations.*

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

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.

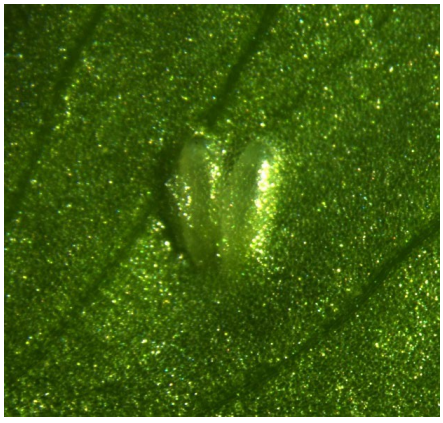

Figure 4. Leafhopper eggs in alfalfa (approximately 1mm long)

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.

Additional Research: Leafhopper Location and Activity

Leafhoppers were most active mid-day and in the evening from 6pm to 10am (Figure 2), although evening catch was much more skewed towards the low trap heights (2' compared to 6' height). We conducted this experiment in part to test the theory that leafhoppers move into the trees at night. However, these data do not seem to support this theory, as in the evening hours most trap capture occurred near the ground cover. In addition, we did not observe evidence that leafhoppers were moving in and out of the orchard during different periods of the day. There was consistent capture at the different distances from the edge in our different time periods (data not shown).

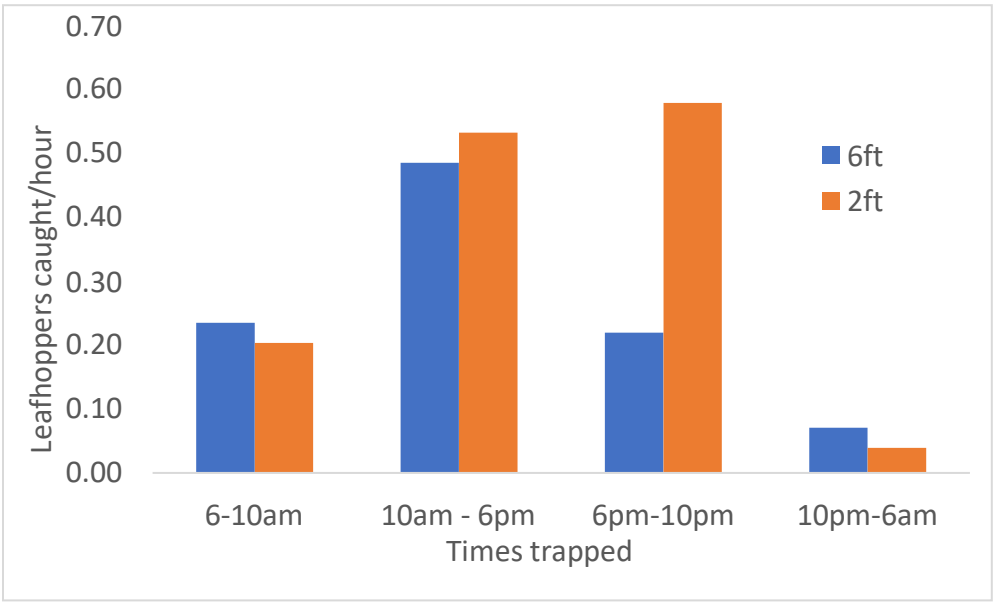

Figure 4 Mean *C. m. reductus* leafhoppers collected per hour (to account for variable trapping intervals) in a commercial Wenatchee region cherry block over a 24 hour period starting on August 5, 2020. No *C. geminatus* were collected. Traps were hung at 6 feet from a cherry tree or placed below the tree on a 2-foot stake. Data summarize 12 traps at each height.

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CONTINUING PROJECT REPORT **YEAR**: No-Cost Extension
WTFRC Project: CH-20-103

Project Title: Insecticidal control of leafhoppers in cherries

PI: Louis Nottingham
Organization: WSU TFREC
Telephone: 509-293-8756
Email: louis.nottingham@wsu.edu
Address: 1100 N Western Ave.
City/State/Zip: Wenatchee, WA 98801

Co-PI: Tobin Northfield
Organization: WSU TFREC
Telephone: 509-293-8789
Email: tnorthfield@wsu.edu
Address: 1100 N Western Ave.
City/State/Zip: Wenatchee, WA 98801

Cooperators: Scott Harper, WSU

Total Project Request: **Year 1:** $81,166 **Year 2:** $84,185

Other funding sources: **None**

WTFRC Budget: ***None***

Budget
Organization Name: Washington State Univ. **Contract Administrator**: Stacy Mondy
Telephone: 916-897-1960 **Email address**: arcgrants@wsu.edu
Station manager: Chad Kruger **Email address**: cekruger@wsu.edu

Item	2020	2021	2022
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	0

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.**
   Future goals: Continue screening products, particularly selective-conventional and organic insecticides. Develop methods for colony rearing to allow testing of insecticides on nymphs.
   Deviations: (1) We did not use stylet sheath analysis because many insecticides tested resulted in high mortality and eliminated the need to perform such time-consuming methods. (2) Particle films were included in 2021 because of their potential to repel adults and prevent feeding. Repellency was assessed using video tracking software and choice tests. (3) We were unable to establish a colony in the lab this year but made significant progress in rearing host plants for leafhopper diet. Knowledge of diet, combined with recently confirmed generation times, will inform a hopefully successful colony establishment in the summer of 2022.
2. **Determine whether X-infected leafhoppers are more susceptible to insecticides than uninfected leafhoppers.**
   Future goals: Continue to store adults that were killed in bioassays, then extract DNA for PCR diagnosis of the presence of phytoplasma.
   Deviations: Instead of performing separate bioassays for this hypothesis, we are utilizing individuals from insecticide screening in Objectives 1 and 3 to gain larger sample sizes, test more materials, and save time.
3. **Determine residual control timelines for the most effective foliar products.**
   Future goals: Continue to perform residual time-line bioassays for more materials, including particle films.
   Deviations: It is too risky to perform insecticide trial in field plots due to 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.**
   Future goals: Test a wider variety of soil-applied materials in 2022.
   Deviations: This objective was successfully tested in 2020, but with unimpressive control of leafhoppers. We decided that testing more effective foliar sprays methods should take priority this year.

Significant Findings:

- We identified conventional and organic insecticides that caused high mortality of *C. reductus* and other leafhoppers in direct contact spray bioassays within 24-36 hours of application.
  - Conventional products resulting in 100% mortality were Scorpion (dinotefuran) and Admire (imidacloprid). Transform (sulfoxaflor, 90%), Asana (esfenvalerate, 96%), and Actara (thiamethoxam, 97%) also resulted in high mortality.
  - Pyganic was the only organic product that resulted in high treatment mortality (98%).
- Both Celite and Surround particle films successfully repelled leafhoppers in bioassays.
- When used alone, insecticides tested in an aged residue assay performed comparably no matter their residue age. However, considerable differences were seen between treatments, with Actara and Asana performing the best overall.
- When combined with IAP 440 Oil (mineral oil), Actara and Asana provided the best control in an aged residue bioassay, and, for Asana and Pyganic, efficacy was reduced as residues aged.
- We were unable to establish a lab colony, but successfully identified and grew herbaceous weeds which will be used for colony diet next year.
- The third leafhopper generation (mid- to late-Sept) was the most abundant this year, indicating control methods must continue throughout the fall.

Methods:

Collection and Transport. Leafhoppers of mixed species were collected from weedy row middles in an organic commercial apple orchard near Rock Island, WA, using sweep nets. Nets were frequently emptied into 12" by 12" mesh cages to avoid sublethal injury to the insects (Fig 1B, background). Once returned to the lab, leafhoppers were chilled to slow their movement and aspirated into vials. They were then promptly divided into replicates and added to assay arenas. Fresh leafhopper collections were made the morning of each experiment.

Fig. 1. 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; field collection cages in background.

Direct Spray Bioassays. Arenas were constructed using 8 oz plastic deli cups with moist soil and excised cherry leaves kept alive by constant contact with water in floral tubes (Fig. 1A). 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. Once leafhoppers were in all arenas, treatments 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 24-36 hours 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. Two spray contact bioassays were conducted. The first tested conventional insecticides (Table 1) and the second tested organic insecticides with the addition of Asana as a positive control (Table 2).

Table 1. Conventional Direct Spray Bioassay

Trt.	Rate per acre
UTC	-
Actara 25 WG	2.75 oz
Admire Pro	2.8 fl oz
Transform WG	2.75 oz
Magister SC	32 fl oz
Scorpion 35SL	36 fl oz

Table 2. Organic Direct Spray Bioassay

Trt.	Rate per acre
UTC	-
Asana XL (pos control)	14.5 fl oz
IAP 440 Oil	1%
Pyganic 1.4 EC	64 fl oz

Particle Film Repellency Assays – Petri Dish Bioassay. Half of a 60mm x 15mm Petri dish was dipped into a prepared insecticide treatment (Table 3). The other half of the dish was left completely untreated. Once the treatment dried, a chilled leafhopper was added to the arena and the dish was lidded (Fig. 2A). Leafhopper movement was recorded via EthoVision for five minutes; this was replicated 18 times per treatment. The cumulative duration spent in each zone (treated vs untreated in seconds) was compared by treatment.

Table 3. Petri Dish Bioassay

Trt.	Rate per acre
Water	-
Celite 610	70 lb
Surround WP	50 lb

Table 4. Excised Leaf Bioassay

Trt.	Rate per acre
Water	-
IAP 440 Oil	1 gal
Celite 610	70 lb
Celite 610 + 440 Oil	70 lb + 1 gal
Surround WP	50 lb
Surround WP + 440 Oil	50 lb + 1 gal

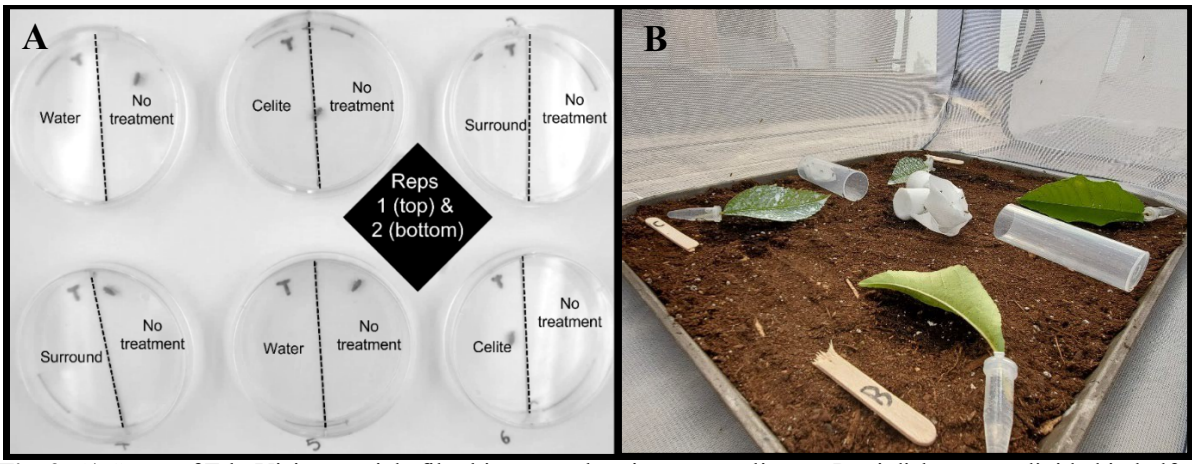

Fig. 2. A) Setup of EthoVision particle film bioassay, showing two replicates. Petri dishes were divided in half (zones) and treatment was applied to one half of the dish. B) Excised leaf bioassay setup.

Particle Film Repellency Assays - Excised Leaf Bioassay. Assay arenas were composed of treated excised cherry leaves evenly spaced on a tray of moist soil in a large mesh cage (Fig. 2B). Leaves were dipped into prepared insecticide solutions (Table 4) and allowed to dry completely. They were then placed into arenas, into which 10-20 leafhoppers were released. The number of leafhoppers harboring on each leaf was assessed at 5h and 22h after release into the arenas and was compared by treatment.

Spray Residue Bioassays. These experiments used Lapins cherry trees (3/4") on Mazzard rootstock planted in 3.6-gallon injection molded pots. Five cherry trees were assigned to each foliar treatment (Table 5, Table 6). Foliar treatments were sprayed using an arm-pump SOLO backpack sprayer. Trees were sprayed to just before runoff, about 0.25 liters per tree. Trees remained exposed to outdoor ambient conditions, including sunlight and occasional rain, for the duration of the experiment. Treated cherry leaves were excised at predetermined intervals; for the first bioassay, after 1 h (fresh residues), 72 h (3 days), 168 h (7 days), and 336 h (14 days), and for the second bioassay, after 1 h (fresh residues), 24 h (1 day), and 122 h (5 days). After excision, leaves were placed in arenas as described in the Direct Spray Bioassay above (Fig. 1), and 5-10 leafhoppers were exposed to the aged residues per replicate (3-5 replicates per treatment per time interval). After 24-36 hours, mortality was evaluated and then compared by treatment.

Table 5. Spray Residue Bioassay 1

Trt.	Rate per acre
Water	-
Actara 25 WG	2.75 oz
Asana XL	14.5 fl oz
Pyganic 1.4 EC	64 fl oz

Table 6. Spray Residue Bioassay 2

Trt.	Rate per acre
Water	-
IAP 440 Oil	1 gal
Asana + 440 Oil	14.5 fl oz + 1 gal
Actara + 440 Oil	2.75 oz + 1 gal
Admire + 440 Oil	2.8 fl oz + 1 gal
Pyganic + 440 Oil	64 fl oz + 1 gal

Results:

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%).

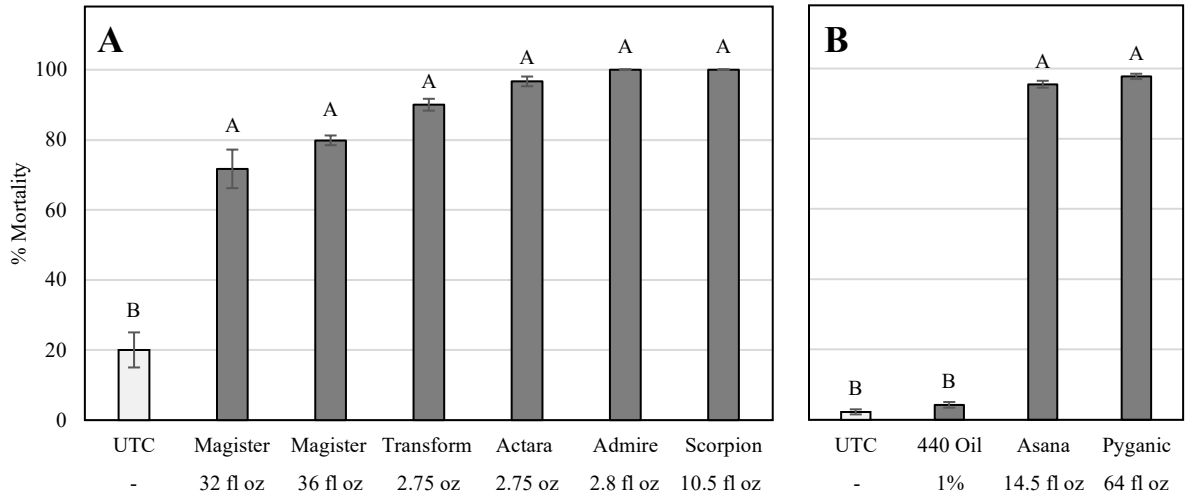

Fig. 3. Conventional (A) and Organic (B) Direct Spray Bioassay. Bars show average leafhopper mortality resulting from each insecticide. Per acre rates shown below each insecticide name. Bars not sharing a letter are significantly different according to Tukey's HSD ($p < 0.05$).

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.

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.

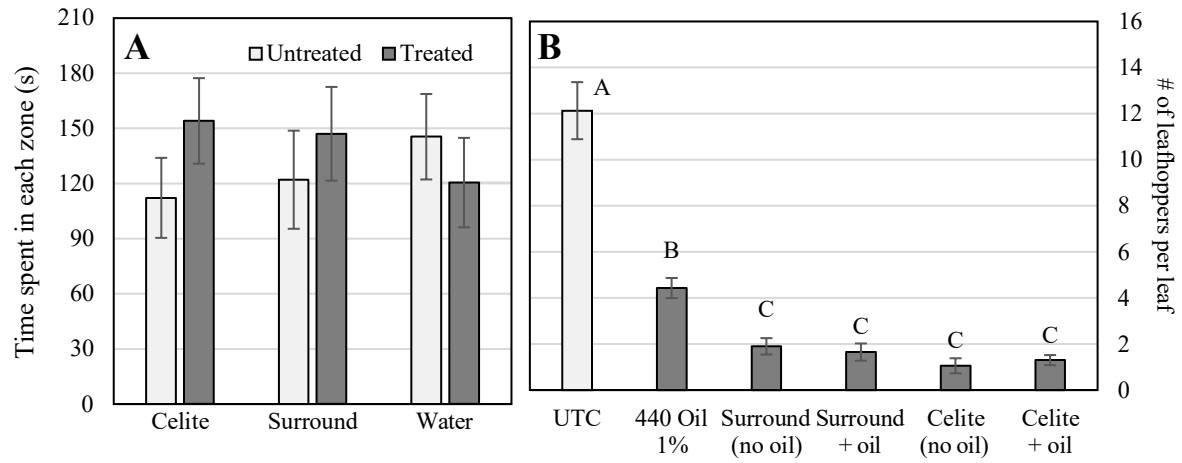

Fig. 4. A) Cumulative duration (s) spent in zones treated with particle films and untreated zones. B) Average leafhopper count on treated and untreated excised cherry leaves. Bars not sharing a letter are significantly different according to Tukey's HSD ($p < 0.05$).

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).

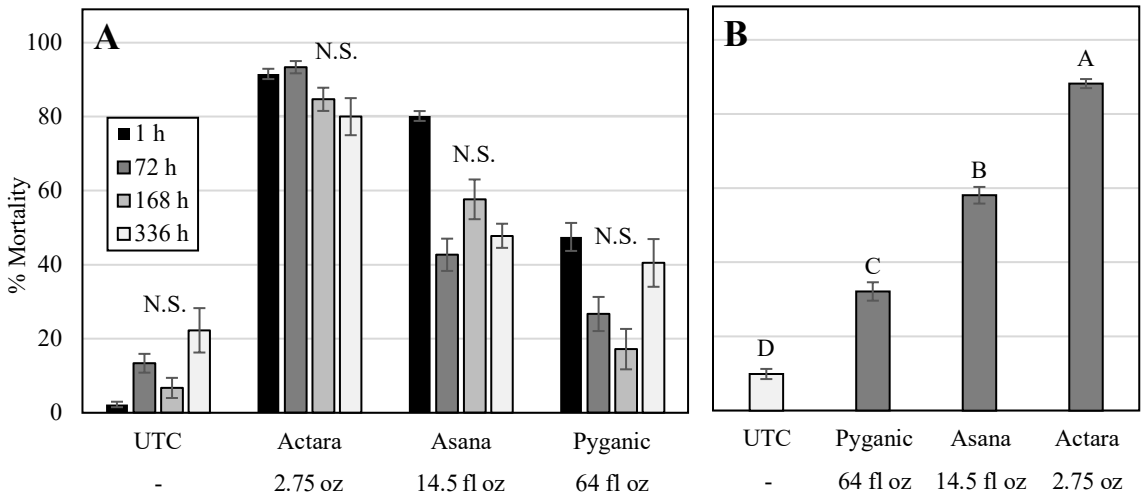

Fig. 5. A) Mortality of leafhoppers exposed to aged insecticide residues. B) Average leafhopper mortality across all residue ages. Per acre rates shown below each insecticide name. N.S. indicates no significant difference, and bars not sharing a letter are significantly different according to Tukey's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

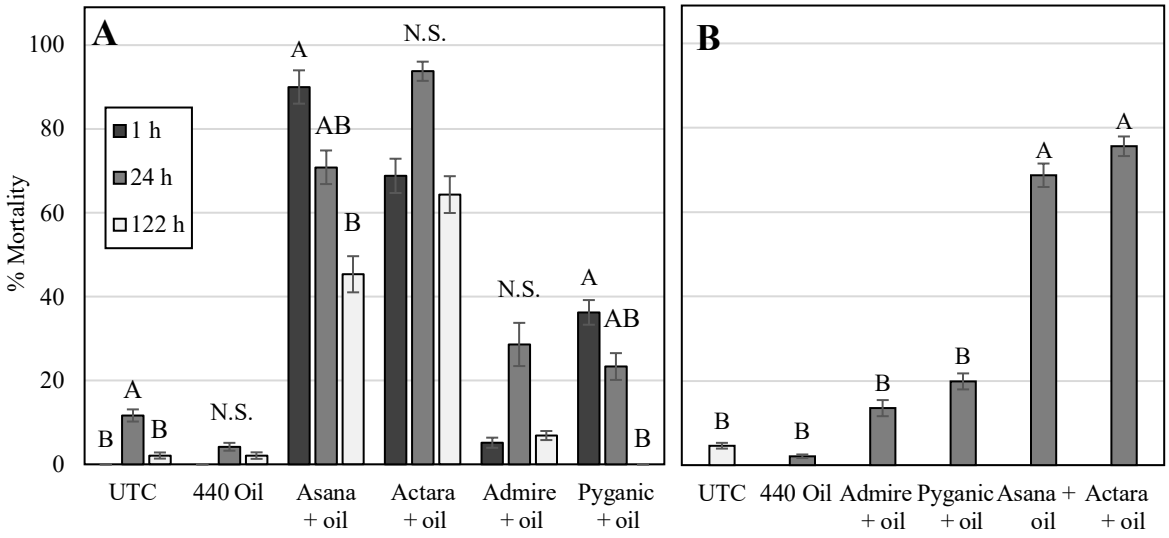

Fig. 6. A) Mortality of leafhoppers exposed to aged insecticide residues. B) Average leafhopper mortality across all residue ages. N.S. indicates no significant difference, and bars not sharing a letter are significantly different according to Tukey's HSD ($p < 0.05$).

Discussion:

Through these 2021 experiments, we have 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.

While rearing attempts were, again, unsuccessful in 2021, considerable knowledge was gained that will be instrumental in 2022 attempts. Several species of weeds known to be host plants for X-disease vectors were easily grown in greenhouse conditions and will allow us to supply leafhopper colonies with fresh food next year. Additionally, we observed that the third and final leafhopper generation of the 2021 season (mid- to late-September) was the most abundant of the 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/Proposal Title:X-disease Vector Identification and Acquisition From Low Titer Trees

Primary PI: Tobin Northfield
Organization: WSU-TFREC
Telephone: 509-293-8789
Email: tnorthfield@wsu.edu
Address: 1100 N Western Ave.
City/State/Zip: Wenatchee, WA, 98801

Co-PI 2: Tianna DuPont
Organization: WSU-TFREC
Telephone: 509-293-8758
Email: tianna.dupont@wsu.edu
Address: 1100 N Western Ave.
City/State/Zip: Wenatchee, WA, 98801

CO-PI 3: Scott Harper
Organization: WSU-TFREC
Telephone: 509-786-9230
Email: scott.harper@wsu.edu
Address: 24106 N Bunn Rd
City/State/Zip: Prosser, WA, 99350

Co-PI 4: Adrian Marshall
Organization: WSU-TFREC
Telephone: 509-293-8800
Email: atmarshall@wsu.edu
Address: 1100 N Western Ave.
City/State/Zip: Wenatchee, WA, 98801

Cooperators: Rodney Cooper, Louis Nottingham, Garrett Bishop

Report Type: Continuing Project Report

Project Duration: 2-Year

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

WTFRC Collaborative Costs: None

Budget 1
Primary PI: Tobin Northfield
Organization Name: WSU-TFREC
Contract Administrator: Anastasia 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	2021	2022
Salaries	$28,260	$29,390
Benefits	$10,206	$10,614
Wages	$0	$0
Benefits	$0	$0
Equipment	$0	$0
Supplies	$13,362	$11,862
Travel	$3,438	$3,438
Miscellaneous	$0	$0
Plot Fees	$0	$0
Total	$55,266	$55,304

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 and plan to conduct them in 2022. We have expanded our efforts to include E. variegatus, given high numbers collected and their greater ability to grow in colony.

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 key vectors, and molecular analyses conducted in the fall/winter of 2021 will determine if these are important vectors or not.

Methods

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

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 (*Col. reductus, Col. geminatus, Scaph. 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.

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

We were unable to maintain a colony to ensure phytoplasma-free individuals for acquisition tests, due to high mortality in the colony and deformities that were emerging from colony-reared nymphs. We are expanding our efforts to include *E. variegatus*, given its high numbers and that it seems to perform better in colonies. In addition, we have three growth chambers with humidity control that we will plan to use for the colony, given that humidity appears to be part of the problem.

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

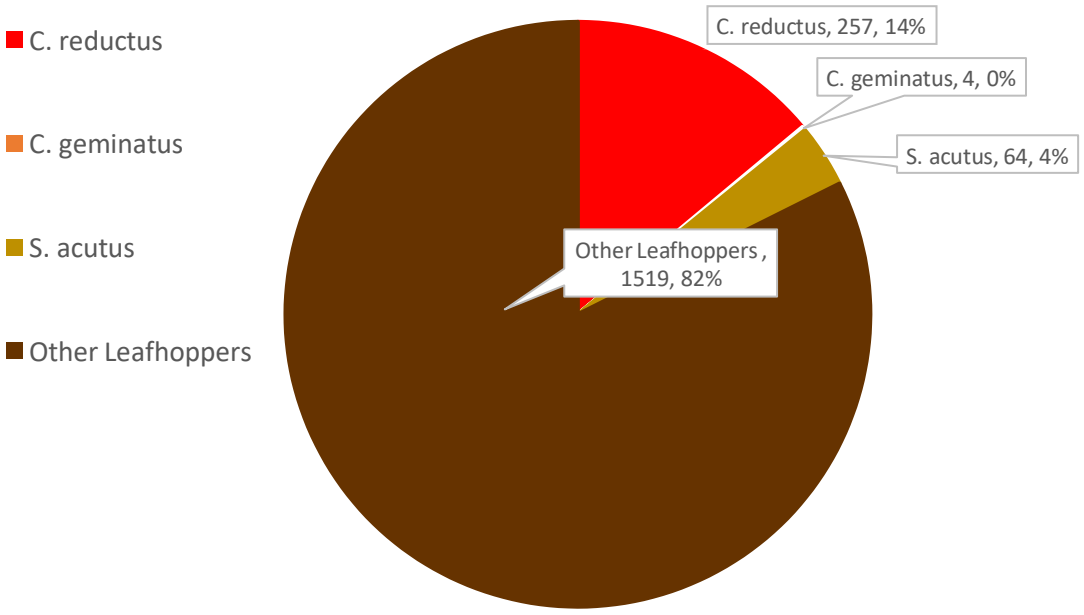

Figure 1. Proportions of leafhopper species collected from the 22 sites. In addition, we collected 406 nymphs, for which the species is unknown.

resemble *E. variegatus*, but appear to be different species (Figure 1). In addition, we collected 406 nymphs for which the species is unknown. We are currently extracting 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. For the other species, we'll evaluate the impacts of mass sampling leafhopper heads and if possible group individuals in the same species to quickly identify species with vector capacity. The *C. m. reductus*, *C. geminatus*, and *E. variegatus* will analyzed using molecular gut content analysis, funded by a WSDA/USDA Specialty Crop Block Grant to identify their feeding hosts. 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.

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)

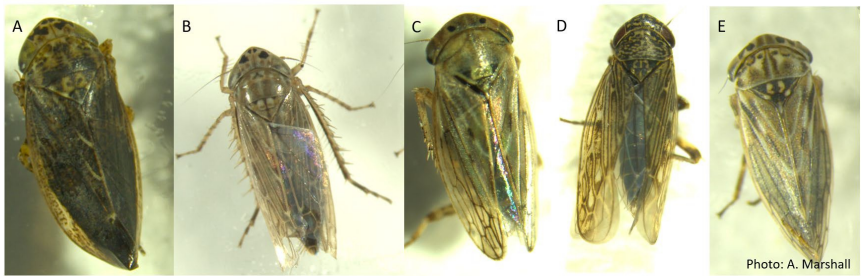

Figure 3. *E. variegatus* (A), and four other types of leafhoppers commonly collected in surveys. Leafhoppers are shown in order of abundance, with A being the highest abundance, and E being the least abundant. Note especially variation in the head shape and patterns, as well as the pronotum (the pirate 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. We plan to conduct acquisition tests in 2022, assuming our colony persists.

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. '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/.

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

References

Galetto, L., S. Abba, M. Rossi, M. Vallino, M. Pesando, N. Arricau-Bouvery, M. P. Dubrana, W. Chitarra, M. Pegoraro, D. Bosco, and C. Marzachi. 2018. Two phytoplasmas elicit different responses in the insect vector *Euscelidius variegatus* Kirschbaum. Infection and Immunity **86**:20.

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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.

FINAL PROJECT REPORT **YEAR**: 1 of 2

Project Title: Rootstock Sensitivity to X disease
PI: Ashley Thompson
Organization: Oregon State University Extension Service
Telephone: 541-296-5494
Email: ashley.thompson@oregonstate.edu
Address: 400 E. Scenic Dr. Ste. 2.278
City/State/Zip: The Dalles, OR, 97058
Cooperators: Stacey Cooper, Steve Castagnoli

Total Project Request: **Year 1:** $35,450 **Year 2:** $34,658

Other funding sources: None

WTFRC Collaborative Expenses: None

Budget 1
Organization Name: OSU ARF **Contract Administrator:** Dan Arp
Telephone: (541)737-4066 **Email address:** Dan.j.arp@oregonstate.edu
Supervisor: Nicole Strong **Email address:** Nicole.strong@oregonstate.edu
MCAREC Director: Steve Castagnoli **Email address:** steve.castagnoli@oregonstate.edu

Item	2020	2021
Salaries[1]	$8,112	$8,112
Benefits[2]	$3,245	$3,245
Wages[3]	$8,320	$8,320
Benefits[4]	$5,824	$5,824
Equipment		
Supplies[5]	$5,693	$4,901
Travel[6]	$756	$756
Miscellaneous		
Plot Fees	$3,500	$3,500
Total	$35,450	$34,658

Footnotes:
[1] 1 month salary for Dr. Thompson
[2]OPE is calculated at 40%
[3]Wages for a BioScience II technician calculated at $16/hr for 40 hours a week for 13 weeks
[4]OPE is calculated at 70%
[5]150 trees at $15 each = $2,250; Grafting infected bud wood $500; pot-in-pot supplies $1985; Screen House; X disease molecular identification in 2020 20 samples at $35 + $258 set up fee = $958, X disease molecular identification in 2021 $3,500 + $258 set up fee; Pot-in-pot upkeep $1,143.
[6]Travel was calculated at $0.55/mile for 25 trips to the MCAREC from the Wasco County Extension office, which is 55 miles round trip.

OBJECTIVES:

1. Evaluate the response of five rootstocks, ‘Maxma 14’, ‘Gisela 5’, ‘Gisela 6’, ‘Krymsk 5’, ‘Krymsk 6’, ‘Lake’ and, ‘Clinton’ to the X disease phytoplasma.
2. Identify hypersensitive rootstocks that can be used to reduce X disease inoculum in cherry orchards.

2021 Goals:
March: Inoculate trees by grafting with X-disease infected buds in March.

July: Test all trees for X-disease and check for pits and grooves at the graft union.
October: Assess tree vigor by measuring the trunk cross sectional area and leader growth of each tree. Report findings from the X-disease molecular and physical tests to OSCC.

Deviation from original schedule:
2020:
I was unable to hire any additional staff due to COVID-19 restrictions put in place by OSU. This made it challenging to plant trees and obtain nursery materials (pots and soil) in a timely fashion. Due to these challenges, I decided to graft trees in March of 2021 to ensure good bud take and the best possible results. I will likely ask for a no-cost extension for this grant for 2022 to make sure we have enough time to observe infection in these trees. In addition, I have been unable to source 'Maxma 14', and I continue to look for this rootstock.

2021:

Scions grafted to 'Mahaleb' died (Figure 1) and the 'Mahaleb' rootstocks were infested by borers (Figure 2). Pits and grooves were not identified at the graft union of any 'Mahaleb' tree. Over half of the young trees grafted to 'Mazzard','MaxMa 14', and 'Lake' planted in Ellepots died during the 2021 heat wave, when temperatures at MCAREC registered 116 F (Figure 3). Pits and grooves were not identified at the graft union of these young trees.

Figure 1 and 2. The scion of the 'Mahaleb' grafted tree died over winter. The rootstock survived the cold conditions, but the above ground portion was infested with a borer.

Figure 3. Despite watering and a shade cover, this 'MaxMa 14' rootstock died due to heat stress.

SIGNIFICANT FINDINGS:

- Based on survey results from a study funded by Columbia Gorge Fruit Growers the following rootstocks are susceptible to X-disease, and will show symptoms:
  - Gi.12
  - Gi.6
  - K.6

METHODS:

Ten of each rootstocks grafted with sensitive varieties (Table 1.) were planted as a completely randomized design in 10-gallon pots filled with general purpose growing medium on 2 June, 2020 at the Mid-Columbia Agricultural Research and Extension Center. Rootstocks were uniformly watered three times weekly. A netted hoop house covering was erected over the trees to prevent the potential movement of X-disease to the surrounding orchard following X-disease infection.

Table 1. Rootstock treatments for this study were selected based on virus susceptibility characteristics and use in the Pacific Northwest.

Rootstock Treatments	**Notes**
'Mahaleb'	Positive control- exhibits a hypersensitive response (death) to X disease
'MaxMa 14'	Related to 'Mahaleb'
'Mazzard'	Negative control- Susceptible to X disease
'Gisela 12'	Negative control- Susceptible to X disease
'Gisela 6'	Susceptible to pollen-borne viruses, but experiences reduced shoot growth when infected

‘Krymsk 5’	Hypersensitive response (death) to pollen-borne viruses
‘Krymsk 6’	Hypersensitive response (death) to pollen-borne viruses
‘Lake’	Open pollenated with parental parent unknown
‘Clinton’	Open pollenated with parental parent unknown

Late summer of 2020, all scions tested negative for X-disease.

In March of 2021, half of the rootstocks will be infected by grafting three infected buds from confirmed X-disease infected trees will be grafted onto the scion. Uninoculated trees will serve as a control. To insure that the infected buds contain adequate X-disease phytoplasma to create an infection, bud wood was collected in the form of semi-hardwood stem cuttings from confirmed X-disease positive trees was collected in late summer of 2020 when higher levels of X-disease phytoplasma are expected to be detected. Wood was rooted in a mixture of perlite and sphagnum moss using Hormodin rooting compound. Cuttings are being cared for indoors at the MCAREC.

RESULTS AND DISCUSSION:

Young trees, particularly those in Ellepots, are very susceptible to temperature fluctuations. In 2021, damage from frost and an extreme heat dome severely injured many of the young trees in our outdoor pot-in-pot planting. Trees killed or significantly damaged included the positive control, ‘Mahaleb’, the negative control, ‘Mazzard’, and the rootstock I was most interested in testing, ‘MaxMa 14’. Future studies like this should be completed in a greenhouse so the temperature can be controlled.

In addition, we can rely on X-disease survey data to rule out rootstocks that are susceptible and symptomatic to X-disease infection. Based on my survey study, we should not include ‘K.6’, ‘Gi.12’, or ‘Gi.6’ in future studies. Data from WSU may have insights into the susceptibility of ‘K.5’ and ‘Gi.5’. I believe it would be more efficient and effective to focus on newer rootstock and more infrequently used rootstocks, like ‘MaxMa 14’.

CONTINUING REPORT (YEAR 1) **PROPOSED DURATION**: 3 Years

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

PI: Ashley Thompson
Organization: OSU Extension Service
Telephone: 541-296-5494
Email: ashley.thompson@oregonstate.edu
Address: 400 E. Scenic Dr. #2.278
Address 2:
City/State/Zip: The Dalles, OR 97058

Co-PI (2): Bernardita Sallato
Organization: WSU-ANR
Telephone: 509-786-9205
Email: b.sallato@wsu.edu
Address: 24106 N Bunn Rd
Address 2:
City/State/Zip: Prosser, WA, 99350

Co-PI(3): Scott Harper
Organization: WSU-Clean Plant Center Northwest
Telephone: 509-786-9230
Email: scott.harper@wsu.edu
Address: 24106 N Bunn Rd
Address 2: Hamilton Hall 201
City/State/Zip: Prosser, WA, 99350

Co-PI: Corina F. Serban
Organization: WSU-Clean Plant Center
Telephone: 509-574-1595
Email: corina.serban@wsu.edu
Address: 2403 S 18th St Suite 100
Address 2:
City/State/Zip: Union Gap, WA 98903

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
Telephone: (541)737-4066 **Email address:** dan.j.arp@oregonstate.edu
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
Telephone: (509) 335-2885
Station director: Naidu Rayapati
Contract Administrator: Katy Roberts
Email address: arcgrants@wsu.edu
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.* In WA and OR, the diversity of replanting strategies utilized by our collaborators suggested we needed to expand the number of replanted sites. Detail strategies on each site is listed in Table 1.

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 will enable to obtain a wider range of conditions and monitor trees for longer period of time after replanting (up to your years). In Washington, five newly planted blocks plus a two-year-old block were selected for the project. An additional site will be added in 2022. In Oregon, three newly planted blocks and one recently removed block were selected for the project. An initial qPCR test of 10 random trees was performed to ensure free virus trees were netted and isolate possible infection rate from nursery or early infection in the field. However, given that titer detection during spring were low, according to Dr. Harper, we randomly selected ten trees for netting prior to testing. Each tree was individually put in cages with insect netting (OVS, 7% shade) attached to pvc pipes or metal t-posts. The inside of the cage was cleaned of weeds and a yellow sticky trap was installed inside each cage to monitor effectiveness of the netting system. Samples from leaf of caged trees were taken during mid-August in Washington and late September in OR, when titer levels started to increase according to Harper. Leaves were collected from the base of the trees (older possible spurs and leaves) to increase detection probability. Positive trees to any LCD pathogen were immediately removed and the cage was re installed in a new free virus tree.

   In 2022, additional sites with confirmed infected trees (tested in 2021) will be incorporated to provide additional scenarios: example. No herbicide, tree removal in the winter, and replanting immediately next spring.

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 will lead the outreach and extension effort of the project starting in 2022.

   Best practice for individual tree removal was published in a short article and video by B. Sallato in collaboration with Good Fruit Grower in English and Spanish (https://www.goodfruit.com/risk-mitigation-strategies-for-little-cherry-driven-removal-and-replant/, https://www.goodfruit.com/es/estrategias-de-mitigacion-de-riesgos-para-la-eliminacion-y-replantacion-motivadas-por-la-enfermedad-de-la-cereza-pequena/). Preliminary results were shared in LCD – Roza field day organized by Corina Serban, LCD-Information Technology Transfer (ITT) Extension organized in October 8th, 2021.

SIGNIFICANT FINDINGS:

- In individual tree replanting sites:
  - Herbicide application over stump cuts during the previous summer killed large roots (> ¼ inch diameter) near the trunk. However, live infected roots were found at 2 foot from removed trunk in 100% sampled sites in WA.
  - Despite the presence of live roots positive to XDP, 100% of the replanted trees were free of infection (LChV1, LChV2 and XDP) when sampled in 2021.
  - Root suckers were not present at WA and OR sites.

- Entire block removal:
    - In 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 WA, in half of the orchards one of ten sampled trees were positive to XDP, with tree age varying from one- to two-year-old. These infected trees cannot be attributed to a particular source of infection: nursery, leafhopper early infection or soil root grafting, as it is the initial prospection prior to installing the netting.

METHODS:

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

Four orchard blocks near The Dalles, OR and six orchards blocks in the Yakima valley, WA were selected for this project. All sites were identified as been removed due to XDP infection, confirmed by qPCR methods done in previous years. These blocks have different age, rootstock, and replanting strategies that reflect current replanting management practices in Oregon and Washington (Table 1.). Most common and distinct practices were selected for comparison.

Table 1. Removal and replanting conditions

Location-Cutivar/Rootstock (previous root)	**Herbicide/Tree cut**	**Removal/Soil**	**Fumigation**	**Period to replant**
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
WA 1* Skeena/Gi.12 (G.12)	**August - Stump herbicide**	Spring - Stump removal. Replace soil in the planting hole	NO fumigation	Next spring
WA 7 Skeena/G.12 (G.12)	**No herbicide**	Fall -remove large	NO fumigation	Next spring
WA 2 Benton/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 Benton/Gi.6 (Peach)	**No herbicide**	Fall-excavator	Spring Fumigation	Next spring
WA 6 Coral/Mazzard (K5)	**No herbicide**	Fall -excavator	Spring Fumigation	Next spring

* Individual tree removal in a block with high pressure and confirmed X-phytoplasma. WA 7 will be added in 2022.

The blocks were monitored for root suckers bi-weekly starting May 1st. If suckers were detected, they were removed and tested for LCD pathogens. On each site, one gallon of soil was collected from five different locations withing the block, where trees were removed due XDP. On site, soil was passed through a soil sieve 0.2 mm to remove soil and collect the remaining roots. Roots were placed in Ziploc bags and covered with the soil, placed in a cooler and taken to Dr. Harper's laboratory for qPCR test of the roots. Soils will be collected again in 2022 - 2023.

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

During 2021, first year of the project, ten random trees per block were selected, labelled, and sampled for LCD during the summer or fall, to verify absence of pathogens (LChV1, LChV2 and XDP). Once negative results were confirmed, these ten trees were be netted to exclude leafhopper vectors. The netting was adjusted to each block training system and tree size, utilizing a low shade grade netting (OVS, shade factor aprox. 7%). To make sure leaf hoppers are not present inside the cage, a yellow sticky trap was placed in each cage.

During second and third year, trees will be monitored 4 times thought the year for XDP and LChV 1and 2, as well as for root suckers around the planting site.

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

We will host a field day or video recording in each state in years 1 and 3. This will allow orchardists to see the short and long term effects of these treatments on sucker development. New findings will be shared in written publications focused on best practices for replanting an X-disease infected orchard. Information will be in English and Spanish.

RESULTS AND DISCUSSION

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

In WA sites (Table 1), the only replant block that had live roots with size larger than ¼ inch sufficient for testing, was the sites with individual tree removal WA 1 (Skeena/Gi.12). All other sites, where the entire block was removed had no significant live roots for LCD infection testing, regardless of the removal method. In WA 1 (individual tree removal of Skeena/Gi.12), trees were monitored for small and colorless cherries during harvest, and symptomatic trees were painted for subsequent removal. After harvest (August) trees were cut at approximately three feet above the ground and Roundup was immediately applied to the cut. Stumps and large roots around the trunk were removed the following spring (May 2021). The new plants were placed in the planting whole with soil from a different area,

with no history of LCD (approximately 5 gallons per planting whole). All roots samples from the recently removed tree were (n= 5), were positive with XDP.
These preliminary results suggest that herbicide application to the stump during the summer tree removal is not effective in killing all the infected roots in G12 seven year old rootstock.
On the other hand, all WA sites with entire block removal, live roots or root suckers were not found, indicating a successful tree and root removal, 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 summer and the following spring, having a direct impact on root survival.

Root survival is currently being evaluated at 4 sites in OR using the same methods described above.

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

Initial prospection of LCD infection in trees prior to installing the netting was carried out in WA and OR. In three out of the six WA sites, one of ten trees came back positive to XDP. In OR, samples have been submitted.
In WA 1 (Skeena/G12 individual tree replanting), despite the presence of live roots positive to XDP in the soil, all new planted trees (n=10) were negative to LChV1, LChV2 and XDP when sampled in August 2021.
The three whole replanted blocks with one of ten trees positive to XDP positive trees in WA correspond to WA 3, WA 4 and WA 6 (Table 1). These trees were discarded immediately by the growers and a new tree was selected for further netting to complete the 10 trees per site needed for 2022 monitoring.

4. Provide orchardists with science-based replant strategies for X-disease infected orchards.
Two videos were developed by B.Sallato in collaboration with GFG on tree removal (English and Spanish) with descriptive article. https://www.goodfruit.com/risk-mitigation-strategies-for-little-cherry-driven-removal-and-replant/
Preliminary results were shared by B. Sallato in the Roza LCD field day coordinated by Corina Serban, ITT LCD extension in October 8th, 2021, where there were 39 attendees.

This project will give us better understanding of (1) level of rate of infection in new plantings (2) infection risk under different tree removal and replanting method, (3) infection risk due to leafhopper versus soil or root grafting. (4) risk versus economic cost of different replanting methods for sweet cherries.

REFERENCES

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Graham, B.F., Jr. and F.H. Bormamm. 1966. Natural Root Grafts. Bot. Rev. 32:255-292.

Lev-Yadun, S. and D. Sprugel. 2011. Why should trees have natural root grafts? Tree Physiology. 31: 575-578.

CONTINUING PROJECT REPORT **YEAR:** 1 of 2

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

Co-PI (1): Cameron Peace
Organization: WSU – Horticulture
Telephone: 509-335-6899
Email: cpeace@wsu.edu
Address: Johnson Hall 39
Address 2: PO Box 646414
City/State/Zip: Pullman/WA/99164

Co-PI (2): Scott Harper
Organization: WSU – Plant Pathology
Telephone: 509-786-9230
Email: scott.harper@wsu.edu
Address: WSU - IAREC
Address 2: 24106 N Bunn Rd
City/State/Zip: Prosser/WA/99350

Co-PI (3): Lyndon Porter
Organization: USDA – ARS Plant Pathology
Telephone: 509-786-9237
Email: lyndon.porter@wsu.edu
Address: WSU-IAREC
Address 2: 24106 N Bunn Rd
City/State/Zip: Prosser/WA/99350

Co-PI (4): Jennifer Watts
Organization: WSU – Molecular Biosciences
Telephone: 509-335-8554
Email: jwatts@wsu.edu
Address: Biotech/LSF 433
Address 2:
City/State/Zip: Pullman/WA/99164

Cooperators: Alexandra Johnson (WSU PhD student, Pullman)
Zoe Sehlke (WSU MS 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
Salaries[a]	$15,250	$15,950
Benefits	$2750	$2900
Wages	$5850	$6050
Benefits		
Equipment		
Supplies[b]	$2150	$2100
Travel[c]	$2000	$2000
Plot Fees	$1000	$1000
Miscellaneous		
Total	$29,000	$30,000

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)
- Four colonies isolated from the solid medium were identified as possible *Ca.* P. pruni
- One of the isolated colonies was determined by DNA sequencing to not be *Ca.* P. pruni; the others have yet to be sequenced

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 were used in this study and all trees were growing in trellised orchards, under standard management practices. In 2021, 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 also 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: Infected stems, spurs, and fruit from 20 infected, symptomatic trees were collected and numerous colonies were produced on phytoplasma-selective medium (Contaldo and Bertaccini 2019), and in a selective environment. Both liquid and solid phytoplasma growth mediums 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, and tubes inoculated with dissected plant material, found to turn yellow after 24 or more hours indicating acidification of medium by bacterial metabolic processes, were presumed positive for bacterial growth. These tubes were quantified for degree of color change and 1 mL of broth was removed from each vial and then spread aseptically across a new plate of solid phytoplasma growth medium. Plates of solid medium inoculated with bacterial broth were then incubated for 48 or more hours at room temperature (26 °C), in a low oxygen environment. Isolated colonies found 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 set forth by Kogej et al. (2020) that detects *Ca.* P. pruni-specific DNA sequences was used. Any colonies with the same genotypic signature as *Ca.* P. pruni were subject to whole genome sequencing to confirm identity.

RESULTS & DISCUSSION

Summary: In 2021, bacteria were isolated from plant tissues known to be infected with *Ca.* P. pruni, the causative organism in X-disease. Most colonies generated were determined to not be *Ca.* P. pruni. However, four colonies were identified as possible *Ca.* P. pruni by PCR-based DNA assays. Sequencing of one of these colonies indicated it was not *Ca.* P. pruni. The remaining three colonies that were also 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 will have portions of their genomes sequenced in late 2021 to early 2022, to verify if any are *Ca.* P. pruni.

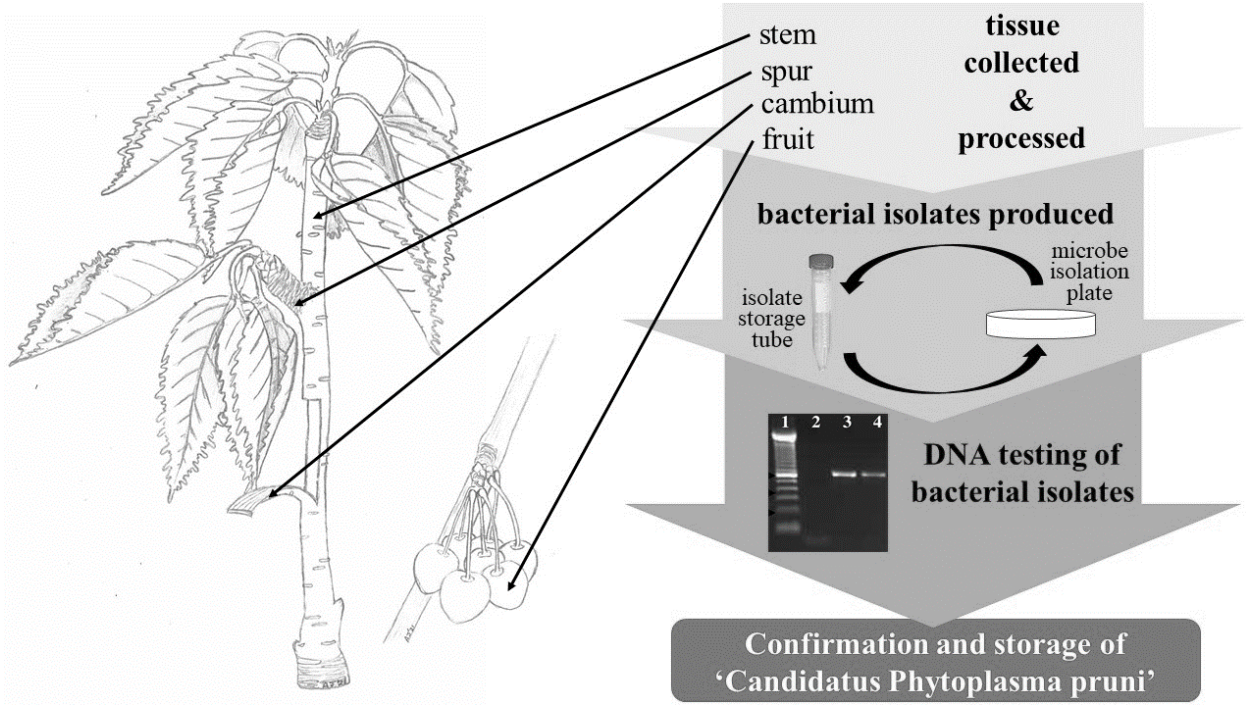


Figure 1: Collection, generation, and identification of bacterial isolates found in trees infected with *Ca.* P. pruni

Bacterial isolation: Initial plates generated from broth cultures produced numerous individual colonies; however, only five to six species of bacteria were identified morphologically. Broths from asymptomatic tissues failed to produce any bacterial colonies. Colonies cultivated in 2022 will be compared with those produced in 2021 to investigate whether a specific subset of other bacteria must be present with *Ca.* P. pruni for X-disease symptoms to appear in sweet cherry trees.

Bacterial confirmation: From PCR testing of 60 colonies, four were putatively identified as *Ca*. P. pruni. One has been sequenced to date and was found to be a contaminant; sequencing of the other three colonies from late 2021 will confirm if any are *Ca.* P. pruni.

Bacterial storage: The three colonies identified as possible *Ca.* P. pruni have been maintained on solid phytoplasma growth medium, in a low-oxygen environment. If later determined to be *Ca.* P. pruni, cells from these colonies will be tested on several growth media and at several temperatures, including ultra-low-temperature (-80 °C) for long-term storage. The culture medium will also be optimized for year-round live growth.

FINAL PROJECT REPORT

Project Title: Canine Detection of Western X Disease in Controlled and Field Settings

PI: Heath Smith
Organization: Rogue Detection Teams
Telephone: (206) 375-2811
Email: Heath.Smith@roguedogs.org
Address: 2011 Clemons Rd
Address 2:
City: Rice
State/Zip: WA/99167

Co-PI (2): Jake Lammi
Organization: Rogue Detection Teams
Telephone: (651) 202-8643
Email: Jake.Lammi@roguedogs.org
Address: 2011 Clemons Rd
Address 2:
City: Rice
State/Zip: WA/99167

Co-PI(3): Suzie Marlow
Organization: Rogue Detection Teams
Telephone: (458) 203-0668
Email: suzie.marlow@roguedogs.org
Address: 2011 Clemons Rd
Address 2:
City/State/Zip: Rice, WA 99167

Co-PI (4): Jennifer Hartman
Organization: Rogue Detection Teams
Telephone: (458) 203-0668
Email: jennifer.hartman@roguedogs.org
Address: 2011 Clemons Rd
Address 2:
City/State/Zip: Rice, WA 99167

Cooperators: Washington Tree Fruit Research Commission (WTFRC); Scott Harper, Washington State University (WSU); Teah Smith, Zirkle Fruit Company; Hannah Walters, Stemilt Growers; Garrett Bishop, G.S. Long Company

Other funding sources:
Amount: $5,053.57
Agency Name: Oregon Sweet Cherry Commission (OSCC)
Notes:

Total Project Funding:
Budget History

Organization Name: Rogue Detection Teams (RDT)
Telephone: 651-307-8415
Station Manager/Supervisor: Heath Smith
heath.smith@roguedogs.org

Contract Administrator: Mike Lammi
Email address: mlammi@c2an.com
Email Address:

Item	Year 1:	Year 2:	Year 3:
Salaries		--	
Benefits		--	
Wages	$4,460	$2700 ($25k)	$29,700
Benefits		--	
Equipment			
Supplies		$392	$500
Travel	$377	$1900	$5,500
Miscellaneous			$5,300
Plot Fees			
Total	$4,837	$29,872	$41,164

OVERVIEW

Little Cherry Disease (LCD) is an emerging problem for Pacific Northwest cherry growers and has been found statewide in Washington since 2010. Trees infected with any of the three diseases must be removed, but detection of symptoms is challenging for human surveyors and not evident until 2-3 weeks before harvest. Molecular detection methods are neither cost-effective nor practical on an orchard-wide scale. Rapid detection methods are needed, and trained detection dog teams (DDTs) may provide a quick screening method for identifying diseased trees for targeted molecular verification.

Rogue Detection Teams (RDT) began utilizing DDTs, a combination of a bounder (commonly referred to as a handler) and a detection dog, on a preliminary pilot study in October of 2019 with Little Cherry Disease 2 (LCD-2). Due to the aggressive nature of Western-X, RDT switched the focus from LCD-2 to Western-X after that initial pilot study.

For the field component, RDT deployed two DDTs in January 2021, during the pruning season to determine if winter is an optimal time of year to survey orchards for LCD. RDT supplemented field deployments with indoor leaf trials and began developing a systematic process for searching unknown leaves and collecting statistics on DDTs' accuracy.

While DDTs have had success detecting viruses both in controlled environments and field visits to orchards, there still remain challenges in determining the best method of deploying detection teams.

RDT requested funding to explore the following objectives in 2021.

- Visit 3 to 4 orchards during January 2021 when trees are being pruned.
- Visit orchards/nurseries to test nursery stock for infections.
- Visit orchards in March/April 2021 just prior to planting new stock.
- Continue testing and developing an efficient method of collection for use in a controlled environment.

Based on current results (see Table 1), RDT is confident that DDTs are able to detect Western-X disease and Little Cherry Virus 2 infected samples in a controlled setting and have completed trials to determine a protocol to systematically test unknown samples with a high level of accuracy.

DDTs tested 540 samples with an overall accuracy (accuracy on healthy and infected samples) of 0.9870 (range = 0.9741 to 1) with a true positive rate (accuracy of dog correctly alerting to infected samples) of 0.95 (range = 0.9048 to 1) and a true negative rate (accuracy of dog correctly passing healthy samples) of 0.9955 (range = 0.9894 to 1).

Timeline of Events: Received samples, Facility training, Field visits.

Date	Type	Notes
7/18/2019	Samples	Received infected and non-infected LCD-2 samples from Teah Smith, Rob Curtiss, Scott Harper
10/21/2019	HQ Trails	First run-through on samples using the new box apparatus, **successful identification of infected LCD-2 samples.**
10/30/2019	Report	Submitted the first progress report
11/12/2019	Presentation	Presentation on the first pilot study
11/21/2019	Samples	Received samples from Teah Smith of healthy and Western-X disease-inoculated leaves, twigs, bark, and roots
December 2019	HQ Work	Training at RDT Headquarters (HQ), Heath Smith with Pips
January 2020	HQ Work	Training at RDT HQ, Jake Lammi, Suzie Marlow, Heath Smith, Collette Yee with Skye, Zilly, Ranger, Pips, and Dio. **Successful detection of samples infected with WESTERN-X.**
02/26/2020	Field Visit	Field visit Othello, Jake, Suzie, and Heath with Skye, Ranger, Zilly, and Pips
03/06/2020	Field Visit	Field visit Rockport, Jake, and Suzie with Skye, Ranger, and Zilly
March 2020	Samples	Received Samples from Zirkle, Teah Smith
March 2020	HQ Work	Training at RDT HQ, Jake, and Suzie with Skye, Zilly, and Ranger
05/17/2020	Report	Follow up report on progress submitted
May-June 2020	HQ Work	Training at HQ
06/02/2020	Funding Request	Out of cycle funding request submitted
06/05/2020	Funding Awarded	Funding award granted ($18,102)
06/08/2020	Field Visit	Field visit at Zirkle - CRO Ranch, Jake and Suzie with Skye, Ranger, Zilly
06/08/2020	Samples	Obtained samples from Zirkle - CRO Ranch, RDT brought to HQ
06/20-21/2020	Field Visit	Field visit at Zirkle - CRO Ranch, Jake and Suzie with Ranger, Zilly, Skye, and Dio
06/24-27/2020	HQ Work	Training at HQ with Ranger, Skye, Dio
06/28-29/2020	Field Visit	Field visit at Zirkle - CRO Ranch, Jake with Ranger and Skye

08/17/2020	Funding Awarded	Additional funding awarded ($25,000)
08/27/2020	Samples	Received samples from Stemlit Growers, Hannah Walters
August 2020	Construction	Obtaining materials for and building the apparatus needed to work through numerous samples
09/28/2020	Samples	Received samples from Zirkle, Teah Smith
09/29/2020	Samples	Received samples from Mattawa Nursery, Andrew Hunsperger
October 2020	Report	Compiled findings into a report
December 2020	Teaching	Continued teaching of samples received from Marrawa and Zirkle
January 2021	Field Visit	Winter field visit to Mattawa, Zirkle - CRO Ranch, Wapato, Jake, and Suzie with Ranger, Zilly, Skye
January 2021	Nursery Tour	Winter nursery tour, with Jake and Suzie
February-March 2021	Testing Methods	Testing of methods to systematically search leaves in a controlled setting, Jake, Suzie, and Heath
March-April 2021	Leaf Trials	**Conducted leaf trials with a combined accuracy of 0.9870**
April 2021	Report	Compile findings into a report

Objectives and Significant Findings:

First Objective: Visit three to four orchards during January 2021 when trees are being pruned.

In January 2021, RDT sent two DDTs to visit three cherry orchards (Mattawa, Zirkle - CRO Ranch, and Wapato) in central Washington one week after pruning. DDTs started at the Mattawa orchard, as it was the only orchard of the three that had lab-confirmed healthy and infected trees. Bounders introduced detection dogs to the infected trees, providing instruction to sit followed with a reward. Throughout the day, both DDTs repeated the process on the infected trees in the orchard.

On the second day of orchard visits, RDT visited Zirkle - CRO Ranch. DDTs presented prunings from healthy trees and one infected tree to the detection dogs. After each odor introduction, the prunings were randomized and the process was repeated. Once the detection dogs were independently alerting to the infected pruning, new prunings were added. Both detection dogs were able to correctly identify the infected prunings the first time they were introduced to them. The bounder then introduced detection dogs to live infected trees to further assist them in understanding the connection between prunings and live trees.

On day three, RDT visited the Wapato orchard. Upon arrival, RDT was informed there were no reliable healthy trees at the orchard. Bounders took the detection dogs to the confirmed infected trees and rewarded them once they alerted at the tree, however DDTs were limited by the lack of healthy trees at the orchard. After visiting the three orchards, RDT decided to spend another day working with the detection dogs at the Mattawa orchard where confirmed healthy and infected trees were present.

Second Objective: Visit nurseries to test nursery stock for infections.

During the January orchard visits, RDT visited two different nurseries to evaluate if it would be practical to use DDTs for the detection of infected nursery stock. RDT met with nursery representatives and were given a detailed tour of the nursery grounds.

Third Objective: Visit orchards in March/April just prior to planting new stock

Washington Tree Fruit Research Commission (WTFRC) decided to forgo the March/April orchard visits. RDT instead focused on continued research and development of a method to detect LCV in a controlled setting.

Fourth Objective: Continue testing and developing an efficient method of collection for use in a controlled environment.

- **Teaching:** Expand on the development of the teaching process by introducing the dogs to new lab-confirmed healthy and infected samples.

  DDTs have the ability to both generalize on an odor or to specialize on specific odors. As such, introducing detection dogs to an assortment of at least 20 laboratory-confirmed healthy and infected samples assists them in learning what is being asked of them, i.e., to generalize on the infected leaf odor and not become specialized on only a small number of samples. Twenty-nine healthy and 20 infected samples, confirmed by Washington State University (WSU) researchers, were utilized for the teaching process. To start, bounders placed one infected sample into a wall apparatus with several holes for a detection dog to sniff, hereinafter referred to as the ring (figure 1) and placed healthy samples in the remaining locations. Bounders instructed the detection dog to search the ring. If the detection dog had a change of behavior at a healthy sample the bounders would give a soft verbal correction and encourage them to continue searching the ring. When the detection dog smelled an infected sample, the bounder instructed them to sit, followed by giving the ball as a reward. This process was repeated with a small subset of healthy and infected leaves until detection dogs were able to correctly identify the infected sample on their own (without a verbal “sit” command).

- **Development:** Develop a systematic process for searching unknown leaves and collect statistics on the DDTs' accuracy.

  Detection dogs are capable of learning patterns. As such, it was important that RDT develop a systematic process for collecting data that ensures the only constant pattern is the infected versus healthy odor. To start, a random, blind scenario was set up in the ring with a mix of healthy and infected samples. A random number generator was used to determine the simulated infection rate (range 0% to 25%) and the location of the infected samples within the ring. In addition to the randomly infected samples that were placed in the ring, one additional infected sample was placed in a location that was known to the bounder. This ensured that if the random infection rate was 0%, the detection dog could still be rewarded. This served three purposes: 1) Since the ring is a circle, detection dogs will continue searching until they find a sample or are removed from the search. Removing a detection dog from the search can cause confusion and the dog may be more inclined to indicate at healthy samples. Having one infected sample where the location is known ensures that the detection dog always has an infected sample to find. 2) If the random infection rate is 0% for multiple sessions in a row a detection dog may become confused at the lack of rewards and indicate at healthy samples. Always keeping one known infected sample in the ring ensures the dog always has a play reward. 3) RDT bounders noticed that within a session, if a detection dog had one error, they are more likely to have two, creating a ripple effect. Keeping one known infected in the ring informs the bounder whether the detection dog is still indicating at infected samples. Next, the bounder chose a starting location and encouraged the detection dog to search the ring. A session ends after a DDT completes searching the ring or has an error. If a detection dog indicates at a blind sample and is rewarded, that sample is pulled, and the DDT continues searching the ring until the DDT detects the known infected sample. As mentioned previously, if a detection dog has one error within a session it can create a ripple effect leading to more errors in the same session. Keeping the same subset of samples in the ring allows detection dogs to quickly cross-examine one another and limit the magnification of errors. The starting and ending point of the search was noted and all samples the detection dog tested were recorded. After each round, every sample was taken out of the ring and a new setup was created.

- **Cost:** Determine how many samples could be done per day and estimate a cost per sample.

  Utilizing the statistics gathered from the leaf trials RDT extrapolated the samples per hour to estimate a cost per sample.

Results:

First Objective: Of the three orchards that RDT visited during the pruning season in January 2021, with the aim to evaluate if the winter season would be optimal for surveys, only one had both healthy and infected trees for the DDTs to work. To truly test whether this season would have ideal working conditions, more orchards with both infected and healthy samples would have been ideal. As such, DDTs were unable to distinguish between healthy and infected trees in the orchard. More research is required to determine if winter is a viable season to conduct orchard surveys.

Second Objective: Also, in January 2021 RDT visited two nurseries to evaluate whether it would be practical to use DDTs to detect infected nursery stock. RDT observed that the nursery warehouses were too densely packed with young trees for DDTs to be able to reliably detect infected trees. When odor becomes as concentrated as it was in the nurseries, DDTs become unable to locate the source of the target odor.

Third Objective: WTFRC decided to forgo the March/April orchard visits to focus on continued leaf testing in a controlled setting.

Fourth objective:

- **Teaching:** During the teaching phase, the DDTs were able to alert to the new infected leaf samples while ignoring the healthy ones. We did not observe detection dogs consistently alerting to any of the new healthy samples. However, there was one infected sample that all the dogs initially had a weak response at during the teaching phase, sample "X", which was a lab-confirmed infected sample ("weak response" defined here refers to a detection dog having a change of behavior more subtle than their alert behavior).

- **Development:** DDTs tested a total of 540 leaf samples to gather statistics and test the systematic process for searching unknown leaves. The DDTs had a combined overall accuracy (accuracy on healthy and infected samples) of 0.9870 (range = 0.9741 to 1) with a true positive rate (accuracy of dog correctly alerting to infected samples) of 0.95 (range - 0.9048 to 1) and a true negative rate (accuracy of detection dog correctly avoiding healthy samples) of 0.9955 (range = 0.9894 to 1) (Table 1). It is worth noting that the known infected sample included in every session was only calculated into the results if the DDTs failed to detect it. If the DDT successfully alerted to the known sample, it was not included as it was not a true blind sample. If the known infected sample was included in the results, the true positive of all four DDTs would increase. More error occurred when DDTs failed to detect infected samples (false negatives) rather than alerting at healthy samples (false positives). There was a total of five false negatives and two false positives. There was no consensus among DDTs on any of the false negatives, or false positives showing the importance of having multiple detection dogs cross-examine one another (Table 2, Table 3).

Table 1: *Detection Dog Team Accuracy*

	True Positive	True Negative	Total Accuracy
Nelson	0.9091 (20/22)	0.9894 (93/94)	0.9741 (113/ 116)
Ranger	0.9655 (28/29)	1.00 (131/131)	0.9938 (159/160)
Skye	1.00 (28/28)	1.00 (119/119)	1.00 (148/148)
Zilly	0.9048 (19/21)	0.9896 (95/96)	0.9744 (114/117)
Total	0.95 (95/100)	0.9955 (438/440)	0.9870 (533/540)

Over a combined 540 samples, DDTs had a total overall accuracy (accuracy on healthy and infected samples) of 0.9870 (range= 0.9741 to 1) with a true positire rate (accuracy of dog correctly alerting to infected samples) of 0.95 (range= 0.9048 to 1) and a true negative rate (accuracy of dog correctly avoiding healthy samples) of 0.9955 (range = 0.9894 to 1).

Table 2: *Instances of False Negatives*

a: ***Sample T632***

Sample T632	Failure to detect sample	Successful Detection	Percent Detected
Nelson	0		100%
Ranger	0		100%
Skye	0	2	100%
Zilly			50%
Total	0	2	100%

c: ***Sample T751***

Sample T751	Failure to detect sample	Successful Detection	Percent Detected
Nelson	0		100%
Ranger			50%
Skye	0		100%
Zilly	0		100%
Total		4	80%

b: ***Sample T635***

Sample T635	Failure to detect sample	Successful Detection	Percent Detected
Nelson	0		100%
Ranger	0		100%
Skye	0		100%
Zilly			50%
Total	0	2	100%

d: Sample T946

Sample T946	Failure to detect sample	Successful Detection	Percent Detected
Nelson	1	0	0%
Ranger	0		100%
Skye	0		100%
Zilly	0		100%
Total	3	9	75%

e: ***Sample T796***

Sample T796	Failure to detect sample	Successful Detection	Percent Detected
Nelson	1	0	0%
Ranger	0		100%
Skye	0		100%
Zilly	0		100%
Total	0		100%

Over a combined 540 samples tested, DDTs had a total of five instances of false negatives (samples T632, T751, T946, T796 and T635). There was no consensus among DDTs on any of the false negatives, showing the importance of having multiple detection dogs cross-examine one anothe,

Table 3: *Instances of False Positives*

a: ***Sample 266***

Dog	Sample 266	Incorrect alert to healthy sample	Successful pass of healthy sample	Percent Detected
	Nelson	1	2	100%
	Ranger	0	2	100%
	Skye	0	2	100%
	Zilly	0	1	100%
	Total	0	2	100%

b: ***Sample 245***

Dog	Sample 245	Incorrect alert to healthy sample	Successful pass of healthy sample	Percent Detected
	Nelson	0	2	100%
	Ranger	0	3	100%
	Skye	0	1	100%
	Zilly	1	2	100%
	Total	0	5	100%

Over a combined 540 samples tested, DDTs had a total of two instances of false positives (samples 245 and 266). There was no consensus among DDTs on any of the false negatives, showing the importance of having multiple detection dogs cross examine one another.

- **Cost:** To estimate samples per day and cost per sample, RDT utilized the statistics gathered from the leaf trials. We estimated that two or three DDTs cross-examining one another for consensus would average ~50 samples per hour. Based on a $65 per hour rate (current as to the writing of this report) and factoring in time for sample processing and proper storage, the estimated cost per sample ranged between $3 to $5 USD. The range in price is attributed to the variance in infection rate among orchards. The higher the infection rate, the fewer samples detection teams are able to work through per round. Each session ends when a detection dog receives a reward. If there is a higher infection rate, a DDT would work through fewer samples before giving a reward. In our leaf trial sessions, the simulated infected rate was randomly chosen (range 0% to 25%). RDT did not notice a drop in accuracy with a higher infection rate, but due to the ripple effect described previously, a higher infection rate has the potential for more errors.

Discussion: RDT obtained promising results in controlled setting experiments. A high success rate of 0.9870 highlights that when utilizing DDTs in a controlled setting, DDTs could become early detectors of diseased plants. In turn, this could serve to inform growers of plants requiring immediate removal in order to reduce the spread of infection. There was no consensus among DDTs on any of the false negatives, showing the importance of having multiple dogs cross-examine one another (Table 2, Table 3). False negatives occur for a variety of reasons ranging from the DDTs moving too quickly out of excitement, detection dogs breathing heavily and thus not smelling each sample effectively, or from detection dogs becoming mentally fatigued due to the intensity of the searches. As with any detection dog work, monitoring the detecting dog's energy is a key factor in success rates, and for this work specifically, towards keeping the occurrence of false negatives at a minimum.

In this study, there were two false positives. There was no consensus among DDTs on any of the false positives, once again highlighting the need for DDTs to cross-examine samples. A false positive may occur for a variety of reasons ranging from accidental influence from their bounder, contamination of the sample/apparatus, early detection of the odor from further up the line, or anxiety of missing a reward. Working in a controlled environment allows bounders to better manage and mitigate the potential for false positives. It is RDTs' professional opinion that the number of false positives would be much higher in a field environment.

The DDTs, Nelson and Zilly had lower overall accuracy than DDTs, Ranger and Skye. Nelson and Zilly accounted for six of the seven total errors. These errors can also account for the differences in total samples tested; Nelson and Zilly tested a combined 233 samples, while Ranger and Skye tested a combined 308 samples. In this study, each session ended following a successful alert, or an error. Nelson and Zilly had more total errors, and as such were able to test fewer samples. While Nelson and Zilly still had high overall accuracies, 0.9741 and 0.9744 respectively, one variable that may have led to lower accuracy is that overall, they have had fewer field deployments than both Ranger and Skye. Ranger and Skye were the primary detection dogs on the project and Zilly and Nelson were brought in to provide a variety of experience levels.

During RDT's previous field visits in summer, bounders observed several limitations to utilizing DDTs in orchards causing RDT to reexamine the efficacy of deploying DDTs in a field setting. Challenges and concerns include; 1) chemicals sprayed in orchards may be harmful to the health of the DDTs and add a layer of confusion for the detection dogs as to which odor bounders are asking them to detect (as an example, if infected plots are sprayed more frequently a detection dog may interpret that they should be detecting trees with fresh spray), 2) cheatgrass seeds (*Bromus tectorum*), as they pose a severe danger to being inhaled by the dogs, which could require expensive and emergency veterinary care, 3) puncture vine (*Tribulus terrestris*) which requires detection dogs to wear special boots to protect their feet during an already hot season (dogs sweat through their feet and when they become overheated they are significantly less efficient and effective), 4) limited movement between rows which would require the bounder to call a detection dog off an odor in order to walk around to enter the adjacent row, 5) limited ability for the DDTs to cross examine one another leading to a potential magnification of errors, and 6) an increased rate of false positives. These limitations are all extremely detrimental to the efficacy of the work, as it hinders the detection dog's ability to maintain focus which ultimately leads to a reduction in accuracy.

During the winter season, orchards do not employ a spray schedule and harmful invasive grasses have yet to emerge. While winter field visits did curb a few of the dangerous environmental conditions encountered in summer, there was still limited movement between rows and decreased ability for the DDTs to cross-examine one another. According to WSU researchers, LCD vacates the above-ground portion of the tree and is dormant in the root system during the winter months, which may pose detection challenges not yet explored.

Working in a controlled setting mitigates all of the above limitations of deploying DDTs to a field setting while maximizing the DDT's work output. DDTs are able to continue working with preserved leaf samples throughout the entire year. The method RDT has developed creates a sustainable and repeatable process. Bypassing field deployments eliminates the dangers that working in the field poses, while simultaneously increasing the efficiency of DDTs and their ability to cross-examine one another. As such, this method could have a wider application as it could be easily taught to growers interested in learning how to have an in-house DDT or other local community-based groups that are interested in learning the methodology. RDT believes that collaboration between growers, research labs, and community dog groups, like a local Kennel Club, would be the ideal solution required for early detection and ultimately, for the eradication of LCD.

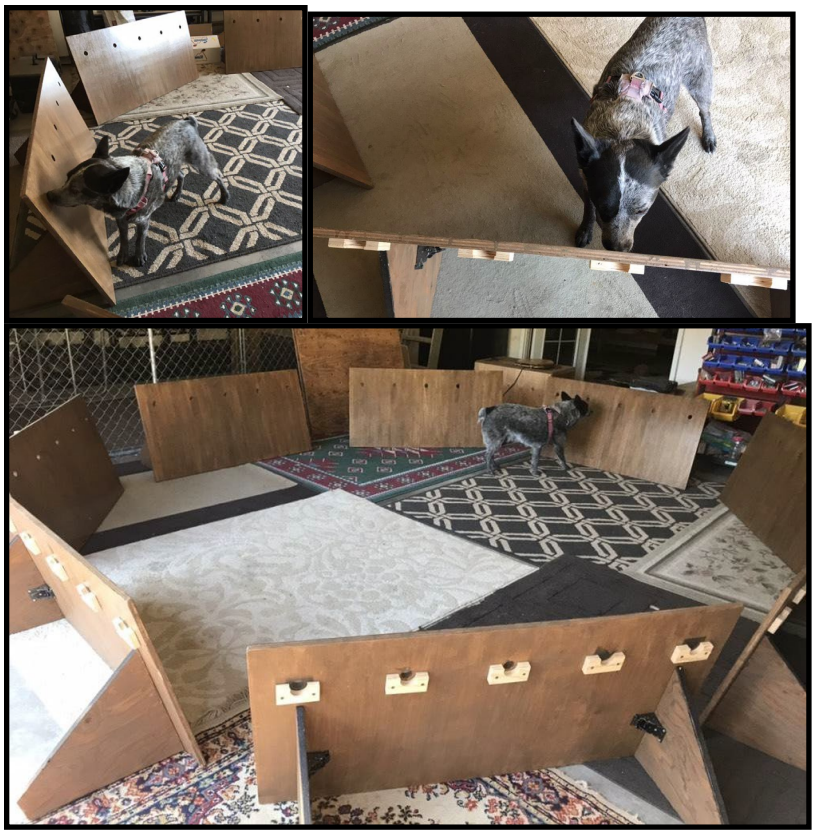


Figure 1. Wall apparatus with detection dog, Dio. Note: These photos were for demonstrating use of the wall apparatus, as such there are no jars with samples in the wall.

KEYWORDS, ABSTRACT AND EXECUTIVE SUMMARY

EXECUTIVE SUMMARY

Project title: Canine Detection of Western X Disease in Controlled and Field Settings

Keywords: Detection, Dog, Detection Dog Team, Canine, Little Cherry Disease, Little cherry virus 2 (LChV2), Western-X phytoplasma (WX)

Abstract: Cherry trees can be detrimentally affected by small size, poor color, and bitter-tasting cherries via one or more of three pathogens in Washington state: Little cherry virus 1 (LChV1), Little cherry virus 2 (LChV2), or Western-X phytoplasma (WX). RDT is exploring the potential to use Detection Dog Teams (DDTs) in the early detection of these diseases before symptoms appear. RDT observed promising results in controlled setting experiments. Over the course of a combined 540 samples tested, DDTs had an overall accuracy of 0.9870. This high success rate highlights that when utilizing DDTs in a controlled setting, DDTs could become early detectors of diseased plants. This would serve to inform growers of plants requiring immediate removal to reduce the spread of infection. RDT believes that collaboration between growers, research labs, and community dog groups, like a local Kennel Club, would be the ideal solution required for the early detection and eradication of LCD.

NEW PROJECT PROPOSAL **PROPOSED DURATION**: 1 Year

Project Title: Towards identification of LCD linked volatile biomarkers

PI: Lav R. Khot
Organization: Washington State University
Telephone: 509-786-9302
Email: lav.khot@wsu.edu

Co-PI: Scott Harper
Organization: Washington State University
Telephone: 509-786-9230
Email: scott.harper@wsu.edu

Co-PI: Bernardita Sallato
Organization: Washington State University
Telephone: 509-786-9205
Email: b.sallato@wsu.edu

Cooperators/Collaborator: Sindhuja Sankaran
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

- LCD symptoms can be detected by a portable FAIMS system at postharvest stage
- A portable FAIMS system could also detect LCD symptoms from field samples at the pre-symptomatic stages--as early as shuck fall growth stage.
- 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 magnitude for the infected samples was consistently higher than the healthy samples at 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. **Aim 1**. The shoot limb samples of cherry trees were collected for the post-harvest growth stage from an orchard located in Buena, WA (*cv*. Bentons). 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.

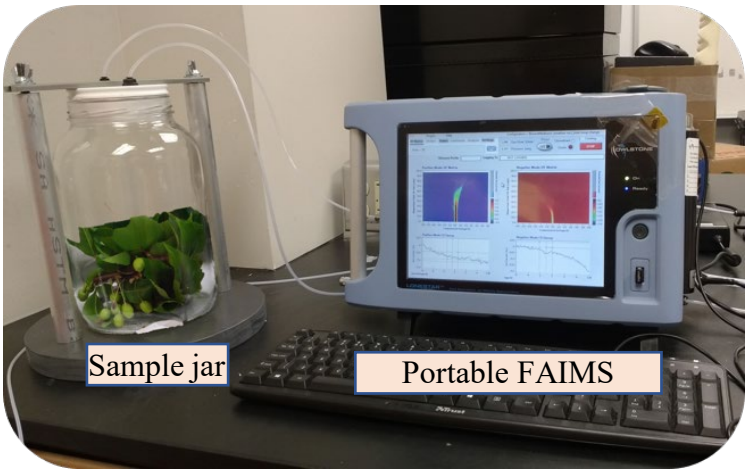


Figure 1. Volatile headspace sampling of cherry samples using a portable FAIMS system.

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.

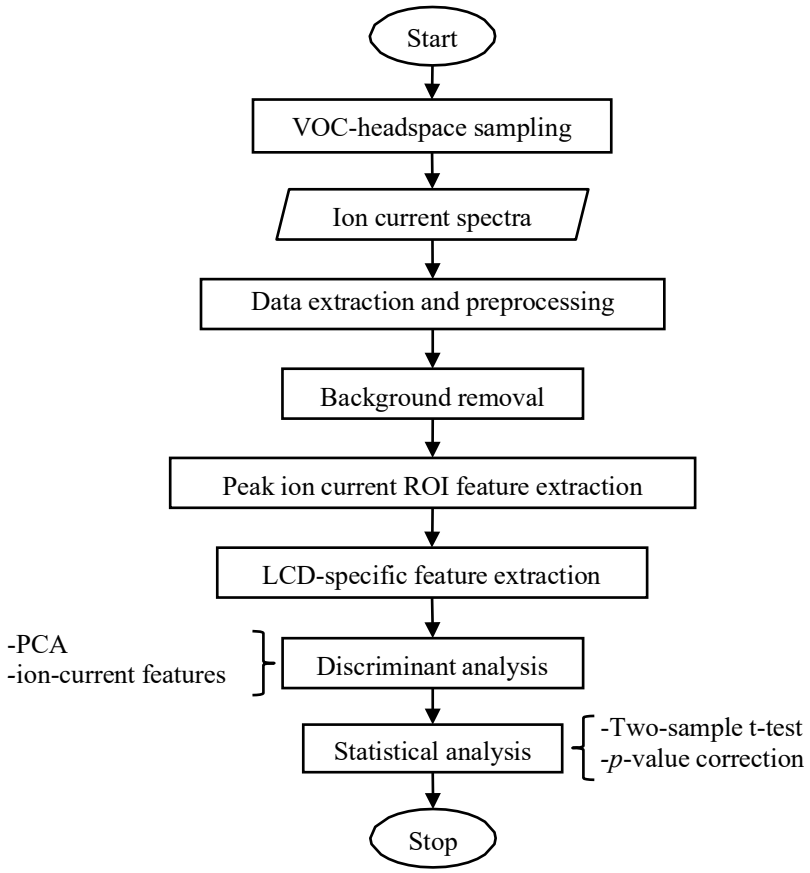


Figure 2. Data analysis pipeline for evaluating FAIMS for LCD detection.

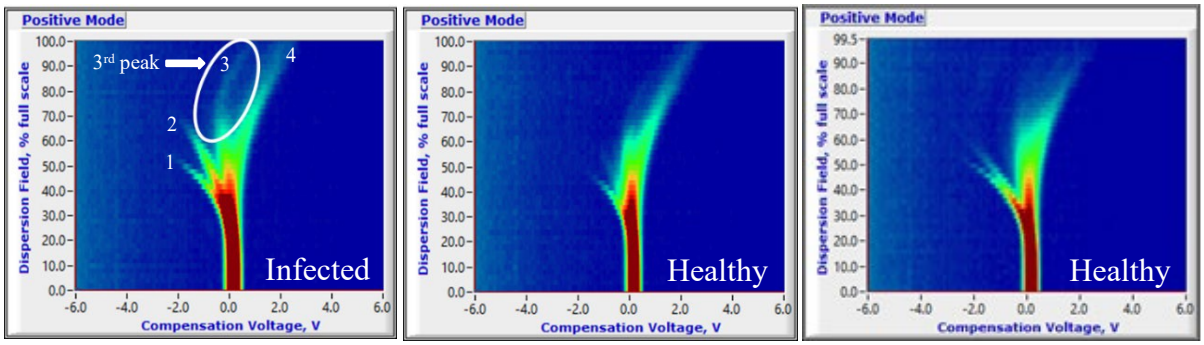
Results and Discussion

Aim 1. 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

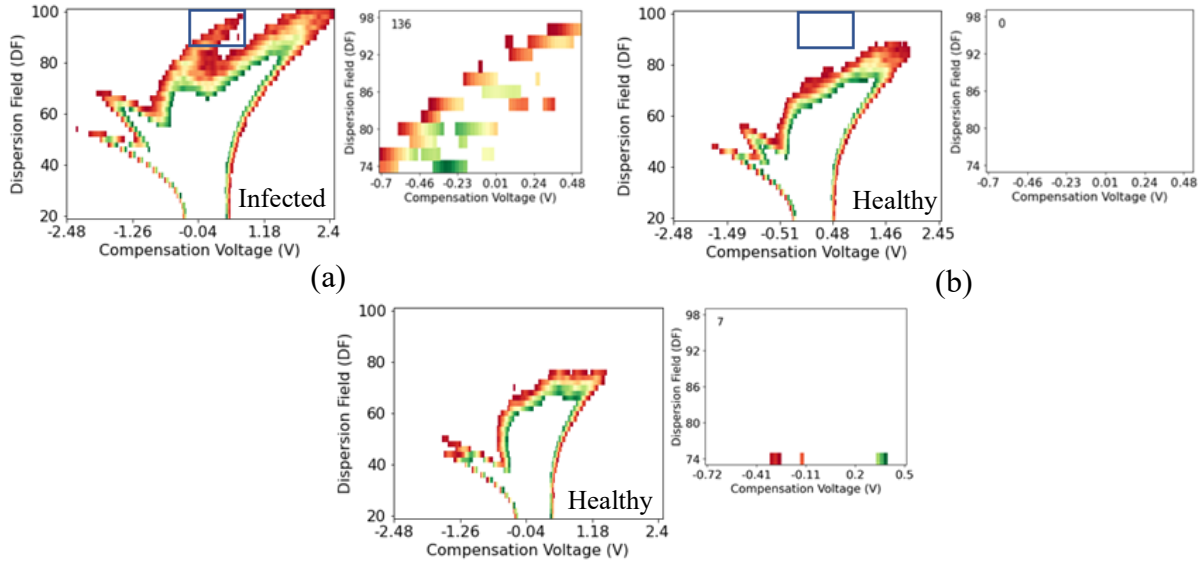
suggests that the healthy samples may not display a third peak in the ion current spectra as was also observed by the ion current spectra for healthy reference samples from greenhouse (fig. 3c).

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.

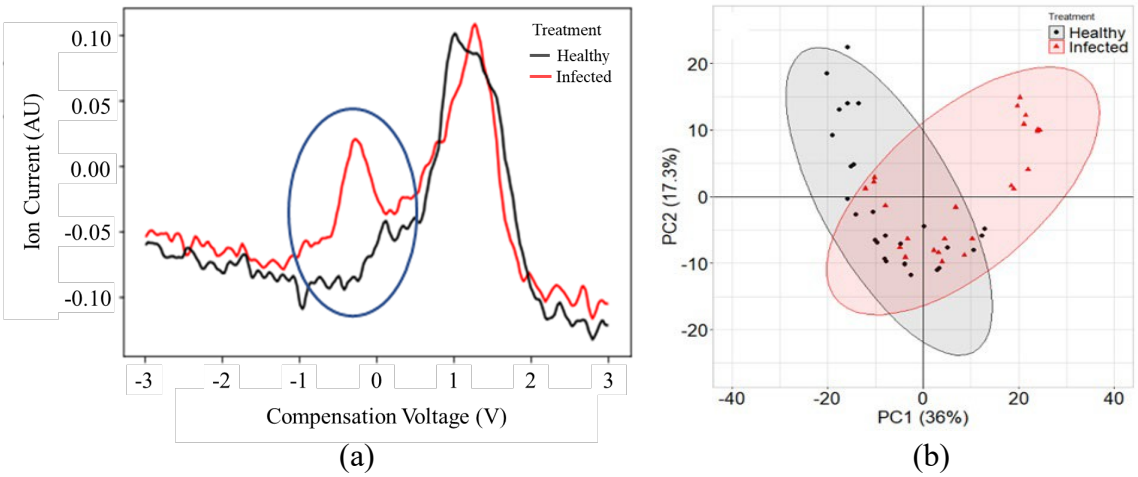


Figure 5. (a) Ion current magnitudes and (b) their pattern distinction for healthy and infected samples using principal component analysis.

<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).

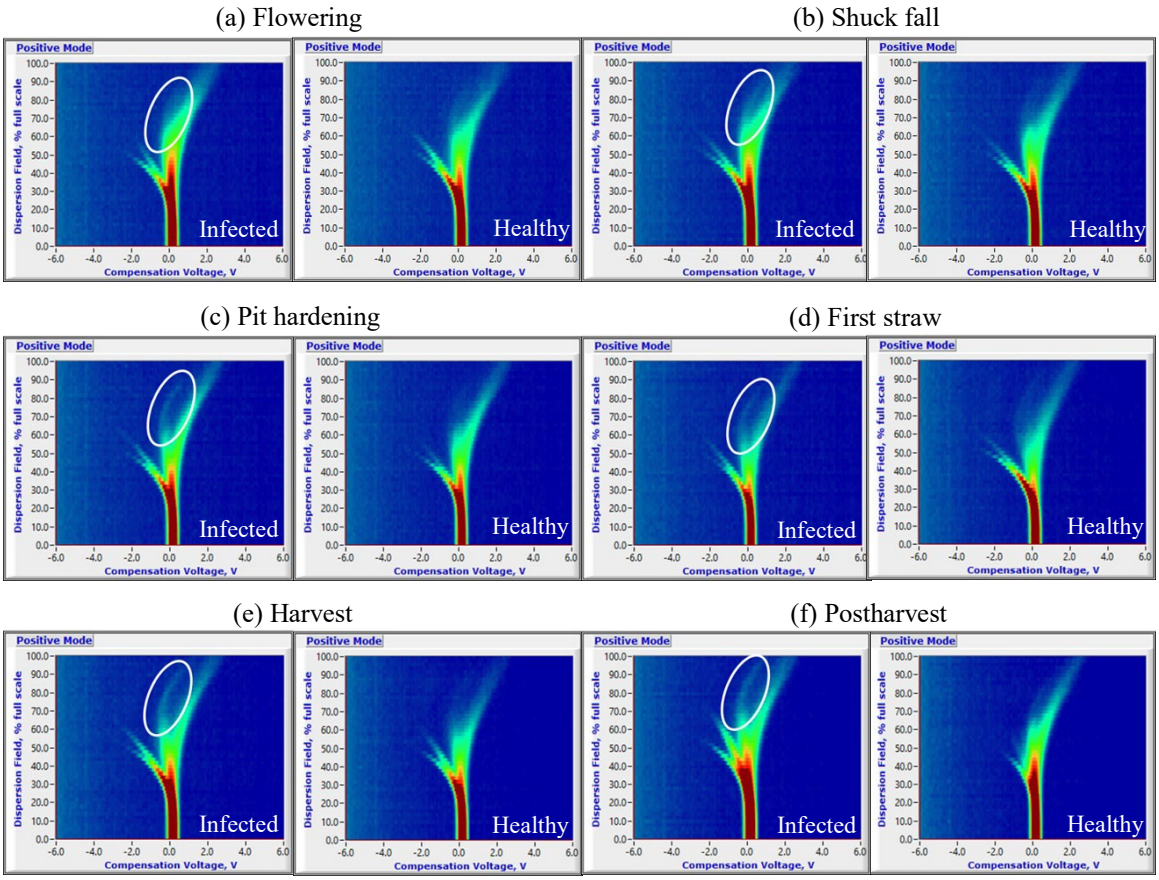


Figure 6. Raw ion current spectra plots for infected and healthy cherry samples collected from the orchard at (a) flowering, (b) shuck fall, (c) pit hardening, (d) first straw, (e) harvest, and (f) postharvest.

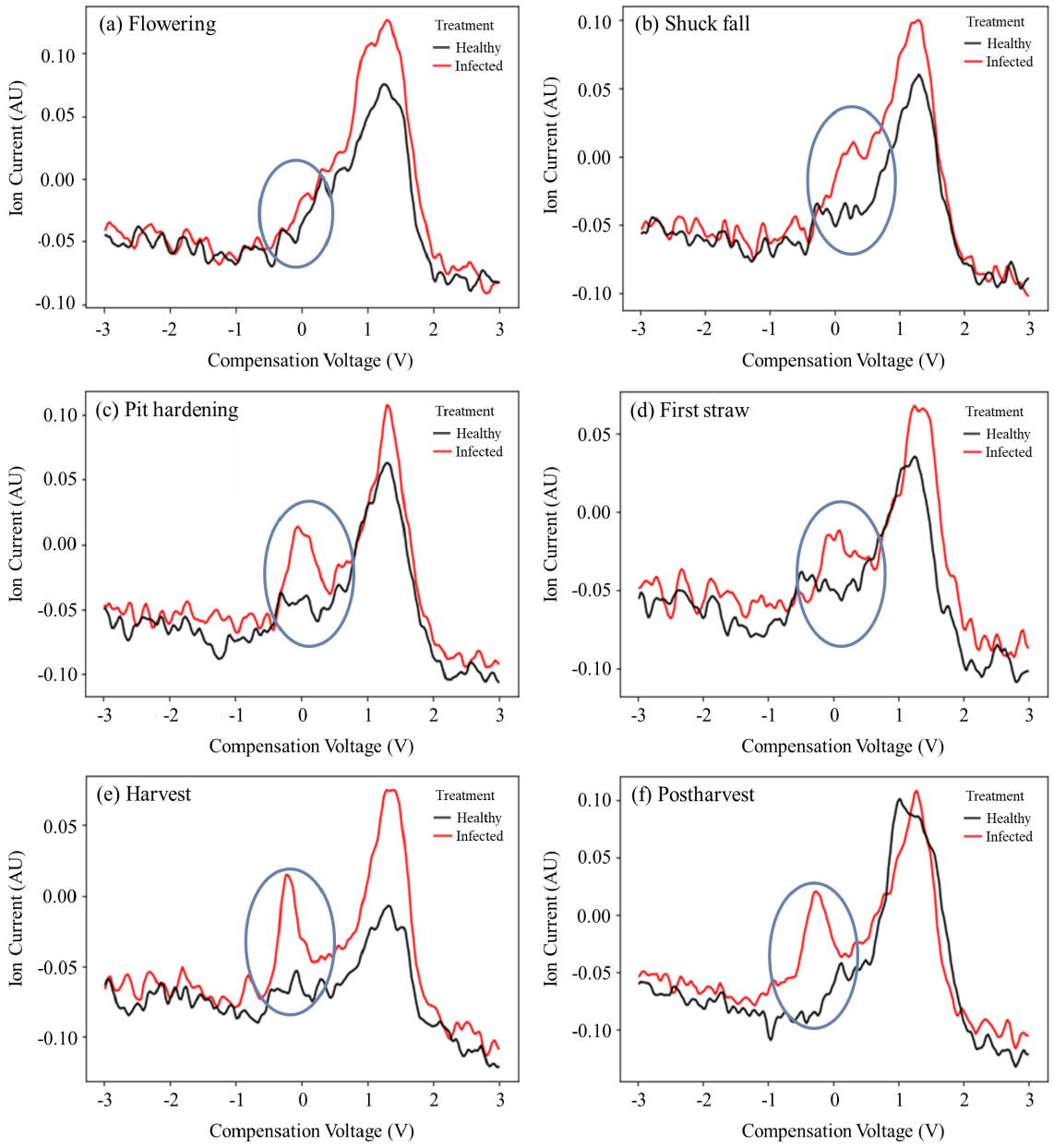


Figure 7. Ion current plots for the infected and healthy samples (differences highlighted in blue ellipse) at (a) flowering, (b) shuck fall, (c) pit hardening, (d) first straw, (e) harvest, and (f) postharvest.

Project Title: Coordinating SWD and X Disease Management

Primary PI: Elizabeth H. Beers
Organization: Washington State University
Telephone: 509-678-1010
Email: ebeers@wsu.edu
Address: WSU-TFREC
Address 2: 1100 N. Western Ave
City/State/Zip: Wenatchee, WA 98801

Co-PI 2: Tobin Northfield
Organization: Washington State University
Telephone: 509-293-8789
Email: tnorthfield@wsu.edu
Address: WSU-TFREC
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

Report Type: Continuing Project Report
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
Equipment			
Supplies	1,500	1,500	1,500
Travel			
Miscellaneous			
Plot Fees			
Total	Total year 1: 24,865	Total year 2: 25,800	Total year 3: 26,772

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
- 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 Yakima site, Extenday applied postharvest reduced SWD adult counts by 47.34% while Surround reduced SWD adult counts by 37.32%.
- At the Yakima site, SWD counts started to increase after late August. At the Wenatchee site, SWD was generally absent or in low abundance until late September.

Methods:
Objective 6
The impacts of post-harvest X-disease vector leafhopper management on SWD populations was assessed in 2021 at two cherry orchard sites near Yakima and Wenatchee respectively. The Yakima 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.

Spotted-wing drosophila trap

At Yakima, 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. Treatments 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).

Extenday ground cover treatment

Kaolin residue on cherry leaves

Results and Discussion:

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 Yakima, 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, Extenday applied postharvest reduced SWD adult counts by 65.83% while Surround reduced SWD adult counts by 66.60% in comparison to the Control (Figure 1, Table 1). Mowed blocks were comparable or hosted more SWD than Control blocks. At the Yakima site, Extenday applied postharvest reduced SWD adult counts by 47.34% while Surround reduced SWD adult counts by 37.32% (Figure 2, Table 2).

The preliminary results from 2021 suggest that postharvest canopy and groundcover based management of leafhopper vectors of X-disease phytoplasma may also 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. These preliminary results suggest that these integrative management options may be viable under a wide scale of potential pest pressure. In the following years, additional trials with Extenday and other forms of weed management will be followed up at the Wenatchee site and at sites comparable to the Yakima site to confirm if these trends hold over multiple seasons and degrees of pest pressure.

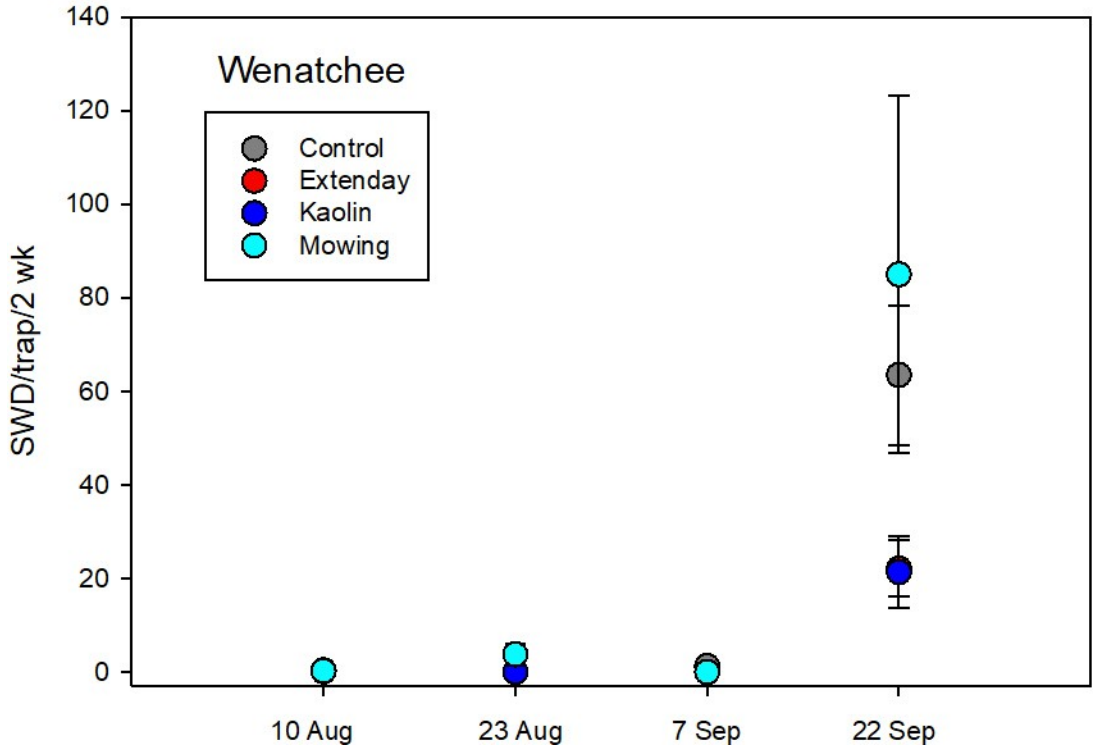


Fig. 1: SWD adult counts by treatment, Wenatchee, 2021

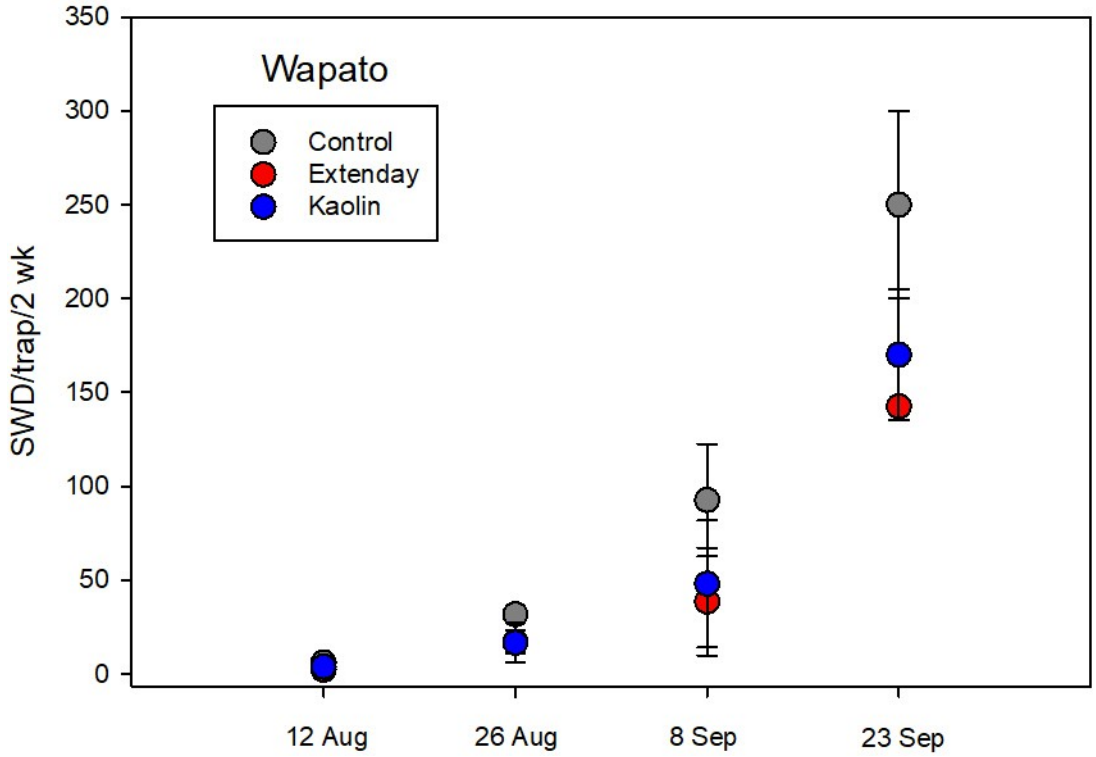


Fig. 2: SWD adult counts by treatment, Yakima, 2021

Table 1: SWD sex ratio, total SWD and *Drosophila* catch by treatment, Wenatchee, 2021

Treatment	n	SWD/trap/2 wk	Percent Reduction
1.Control	4	16.28 ab	-
2.Extenday	4	5.56 b	65.83 %
3. Kaolin	4	5.44 b	66.60 %
4. Mowing	4	22.25 a	-36.66 %

Data collection still in progress.

Table 2: SWD sex ratio and total *Drosophila* catch by treatment, Yakima, 2021

Treatment	n	SWD/trap/2 wk	Percent Reduction
1.Control	2	95.13 a	-
2.Extenday	2	50.09 b	47.34 %
3. Kaolin	2	59.63 ab	37.32 %

Data collection still in progress.

Project/Proposal Title: Understanding phytoplasmas infecting stone fruit trees in Washington state.

Primary PI: Dr. Scott Harper
Organization: Washington State University
Telephone: 509-786-9230
Email: scott.harper@wsu.edu
Address: 24106 N Bunn Rd
Address 2:
City/State/Zip: Prosser, WA 99350

Co-PI 2: Dr. Alice Wright
Organization: Washington State University
Telephone: 509-786-9210
Email: alice.wright@wsu.edu
Address: 24106 N Bunn Rd
Address 2:
City/State/Zip: Prosser, WA 99350

Cooperators: WA stone fruit growers.

Report Type: Final Project Report

Project Duration: 2-Year

Total Project Request for Year 1 Funding: $46,380
Total Project Request for Year 2 Funding: $45,455

Other related/associated funding sources None

WTFRC Collaborative Costs: None

Item	2019	2020	2021 No-Cost Extension
Salaries	19,370	20,145	0
Benefits	7,510	7,810	0
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	19,000	17,000	0
Travel	500	500	0
Plot Fees			
Miscellaneous			
Total	46,380	45,455	0

Footnotes:
Salaries and benefits for one 0.4X FTE postdoctoral researcher.
Supplies include laboratory consumables and sequencing services.
Travel is estimated mileage for field sampling.

Objectives:

1. Determine which phytoplasmas are infecting stone fruit trees in Washington state and determine if multiple isolates are present by high throughput sequencing.

   Preliminary work has shown that cherries and peaches in the Columbia basin are infected with X-disease phytoplasma (*Ca*. P. pruni) and that peaches and nectarines are also infected with peach yellow leaf roll phytoplasma (*Ca*. P. pyri). As of 2018, the incidence in peach and nectarine was unknown as they were detected that year in a brief survey in response to grower inquiries. This is a particular problem given the movement of material into and within the state. Therefore, we propose to survey stone fruit trees, including peaches, nectarines, apricots, and plums, to identify which phytoplasmas are present in Washington state. Sequencing will be performed to obtain draft genomes for these phytoplasmas, and marker-directed genotyping performed to see whether there is active movement of phytoplasmas from one stone fruit crop to another or from one county to another. These data will answer the questions of 'what' and 'where'.

2. Identify physiological markers associated with the disease by comparing fruit of infected and healthy trees.

   Both of the presently identified phytoplasmas can affect the quality of infected stone fruit trees, yet previous research is limited to a few varieties or species, and, for peaches and nectarines, is primarily from California. Moreover, no data has been collected on the effects of infection by multiple phytoplasmas, as we have observed in both peaches and nectarines in the Columbia basin. Here we propose to examine symptoms in fruit and phloem tissue of infected trees, and by comparing these to healthy trees in the same location, determining type and severity of disease caused by endemic phytoplasmas. This will identify which phytoplasma species, aside from *Ca*. P. pruni, are particularly problematic for the tree fruit industry in Washington.

3. Determine how the presence of multiple phytoplasmas affects symptom development by using transcriptomics to identify affected pathways.

   It is unknown how these phytoplasma species cause disease in infected stone fruit. Using transcriptomics, we will be able to determine which pathways have altered regulation in diseased trees and may be important to symptom development. Understanding which pathways are important to symptom development may one day help with breeding for tolerant trees.

Significant Findings:

- The X-disease (*Ca*. P. pruni) and Peach yellow leaf roll (*Ca*. P. pyri, aka 'Pear Decline') phytoplasmas were confirmed to infect in fruit trees in Washington state. While *Ca*. P. pruni was found to infect all prunus grown, *Ca*. P. pyri was found in peaches, nectarines, and plums, as well as in pears and apples.
- X-disease can change sugar (sucrose, glucose, fructose) and secondary metabolite content of the fruit on infected trees, although the precise changes are cultivar specific.
- The pathogens reduce leaf chlorophyll content in stone fruit, a probable cause for lower sugar content in fruit.
- There are two genetic groups of the X-disease phytoplasma found in Washington cherries and other stone fruit; both are distinct from the X-disease phytoplasma found in California and the east coast states. Biological differences have yet to be determined.

Results and Discussion:

1. Determine which phytoplasmas are infecting stone fruit trees in Washington state and determine if multiple isolates are present by high throughput sequencing.

In 2020 fruit trees were surveyed for the presence of phytoplasma. These trees included not only stone fruit, but also apples and pears as these are often grown in close proximity to stone fruit. For each tree sampled, DNA was extracted from woody stem tissue and screened for the two phytoplasmas with species-specific assays. *Ca*. P. pruni was detected far more frequently in stone fruit than *Ca*. P. pyri. (Table 1).

Table 1. A summary of phytoplasma positive stone fruit trees identified in 2020.

Host	No. Trees Sampled	PYLR Positive	XDP Positive
Apricot	9	0	2
Nectarine	49	1	9
Peach	121	7	48
Plum	20	4	5
Pluot	1	0	1

Draft genomes have been obtained for both phytoplasmas, however they are highly fragmented. The *Ca*. P. pruni genome consists of 371 contigs with 484 genes identified. To date we have approximately 580 kb of sequence in eight large fragments, from an estimated 680 kb. The low GC-content and high level of sequence repeats has made completing the circular genome difficult. Nevertheless, we now have nearly 200 kb more sequence than the earlier *Ca*. P. pruni genomes from other parts of the country and have annotated a total of 484 genes. This winter we hope to complete this genome using the Nanopore long read sequencing platform. This technology may also be used to obtain a complete *Ca*. P. pyri genome, as to date this genome consists of 179 short contigs containing 271 genes.

We have eight additional fragmented genome sequences of the X-disease phytoplasma from peach, sweet cherry, and sour cherry samples, phylogenetic analysis of which suggests that there are two major groupings that differ by up to 3%. However, isolates from both groups are found in both stone fruit and cherries suggesting frequency movement within and between the crops. Interestingly, the isolates of *Ca*. P. pruni present in Washington state and Oregon are noticeably different from the partial sequences of Californian and Canadian isolates, as well as smaller fragments from Utah and Pennsylvania (Figure 1). These differences might account for the more transmissible, and more virulent phenotype we are observing relative to the older Californian reports from the 1970s and 80s.

Lastly, in 2020 we investigated a case of rapid bleaching and dieback of peach trees in Washington. The dieback was restricted mostly to one block and was similar in appearance both to iron deficiency and symptoms produced by a group Vb phytoplasma in Asia. In 2021, a small seedling that was exhibiting these symptoms was collected and transferred to our greenhouse and recovered from these symptoms. This, and the inability to detect phytoplasma in most of these plants suggests that the bleaching and dieback is likely to be an caused by an environmental condition.

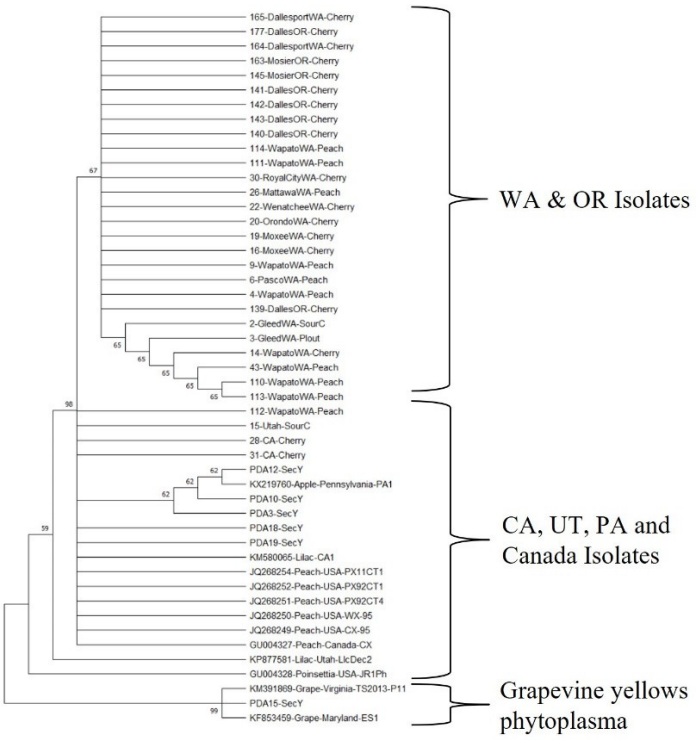


Figure 1. Phylogeny of the *Ca*. P. pruni isolates present in peach and cherry in Washington and Oregon based on the SecY gene, as compared to isolates from other states.

2. Identify physiological markers associated with the disease by comparing fruit and phloem tissues of infected and healthy trees.

In 2020, symptoms were assessed on a total of seven peach, five nectarine, two plums and one apricot cultivar, and then correlated with the presence and titer of the two pathogens (Table 2). We found that for both pathogens, the severity of foliar and fruit symptoms increased with pathogen titer.

Here we observed that, as per previously published studies, the major difference between X-disease and Peach yellow leaf roll disease in peaches and nectarines was the presence of shot-holing in the leaves of X-disease infected peaches and nectarines, whereas those infected with *Ca*. P. pyri showed only yellowing. However, shot holes were only frequently found in trees with increasing *Ca*. P. pruni titer; low titer containing plants showed only yellowing. Plums and apricots showed only slight yellowing with *Ca*. P. pruni, and that at increasing titers. Low level infections by either phytoplasma produced no appreciable foliar effects.

Fruit was observed to decrease in size, and in some cases show distortions or malformations with higher *Ca*. P. pruni titer. Malformations included bumps or lumps, and rarely, wrinkled or rippled smaller lumps under the skin of nectarines which may be due to location-based effects. The reduction in fruit size and delayed maturity was observed with peaches and nectarines as well as in plums. In contrast, no appreciable symptoms were found on infected apricots. As fewer *Ca*. P. pyri positives were found, and those were predominantly at lower titer, less data could be collected for analysis.

Finally, dieback was observed with both pathogens, although this was cultivar and location specific, and correlated with increasing *Ca*. P. pruni titer. It was common to see one leader of a tree on a v-trellis setup showing severe foliar symptoms and poor fruit set, begin to decline and die back near harvest, containing

high levels of the X-disease phytoplasma, while the other leader that contained little to no phytoplasma show mild foliar symptoms and continued to grow normally. However, revisiting those trees the following year we found that the remaining leaders began to show severe symptoms then decline and die.

Table 2. Effects of phytoplasma infection observed on peach cultivars in Washington state.

Species	Cultivar	Pathogen	Titer	Avg. Symptom rating[1]	Leaf Yellowing	Enlarged Midveins	Leaf Shotholes	Dieback	Fruit Size[2]	Fruit Deformation[3]
Peach	Country Sweet	XDP	Low	1	Yes	No	No	None	2.5	2
			Medium	2	Yes	Yes	Yes	None	2.1	2.2
	Diamond Princess	XDP	Low	1	Yes	No	No	None	3	1
			Medium	2.17	Yes	Yes	Yes	Some	2.25	2
	Elegant Lady	XDP	Medium	2.5	Yes	Yes	Yes	None	2.08	2.17
	O'Henry	XDP	Low	2	Yes	Yes	Yes	None	2.83	2
		Both	Low/Low	2	Yes	Yes	Yes	None	2.5	2
	Regina	XDP	Low	1.5	Yes	No	No	None	2.25	2
			Medium	2	Yes	Yes	Yes	None	2.13	2
	Sierra Rich	XDP	Low	1	Yes	No	No	None	1	2
			Medium	2	Yes	Yes	Yes	None	2	2
		PYLR	Low	0	Yes	No	No	None	2.5	2
	Zee Lady	XDP	Low	1.5	Yes	Some	Some	Some	3	2
			Medium	2	Yes	Yes	Yes	Some	2.71	2
		PYLR	Low	1	Yes	No	No	None	3	2
		Both	Med/Low	2	Yes	Yes	Yes	None	3	2
			Med/Med	2	Yes	Yes	Yes	Some	3	2
Nectarine	Grand Bright	PYLR	Low	2	Yes	Yes	No	None	2.5	2
	August Bright	XDP	Low	1	Yes	Yes	Yes	None	N/A	N/A
	Honeyhaven	XDP	Low	1	Yes	No	No	None	2.75	2.5
			Medium	2	Yes	Yes	Yes	None	2.17	2
	Summer Flair	XDP	Low	2	Yes	Yes	1	None	N/A	N/A
		PYLR	Low	2	Yes	Some	No	None	N/A	N/A
		Both	Low/Low	2	Yes	Yes	No	None	N/A	N/A
	Unspecified	XDP	Low	3	Yes	Yes	Yes	Some	N/A	N/A
		PYLR	Low	3	Yes	Yes	Yes	None	N/A	N/A
Plum	Friar	XDP	Low	0	0	No	No	None	2.5	1
			Medium	2	Yes	Some	Yes	None	2	1.67
		PYLR	Low	1	0.5	0.5	0.5	None	2.25	1
	Unspecified	XDP	Low	1	0	No	No	None	N/A	N/A
		PYLR	Low	2.67	Yes	Yes	Yes	N/A	N/A	N/A
Apricot	Unspecified	XDP	Low	1	0.5	No	No	Some	N/A	N/A

1. Symptom rating: 0 – Asymptomatic, 1 = mild, 2 = moderate, 3 = severe.
2. Fruit Size rating: 3 = normal, 2 = 25% reduction, 1 = 50% reduction
3. Fruit Deformation rating: 3 = normal, 2 = bulge or lump on one side, 3 = complete deformation

We also examined the sugar and secondary metabolite content (citric and malic acid content, and total phenolics) of fruit from infected trees. We found that while there were differences between asymptomatic and symptomatic trees, it was highly cultivar dependent (Figure 2). For example, looking only at sugar content 'Country Sweet,' 'Diamond Princess,' and 'Honeyhaven' cultivars showed a decrease in sucrose and an increase in fructose, while 'Elegant Lady' also exhibited an increase in fructose. No cultivars were found to have a significant difference in glucose or sorbitol content between asymptomatic and symptomatic fruit.

Examining secondary metabolites, we found a similar effect, with citric acid and malic acid content decreased in X-disease infected 'Country Sweet' and 'Friar plum,' whole malic acid was found to decrease in 'Diamond Princess'. In contrast, both 'Elegant Lady' and 'O'Henry' peaches showed a significant increase in total phenolics content of infected fruit.

These differences in sugar content and secondary metabolites would likely affect taste and suggest that there are underlying fruit maturation and developmental pathways affected by *Ca*. P. pruni. Most interestingly, these effects were not recorded in all cultivars, or at least did not produce significant differences. This suggests that some cultivars are more sensitive or susceptible to the pathogen than others, and that different pathways are being affected by the same pathogen in different cultivars.

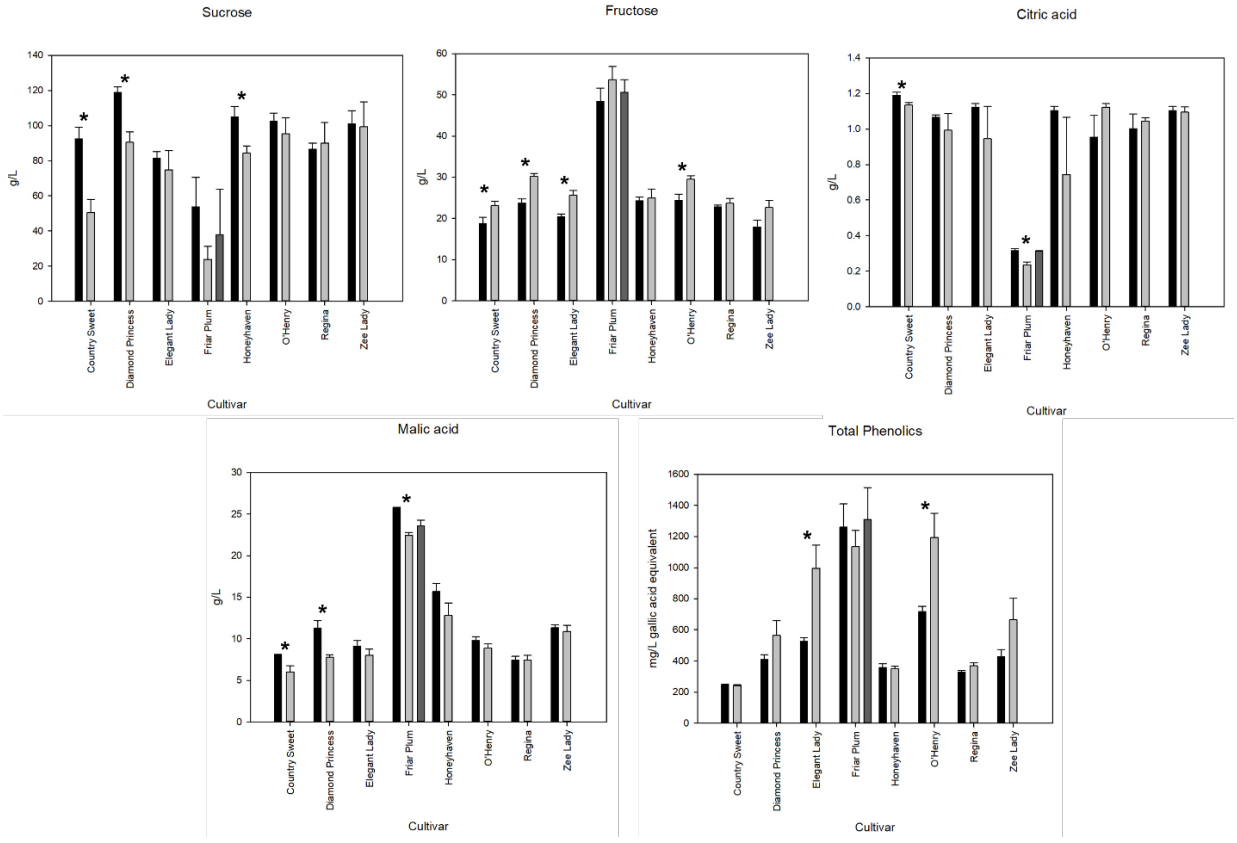


Figure 2. Sucrose, fructose, citric acid, malic acid, and total phenolics content across peach, nectarine, and plum cultivars for XDP infected, PYLR infected, and healthy trees. Asterisks indicate statistically significant differences.

Finally, as sugar content was lower in many of the *Ca*. P. pruni affected cultivars, we examined what effect the X-disease phytoplasma was causing on leaf chlorophyll. For this experiment we collected leaves from symptomatic and asymptomatic 'Honeyhaven' nectarine trees at the beginning of July 2021, when foliar

symptoms first appeared, and then every two weeks after that for a total of four time points. On infected trees, leaves were collected from the most symptomatic leader and the least symptomatic leader. Symptoms tended to occur on the eastern side of the tree, so on asymptomatic trees, leaves were collected and tested from both the east- and west-facing leaders to account for any effect positioning within the tree might have.

At all four time points, there was a significant decrease in content for chlorophyll a and b in the leaves from the most symptomatic leader (Figure 3). For all but the second time point, the decrease in content for carotenoids was also significant. As one of the primary symptoms of X-disease infection in peach and nectarine is leaf yellowing, which is characteristic of breakdown of leaf chloroplasts, this is perhaps not surprising, but does correlate with lower sugar concentration in the fruit. This phenomenon has been observed in other chlorosis-inducing phytoplasmas, although interestingly this does not occur in similarly X-disease phytoplasma infected cherries.

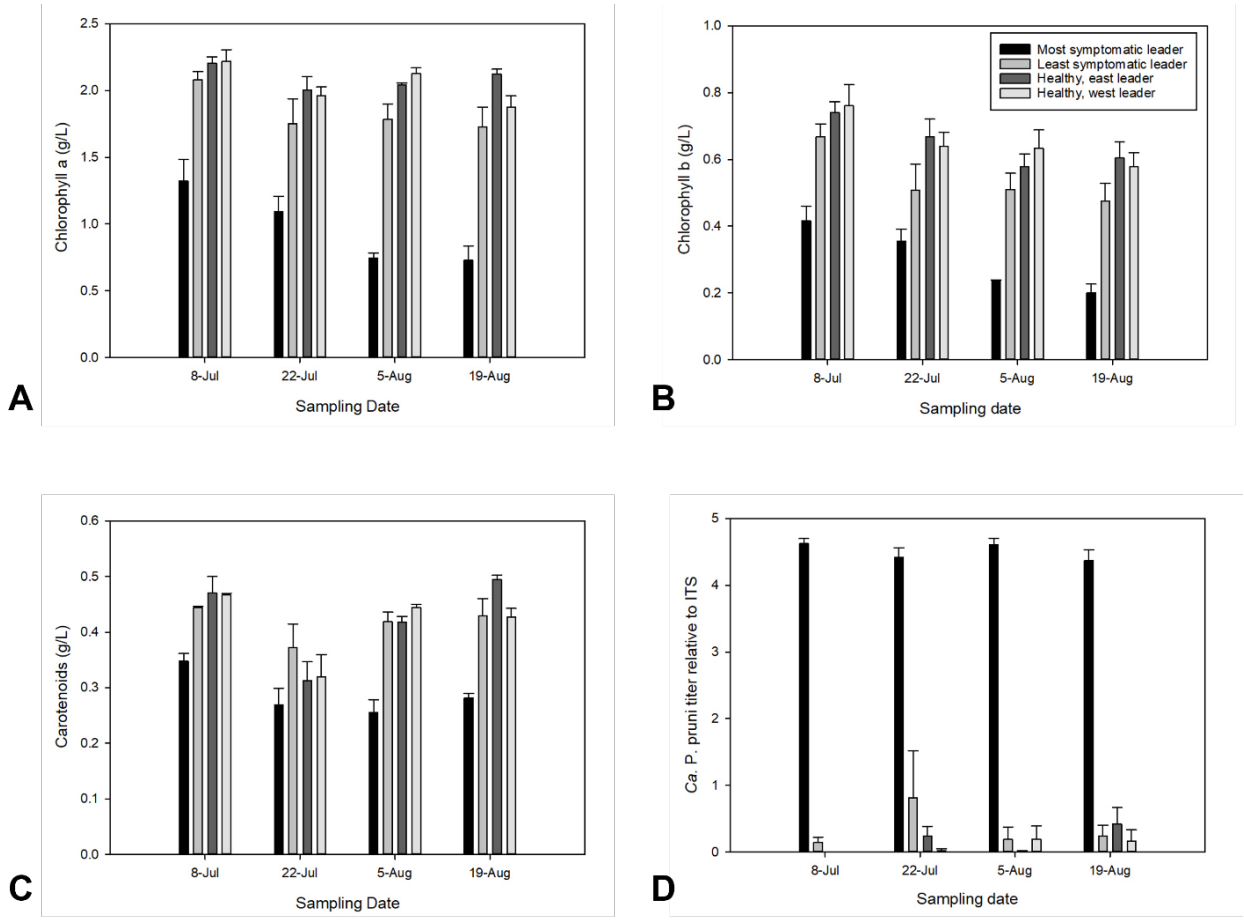


Figure 3. Concentrations of a) chlorophyll a, b) chlorophyll b, and c) carotenoids in leaves from symptomatic and asymptomatic 'Honeyhaven' nectarine trees compared to the concentration of d) X-disease phytoplasma, over time.

3. Determine how the presence of multiple phytoplasmas affects symptom development by using transcriptomics to identify affected pathways.

During the 2020 field season it was found that the onset of foliar symptoms caused by *Ca*. P. pruni interfered with the extraction of viable RNA suitable for gene expression analysis. Therefore, for the 2021 field season we began collecting samples from a 'Honeyhaven' nectarine block prior to symptom onset, and when symptoms began to appear in July, confirmed *Ca*. P. pruni infection by qPCR. Symptomatic samples were collected from trees that were beginning to express foliar chlorosis, and total RNA was extracted from these and asymptomatic samples and at the time of writing has been submitted to a third-party sequencing provider. Differential gene expression analysis will be performed once the sequence files are received, and addendum to this report will be submitted once complete.

Executive Summary:

Project Title: Understanding phytoplasmas infecting stone fruit trees in Washington state.

Keywords: X-disease phytoplasma, peach, transcriptomics

Abstract

With the onset of X-disease caused by *Candidatus* Phytoplasma pruni in Washington sweet cherry orchards, we asked whether this, and other phytoplasmas, were present in other stone fruit, particularly peach and nectarine blocks in the state. We surveyed peach, nectarine, plum and apricot blocks and found high incidence of the X-disease phytoplasma, with lower incidence of the Peach yellow leaf roll phytoplasma (*Ca*. P. pyri). Genetic analysis of the former found two distinct groups, both of which were present in both peaches/nectarines, and in sweet cherry, suggesting frequent transmission between the two species. This is an important finding because peach had previously been thought to be a dead-end host, but the high titer of *Ca*. P. pruni in this host would suggest otherwise. In addition, both were found in apples and pears, which are often grown adjacent to stone fruit orchards. No other phytoplasma species were found in stone fruit in Washington state in this survey.

Having identified the phytoplasmas present, we examined the disease they caused on stone fruit in Washington state. We found that both caused leaf yellowing, although the X-disease phytoplasma was generally more severe as infection progressed and caused shot holes on the leaves. We confirmed this visual observation by examining leaf chlorophyll and carotenoid content and found that high X-disease phytoplasma titers correlated with reduction in these compounds in symptomatic leaves. Next, we examined their effect on fruit and found that both caused a reduction in fruit size and malformations (bumps or lumps). Biochemical analysis revealed changes in sucrose and fructose content, citric acid and malic acid content, and total phenolics content in infected trees compared to healthy, however these changes were highly cultivar dependent in that not all cultivars showed the same response.

Cumulative these data suggest that the X-disease phytoplasma in particular changes fruit maturation pathways. To understand how this occurs we prepared and submitted samples for RNAseq. Differential gene expression analysis will be performed once the sequence files are received, and addendum to this report will be submitted once complete.

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

Project Title: Engineering transgenic *Drosophila suzukii* for wild population suppression & eradication

PI: Omar Akbari
Organization: UC San Diego
Telephone: 858-246-0640
Email: oakbari@ucsd.edu
Address: 9500 Gilman Drive
Address 2:
City/State/Zip: La Jolla, CA 92093

Co-PI (2): None
Organization:
Telephone:
Email:
Address:
Address 2:
City/State/Zip:

Cooperators: None

Objectives: Spotted wing Drosophila, *D. suzukii*, is a major worldwide crop pest of various soft-skinned fruits(Walsh et al. 2011). A highly promising approach to *D. suzkuii* control that could complement existing control methods is genetic pest management, which includes strategies such as gene drive and precision-guided sterile insect technique (pgSIT)(Kandul et al. 2018; Ant et al. 2012). SIT has been a successful technology for insect population suppression, which is achieved by introducing large numbers of sterile males into a target population. While the classic irradiation-based SIT presents an environment-friendly method of local population suppression, it is not technically feasible or scalable for the control of most insects. PgSIT, on the other hand, is a simplified way to generate sterile males and should be less expensive and labor intensive than irradiation-based SIT even at scale.

We also propose engineering *D. suzukii* gene drive strains, which can be utilized to more rapidly spread desirable genes (e.g., susceptibility to a novel bio-friendly pesticide) throughout, or to entirely suppress/eradicate, wild *D. suzukii* populations. Such an approach is catalytic, with release of only modest numbers of engineered insects required to spread desirable genes or achieve population suppression. Additionally, since such a system relies on only a few releases of transgenic insects to do all of the work on an ongoing basis, it is affordable as compared to the use of insecticides, which need to be applied regularly. Finally, such an approach is environmentally friendly and entirely insect-specific and would have no effect on crops or on beneficial organisms.

Our objective is to therefore engineer *D. suzukii* gene drive strains that could be utilized as part of current integrated pest management programs to control wild *D. suzukii* populations. Specifically, out of the multiple types of gene drive systems that can be utilized in a genetic pest management program(Champer, Buchman, and Akbari 2016; Scott et al. 2018). We aim to develop a pgSIT system in *D. suzukii* using the design principles we have optimized in *D. melanogaster(Kandul et al. 2018)*. We also aim to develop synthetic *Medea* elements that can be used to suppress wild *D. suzukii* populations. Ultimately, our goal is to develop a product (a genetically modified *D. suzukii*) that can be mass-reared and deployed into the wild to catalytically suppress, and completely eliminate, the wild populations of this significant pest.

Objective A - Refinement of a *Medea* drive system for *D. suzukii* population suppression. We have developed a synthetic *Medea* gene drive system for population suppression(Buchman et al. 2018). Engineered *Medea* systems rely on a *Medea* element consisting of a toxin-antidote combination. The toxin consists of a miRNA that is expressed during oogenesis in *Medea*-bearing females, disrupting an embryonic essential gene. A linked antidote is expressed early during embryogenesis and consists of a recoded version of the target gene that is resistant to the miRNA. This combination results in the survival of half of the embryos originating from a *Medea*-bearing heterozygous female, as those that do not inherit the *Medea* element perish. If a heterozygous *Medea* female has mated with a heterozygous

Medea male, the antidote from the male will also take effect in the embryo, resulting in 3/4 of the embryos surviving. Therefore, *Medea* will rapidly spread through a population, carrying any linked genes with it.

We have already engineered a first-generation *Medea* system in *D. suzukii* (Buchman et al. 2018), which is the first functional gene drive developed in this pest. We had rigorously tested it in laboratory cage populations, and had characterized it in different genetic backgrounds to determine effectiveness and fecundity. We found that this first-generation *Medea* system was capable of biasing Mendelian inheritance rates with up to 100% efficiency and could maintain itself at high frequencies in a wild population; however, drive resistance, resulting from naturally occurring genetic variation and associated fitness costs, was present and could hinder the spread of such a drive. Therefore, since mathematical modeling indicates that our *Medea* drive system could spread to fixation if resistance was reduced(Buchman et al. 2018), we need to engineer a second-generation *Medea* system that should obviate the specific resistance that we observed. To safeguard, reduce risk, and mitigate the spread of the *D. suzukii Medea* system into wild populations, we also aim to develop a reversal *Medea* (RM) system that can be used to replace the original *Medea* in case a recall is necessary. Reversing the drive of a *Medea* system has been theorized; however, it has never been experimentally demonstrated. Finally, in order to use *Medea* to bring about population suppression, we need to link it to a cargo gene capable of killing *D. suzukii* under specific conditions to bring about a population crash. We have already identified several promising putative cargo genes and are testing them in *D. melanogaster*, a closely related species to *D. suzukii* that is easier to work with and provides a useful testing platform for transgenes. However, we will still need to build and test them in *D. suzukii*. Successful completion of the above objectives would lead to the development of a genetically modified *D. suzukii* strain (carrying a synthetic *Medea* element) that can be mass-reared and deployed into the wild to catalytically suppress, and completely eliminate, wild populations of *D. suzukii*.

Objective B: Precision guided sterile insect technique (pgSIT) for *D. suzukii* population suppression. The Sterile Insect Technique (SIT) is an alternative, proven pest management approach that could complement existing control methods(Nikolouli et al. 2018; Alphey and Bonsall 2018; Lees et al. 2015). SIT involves the mass-production and release of sterile males, and has historically been used to control, and eradicate, insect pest populations dating back to the mid-1930s(Knipling 1955; Bushland, Lindquist, and Knipling 1955; Klassen and Curtis 2005; Vanderplank 1944; Dyck, Hendrichs, and Robinson 2005). Traditional SIT methodologies have relied on DNA-damaging agents for sterilization, substantially reducing overall fitness and mating competitiveness of released males. A next-generation highly-efficient technology that can be used for biocontrol of *D. suzukii* is precision guided SIT (pgSIT). PgSIT functions by exploiting the precision and accuracy of CRISPR to simultaneously disrupt genes essential for either female viability or male fertility. It utilizes a simple breeding scheme requiring two homozygous strains - one expressing Cas9 and the other expressing double guide RNAs (dgRNAs). A single mating between these strains mechanistically results in synchronous RNA-guided dominant biallelic knockouts of both target genes throughout development, resulting in the complete penetrance of desired phenotypes in all progeny. We have previously built pgSIT in *Drosophila melanogaster*, a model organism that is closely related to *D. suzukii*, and shown that it is extremely robust at genetically sexing and simultaneously sterilizing resulting progeny reproducibly with 100% efficiency, and that pgSIT sterile males are fit and can compete for mates(Kandul et al. 2018). We therefore aim to develop pgSIT technology in *D. suzukii* (Objective B). Successful development of this technology would produce a genetic-based sterile insect strain that can be mass-reared and released to reduce populations of *D. suzukii* in a straightforward manner with respect to regulations.

Significant Findings:

Objective A:

● We have developed a modified version of our original *Medea* system that is designed to reduce resistance to the drive.
● We have developed a second-generation "reversal" *Medea* system that should be more robust in the face of genetic diversity in general and could be used to replace the original *Medea* in case a recall is necessary.
● We have identified several promising putative cargo genes that could be spread with the *Medea* gene drive to cause population suppression.

Objective B:
● We developed a pgSIT system that is competitive, fit and consistently produces sterile males.
● Plans for a confined field trial of the pgSIT technology is planned at a USDA facility in Corvallis, Oregon.

Results and Discussion:

Objective A - Refinement of a *Medea* drive system for *D. suzukii* population suppression.

Second generation *Medea* drives: We have developed the first proof of concept *Medea* drive in *D. suzukii(Buchman et al. 2018)*. Given our observations regarding resistance and its effect on *Medea* function, we have engineered improved *Medea* systems that could reduce the chances of resistance acting as an impediment to spread. We first performed some sequencing-based characterization of naturally occurring genetic variation in various geographically distinct target populations to help guide selection of target sites that are well conserved across all populations in which the drive is intended to function. We then designed a modified version of the original *Medea* system that targeted different, conserved sequences (still in the 5'UTR of the *myd88* target gene), reasoning that such a *Medea* element should function very similarly to the original element but not be impeded by the resistance we previously observed. We obtained transgenic lines for this improved *Medea* element. Preliminary data indicated that it work better than the original *Medea*, producing 100% inheritance bias, but due to COVID19 related work delays we are still continuing to rigorously test this second-generation *Medea* element to characterize its function and ability to bias inheritance 100% in geographically distinct populations. Due to the higher likelihood that the pgSIT will be approved for field use before a *Medea* drive, since pgSIT is simply a new and improved version of the long used sterile insect technique (SIT), when forced to work at a lower capacity to adhere to the state of California and University of California COVID19 safety guidelines, we prioritize work on pgSIT.

Reversal *Medea* systems: Additionally, we hypothesized that to reduce resistance, miRNA target site selection could be limited to the coding DNA sequence regions of a genome, which tend to be strongly conserved, as opposed to regions such as the 5'UTR, which canonically have higher tolerance for sequence variation. We have therefore also developed a second-generation "reversal" *Medea* system in *D. suzukii* that should be more robust in the face of genetic diversity in general (because it targets coding DNA regions as opposed to the 5'UTR) and could be used to replace the original *Medea* in case a recall is necessary. Specifically, to reduce risk and mitigate the spread of the *D. suzukii Medea* system into wild populations, it is important to develop a reversal *Medea* (RM) system and demonstrate that it can function as predicted. Reversing the drive of a *Medea* system has been theorized; however, it has never been experimentally demonstrated. We finished designing and building a reversal *Medea* system capable of spreading on its own and of replacing the first *Medea* described above, but due to COVID19 related work delays we did not have time to develop transgenic lines for this construct. We focused our efforts instead on completing the pgSIT work, which should have a faster and more predictable path to approval for field implementation.

Identification of putative "cargo" genes: For *D. suzukii*, elimination of the pest populations is ultimately the goal. An engineered *Medea* system could achieve this by spreading a "cargo" gene proffering susceptibility to a particular pesticide, or a conditional lethal gene that would be activated by some substance or environmental cue such as high temperature or diapause. One promising type of candidate "cargo" gene is a thermally activated TRPA1 cation channel(Castillo et al. 2018).

Specifically, TRPA1 is an ion channel located on the plasma membrane of many human and animal cells, and is finely tuned to detect specific temperatures ranging from extreme cold to noxious heat(Castillo et al. 2018). Upon exposure to a critical "threshold" temperature, this cation channel can "open" and modulate Ca2+ and Mg2+ entry into the cell(Guimaraes and Jordt 2011); when TRPA1 is overexpressed in an exogenous tissue (such as the fly brain, for example), this "opening" can lead to total fly paralysis and death. We therefore have started to engineer *D. suzukii* to express a specific TRPA1 channel in the brain, so that exposure of the engineered individuals to a threshold temperature (determined by the specific TRPA1 channel used) would paralyze/kill the flies. We have designed these lines, but due to COVID19 related work delays, we were unable to test whether these transgenic lines are able to spread this temperature-activated "cargo" gene through wild populations. The goal would have been to couple this with a *Medea* system whereby at cooler temperatures the cargo is inactive, but at higher temperatures the TRPA1 and achieve population suppression when the TRPA1 gene is activated at warmer temperatures.

Objective B: Precision guided sterile insect technique (pgSIT) for *D. suzukii* population suppression.

Precision guided SIT: In order to construct a pgSIT system, we need functional Cas9 tools (including gRNA lines that target genes essential for female viability and male sterility and Cas9 expressing lines (**Fig. 1-2**) in *D. suzukii*. We developed multiple transgenic lines that express Cas9 (*bicC-cas9, vasa-cas9, nanos-cas9, ubiq-cas9*)(Kandul et al., n.d.). To robustly express and import Cas9 into nuclei, we used *Streptococcus pyogenes* Cas9 (Cas9) with a nuclear localization sequence (NLS) on either the C-terminal end only (Cas9-NLS), or on both terminals (NLS-Cas9-NLS (Fig. 1A). To drive expression of Cas9, we used *D. melanogaster* promoters expressed in either early germ cells, *vasa* (*vas*), or *nanos* (*nos*), or in late germ cells, *Bicaudal C* (*BicC*), or in both germ and somatic cells, *polyubiquitin 63E* (*ubiq*). Using each promoter, we built four piggyBac constructs that express NLS-Cas9-NLS terminated by a p10 3′-UTR derived from the *Autographa californica* nucleopolyhedrovirus (AcNPV) or strong translation of Cas9, and a red (Opie2-dsRed) transgenesis marker. We also built two alternative piggyBac constructs that contain either the *vas* or *nos* promoters driving expression of the Cas9-NLS terminated with *vas* or *nos* 3'UTR's from *D. melanogaster*, with a green (ubiq-ZsGreen) transgenesis marker (**Fig. 1A**). In total, six Cas9 constructs were engineered that were used to generate 8 homozygous transgenic strains (at least one homozygous transgenic strain per construct), two of which were X-linked (**Fig. 1B**). Initially, the efficiency of these promoters was tested by genetically crossing two gRNA lines targeting the *white* and *yellow* phenotypic genes (**Fig. 1C, 2**). To assess the functionally of the lines produced, we genetically crossed homozygous Cas9 females to homozygous gRNA males and examined expected eye and/or body coloration phenotypes in the resulting F_1 progeny (**Fig. 2A-F**). This cross was performed to explore the rates of mutagenesis in the F_1 somatic tissues, which is augmented by maternal deposition of Cas9. High percentages (61.1%-100%) of F_1 trans-heterozygous progeny generated by dsRed+ Cas9 females crossed to *yellow* targeting gRNA lines (gRNAy) males had visible yellow, instead of brown, body coloration indicating robust somatic *yellow* gene disruption (y– phenotype) (**Fig. 2B,C**).

Also, essential to building a pgSIT system are guide RNA (gRNA) lines that target genes essential for female viability and male fertility. We have previously identified genes essential for female viability or male fertility in *D. melanogaster* and have shown that disrupting these genes via CRISPR/Cas9 produces the desired results (e.g., female death or conversion of females into sterile intersex individuals for the former group, male sterility for the latter(Kandul et al. 2019). Since *D. melanogaster* is closely related to *D. suzukii*, we reasoned that disruption of these same genes would have a similar effect in *D. suzukii*. Specifically, to disrupt female viability in *D. suzukii*, we targeted several sex-specifically alternatively spliced sex-determination genes including *sex lethal* (*sxl*), *transformer* (*tra*), and *doublesex* (*dsxF*), as well as *zero population growth* (*zpg*), a germline-specific gap junction gene. We identified *D. suzukii* homologues of all of these genes and have carefully selected two gRNA target sites in each gene that are highly conserved and thus unlikely to have high sequence

variation in the wild. We generated multiple transgenic lines for each gRNA target and crossed to the Cas9 strains to see whether the combinations of Cas9+gRNA will produce female lethality and male sterility. To track transgenes, gRNAdsx, gRNAtra, and gRNAβTub plasmids also harbored a green fluorescent tag, Opie2-mVenus, while the gRNAsxl plasmid carried Ubiq-dsRed (**Fig. 3**). Each gRNA plasmid was injected into embryos harboring hsp-pBac intergrase, and one or two gRNA transgenic lines were generated for each construct. Then, virgin transgenic flies from each lines were repeatedly backcrossed to establish homozygous stocks of gRNAsxl.L1, gRNAsxl.L2, gRNAdsx, gRNAtra.L1, gRNAtra.L2, gRNAβTub.L1, and gRNAβTub.L2 lines. The *vas* and *BicC* Cas9 lines had the highest and most consistent gene knockout efficiencies with the least fitness costs, so we used these lines to evaluate the targeting efficiency of each gRNA line (**Fig. 4)**. This evaluation was done by genetically crossing each gRNA strain to both vasCas9 and BicC.Cas9 strains, scorring sex frequency and fertility of F1 trans-heterozygous progeny, and then sequencing target loci.

The lines that most efficiently killed or masculinized females, targeted *sxl*, which is a sex determination gene essential for female-specific development in *D. melanogaster* and other insect species and disruption of this gene has been shown to cause female lethality or masculinization in other studies. (Kandul et al. 2019). Two lines that target *βTub* showed the high rate of male sterility. which arrests spermatid elongation and sperm mobility resulting in the male-specific sterility *(*Kandul et al. 2019; Li et al. 2021). We then built multiple lines with, which targeted both *sxl* and *βTub* (**Fig. 3**). The most efficient and healthy lines (gRNA$^{sxl,\beta Tub\#4.L1}$, gRNA$^{sxl,\beta Tub\#4.L2}$, and gRNA$^{sxl,\beta Tub\#5.L1}$) were chosen for further characterization and to ensure these results were reproducible over many generations. When crossed to vasCas9 we demonstrated complete female-specific lethality and nearly complete male sterility in the F_1 trans-heterozygous progeny (**Fig. 5**). To further quantify the fertility of pgSIT males, we crossed batches of 5 pgSIT ♂ to 20 *wt* virgin ♀ and scored numbers of laid and hatched eggs. Not a single egg out of 952 and 321 eggs laid by females mated with *gRNA$^{sxl,\beta Tub\#4}$.L1*/+; *vasCas9*/+ and *gRNA$^{sxl,\beta Tub\#5}$.L1*/+; *vasCas9*/+ males respectively, hatched (Table 1). Five eggs hatched out of 518 eggs laid by females mated with *gRNA$^{sxl,\beta Tub\#4}$.L2/+; vasCas9*/+ males (0.9% egg hatching rate, Table 1). Therefore, these results indicate that genetic crosses of the *vasCas9* strain to any of three autosomal *gRNA$^{sxl,\beta Tub}$* strains result in complete lethality or masculinization of females into sterile intersexes while the generated pgSIT males are nearly 100% sterile. The induced pgSIT phenotypes, i.e. female lethality / masculinization and male sterility, correlated with mutagenesis at *slx* and *βTub* target loci induced by Cas9/gRNA in trans-heterozygous flies (**Fig. 6**). Then we chose one line to be rigorously tested in male competition and fitness studies as well as laboratory population cages to ensure the sterile males are fit to compete in field conditions (**Fig. 7-8**). pgSIT males were able to compete for mates with wildtype males (**Fig. 7**) and were able to successfully suppress small laboratory populations of *D. suzukii*.

Developing a field-ready strain: We have developed pgSIT strain ready for use in the field for *D. suzukii* biocontrol. We are currently using this data to inform mathematical models to predict the introduction frequencies we will need to use to achieve suppression and this strain has been transferred to Agragene, a local biotechnology company for future confined field assessments at a USDA facility in Corvallis, Oregon. Our collaborator has received a field cage study permit from USDA-APHIS BRS/PPQ. APHIS is responsible for issuing permits for the import, transit and release of regulated animals, animal products, veterinary biologics, plants, plant products, pests, organisms, soil, and genetically engineered organisms. They have also applied for a BRS 2000 (Application for Permit or Courtesy Permit for Movement or Release of Genetically Engineered Organisms). These permits have been successfully issued for the release of transgenic insects in the USA. For example, in 2009 the USDA approved the integration of genetically engineered pest insects (including pink bollworm moth (*P. gossypiella*), Mediterranean fruit fly (*Ceratitis capitata*), Mexican fruit fly (*Anastrepha ludens*), and oriental fruit fly (*Bactrocera dorsalis*)) into ongoing SIT programs(Reeves et al. 2012). Some key advantages of the pgSIT approach will be that only males will need to be released (so crops will not be damaged); that it is very species-specific, since the released males will be sterile and not capable of mating with wild *D. suzukii* or any other species; and that the approach is self-limiting, which makes it a safer alternative than self-sustaining approaches and thus more likely to win public and regulatory

approval. Therefore, the key point here is that obtaining regulatory approval for releasing transgenic insects in the USA, that are engineered to reduce wild populations and prevent crop damage, has been achieved in the past, and therefore we do not envision it to be a limitation with our approach.

Table 1: Fertility of pgSIT (trans-heterozygous) males measured by the hatching rate of sired eggs.

gRNA strain	Groups of 5 ♂ and 20 virgin ♀	Total number of laid eggs	Total number of hatched eggs	Hatching rate
gRgRNA$^{sxl,\beta Tub\#4}$.L1	5	952	0	0%
gRgRNA$^{sxl,\beta Tub\#4}$.L2	3	518	5	0.9%
gRgRNA$^{sxl,\beta Tub\#5}$.L1	3	321	0	0%

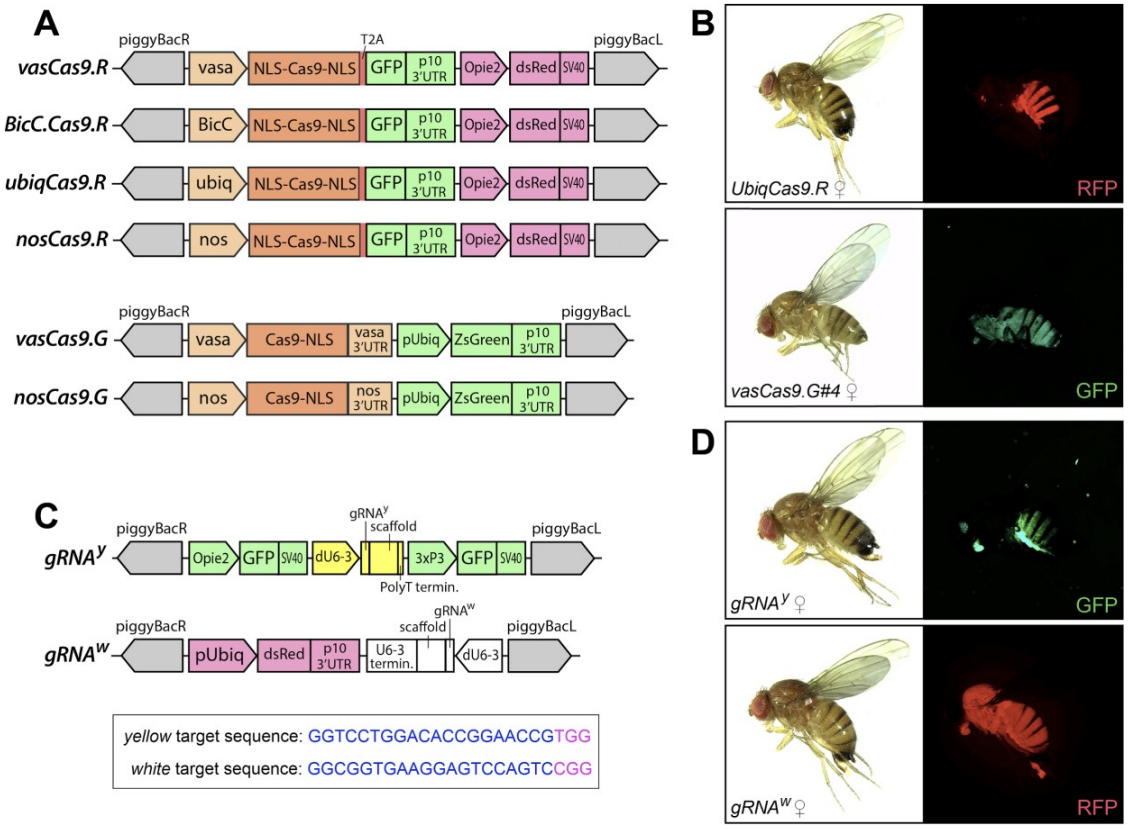


Figure 1. Schematic maps of genetic constructs and images of transgenic SWD. (A) Schematic maps of two sets of Cas9 constructs. The first four Cas9 constructs harbor a human-codon-optimized SpCas9 (Cas9) (61) coding sequence (CDS) surrounded by two nuclear localization sequences (NLS-Cas9-NLS), linked to the eGFP CDS at its C-end via a self-cleaving T2A sequence, and terminated by the p10 3′-UTR from the *Autographa californica* nucleopolyhedrovirus (AcNPV). The SpCas9 is expressed in early germ cells under vasa (vas) and nanos (nos) promoters, in late germ cells with *Bicaudal C* (*BicC*), and in both germ and somatic cells with *Ubiquitin 63E* (*ubiq*) promoter. These constructs also contain a red transgenesis marker (Opie2-dsRed). The second group of Cas9 constructs carry a human-codon-optimized SpCas9 expressed under the *vas* or *nos* promoter, and terminated with a single NLS (Cas9-NLS) and the corresponding *vas* and *nos* 3'UTR, as well as a green transgenesis marker (Ubiq-ZsGreen). (B) Images of homozygous transgenic SWD Cas9 ♀ flies generated with UbiqCas9.R and VasCas9.G. (C) Schematic maps of two gRNA constructs, and the targeted sequences

in both yellow and white loci. The gRNAy construct harbors the yellow gRNA (gRNAy) with a scaffold expressed with the Dmel pU6-3 promoter and terminated by a PolyT terminator, and two green transgenesis markers, Opie2-GFP and 3xP3-GFP. The gRNAw construct harbors the white gRNA (gRNAw) with a scaffold expressed with the same Dmel pU6-3 promoter and terminates the pU6-3 terminator sequence, and a red transgenesis marker, Ubiq-dsRed. (D) Images of homozygous SWD gRNAy and gRNAw ♀. Both sets of RGB images for each ♀ fly were taken under the white light and corresponding fluorescent light illumination.

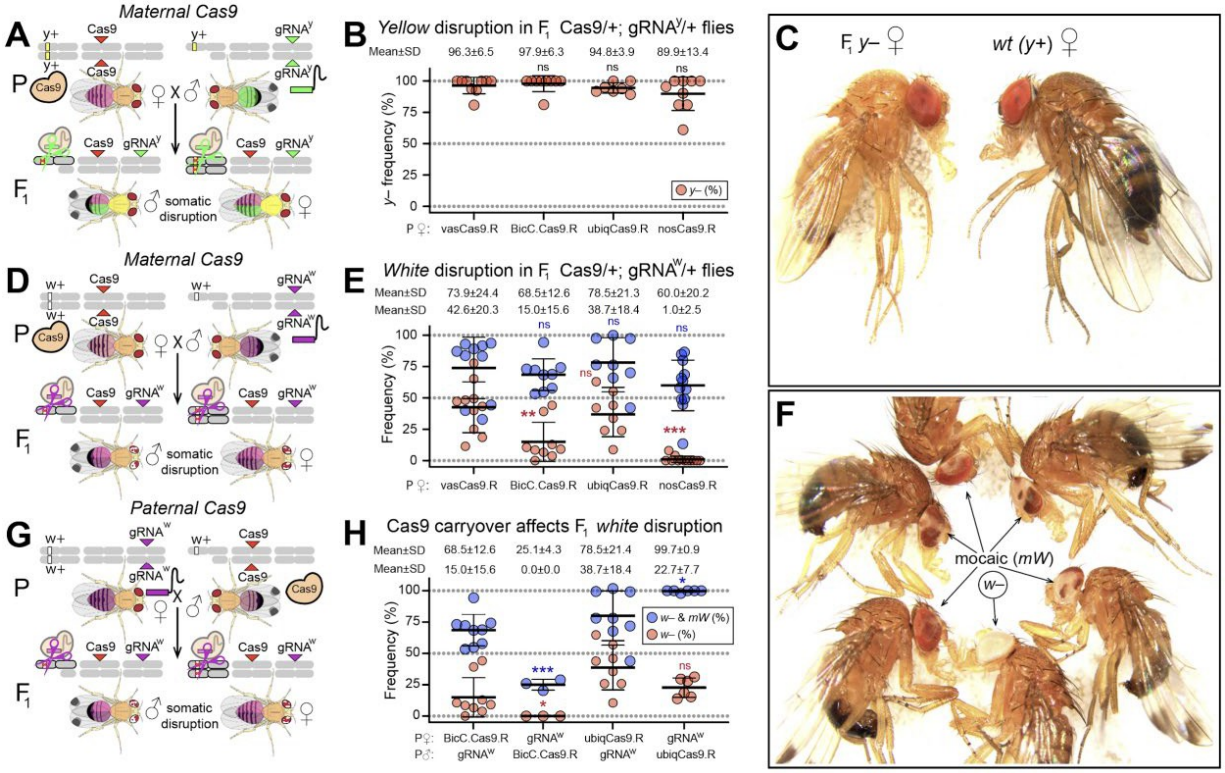


Figure 2. Disruption of *yellow* and *white* loci in F1 trans-heterozygous flies. (A) The schematic of a genetic cross between Cas9 and gRNAyellow(y) flies. To generate F_1 trans-heterozygous flies, homozygous Cas9 ♀ (red marker) crossed to homozygous gRNAy ♂ (green marker). The targeted wildtype (wt) *yellow* gene (y+ alleles, yellow stripes on X chromosome) is on the X chromosome. Yellow colored thoraxes in F_1 flies indicate disruption of the yellow gene (y-). (B) Dot plot depicting the results of disruption of the yellow gene in somatic cells from F_1 trans-heterozygous (Cas9/+; gRNAy /+) progeny using the Cas9.R strains inherited maternally. (C) Images of F_1 y– trans-heterozygous and wt y+ ♀. (D) The schematic of a genetic cross with maternal homozygous Cas9 (red marker) and paternal homozygous gRNAw (purple marker). The white gene is on the X chromosome (w+ alleles, white stripes on X chromosome). Red and white eye coloration in the F_1 flies indicate somatic disruption of the *white* gene (mW). (E) Dot plot depicting the results of disruption of the *white* gene in somatic cells from F_1 trans-heterozygous (Cas9/+; gRNAw/+) progeny using the Cas9.R strains inherited maternally. (F) Images of F1 trans-heterozygous mosaic white disruption (mW) and complete disruption (w–) phenotypes. (G) The schematic of a genetic cross between paternal Cas9 and maternal gRNAw flies. To generate F1 trans-heterozygous flies, homozygous Cas9 ♂ (red marker) crossed to homozygous gRNAw ♀ (purple marker). The *white* gene is on the X chromosome (w+ alleles, white stripes). (H) Dot plot depicting the results of disruption of the *white* gene in somatic cells from F_1 transheterozygous (Cas9/+; gRNAy /+) progeny using the Cas9.R strains inherited paternally. Plots show the mean ± SD over at least three biological replicates. Statistical significance was estimated using a two-sided Student's t test with unequal variance. ($p \geq 0.05$ns, $p < 0.05$*, $p < 0.01$**, and $p < 0.001$***).

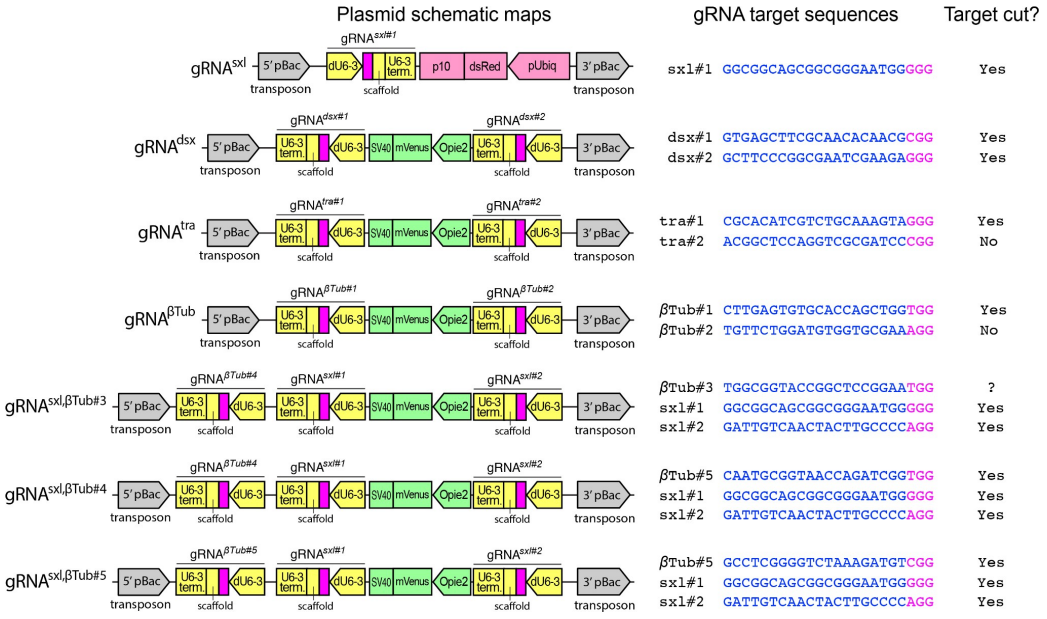


Figure 3. Schematic maps of pgSIT plasmids and gRNA target sequences. Different gRNA lines expressing one or two gRNA targeting *sex lethal* (*sxl*), *double sex* (*dsx*), *transformer* (*tra*), or *βTubuline 85D* (*βTub*). To track transgenes, the gRNAdsx, gRNAtra, and gRNAβTub plasmids also harbored a green fluorescent tag, Opie2-mVenus, while the gRNAsxl plasmid carried Ubiq-dsRed. The gRNA was driven by the constitutively expressed *D. melanogaster small nuclear RNA U6-3* promoter.

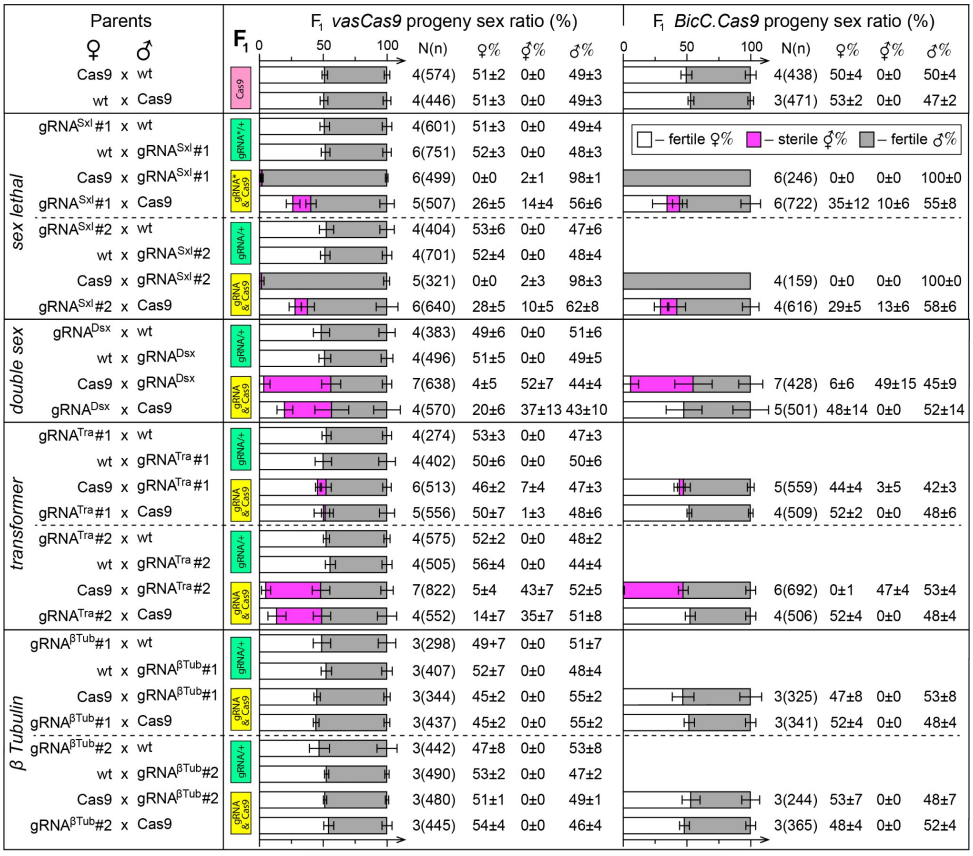


Figure 4. Assessment of Cas9/gRNA-mediated disruption of single-gene. Cas9/gRNA-mediated knockout causes lethality and/or masculinization of F_1 trans-heterozygous females. Different gRNA lines expressing one or two gRNA targeting *sex lethal* (*sxl*), d*ouble sex* (*dsx*), *transformer* (*tra*), or *βTubuline 85D* (*βTub*) were assessed in combination *vasCas9* and *BicC Cas9* lines.

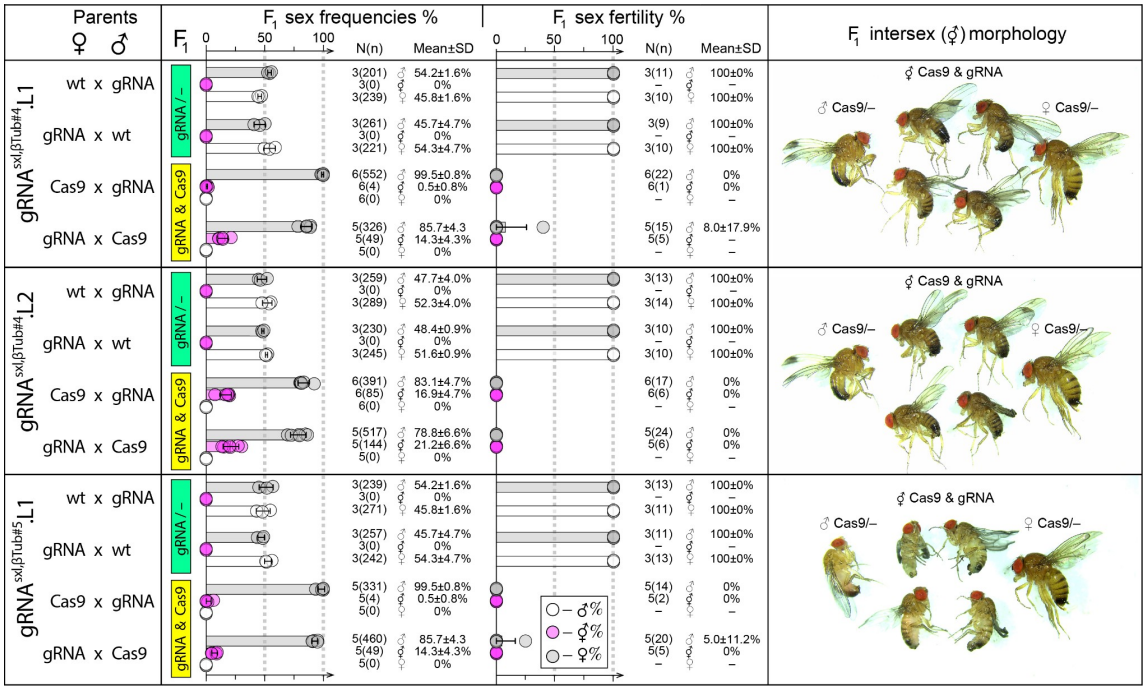


Figure 5. Three active *D. suzukii* pgSIT systems. Cas9/gRNA-mediated knockout causes lethality and/or masculinization of F_1 trans-heterozygous females. Different gRNA lines expressing one or two gRNA targeting *sex lethal* (*sxl*), d*ouble sex* (*dsx*).

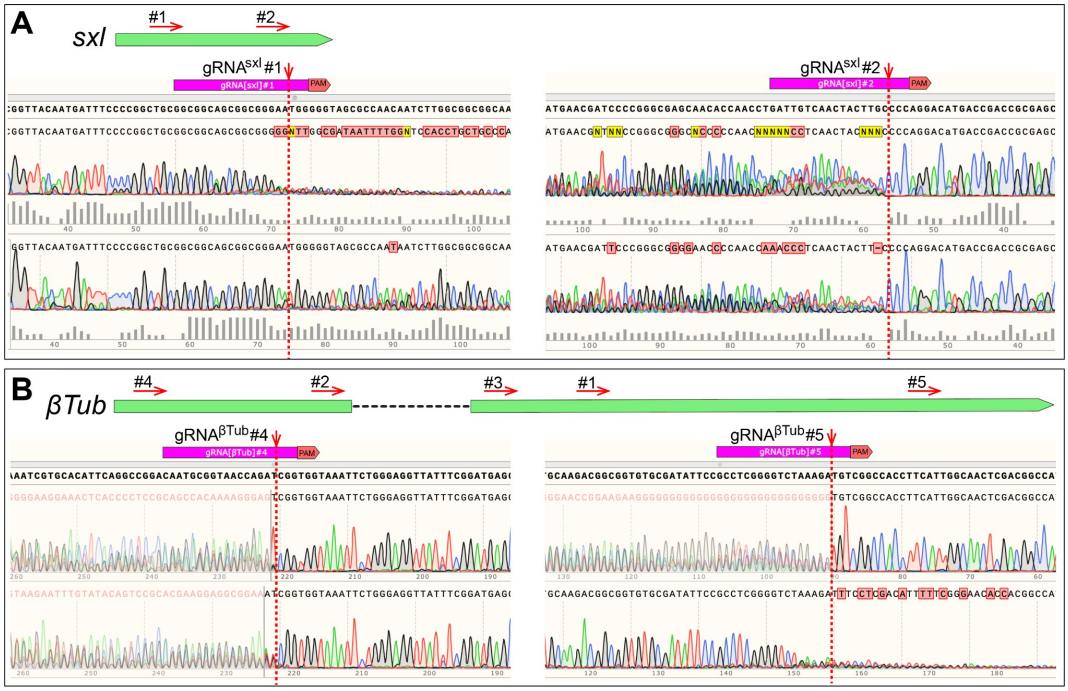


Figure 6. Double-gene KO Sanger Sequence reads of targets in *Sxl* **(A)** and *βTub* **(B)**.

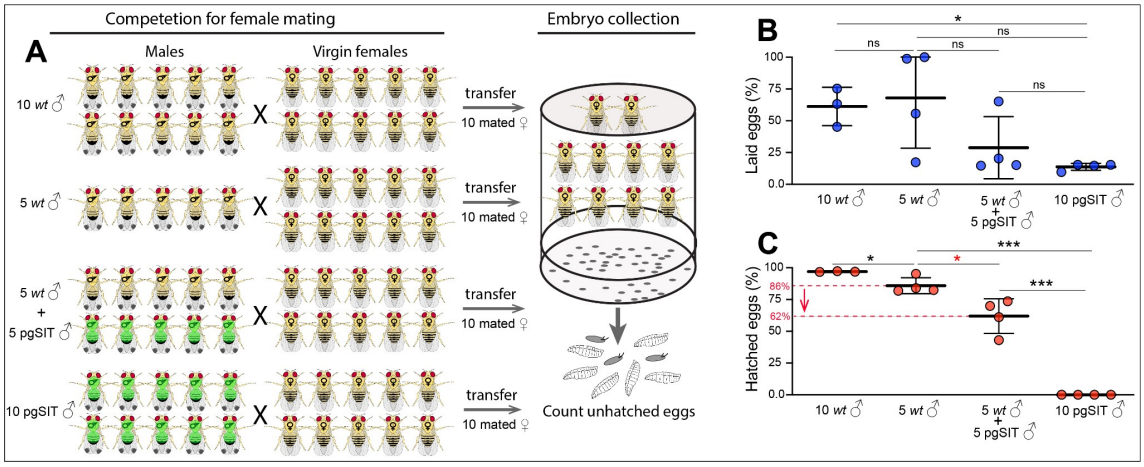


Figure 7. pgSIT males are competitive with wildtype males. In small population cage studies, male pgSIT flies (green flies) were released in 1:2 or 1;1 male to female ratios. All wildtype and all pgSIT male controls were also evaluated. As expected, there was no difference in the number of eggs laid in any of the groups containing wildtype males. However, in experimental groups with pgSIT there were significantly less eggs hatched and in the pgSIT only control group, few eggs were laid and none hatched.

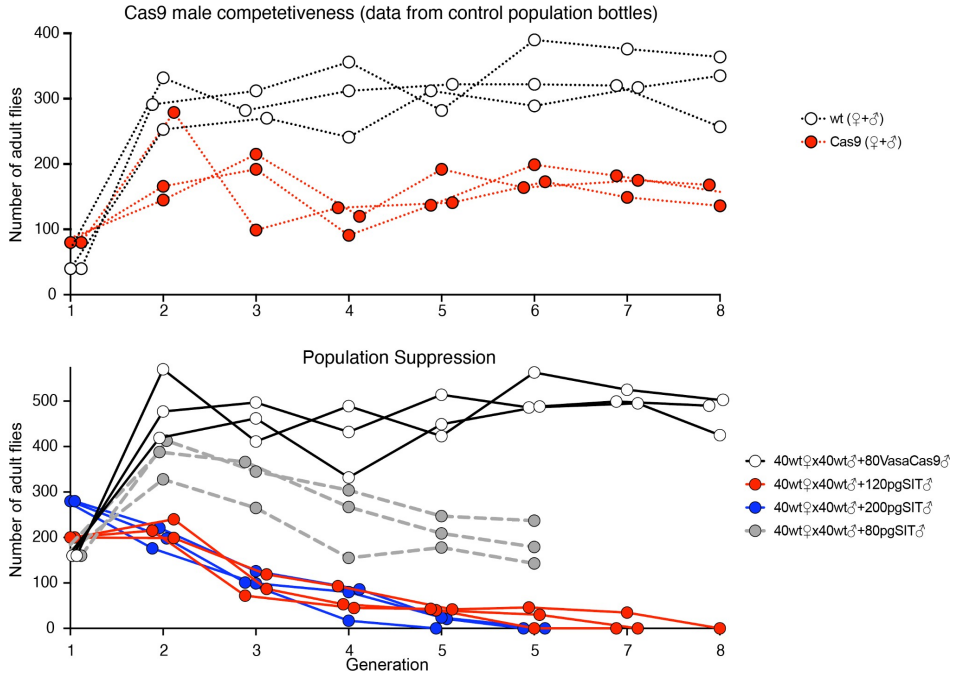


Figure 8. pgSIT males suppress discrete populations. To establish the fixed size populations of *D. suzukiii*, were seeded groups of forty 4-5-day-old virgin wt females in 0.3 L plastic bottles (VWR Drosophila Bottle 75813–110). The mixture of 4-5-day-old 40 wt and 80 vasCas9 (1:2 ratio) males was added to control bottles (white circles). For test bottles, we added 40 wt and 80 pgSIT (1:2 ratio- gray circles) or 40 wt and 120 pgSIT (1:3 ratio- rec circles) or 40 wt and 200 pgSIT (1:5 ratio-blue circles) males per bottle. After 4 days at 21˚C/70˚F, parent flies were transferred into fresh bottles and the first bottles were discarded. In 5-7 days, depending on the food condition, the parent flies were transferred again into the fresh bottles before being removed permanently in another 5 days. After a total of 18-25 days, progeny adults that emerged from the second and third bottles were collected, sexed, sorted for the presence or absence of the fluorescent protein marker, and then counted. The control bottles were set with the same number of flies, 40 wt pairs and 80 vasCas9 males, per bottle each generation. For the test bottles, the number of virgin wt females per bottle was based on the average number of females produced in the previous generation in the text bottles normalized that in the control bottles. The number of added females was calculated according to the formula N = 40 × (T/C), where N is the number of females added to the current generation of test bottles, T is the average number of females produced from the test bottles in the previous generation and C is the average number of females pro- duced from the control bottles in the previous generation. To keep the release ratio of pgSIT males constant,

N wt males mixed with 2N or 3N or 5N pgSIT males were added to the corresponding test bottles. The experiment was continued until no females were produced from the test bottles with the 1:3 pgSIT male release ratio.

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Executive Summary

Project Title: Engineering transgenic *Drosophila suzukii* for wild population suppression & eradication

Keywords: *Drosophila suzukii*, gene drive, precision guided sterile insect technique

Abstract: The spotted wing Drosophila (SWD) is a major worldwide crop pest of soft-skinned fruits. Current methods to control the SWD rely considerably on the use of expensive, broad-spectrum insecticides (e.g., malathion), which have variable efficacy, are difficult to use due to timing of fruit infestation, and face the risk of *SWD* evolving resistance. Additionally, use of broad-spectrum insecticides has led to disruption of integrated pest management systems developed for crops such as cherries and berries, and has had a serious impact on beneficial arthropods. However, there are no effective alternatives to managing the SWD infestation, and it is likely that, unless more effective control measures are developed, this pest will continue to spread.

Gene drives can be utilized to more rapidly spread desirable genes (e.g., susceptibility to a novel bio-friendly pesticide) to suppress/eradicate wild SWD populations. Such an approach is catalytic, with release of only modest numbers of engineered insects required to spread desirable genes or achieve population suppression. Additionally, since such a system relies on only a few releases of transgenic insects to do all of the work on an ongoing basis, it is affordable as compared to the use of insecticides, which need to be applied regularly. Finally, such an approach is environmentally friendly and entirely insect-specific and would have no effect on crops or on beneficial organisms. We initially built a *Medea* drive to support the rapid modification of SWD populations and in this project we made improvements to this drive system. Engineered *Medea* systems rely on a *Medea* element consisting of a toxin-antidote combination. The toxin consists of a miRNA that is expressed during oogenesis in *Medea*-bearing females, disrupting an embryonic essential gene. A linked antidote is expressed early during embryogenesis and consists of a recoded version of the target gene that is resistant to the miRNA. This combination results in the survival of half of the embryos originating from a *Medea*-bearing heterozygous female, as those that do not inherit the *Medea* element perish. If a heterozygous *Medea* female has mated with a heterozygous *Medea* male, the antidote from the male will also take effect in the embryo, resulting in 3/4 of the embryos surviving. Therefore, *Medea* will rapidly spread through a population, carrying any linked genes with it. However, since there is much uncertainty about whether gene drives will be acceptable for field use, or even needed, we also developed alternative technologies to control SWD, but will have a higher likelihood of gaining public and regulatory approval.

An alternative, highly promising approach that could complement existing control methods is genetic pest management, which includes strategies such as gene drive and transgenic-based precision-guided sterile insect Technique (pgSIT). SIT has been a successful technology for insect population suppression, which is achieved by introducing large numbers of sterile males into a target population. Over time, repeated mass releases of sterile males suppresses and can even eliminate the target population. This technique has been used to control other insect pests, so it has a clear regulatory pathway, but it uses irradiation-based methods, which are not technically feasible or scalable for the control of many insects. PgSIT, on the other hand, is a simplified way to generate sterile males and should be less expensive and labor intensive than irradiation based SIT even at scale. We therefore engineered pgSIT and synthetic *Medea* gene drives that can be used to suppress wild SWD populations. We developed multiple pgSIT strains that were able to efficiently generate 100% sterile male SWDs that were fit, competitive to wildtype males. When released into small laboratory populations of SWD, the pgSIT technology was able to eradicate SWD populations in only a few generations. Plans are now underway to test the pgSIT technology in confined field trials and if successful we hope this technology can be evaluated in the field in the next few years.

FINAL PROJECT REPORT **YEAR: 2021**

Project Title: A novel attract-and kill technique to manage Spotted-Wing Drosophila

PI: Dr. Vaughn M. Walton
Organization: Oregon State University
Telephone: 541-740-4149
Email: vaughn.walton@oregonstate.edu
Address: 2750 SW Campus Way
City/State/Zip: Corvallis/Oregon/97331

Co-PI (2): Dr. Gabriella Tait
Organization: Oregon State University
Telephone: 541-829-0860
Email: gabriella.tait@oregonstate.edu
Address: 2750 SW Campus Way
City/State/Zip: Corvallis/Oregon/97331

Co-PI (3): Steve Castagnoli
Organization: Oregon State University
Telephone: 541-386-2030
Email:steve.castagnoli@oregonstate.edu
Address: 3005 Experiment Station Drive,
City/State/Zip: Hood River, OR 97031

Contact information: Vaughn M. Walton, vaughn.walton@oregonstate.edu, 541-740-4149

Total Project Request: **Year 1:** $63,778 **Year 2:** $65,422 (none received)

Other funding sources: None
WTFRC Budget: None

Budget 1:
Organization Name: Oregon State University **Contract Administrator:** Charlene Wilkinson
Telephone: 541-737-3228. **Email address:**charlene.wilkinson@oregonstate.edu

Item	2020	2021 (no funding received)
Salaries	15,000	15,450
Benefits	9,375	9,656
Wages	14,000	14,420
Benefits	1,000	1,030
Equipment		
Supplies	9,443	9,726
Travel	6,000	6,180
Miscellaneous		
Plot Fees	8,960	8,960
Total	63,778	**65,422**

Footnotes: We submitted the original proposal for 2 years of funding i.e., 2020 and 2021. We completed work during 2020. This report is a summation of work conducted during 2020 only. Some additional details of trials in small fruit are provided in the discussion section.

Conflict of Interest statement: Vaughn Walton and Gabriella Tait are major shareholders of TerrAmor Inc., who obtained the Intellectual Property of the A&K technology from Oregon State University. TerrAmor Inc is currently seeking commercialization through EPA and state registrations.

ORIGINAL GOALS AND SIGNIFICANT FINDINGS

Spotted-wing drosophila (*Drosophila suzukii*, SWD) has emerged as a major pest of cherry since its establishment in the Pacific Northwest (PNW) in 2009. Due to the lack of effective alternatives, insecticides have been the mainstay of SWD management programs for PNW cherry growers. Multiple factors associated with reliance on insecticides (high cost, potential for resistance development, disruption of natural enemies, MRLs, etc.) make development of alternative approaches to SWD management imperative.

Behavioral controls offer an alternative to insecticides, especially if they can have a longer-term impact on SWD pest populations. Historically, studies on attractants have focused on fruit blends, which are believed to outcompete synthetic blends. Several commercially available lures focus on attraction based on fruit-derived volatiles, while little attention has been given to the manipulation of oviposition behavior (Haye et al. 2016; Cloonan et al. 2019). There are several technologies making use of behavior manipulation to control insect pests (Lee et al. 2011; Iglesias et al. 2014; Evans et al. 2017; Kirkpatrick et al. 2017), including a recently developed novel, pesticide-free behavioral disruptor technology, which can compete with ripening fruit in modulating the SWD oviposition behavior (Tait et al. 2018; Rossi-Stacconi et al. 2020). Previous laboratory and field tests on cherry and soft-skin fruits showed that the disruptor technology, which is composed of a proprietary matrix, causes an alteration of SWD behavior. This alteration has been described as an arrestant, with the altered behavior ultimately resulting in SWD adults being sequestered and arrested close to and on the matrix, and away from fruit.

The goal of this proposed project is to develop an effective attract-and-kill (A&K) technology for SWD for PNW cherry growers that are effective under field conditions beyond 21 days. Our trials will provide direct comparisons with the current grower standard. We anticipate the A&K technology will allow growers to reduce insecticide use by ~50%. The A&K technology will significantly reduce costs, with estimated savings ranging from 40-60%, but also other negative consequences of current practices, genetic resistance for instance (Gress & Zalom et al. 2019).

1) Evaluate multiple conventional and organic toxicants in combination with the arrestant under *laboratory* conditions in order to create an A&K tool for SWD.
2) Validate the new A&K formulation under *greenhouse conditions*.
3) Conduct long-term (21-day and beyond) *open-field efficacy trials* of the refined A&K tool, grower-standard (GS) pesticide applications, and integrated (INT, reduced insecticide reliance) as a direct comparison. We will assess the efficacy of this technology through fruit damage levels in cherries.

SIGNIFICANT FINDINGS

- We found in laboratory tests that the use of pesticide can be reduced by ~2,000 times when using the A&K in combination with Entrust, or any other conventional insecticide (data not shown). For Entrust combined with the A&K, we recorded statistically similar and numerically higher mortality fruit protection levels, even though the quantity of toxicant is magnitudes lower than toxicant applied as a full cover directly on fruit. When combining the A&K with toxicant, no direct applications of toxicant was applied on the fruit.
- Similar levels of fruit protection and SWD mortality was found under controlled laboratory conditions for the toxicants Grandevo and Venerate, with slight numerical improvements of toxicity and fruit protection. For Erythritol we did not observe any synergistic benefit combining these materials.
- In open field trials in Hood River, we found a trend of lower damage levels under open field conditions during the experimental period between untreated control plots and A&K plots.

- In open field trials in Salem, we found a significant reduction (~90% and ~50%) in damage levels in Buffer and A&K plots during the experimental period.
- Other open field trials in California and Oregon in small fruit resulted in a synergistic effect of using the A&K under standard commercial conditions with crop losses being ~45% lower in large scale plots where A&K was combined with conventional toxicants (data not shown).
- Additional field data show ~21-day activity/benefit of the A&K when used in such a systems.

METHODS

1) Laboratory evaluation of toxicant/arrestant combinations. This experiment consisted of three treatments: Berries dipped in pesticide (a), A&K treated with pesticide (b), and the untreated control (UTC) (c).

a. Berries dipped in pesticide. We tested four toxicants: Spinosad, Grandevo, Erythritol and Venerate in order to allow for direct comparison with the A&K solution. Here, concentrations of insecticides were conducted at field rate (Al/ha): Entrust 105.4 g (Al/ha), Grandevo 1,005.9 g (Al/ha), Venerate 17.7 kg (Al/ha) and Erythritol (1.75 M) (Al/ha).

b. Attractant treated with pesticide. To obtain the optimal A&K formulation, the arrestant was combined with the four toxicants trialed above. Each toxicant was tested by mixing it at the equivalent of ~1/2,000th of field rate.

c. UTC. No pesticide or A&K.

Pesticide-dipped berries and the A&K formulations were allowed to dry (1-1.15 hours) before they were placed within the test arenas.

The egg laying/mortality tests were conducted in the laboratory (72 ± 2 $^{\circ}$F, 62 ± 8 % R.H., and 14:10 L: D photoperiod) using 20 arenas (6-7 treatment containers per treatment) using ½ gal transparent Griffin-style graduated low-form plastic beakers (Nalgene, Rochester, NY), each with 9 ventilation holes (1/2 inch diameter) (Tait et al. 2018). The holes were covered with fine white mesh in order to prevent SWD individuals from escaping. The top of each beaker was drilled and were connected to a 0.5 cm diameter plastic tube providing a vacuum in order to create a constant and uniform air flow (1.5 L min^{-1}) within the containers. Beakers were placed upside down on a flat surface covered by white paper sheets. We placed three berries and one 3 oz plastic cup (Dart Container Corporation, Mason, MI) containing 3 gm of the A&K formulation containing each of the respective toxicants. Inside the arena, a ball of cotton soaked with water were provided as hydration to the test flies. Each container had ten mated females and ten mated males aged between 7 and 12 days. At 24h after the initiation of the experiment, the berries were inspected for oviposition, and the number of eggs per berry and dead insects were reported. UTC treatments (treatment c) consisted only of three berries inside each arena washed in water. For the cherries dipped in pesticide (i.e. Entrust, treatment a), or the A&K treated with pesticide (treatment b), the residual effect of the toxin in the A&K was monitored for 6 days by recording the oviposition in the berries and the number of dead flies. For treatments a and b each container also had three berries.

2) Controlled greenhouse evaluations. These experiments were not conducted during 2020 because of COVID 19 restrictions during this period.

3) Field evaluations 2020.

Hood River: We conducted field trials to determine field-efficacy under standard commercial cherry production conditions at the Mid-Columbia Agricultural Research and Extension Center (MCAREC, 45°68’515’’N, 121°51’67’’W). There were two treatments with the experiment conducted over a period of 35 days starting on June 16 through July 22, 2020.

1) Untreated Control (UTC). No insecticide was applied during the duration of the experiment.

2) Attract-and-kill (A&K). A&K treatments applied at day 0 (June 16), at the rate of 50 per acre. No additional chemical treatments.
There were 8 plots for the two treatments, each ~0.18 acres in size (~41 trees, Regina cv. sweet cherry each) within a total orchard of 2.8 acres (8 plots left blank). Each A&K plot therefore received a total of 8 dispensers. All plots were assigned in a gridded pattern for each treatment. Because volatile plumes from the A&K plots can be influenced by air movement, UTC plots will be situated upwind from the A&K plots to minimize interference caused by drifting volatile plumes originating from the dispensers placed in those plots. At first fruit color, we did supplemental releases of SWD in each plot. We released 200 mated 8–12-day-old SWD in the center of each plot (800 total, 400 females and 400 males) on a weekly basis in order to create a relatively even distribution of populations. These populations were released four times, on June 23, July 1, July 8, and July 15 of the experimental period. We collected cherries once per week. Each collection contained 10 cherries, respectively from the lower (3 ft), middle (5ft) and high (7ft) portion of the central two plants in each plot (30 per plant, 60 total per plot and 240 per treatment). Assessment of oviposition was determined considering the number of eggs laid per berry and percent of infected berries.

Willamette Valley: Field trials were conducted at a grower orchard (8-year-old Regina cv. sweet cherry) in Salem, Oregon from June 23-July 21, 2020, with the last sampling date concluding at harvest. There were three treatments and the experiment was conducted over a period of 29 days. There were five evaluation dates i.e. June 23, 29, and July 12 and 21. Five berries each were collected from each of four trees within the center of each of the experimental plots (total 20 berries collected from each experimental plot). Experimental plots were ~0.2 acres each, replicated ten times within a randomized block design (30 plots total, 6 acres total). Plots were oriented so that A&K were downwind from the prevailing wind direction.
1) Grower standard (GS) (2 acres total). Two insecticide applications i.e. Rimon/Delegate were applied on all plots (GS, Buffer and A&K) on June 24, and July 4, 2020. The pesticide applications were done as a tank mix of Rimon/Delegate at registered field rates, concluding at the appropriate preharvest interval before harvest.
2) Buffer (2 acres total). Two insecticide applications i.e. Rimon/Delegate were applied on all plots on June 24, and July 4. These plots were 40-60 feet away from the dispensers placed in the A&K plots.
3) Attract-and-kill (A&K, 2 acres total). Two insecticide applications i.e. Rimon/Delegate were applied A&K plots on June 24, and July 4. A&K dispensers were applied on June 23, 2020. The A&K treatments consisted of placing the hemp fiber substrate (10 x 10 x 0.5 cm, BioComposit, Alberta, Canada) at the base of every 4th tree in a shaded position. The treatments were applied at the rate of 50 per acre (10 per 0.2 acres). Drip irrigation was supplied every day ~5pm from the initial placement up to July 12. There were 30 plots, each ~0.2 acres in size for a total of ~6 acres. Because volatile plumes from the A&K plots can be influenced by air movement, GS plots were situated upwind from the Buffer and A&K plots to minimize interference caused by drifting volatile plumes originating from the dispensers placed in those plots. Assessments of oviposition were determined by counting the number of eggs laid per berry, enabling determination of percentage of infected berries. Environmental data was collected during the field trials using data loggers (HOBO U23 Pro v2 Temperature/%RH; Onset Computer Corp., Bourne, MA) placed in the bottom, middle and top part of the trees. The data loggers measured ambient air temperature (°F), and relative humidity (%RH).

Statistical Analysis
Data from laboratory double-choice experiments and oviposition trials was analyzed using a Kruskall Wallis test. They were applied to separate differences at $\alpha < 0.05$. Field trial data were analyzed using factorial ANOVA tests in R-studio.

Results:

1) Evaluate toxicants in combination with the A&K under *laboratory* conditions to create an A&K tool for SWD.

Erythritol: Erythritol alone and A&K plus Erythritol (17.86, 33.14 eggs per berry respectively) resulted in significantly lower oviposition compared to the control (52.28 eggs per berry) treatments ($\chi^2 = 21.63$, $P < 0.001$). No dead flies were recorded after 24 hours of exposure in any of the controls or treatments.

Entrust: Results from 1 day-exposure periods showed a significantly lower oviposition rate in Entrust and A&K plus Entrust compared to the UTC ($\chi^2 = 42.32$, $P < 0.001$). No statistical differences were recorded between the two treatments containing toxicant. Entrust alone and A&K plus Entrust (17.76, 15.78 eggs per berry respectively) resulted in significantly lower oviposition compared to the UTC (49.82 eggs per berry) treatments ($\chi^2 = 3.655$, $P = 0.05$) over the experimental period. The number of dead flies was 1.28 in the UTC, compared to Entrust and A&K plus Entrust that had much higher mortality (9.03 and 8.32 respectively).

Grandevo: Treatments containing toxicant (Grandevo and A&K plus Grandevo) resulted in no differences compared to UTC treatments. Grandevo alone and A&K plus Grandevo (24.14, 17.71 eggs per berry respectively) resulted in numerically lower oviposition compared to the UTC (27.11 eggs per berry) treatments ($\chi^2 = 4.878$, $P = 0.086$) over the experimental period. No statistical difference in mortality rates were recorded between the two treatments containing Grandevo either ($\chi^2 = 1.949$, $p = 0.178$). The number of dead flies at 24 hours was 0 in the UTC, 4 in the Grandevo and 0 in the A&K plus Grandevo. At 48 hours the number of death flies was 0 in the UTC, 46 in the Grandevo and 56 in the A&K plus Grandevo.

Venerate: Here, Venerate and A&K plus Venerate resulted in lower numbers of eggs compared with UTC treatments ($\chi^2 = 7.691$, $P = 0.021$). Venerate alone and A&K plus Venerate (19.85, 18.42 eggs per berry respectively) resulted in lower oviposition compared to the UTC (30.14 eggs per berry) No statistical mortality difference was recorded between the two treatments containing Venerate ($\chi^2 = 0.126$, $p = 0.724$). The number of dead flies at 24 hours was 1 in the UTC, 0 in the Venerate and 3 in the A&K plus Venerate. At 48 hours the number of dead flies was 0 in the UTC, 0 in the Venerate and 3 in the A&K plus Venerate.

2) Validate the new A&K formulation under *greenhouse conditions*.

This portion of the experiment was not conducted because of COVID19 greenhouse facility shutdown during the planned experimental period.

3) Conduct long-term (21-day and beyond) *open-field efficacy trials* of the refined A&K tool, grower-standard (GS) which include pesticide applications, and buffer plots as a direct comparison.

Hood River:

Field experiments indicated a numerical (no statistical differences recorded) reduction of eggs laid. The overall reduction of eggs laid in fruit during the experimental period was 11% in the A&K plots compared to the UTC plots during the entire experimental period ($F_{2,4} = 0.093$, $p = 0.91$, Table 1). When looking at the respective sampling dates, the A&K treatment plots resulted in a numerically lower level of eggs compared to the UTC plots (Figure 1). On June 23 (7 days after placement), July 8 (21 days after placement) and July 23 (28 days after placement) the reductions in SWD egg laying were 44, 30 and 28% respectively.

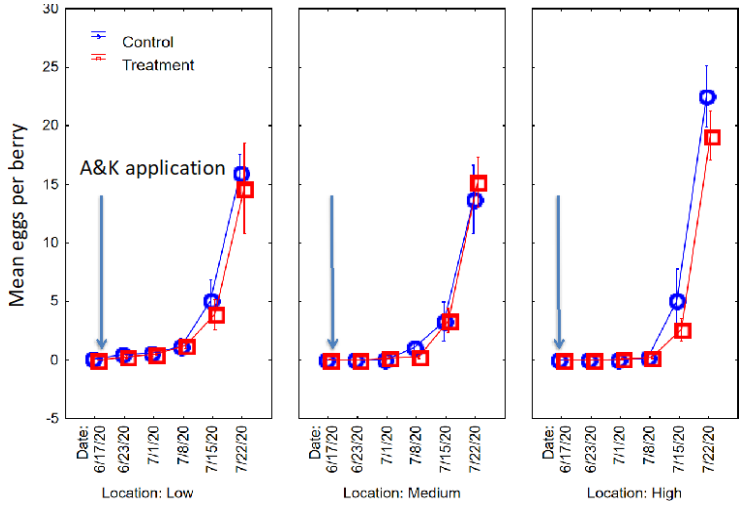

Figure 1. Mean number of *Drosophila suzukii* eggs per berry (±SEM) in a research cherry production block in Hood River, Oregon from June 17-July 22, 2020. The Attract and Kill (A&K) applications was done on June 17 (indicated by arrows).

Willamette Valley:

Field experiments indicated a statistical difference in the reduction of eggs laid in the buffer compared to the UTC plots. During the overall experimental period, reduction of eggs laid in fruit was 92.8 and 50% lower in the buffer and A&K plots respectively compared to the Grower Standard plots ($F_{1,3} = 2.88$, $P < 0.022$, Table 1). There were 6.5 and nearly 2X times less infested fruit in buffer and A&K plots compared to the Grower Standard plots. The majority of *D. suzukii* infestation happened during the last week before crop harvest, with 82% of eggs laid during this period.

Table 1. Mean number of *Drosophila suzukii* per berry (±SEM) and percent infested berries in a conventional cherry production block in Hood River and Salem, Oregon from June 17-July 22, and June 23-July 21, 2020 respectively. Numbers with different letters are statistically different.

Treatment	**Mean eggs/berry**	**% Infested**
Hood River		
Untreated Control	3.8±0.81 ns	36
Attract and Kill	3.4±0.76 ns	34.9
Salem		
Buffer	0.011±0.004 B	0.8
Grower Standard	0.152±0.058 A	5.2
Attract and Kill	0.076±0.038 A	3.1

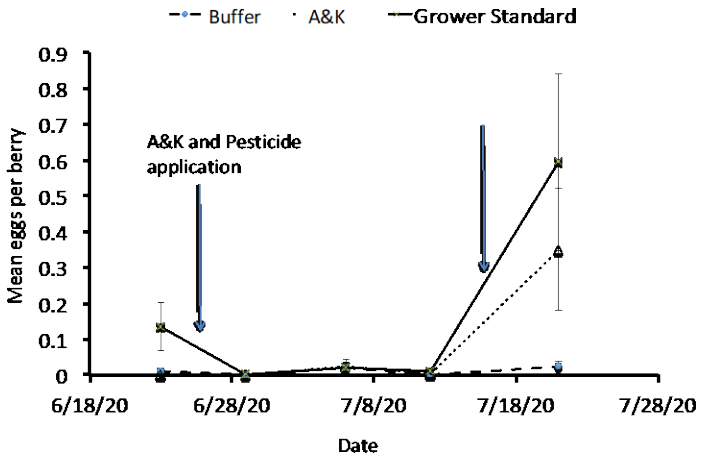

Figure 2. Mean number of *Drosophila suzukii* eggs per berry (±SEM) in a conventional cherry production block in Salem, Oregon from June 23-July 21, 2020. Pesticide and A&K applications are indicated by arrows. One A&K application was applied on June 24.

The combination of A&K containing insecticide shows promise, potentially resulting in similar levels of control of SWD under field conditions. The A&K used alone under high pressure conditions in Hood River resulted in a trend of reduced damage due to SWD. In Salem, where growers used pesticides in combination with the A&K, the damage was lower in and adjacent to plots containing the matrix. Data (not shown) generated by third parties in California, and Georgia resulted in similar reductions in SWD damage, attributable to the matrix.

Additional large-scale open-field trials (200-2,000 acres total, 2020-2021) in California and Oregon small fruit using the A&K technology plus insecticide resulted in an average of 45% savings in crop when compared to insecticide only. Crop rejections due to SWD damage were eliminated using this technology in multiple cases. The A&K is currently undergoing EPA registration and commercialization.

References

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Tait, G., Kaiser, C., Stacconi, R., Dalton, D.T., Anfora, G. and Walton, V.M., 2018. A food-grade gum as a management tool for *Drosophila suzukii*. 71(2), pp.295-307.

EXECUTIVE SUMMARY

Project Title: A novel attract-and kill technique to manage Spotted-Wing Drosophila

Key words: Synergistic impacts, production efficacy, pest management

Abstract: We tested a newly licensed and patent pending technology from Oregon State University to determine potential benefits for the management of Spotted-Wing Drosophila in cherry. The active ingredient is dispensed in commercial field conditions at the rate of 50/acre directly under the dripline. Previous work indicated placement rate, location and field longevity (~21 days). Laboratory tests helped explain why growers found a synergistic impact of the technology when used in combination with insecticides. Use of A&K can be aimed either to reduce pesticide dependency, or increase efficacy over extended periods up to 21 days. Fruit dipped in toxicant only were protected from SWD at similar levels compared to A&K where no residues are sprayed onto berries. In open field trials in Hood River, we found a trend of lower damage levels under open field conditions during the experimental period between untreated control plots and A&K plots (no toxicants used). In open field trials in Salem, we found a significant reduction (~90% and ~50%) in damage levels in Buffer and A&K. Here it is likely the full cover pesticide that acted synergistically to reduce damage levels. Previous work showed that commercial toxicants can be used synergistically with the A&K technology to lower crop damage by ~45% over larger areas.

CONTINUING PROJECT REPORT **PROPOSED DURATION**: 2 Years

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

PI: Nik Wiman
Organization: Oregon State University
Telephone: 541-250-6762
Email: nik.wiman@oregonstate.edu
Address: North Willamette Research and Extension Center
Address 2: 15215 NE Miley Rd
City/State/Zip: Aurora, OR

Co-PI (2):
Organization:
Telephone:
Email:
Address:
Address 2:
City/State/Zip:

Cooperators: JD Walker, Les Stephens, Chris Adams

Total Project Request: **Year 1:** $21,443 **Year 2:** $18,249 **Year 3:** (Not originally proposed, but potentially beneficial to extend)

Other funding sources: **None**
Amount:
Agency Name: Oregon Sweet Cherry Commission
Notes:

WTFRC Budget: None

Budget 1
Organization Name: Oregon State University, Agricultural Research Foundation
Contract Administrator: Charlene Wilkinson
Telephone: 541-737-3228
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 6 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**).

- We examined the capture of SWD on dry traps to evaluate the relationship between captures and SWD fruit infestation data. Two Trece lure formulations were evaluated along with one AlphaScents trap. Three reps of each trap were deployed at each site.
    - SWD were caught in Trece dry traps starting in mid-June. The highest numbers were found at the higher elevation site (potential heat evasion strategy), and some interesting trends were detected (**Fig. 2**)
        - 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. (**Fig. 3**)
            - 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.

- Firmness and size of fruit were captured by the FirmTech machine on a weekly basis for 6 cherry cultivars, both blush and dark.
    - These firmness data followed distinct trajectories that can be easily modeled (**Fig. 4**)
    - If firmness turns out to be one the better predictors of fruit infestation, it may be predictable with a phenology model.

- 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 (**Fig. 5**).

- SWD populations were very low, with a very low level of infestation of fruit detected by A) the salt test to force larvae from fruit (no larvae ever found using this method), and B) rearing insects from harvested fruit (**Fig. 6**).
    - Extremely dry and hot conditions were a big factor.
        - Fortunately, fruit were largely harvest by the major 'heat dome event' at the end of June.
        - Fruit were severely burned after temperatures reach 112 °F.
    - Another tactic we used to evaluate fruit susceptibility was to artificially infest fruit harvested fruit with SWD reared in the lab.
        - Experimental protocols to improve natural and artificial infestation of fruit will be made to better take advantage of this tactic to evaluate fruit susceptibility.

- 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

- 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.

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

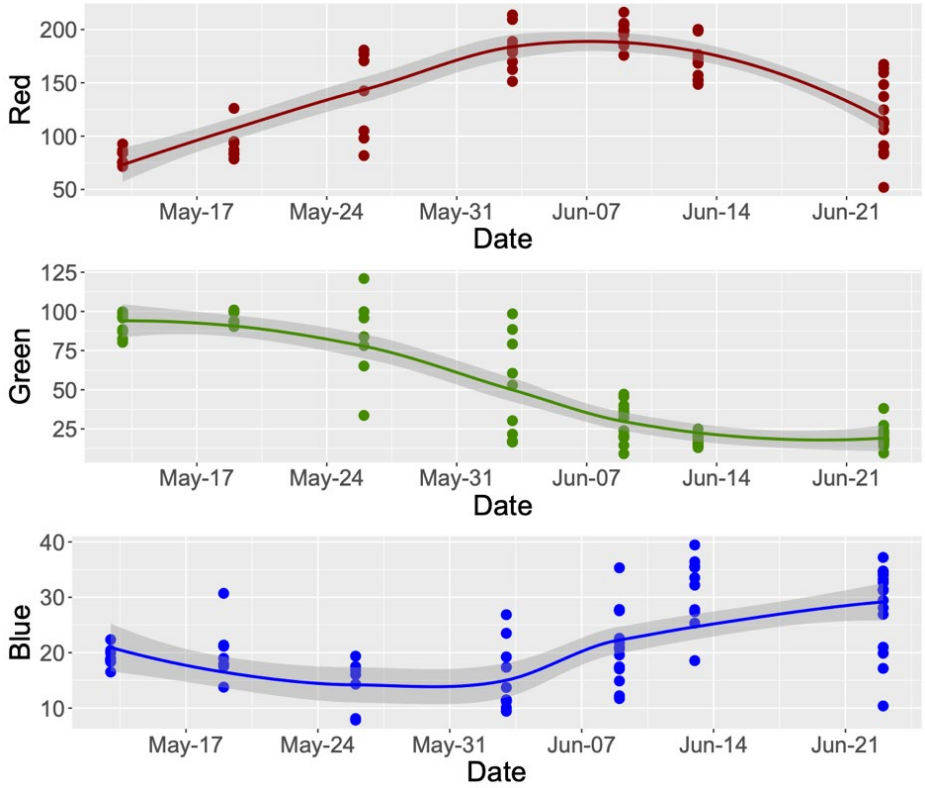


Figure 1. *RGB analysis of color in cv. 'Rainier' as fruit matures and ripens. RGB data was taken from individual photographs of fruit. Cell phone apps can also provide this information,*

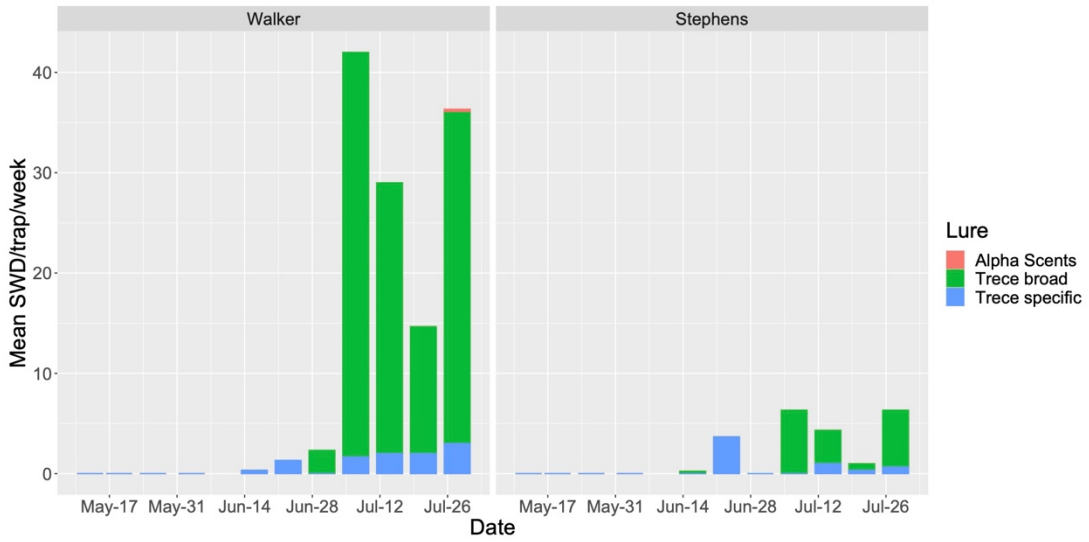


Figure 2. *Captures of SWD on 'dry' traps at the two research sites.*

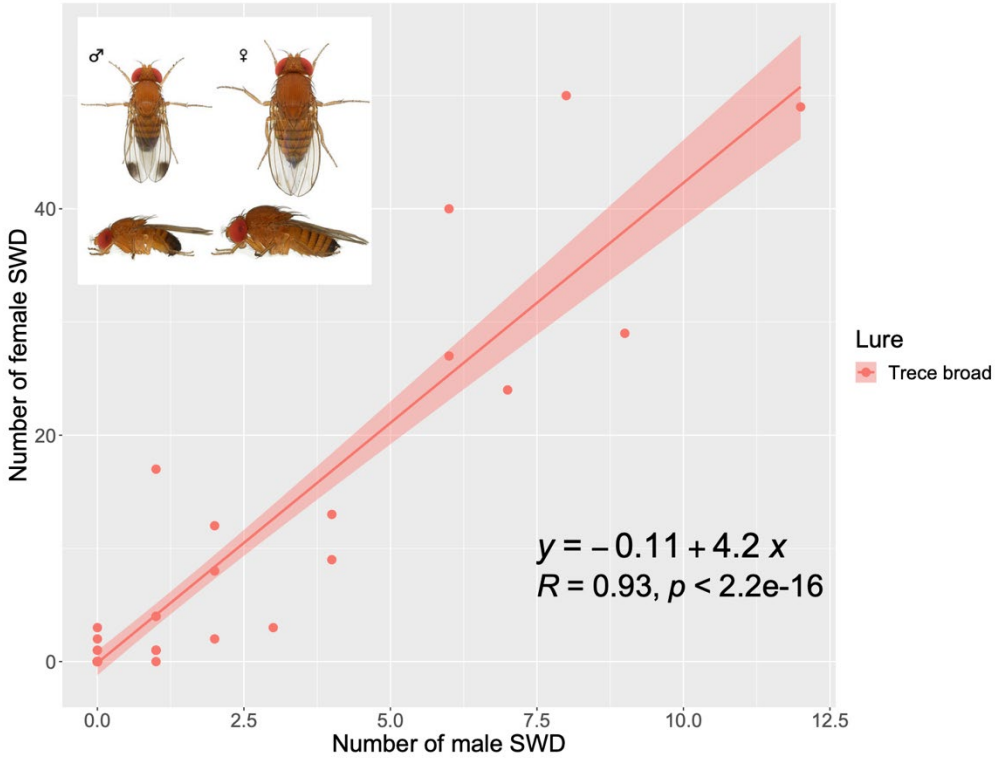


Figure 3. *More females than males were caught on the Trece broad spectrum trap, but the number of males caught was very predictive of the number of females caught.*

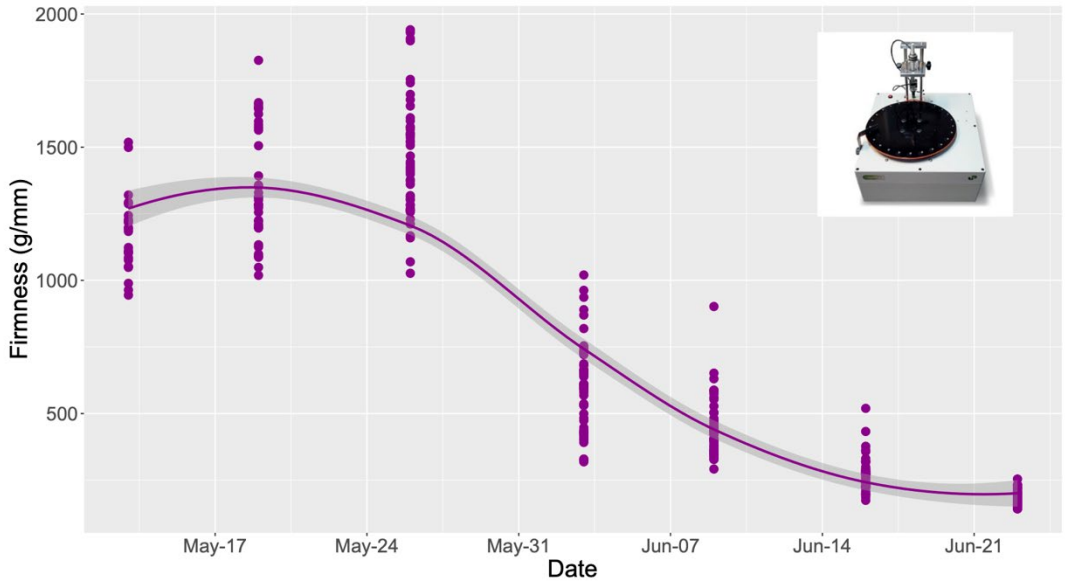


***Figure 4**. Firmness of cv. 'Rainier' over the fruit maturation period as determined by the FirmTech. Firmness followed a very well-defined pattern that can be modeled with high confidence.*

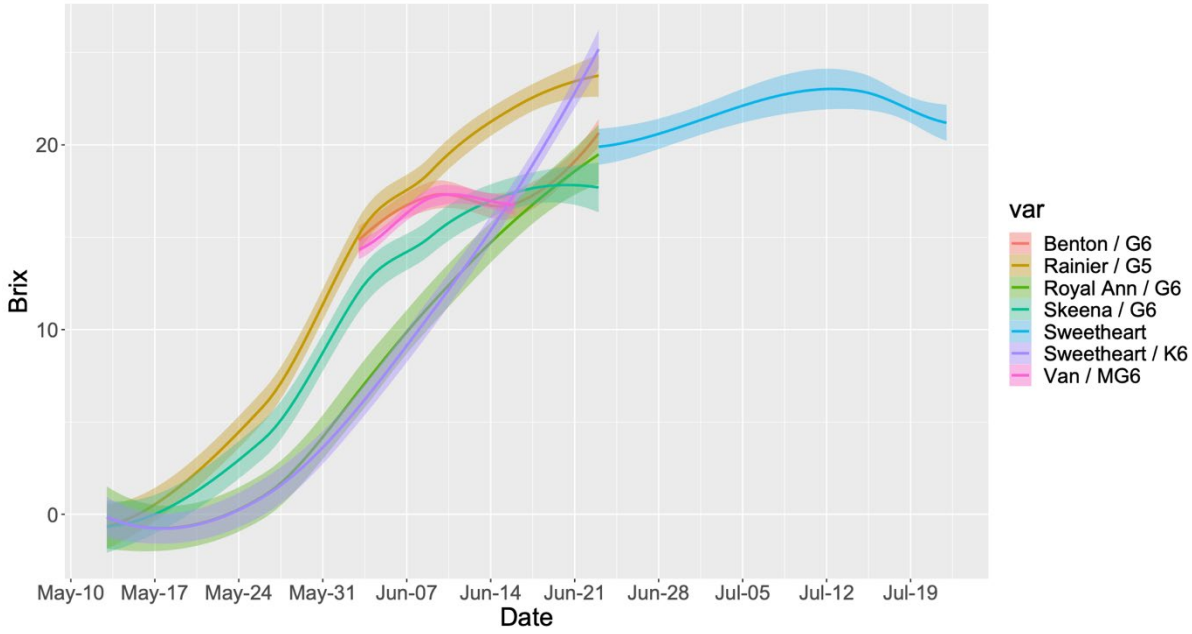


***Figure 5**. Brix and pH (not shown) data for six cultivars shows important cultivar differences that could potentially affect susceptibility to SWD at different times of the season.*

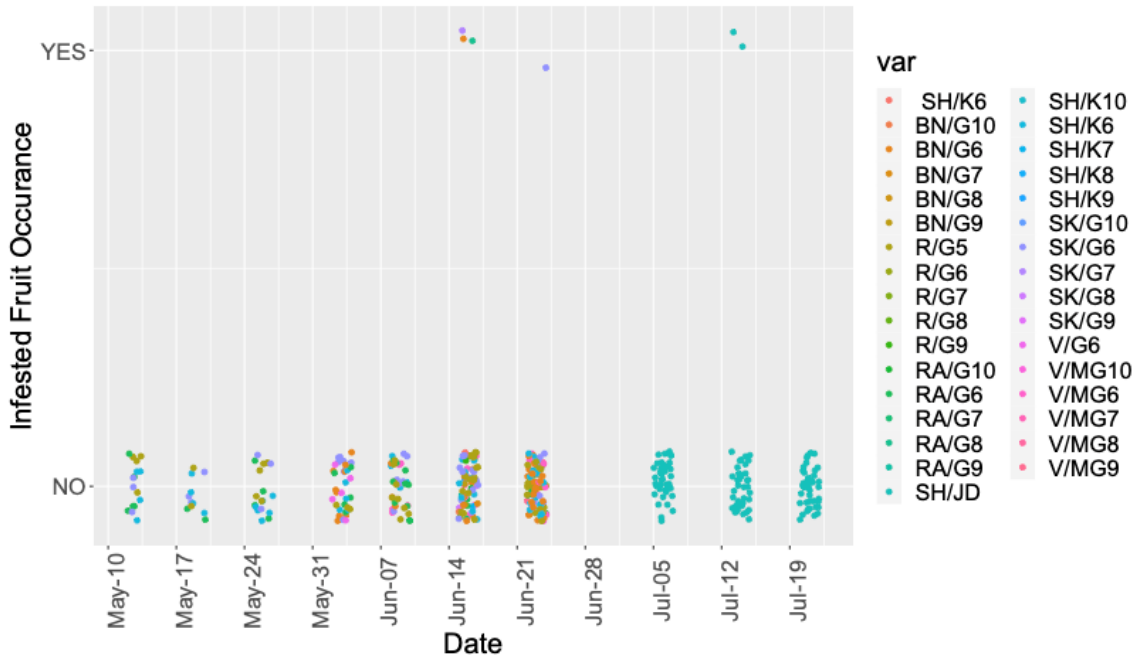


***Figure 6**. Fruit infestation was uncharacteristically low due to excessive heat and drought. Only five cases of fruit infestation were noted out of thousands of checked samples. We are considering increasing sample sizes if dry conditions persist in year 2.*

CONTINUING PROJECT REPORT **YEAR**: 2 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

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 (est.)
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 10% 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

2021 WTFRC CHERRY PESTICIDE RESIDUE STUDY

Since 2011, the WA Tree Fruit Research Commission has conducted annual studies of residues of commonly used pesticides on cherry fruit at harvest. Digital versions of this report and similar studies on apple and cherry including comprehensive summaries of multiple years' results are available at www.treefruitresearch.org. For current information on maximum residues levels (MRLs) and other regulatory issues, please consult the Northwest Horticultural Council website at http://nwhort.org/export-manual.


New Skeena trial block

TRIAL DETAILS

- Mature 'Skeena'/Mazzard multiple leader open vase trees on 10' x 16' spacing near Orondo, WA
- 12 insecticides/acaricides & 7 fungicides applied at or near maximum rates and minimum pre-harvest and re-treatment intervals
- Ground applications made by Rears PakBlast PTO-driven airblast sprayer of the same rate of product per acre with 8 oz Regulaid surfactant/100 gal water at 200 gal water/acre
- No measurable precipitation fell on trial block over the course of the study except 0.14" of rain roughly 10 days before harvest (June 13-14)
- Grower applied RainGard 3 times during the study (5/25, 6/4, 6/11) to reduce cracking, which may have helped preserve residues of pesticides applied by WTFRC staff
- Samples submitted overnight to Pacific Agricultural Labs (Sherwood, OR) for chemical analysis

RESULTS & DISCUSSION

These studies have historically been conducted in mature 'Bing' blocks in a commercial orchard near Orondo, but those older blocks have been removed, forcing the relocation of test plots to a mature 'Skeena' block in the same orchard. As in previous years, this study generally simulates a *worst case scenario* for residues of legally applied pesticides by using aggressive rates, timings, and spray intervals. Most materials were applied twice as allowed by product labels, whether or not typical commercial use patterns would do the same. With that approach, all residues complied with domestic tolerances but most **exceeded some foreign tolerances,** whether from published MRLs or national default values:


Dried residues on fruit at harvest

Insectides/acaricides: Centaur WDG, Bexar, Agri-Mek 0.15SEC, Perm-Up 3.2EC, Verdepryn 100SL, Danitol 2.4EC, Carbaryl 4L, Onager

Fungicides: Torino, Gatten, Orbit, Topsin 4.5FL, Miravis

Some 2021 residue levels may be somewhat elevated due to grower applications of a rain protectant; previous WTFRC studies (2013-2015) demonstrated a tendency for rain protectants (RainGard, Parka) to preserve residues on cherry. MRLs are known to change frequently and cherry producers should routinely monitor the most current information (http://nwhort.org/export-manual) to facilitate compliance with constantly shifting foreign standards.

Measured residue levels vs. MRLs for pesticides applied to cherry fruit at 200 gal water/acre. 'Skeena'/Mazzard, Orondo, WA. WTFRC 2021.

Common name	Trade name	Application rate[1]	Application timing(s)	Measured residue	US tolerance[2]	Lowest export tolerance[3]
		per acre	*days before harvest*	*ppm*	*ppm*	*ppm*
buprofezin	Centaur WDG	34.5 oz	28, 14	**1.8**	2	0.01 (EU/UK)
tolfenpyrad	Bexar	27 oz	28, 14	**0.54**	2	0.01 (many)
abamectin	Agri-Mek 0.15SEC	20 oz	21	**0.043**	0.09	0.01 (EU/UK)
thiamethoxam	Actara	5.5 oz	21, 14	0.18	0.5	0.5 (many)
chlorantraniliprole	Altacor	4.5 oz	21, 10	0.098	2.5	0.5 (Korea)
acetamiprid	Assail 70WP	3.4 oz	21, 7	0.49	1.5	1 (Taiwan)
permethrin	Perm-Up 3.2EC	8 oz	14	**0.30**	4	0.05 (EU/UK)
flutriafol	TopGuard	14 oz	14, 7	0.34	1.5	0.8 (many)
cyclaniliprole	Verdepryn 100SL	11 oz	14, 7	**0.18**	1	0.01 (EU)
sulfoxaflor	Transform WG	2.75 oz	14, 7	0.73	3	1.5 (many)
cyflufenamid	Torino	8 oz	14, 7	**0.13**	0.6	0.02 (Australia)
fenpropathrin	Danitol 2.4EC	21.3 oz	14, 3	**0.74**	5	0.01 (EU/UK)
carbaryl	Carbaryl 4L	96 oz	10, 3	**2.5**	10	0.01 (EU/UK)
flutianil	Gatten	8 oz	10, 3	**0.051**	0.4	0.01 (EU/UK)
myclobutanil	Rally 40WSP	6 oz	10, 1	0.41	5	1 (Can, Taiwan)
propiconazole	Orbit	4 oz	10, 1	**0.24**	4	0.01 (EU/UK)
thiophanate-methyl*	Topsin 4.5FL	30 oz	10, 1	**0.42**	20	0.3 (EU/UK)
pydiflumetofen	Miravis	5.1 oz	10, 1	**0.093**	2	0.01 (EU, Japan)
hexythiazox	Onager	24 oz	7	**0.27**	1	0.1 (Korea)

[1] All materials were applied by Rears PakBlast sprayer with 8 oz Regulaid/100 gal water
[2] 13 Aug 2021. http://nwhort.org/export-manual/comparisonmrls/cherry-mrls/
[3] Major export markets for Pacific Northwest cherries; 13 Aug 2021; tolerances may be based on published MRLs or default values. http://nwhort.org/export-manual/comparisonmrls/cherry-mrls/
* Reported thiophanate-methyl values reflect sum total of thiophanate-methyl and carbenzadim residue levels

For more information, contact Tory Schmidt (509) 669-3903
or email tory@treefruitresearch.com

WASHINGTON TREE FRUIT RESEARCH COMMISSION

Results of this lone unreplicated trial are shared for informational purposes only and should not be construed as endorsements of any product, reflections of their efficacy against any arthropod or fungal pest, or a guarantee of similar results regarding residues for any user. Cherry growers should consult with extension team members, crop advisors, and warehouses to develop responsible pest control programs.

Project/Proposal Title: Supporting a robust PNW sweet cherry breeding and genetics program

Primary PI: Per McCord
Organization: Washington State University
Telephone: 509-786-9254
Email: phmccord@wsu.edu
Address: WSU IAREC
Address 2: 24106 N. Bunn Road
City/State/Zip: Prosser, WA 99350

Co-PI 2: Cameron Peace
Organization: Washington State University
Telephone: 509-335-6899
Email: cpeace@wsu.edu
Address: Johnson Hall 39
Address 2: PO Box 64614
City/State/Zip: Pullman, WA 99164

CO-PI 3: Bernardita Sallato
Organization: Washington State University
Telephone: 509-786-9201
Email: b.sallato@wsu.edu
Address: WSU IAREC
Address 2: 24106 N. Bunn Road
City/State/Zip: Prosser, WA 99350

Co-PI 4: Steve Castagnoli/Kelsey Galimba
Organization: Oregon State University MCAREC
Telephone: 541-386-2030 x38220
Email: steve.castagnoli@oregonstate.edu, kelsey.galimba@oregonstate.edu
Address: OSU MCAREC
Address 2: 3005 Experiment Station Dr
City/State/Zip: Hood River, OR 97031

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

Report Type: Final Project Report

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $48,623
Total Project Request for Year 2 Funding: $174,559
Total Project Request for Year 3 Funding: $183,584

Other related/associated funding sources:

Awarded
Funding Duration: 2014-2019
Amount: $10 million
Agency Name: USDA NIFA—SCRI
Notes: "RosBREED: Combining disease resistance with horticultural quality in rosaceous cultivars". PI: Amy Iezzoni. Multiple Co-PI's including Cameron Peace and Per McCord

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
Funding Duration: 2019
Amount: $79,000
Agency Name: WTFRC/OSCC
Notes: "Equipping the re-launched PNW cherry breeding program"

Awarded
Funding Duration: 2019-2020
Amount: $88,000
Agency Name: WTFRC/OSCC
Notes: "Durable genetic solutions to powdery mildew infection in sweet cherry". PI: Cameron Peace. Co-PIs: Per McCord, Prashant Swamy.

Awarded
Funding Duration: 2020-2022
Amount: $458,022
Agency Name: WTFRC/OSCC
Notes: "Understanding little cherry disease pathogenicity". PI: Scott Harper. Co-PIs: Alice Wright, Per McCord.

WTFRC Collaborative Costs: None

Budget 1
Primary PI: Per McCord
Organization Name: Washington State University
Contract Administrator: Katy Roberts
Telephone: 509-335-2885
Contract administrator email address: arcgrants@wsu.edu
Station Manager/Supervisor: Naidu Rayapati
Station manager/supervisor email address: naidu.rayapati@wsu.edu

Item	(2019)	(2020)	(2021)
Salaries[1]	$45,760	$37,440	$38,938
Benefits	$19,493	$16,230	$17,327

Wages[2]	$31,200	$32,450	$33,750
Benefits[3]	$10,564	$5,390	$5,606
Equipment			
Supplies[4]	$9,760	$33,325	$52,363
Travel	$4,000	$5,500	$6,100
Miscellaneous[5]	$40,000	$19,259	$2,500
Plot Fees	$4,275	$7,630	$8,800
Carryover from 2018 request	-$132,665		
Total	$32,387	$157,224	$165,384

Footnotes: [1]Includes Horticultural Support in 2019 (only), plus 1.0 FTE research technician. [2]Includes temporary labor for crossing, harvesting, seed extraction/transplanting, plus farm crew wages. [3]Reduction of benefit costs for 2020-21 reflects a more accurate estimate based on actual 2019 expenses. [4]Supplies for fruit evaluation, DNA extraction/genotyping, embryo rescue, propagation supplies/services, orchard maintenance. Amount is increased from original request as a result of more detailed expense information. [5]Irregular expenses. Amount is reduced from original request as a result of more detailed expense information.

Budget 2
Co PI 2:
Organization Name: Oregon State University MCAREC
Contract Administrator: Charlene Wilkinson
Telephone: 541-737-3228
Contract administrator email address: charlene.wilkinson@oregonstate.edu
Station Manager/Supervisor: Steve Castagnoli
Station manager/supervisor email address: steve.castagnoli@oregonstate.edu

Item	Type year of project start date here	(Type year start date of year 2 here if relevant)	(Type year start date of year 3 here if relevant)
Salaries[1]	$5,405	$6,005	$6,305
Benefits	$4,486	$4,985	$5,234
Wages[2]	$3,840	$3,840	$4,032
Benefits	$384	$384	$403
Equipment			
Supplies			
Travel			
Miscellaneous[3]	$2,121	$2,121	$2,226
Plot Fees			
Total	$16,236	$17,335	$18,200

Footnotes: [1] Estimated salary for technician to complete pruning, thinning and data collection. [2] Wages for one part-time employee ($16/hr) to assist with orchard activities. [3] Fees include per-acre research plot fees ($3104/acre), 2 months cold storage room fee ($1.24/square foot) and miscellaneous lab supplies.

Original Objectives & Significant Findings

1. *Build a well-trained support team to maintain and improve horticultural practices in the breeding orchard and maximize breeding efforts*
    - WTFRC/OSCC and WSU startup-funded technicians hired in January 2019
        - New WSU-funded technician hired October 2020
        - WTFRC/OSCC funded technician voluntarily separated July 2021
        - WSU-funded technician voluntarily separated September 2021
        - Moving forward, will request support for one WTFRC/OSCC funded technician, second technician (approximately 0.3 FTE) supported via breeding program royalties
    - Solid training in horticultural practices received from Bernardita Sallato, Matt Whiting, Mark Hanrahan, Tom Auvil, and other industry partners.
    - Breeding program transitioning from the Roza to IAREC headquarters (all plantings since 2019 have been here)
    - Orchard blocks fertilized according to results of soil and foliar analyses. Fertigation for new P1 plantings
    - Parental trees and P2 selections screened for presence of *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV), *little cherry virus 1,2* (LChv-1,2), and X-disease phytoplasma (XDP)
        - PDV is common in breeding blocks, PNRSV and XDP rare (but not absent)
        - No known infections with LChv-1,2
        - All trees known to be infected with XDP removed (including seedlings)

2. *Continue to rigorously evaluate existing selections in Phase 2 (P2) and seedlings in Phase 1 (P1). Advance selections as warranted to Phase 3 (P3)*

    - Incorporated modified atmosphere packaging for postharvest samples
    - Digital data collection at Prosser (no more paper forms)
    - Evaluated 17 P2 selections (10 multi-location, 7 only at Prosser)
    - Three selections advanced to Phase 3 trials: 'R3', 'R19', 'R29'
    - P1 seedlings advanced to P2: 2 (2019), 2 (2020), 3 (2021)
    - Removed oldest seedlings from C52 (361 trees)
    - New P2 plantings made at Prosser, Pasco (Sagemoor), and Hood River (MCAREC)
        - Replicated, randomized trials (12-15 trees per selection per site)
3. *Increase the number of targeted crosses made, seeds germinated, and seedlings transplanted*
    - 230 crosses made since program restart (2018) resulting in more than 22,400 seed
        - More than 68% of seed from bi-parental crosses
        - Over 4,170 new seedlings transplanted to the field, guided by DNA tests for self-fertility and powdery mildew resistance
    - Successfully employed embryo rescue for crosses targeting early ripening. Recovery rate increased via seed coat removal.
4. Enhance precocity and reduce external variation in the seedling blocks (*delayed 1 year*)
    - Low success rate (<10%) with initial attempts in 2020
    - In 2021, test budding of greenhouse-grown, cold-acclimated seedlings onto Gisela-12 rootstock (in the field) had success rate of 69% (single budding)
    - Applied limb bending and girdling in 2019 seedling planting (own roots); will evaluate for presence of floral buds after leaf drop in 2021 and during bloom in 2022.

Results & Discussion

1. *Support team and horticultural practices*

Michael Stein (WSU-funded) and Corina Serban (WTFRC/OSCC-funded) both began working for the CBP as full-time technicians in January 2019. Dr. Stein left in June 2020, and was replaced in October 2020 by Juhi Chaudhary. Ms. Serban left in July 2021 to work for WSU Extension, and Dr. Chaudhary left in September 2021 to work for Syngenta. Moving forward, the CBP will be requesting funds for a new 3-year project to support one technician at 1.0 FTE. A second technician will be hired on a part-time basis (approximately April-August), supported by royalty income. The breeding program has built a good working relationship with the IAREC farm manager and farm crew, allowing them to accomplish the majority of orchard maintenance under Dr. McCord's overall direction. This relationship should allow the program to continue to function at an adequate level without two full-time technicians.

Throughout the project, training in orchard management and horticultural practices has been provided by Bernardita Sallato, Dr. Matt Whiting, Mark Hanrahan, Tom Auvil, and other BPAC and industry members. Through the efforts of Dr. McCord, Ms. Serban, and close cooperation with our crop consultant (Jeff Sample) and the IAREC farm manager and crew, we have been able to maintain good orchard management practices. Control of insect pests and powdery mildew was acceptable, and irrigation has been accomplished in a timely manner. In 2021, postharvest sprays to control leafhoppers have been applied at 2-3 week intervals, and an October application of urea and zinc sulfate should speed defoliation, further discouraging leafhopper feeding.

As in prior years, the Hood River (MCAREC) and Pasco (Sagemoor) blocks were pruned during the winter, as well as the main parental block at the Roza (B53), and the 2019 P1 planting at IAREC headquarters. The RosBREED block (C53) and younger P1s at the Roza were pruned during the summer, as well as the 2020 P1 planting at IAREC.

Throughout the project, trees were evaluated for disease via bioassays (on 'Shirofugen' indicator rootstock), ELISA, or PCR. The main diseases of interest were the ilarviruses (Prune dwarf virus (PDV) and *Prunus* necrotic ringspot virus (PNRSV), little cherry viruses 1 and 2 (Lchv-1,2), and X-disease phytoplasma (XDP). Presence of PDV is high in the breeding program. We have removed the majority of known PDV-infected trees, but since there are rarely if ever symptoms of PDV (aside from lower bud take), we are focusing our efforts more on avoiding the use of infected material in making crosses. All trees known to be infected with PNRSV and XDP have been or will be removed by the end of October 2021 (no trees have been found to be infected with Lchv-1 or 2). PCR inhibitors in samples taken by CBP personnel for XDP/Lchv1,2 screening have limited our in-house ability to screen for these pathogens. We will continue to work with the Clean Plant Center NW (CPCNW) to refine our protocols, including cleanup and retesting of archived samples.

2. *P1 and P2 evaluations*

As in prior years, BPAC members were invited to inspect P1 seedlings during the fruiting season. Walkthroughs were conducted 1-2 times per week, with BPAC members visiting once per week. Selection criteria in the field was based on fruit size, firmness, and flavor. Fruit from selected P1 seedlings, all current P2 selections, and standard cultivars were evaluated in the laboratory for defects (harvest and post-harvest), weight, diameter, firmness, stem pull force (P2 only), color, Brix, and titratable acidity.

In order to maximize efficiency, P1 selections that did not meet the thresholds of weight (minimum 9 grams) or firmness (minimum 270 g/mm) generally were not evaluated for downstream traits. An 'induced pitting' protocol for post-harvest analysis was implemented by putting fruit in a bucket on an orbital shaker platform for 3 minutes at 200 rpm. When sufficient fruit was available, we also performed an induced cracking test based on a 4-hour soak in deionized water. 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.

In 2021, a total of 106 P1/P1.5 selections passed field criteria and were evaluated in the lab. This was a good deal lower than in 2020 (Table 1), likely due to heat stress. Three selections showed good performance over multiple years, and were advanced to P2 for planting in 2023. Summary data can be seen in Table 2, which also includes data from selections advanced to P2 in 2019 and 2020. Over the course of this project, we have advanced seven P1/P1.5's to P2.

From 2019-2021, 14 P2 selections were evaluated, including 7 only present at Prosser. From this group, three were advanced to P3 (Table 3). R3 and R19 are early ripening cherries with superior fruit quality vs. 'Chelan', the early standard variety.

Of the 7 P2's those only at Prosser, four were advanced to a full (multi-location) P2 trial. The new P2 trial (including the first P1/P1.5 selections in Table 2) was planted in 2021 at Prosser (IAREC headquarters), Pasco (Sagemoor), and Hood River (MCAREC). Each location was planted in a completely randomized design with 4-5 replicates per selection, and 3 trees per replicate. Standard varieties ('Bing', 'Rainier', and 'Skeena') were planted with 3 replicates per variety, and 2 trees per replicate. The improved replication and randomization in the new P2 trials should yield higher quality data to guide advancement decisions, and provide sufficient budwood for propagation.

Table 1. Numbers of P1/P1.5 selections passing field criteria (size, firmness, flavor) and evaluated in the laboratory.

Year	Laboratory Evaluation	Rate of Field Selection
2019	143	7.5%
2020	169	11%
2021	106	7%

Table 2. Characteristics of P1/P1.5 selections advanced to Phase 2.

ID	Color	Timing	Fruit Weight (g)	Row Size/Diameter (mm)	Firmness (g/mm)	Brix/ TA	Advancement Year
CR01T078	Mahogany	Bing +8	11.5	~9/29.5	354		2019
R35	Blush	Bing +15	12.7	9 /30.1	324		2019
FR09T084	Mahogany	Bing -10	9.7	9.5/27.6	352	18.7/ 0.58	2020
CR11T019	Mahogany	Bing +26	9.6	9.5/27.6	335	23.1/ 0.72	2020
FR31T011	Light Mahogany	Bing +1	14	8.5/31.4	294	20.0/ 0.5	2021
R37	Blush	Bing + 12	12.6	8.5/32.0	280	23.4/ 0.89	2021
CR21T043	Mahogany	Bing +26	10.1	9.5/28.7	272	26.4/ 0.60	2021

Table 3. Characteristics of selections advanced to Phase 3.

ID	Color	Timing	Fruit weight (g)	Row size/Diameter (mm)	Firmness (g/mm)	Brix/TA	Notes
R3	Mahogany	Chelan +3-4	10.0	9.6/28.1	326	20.5/0.51	Meaty texture, ripen fully for best flavor
R19	Mahogany	Chelan + 0	9.0	9.8/27.6	335	23.3/0.59	Early, sweet and firm, nose cracking, self-fertile but light crops, storage challenges
R29	Mahogany	Bing + 3	12.1	8.9/30.3	302	21.1/0.40	Very large, self-fertile, mild flavor

3. *Crossing and seedling production*

In 2021, the CBP made 65 crosses, producing an estimated 5,854 seed. Seventeen of these crosses (producing 854 seed) were made using potted trees in the new hoop house, which protects trees from frost. Since the CBP began making crosses again in 2018, 230 crosses have been made, resulting in more than 24,000 seed. We have increased the proportion of seed resulting from bi-parental crosses, where identity of both parents is known (Figure 1). Crosses have been made targeting industry-relevant traits, including maturity (early/late, with a focus on early ripening), size, firmness, self-fertility, powdery mildew resistance, and resistance to X-disease phytoplasma. Cross combinations were guided by a combination of DNA information and field performance. Pollen viability testing, begun in 2019, was augmented in 2021 with testing for PDV and PNRSV in an effort to eliminate infected pollen donors from the breeding program. We built upon our previous research using ReTain to increase fruit set, by testing additional plant growth regulators (PGRs). Although late frosts limited the number of hand pollinations, we did see a beneficial effect on fruit set using a combination of ReTain and Harvista (a formulation of 1-MCP). DNA testing was used each year of the project to focus on seedlings with desired traits. Self-fertility (S4') and powdery mildew resistance (*pmr*-1) were the most-used tests. Additional tests were developed in 2021 for cracking and firmness, and will also be employed for seedling selection.

Embryo rescue for seeds from early-ripening crosses was begun in 2019, with approximately 780 embryos cultured under sterile conditions. Although we cultured essentially the same number of embryos in 2020, recovery of viable seedlings was higher (and germination more rapid) via removal of the seed coat. (Table 4). In 2021, the procedure was greatly expanded, and we began testing new growth media and the addition of PGRs. Data collection from 2021 is ongoing.

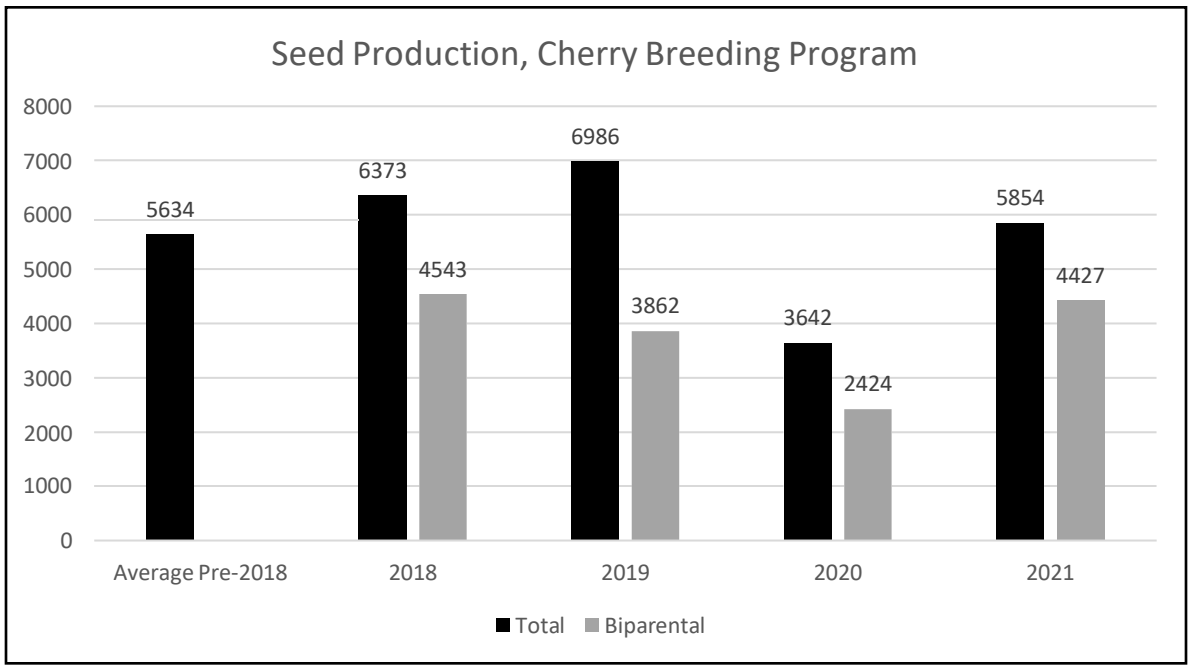
During the course of the project, the CPB transplanted more than 4,170 seedlings to the field at IAREC headquarters. The number transplanted each year varied depending on seed production and germination (752 in 2019, 2,488 in 2020, 933 in 2021). Moving forward, we have established an upper limit of 2,000-2,500 transplanted seedlings per year to fit with available land and personnel resources. The use of microsprinklers and drip tape for prompt irrigation and fertigation has allowed for strong establishment and healthy growth of seedlings.

Table 4. Progress in use of the embryo rescue technique by the CBP.

Year	Embryos rescued	Percent recovery	Notes
2019	780	33	
2020	800	50	Seed coat removed
2021	1550	33 (in-process)[1]	New media, PGRs

Footnotes: [1]The largest family in the group, with more than 500 seed, contained a large number of albino seedlings which germinated but will not survive.

Figure 1. Seed production in the CBP.



4. *Enhancing precocity and reducing variation in seedling blocks*

Our initial efforts to bud cherry seedlings onto precocious rootstocks were unsuccessful, most likely due to inexperience. We attempted to bud Gisela-12 rootstocks in the greenhouse during the winter of 2019-2020, using greenhouse-grown seedlings (2019 crosses) as scions. Despite using the more mature wood at the base of the seedling, none of the buds took. In the spring of 2020, our attempt to spring bud Gisela-6 rootstocks in the field using winter-collected wood from 2018 crosses had a bud take of only 9%. In spring of 2021, we single-budded a small number (13) of greenhouse-grown seedlings on Gisela-12 rootstocks that had been planted in the field the previous year. Prior to budding, the seedlings were given an 8-week cold treatment after reaching a height of 2-3 feet. Bud take was 69%, which is very encouraging. In addition, we applied girdling and limb bending to the cohort of seedlings transplanted in 2019 to test these effects on seedling precocity. As these trees begin to flower in 2022, we will be able to evaluate the results of these training methods compared to the traditionally pruned control seedlings.

In conclusion, the CBP has devoted significant efforts to develop superior sweet cherry cultivars for the Pacific Northwest. We have engaged university and industry experts to establish sound orchard management practices. We have re-started the crossing and P1 portion of the breeding pipeline, which is critical for long-term success, and are employing techniques such as embryo rescue and DNA-informed breeding to target important traits and make the process more efficient. We have also identified and advanced promising selections in the program, including three selections now in P3, two of which are targeting the critical early-ripening sector.

Executive Summary

Project Title: "Supporting a robust PNW sweet cherry breeding and genetics program"

Key words: breeding, embryo rescue

Abstract: The Pacific Northwest sweet cherry breeding program (CBP) is devoting significant efforts in its mission to develop superior cherry cultivars for the Oregon and Washington industries. Since the re-launch of the program in 2018, significant improvements have been made. The breeding pipeline has been re-started in earnest. The majority of seed produced is from bi-parental crosses targeting industry-relevant traits, and embryo rescue has been successfully implemented for early ripening and interspecific crosses. More than 4,000 new seedlings (Phase 1/P1) have been planted over the past three years. Six new selections have been planted in replicated and randomized Phase 2 (P2) trials at Prosser, Pasco, and Hood River, and three selections have been advanced to on-farm Phase 3 (P3) trials for pre-commercial evaluation, including two early-ripening selections (R3 and R19), which have the potential to increase the profitability of the Pacific Northwest cherry industry. Efforts are also being made to shorten the breeding cycle by budding seedlings onto precocious rootstocks, and training own-rooted seedlings to enhance fruiting.

ADDENDUM

Final report for 1-year project, "Equipping the Relaunched PNW cherry breeding program"

PI: Per McCord
Organization: WSU Dept. Horticulture
Telephone: 509-786-9254
Email: phmccord@wsu.edu
Address: WSU-IAREC
Address 2: 24106 N. Bunn Rd
City/State/Zip: Prosser, WA 99350

Total Project Request: **Year 1:** $79,000

Budget 1
Organization Name: Washington State University **Contract Administrator:** Katy Roberts
Telephone: (509)-335-2885 **Email address:** arcgrants@wsu.edu

Item	2019		
Salaries			
Benefits			
Wages			
Benefits			
Equipment	$79,000		
Supplies			
Travel			
Miscellaneous			
Plot Fees			
Total			

Original Objectives

The objectives of this proposal were to purchase/build critical equipment and infrastructure needed for a successful cherry breeding program. Specifically, funds were requested for the following:

- Laminar flow hood to generate sterile conditions for embryo rescue of seeds from crosses targeting early ripening, a critical trait for the industry
- Growth chamber for initial culturing of rescued embryos
- Tissue grinder to prepare leaf samples for DNA extraction prior to DNA marker testing
- A greenhouse (approximately 90 X 30 feet) for making crosses using potted trees

Results and Discussion

After determining that a combination of a walk-in cooler (for stratification) and a simple light cart was sufficient for initial growth of embryo-rescued seedlings, the CBP has procured two laminar flow hoods and LED grow lights instead of a single hood and a single growth chamber. In 2021 alone, 1550 embryos were rescued, resulting in more than 500 viable seedlings targeting early ripening.

A 'Beadbeater-96' tissue homogenizer has been used to extract DNA from more than 4,000 seedlings, as well as processing samples for pathogen detection and DNA marker discovery and test development.

After delays in design/procurement and the COVID-19 pandemic, a 96 X 30 foot hoop house was constructed in early 2021. It is equipped with double layer polyethylene sheeting and an inflation fan, as well as exhaust fans and a cooling wall. In addition to protecting potted trees from frost, the double sheeting and cooling wall provided adequate cooling even during the summer. A total of 854 seed were produced during the first season of using the hoop house, and its use in crossing will continue to expand.

These critical investments in the cherry breeding program are enabling the techniques and throughput required to more rapidly produce superior sweet cherry varieties for the Pacific Northwest.

FINAL PROJECT REPORT

Project Title: Advancing precision pollination systems to improve yield security

PI:	Matthew Whiting	**Co-PI (2):**	
Organization:	WSU-IAREC	**Organization**:	
Telephone:	786-9260	**Telephone**:	
Email:	mdwhiting@wsu.edu	**Email**:	
Address:	24106 N. Bunn Road	**Address**:	
Address 2:		**Address 2**:	
City/State/Zip:	Prosser WA 99350	**City/State/Zip**:	

Cooperators: Finley Cherries, Olsen Brothers, Hayden Farms, Russ LeSage, David Green, Chisa Fruit Co., Firman Pollen Co., On Target Spray Systems, Cameron Peace, Katie Taylor, Connor Dykes

Other funding sources
Firman Pollen company provided pollen for this project at no cost – estimated at $5,000; OnTarget Spray Systems is providing a technician (estimated 140 hrs/ year for this project) and a sprayer (retail value of $20,000) for this research

Total Project Funding: **Year 1:** 74,566 **Year 2:** 74,624

Budget History
Organization Name: Washington State University Contract Administrator: Katy Roberts
Telephone: 509.335.2885 Email address: katy.roberts@wsu.edu

Item	2017	2018	2019
Salaries	$40,856		$44,191
Benefits	$5,482		$6,053
Wages	$12,480		$13,500
Benefits	$1,248		$1,350
Equipment	$0		$0
Supplies	$8,000		$2,500
Travel	$6,500		$7,030
Plot Fees			
Miscellaneous			
Total	$74,566		$74,624

Objectives:
Our long term goal is to improve yield security and yield by developing and deploying a reliable precision pollination system that can 1) supplement current grower pollination practices or, 2) replace the current (ancient) system of planting pollinizers and renting pollinators. We will continue to work with grower collaborators, Firman Pollen Company, and On Target Spray Systems to be sure that research progress is easily translatable to commercial-scale solutions.

1. Refine pollen rate and application timing to improve efficiency of precision pollination systems
2. Optimize pollen suspension constituents to preserve pollen viability and improve solubility
3. Investigate pollen production systems
4. Use commission funded work to strengthen regional and national research proposals

Significant findings:

Overall:

- There is tremendous variability within and among orchards in fruit set
- Supplemental pollination treatment effects should be assessed on large-scale
- Supplemental pollination treatments can reduce variability in fruit set
- Supplemental pollination treatments, at 15 – 40 g/acre, can improve fruit set and yield
- Under favorable environmental conditions, pollination may be maximized with 4-5 hives per acre
- Pollen viability can be maintained in suspension media, in a commercial sprayer, for 100 minutes
- Pollen germinability is improved in the suspension media for up to 90 minutes
- There is high variability in pollen viability among genotypes, both within a year and among years

No-cost extension (2021):
Pasco 'Coral Champagne'

- Low fruit set overall, ca. 12% in control
- No change in fruit set from supplemental pollination

Pasco 'Early Robin'

- Very low fruit set in control, ca. 5%
- Supplemental pollination improved fruit set to 8.8%

Prosser 'Coral Champagne'

- Low fruit set overall, ca. 15% in untreated control
- Supplemental pollination improved fruit set to 19%

Large-scale trials

- In two trials in CA, yield was improved by supplemental pollination by 23% and 27% (+2,000-3,300 lbs/acre)

RESULTS & DISCUSSION

This report encompasses the results from trials conducted in 2021 on a no-cost extension to the original project.

In 2021, we conducted three trials evaluating artificial, supplemental pollination. Two trials were in 'Coral Champagne' blocks, and another was setup in 'Early Robin'. In addition, with collaborators Firman Pollen Company, and independent growers, two large-scale trials were setup in California in 'Bing' and 'Coral Champagne' orchards.

'Coral Champagne' in Pasco: This orchard was a 7th leaf block north of Pasco on 'Gisela®6' and trained to a Y-shaped, trellised architecture. The orchard was established with 4 rows of 'Coral Champagne' alternating with a single row of 'Early Robin'. Additional pollenizers were subsequently distributed throughout the block – these included 'Santina', 'Black Pearl', and 'Chelan'. All applications of supplemental pollen were made by the grower collaborator using an OnTarget Spray Systems electrostatic sprayer at ca. 20 gallons per acre. Pollen applications were made twice, the first at ca. 50% full bloom, and the second at ca. 90% full bloom. Two sections of 5 rows were left unsprayed as controls.

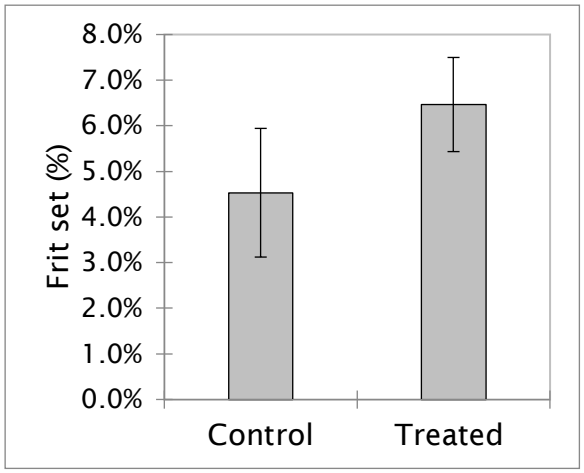


Figure 1. Effect of supplemental pollination treatment on fruit set (% of available flowers) (mean+/- std error) in 'Early Robin' sweet cherry trees. n=26

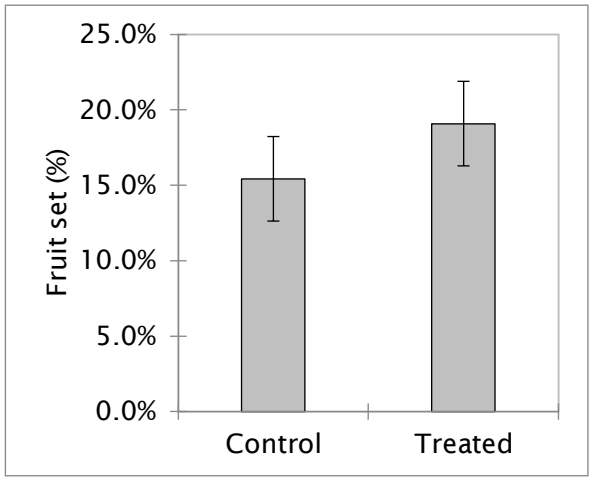


Figure 2. Effect of supplemental pollination treatment on fruit set (% of available flowers) (mean+/- std error) in 'Coral Champagne' sweet cherry trees. n=25

Fruit set of untreated control was low at 12.4%. Variability among sample limbs for fruit set was high, ranging from 4% - 26%. Supplemental pollination did not improve fruit set statistically – mean set was 12.7% in treated limbs, with a similarly wide range (3% - 28%) (data not shown).

'Early Robin' in Pasco: This orchard was a 7th leaf block north of Pasco on 'Gisela®6' and trained to a Y-shaped, trellised architecture. Data were collected from the single rows of 'Early Robin' in the same block as the 'Coral Champagne', described above.

Fruit set in untreated limbs was very low, at 4.5%, however, this is not unusual for 'Early Robin' (see previous reports). The supplemental pollination treatments significantly ($p>0.07$) improved fruit set though it remained low at 6.5% (Fig. 1). The range in fruit set from individual limbs varied from 2% - 8% for untreated control, and from 2% - 17% in treated limbs.

'Coral Champagne' in Prosser: A trial was setup in a 6th leaf 'Coral Champagne'/'Gisela®6' orchard north of Prosser. The block was trained similarly to the Pasco orchard – to a Y-trellised architecture with horizontal fruiting wood. Fruit set of untreated was slightly greater than the Pasco block at 15.4%, with a range of 2% - 27% for individual limbs. Fruit set of treated limbs was greater ($p>0.12$) at 19.1% (Fig. 2), and a range of 5% - 45% among individual limbs.

Large-scale 'Bing'/Mazzard: This trial was setup near Linden, CA in a uniform, mature 'Bing'/Mazzard block. A single application of liquid pollen suspension (30 g/acre) was made at ca. 75% of full bloom to a 6.3-acre section of the orchard. The remainder of the block (16.2 acres) was left untreated. Total yield per block was determined at the packing house receiving. Yield

in the untreated control section of the block was 8,774 lbs/acre. The pollination treatment improved yield per acre to 10,763, about a 23% increase (ca. 2000 lbs/acre increase).

Large-scale 'Coral Champagne'/Mazzard: In this trial supplemental pollination was made to about 60% of a mature block of 'Coral Champagne', also in the Linden area. In this case, two applications of 15g/acre were made at ca. 50% and 90% of full bloom. Again, total yield was determined at receiving for each block separately, and used to estimate yield per acre. The yield from the untreated portion of the orchard was 40,100 lbs from 3.3 acres (ca. 12,150 lbs/acre). The yield from the portion of the orchard receiving supplemental pollination was 72,500 lbs from 4.7 acres (ca. 15,425 lbs/acre). Therefore, the artificial pollination improved yield by nearly 3,300 lbs/acre, an increase of ca. 27%.

FINAL PROJECT REPORT **YEAR: 2021**

Project Title: Modeling for the PNW cherry bud phenology/cold hardiness

PI: Dave Brown

Report is forthcoming.

Project Title: Increased Sampling for the PNW Sweet Cherry Bud Phenology and Cold Hardiness Model

Primary PI: Kelsey Galimba
Organization: Oregon State University
Telephone: 541-386-2030 Ext. 38218
Email: kelsey.galimba@oregonstate.edu
Address: 3005 Experiment Station Dr.
City/State/Zip: Hood River, OR, 97031

Co-PI 2: Clark Kogan
Organization: Washington State University
Email: clark.kogan@wsu.edu
Address: PO Box 643113
City/State/Zip: Pullman, WA, 99164

CO-PI 3: David Brown
Organization: WSU AgWeatherNet
Telephone: 509-335-1859
Email: dave.brown@wsu.edu
Address: PO Box 646420
City/State/Zip: Pullman, WA, 99164

Cooperators: Eric Shrum, Western Ag Improvement; Mike Omeg, Orchard View

Report Type: Final Project Report

Project Duration: 1-Year

Total Project Request for Year 1 Funding: $33,398

Budget 1
Primary PI: Kelsey Galimba
Organization Name: Oregon State University
Contract Administrator: Charlene Wilkinson
Telephone: 541-737-3228
Contract administrator email address: charlene.wilkinson@oregonstate.edu
Station Manager/Supervisor: Steve Castagnoli
Station manager/supervisor email address: steve.castagnoli@oregonstate.edu

Item	Type year of project start date here
Salaries[1]	$13,408
Benefits	$8,424
Wages	$0
Benefits	$0
Equipment	$0

Supplies	$680
Travel[2]	$504
Miscellaneous	$0
Plot Fees	$0
Total	$23,016

Footnotes:
[1] Salary and Benefits include 0.20 FTE Bio Sci Research Tech.
[2] Travel budgeted for travel to field sites for sampling and phenology assessment.

Budget 2
Co PI 2: Clark Kogan
Organization Name: Washington State University
Contract Administrator: Katy Roberts
Telephone: 509-335-2885
Contract administrator email address: ARCGrants@wsu.edu

Item	Type year of project start date here
Salaries	$7,663
Benefits	$2,319
Wages	$0
Benefits	$0
Equipment	$0
Supplies	$0
Travel	$0
Miscellaneous	$400
Plot Fees	$0
Total	$10,382

Footnotes:
[1] Salary and Benefits include statistician time provided by the WSU Center for Interdisciplinary Statistical Education and Research.
[2] Miscellaneous includes facilitation of weather data and processing by AWN.

Recap of Original Objectives

The main purpose of this project was to increase the amount of cold hardiness data collected in the 2020-2021 season. Additional data, particularly from OR, was deemed critically important once the analysis of all previously collected data began to indicate that it was unreliable, and it became apparent that the model would be constructed using the 2020-2021 data, only being collected in WA. A second goal of this project was to directly compare the two methods that had been used for determining lethal temperature (LT); a differential thermal analysis (DTA) method that had been used for previous data, and a "freeze and dissect method" (F&D), that was being used for 2020-2021. The goal of this comparison was to 1 – potentially understand discrepancies in the previous data, and 2 – determine which method would be the best to use in any future data collections. Specific areas of interest included how well predicted LTs from both methods matched, whether one method had greater error in its prediction, and whether high temperature exotherms (HTEs) could be used to estimate LT, as has been indicated in recent publications.

Objective 1. Increase the amount of lethal temperature and relative water content data collected in spring 2021, in order to support the completion of an accurate model this year.

Objective 2. Compare the lethal temperature results obtained from the Differential Thermal Analysis (DTA) method and the traditional freezing/cutting method.

Significant Findings

Objective 1
Completion = 100%

- Data collected from Sweetheart, Chelan, Bing (The Dalles, OR) and Regina (Hood River, OR and The Dalles, OR) was combined with WA data to construct cold hardiness models.
- When plotted against growing degree days (GDD), OR and WA data was tightly correlated, supporting the legitimacy of the methods used.

Objective 2
Completion = 100%

- DTA and F&D methods showed strong correlation (.97 for LT25, 0.96 for LT50, 0.97 for LT25) overall.
- Mean absolute difference between DTA and F&D methods is 1.08°C (1.94°F)
- DTA data reliability degrades (error increases) as the season progresses, to a greater degree than F&D data.
- Buds appear to begin to lose the ability to super cool as early as stage 1.
- High temperature exotherms (HTEs), do not appear to be useful for calculating LT.

Methods

Objective 1: Increase the amount of lethal temperature and relative water content data collected in spring 2021, in order to support the completion of an accurate model this year.

The four cultivars currently being collected (Sweetheart, Regina, Chelan and Bing) in Washington or Hood River will also be sampled in The Dalles, OR, starting at the beginning of February and extending through the end of April. Collections will be made once a week to thoroughly cover the transition from dormancy to full bloom.

The traditional freezing and dissecting method will be used to determine lethal temperatures, in an identical manner as the data currently being collected in Prosser, WA and Hood River, OR. Spurs from randomly selected shoots on trees of similar age and rootstock will be collected from cooperating growers in The Dalles, OR, in locations with proximity to AgWeatherNet (AWN) stations. Forty spurs will be frozen at decreasing 1° C increments using the Tenney Temperature Cycling Test Chamber at MCAREC, to a temperature low enough to guarantee 100% flower death. Four spurs will be removed in each of the last ten increments in the freeze series, and allowed to thaw slowly in the refrigerator (4° C) for a minimum of one hour. They will then be incubated at 21° C for 24 hours to allow enzymatic activity to result in oxidative browning. Two to three buds from each cluster, for a total of ten buds from each temperature, will be dissected to determine individual flower mortality within buds. An additional 25 unfrozen buds will be dissected to assess the level of field mortality present before our experiments and

will be used to adjust mortality rates. Every bud will have date, site, phenology, and flower mortality documented.

In addition to phenology assessments made by visual inspection, relative water content will be measured for all collections. This will help identify when the buds leave dormancy and begin to develop, even if visual clues (e.g. bud swelling) are not apparent. Fifty buds will be weighed fresh, dried for four days at 130° C and then weighed again to calculate water content.

This data will be combined with weather data from the closest AWN stations and used to construct statistical models to estimate sweet cherry bud phenology and predict related lethal temperatures

Objective 2: Compare the lethal temperature results obtained from the Differential Thermal Analysis (DTA) method and the traditional freezing/cutting method.

The sweet cherry bud phenology and cold hardiness model will utilize two types of lethal temperature data: current measurements collected by the traditional method of freezing and dissecting buds, as well as past measurements made by differential thermal analysis (DTA). Both of these methods are used widely to determine lethal temperature of floral buds in many species, and each has its own set of disadvantages. The traditional dissecting method is time consuming and labor-intensive, requiring at least three consecutive days of set-up, sample removal, and dissection/data collection. The DTA method in comparison is much easier, requires only one day of set up, and can use a much larger sample size. However, the DTA method has long been considered inappropriate in certain species or bud stages that do not supercool. In sweet cherries, the ability to supercool is thought to be lost by first swell, and so DTA data collected in previous years for the Cherry Cold Hardiness Model has not been used past this stage.

In recent years, some research on fruit tree cold hardiness has indicated that the DTA method is actually a viable method in older, more developed buds and even open flowers, if the single exothermic peak produced upon freezing is used to determine the floral death point. If this could be shown to be true in sweet cherry, it would allow a larger amount of previously-collected data to be used in the model. Perhaps more importantly, it would justify the use of this more efficient method in any future data collection as the model is validated and expanded for other cultivars and locations.

In order to compare these two methods, DTA will be performed simultaneously alongside the freeze/dissection run. For each cultivar, 60 buds will be removed from 20 spurs collected from the same trees and placed in four modules on two DTA plates. These will be run at the same time, in an identical Temperature Cycling Test Chamber, using the same freeze protocol as in Objective 1. At the end of the run, DTA data will be gathered and statistically analyzed to determine whether it correlates to the lethal temperature data gathered by the freeze/dissection method throughout development.

Results and Discussion

Both objectives of this project were accomplished in 2021. We collected F&D data from three cultivars (Sweetheart, Chelan, Bing) from The Dalles, OR, and one cultivar (Regina) from Hood River and The Dalles, OR, starting at the beginning of February and extending throughout full bloom in April. These collections were combined with the data from the three cultivars (Sweetheart, Chelan, Bing) gathered in Prosser, WA, giving us two datasets for each. This data was used for model construction (see final report entitled *Modeling PNW sweet cherry bud phenology and cold hardiness* for more details regarding the models).

In addition to gathering additional datasets using the F&D method, we also simultaneously ran DTA analyses on samples collected at the same time, from the same trees. This allowed us to directly compare the outputs of both methods. In previous years, raw DTA data gathered at MCAREC was processed in excel in order to determine low temperature exotherms (LTEs) and high temperature exotherms (HTEs). LTEs are designated as voltage peaks that occur when water inside a floral initial that is capable of super-cooling freezes. HTE voltage peaks occur when water outside of the floral initial

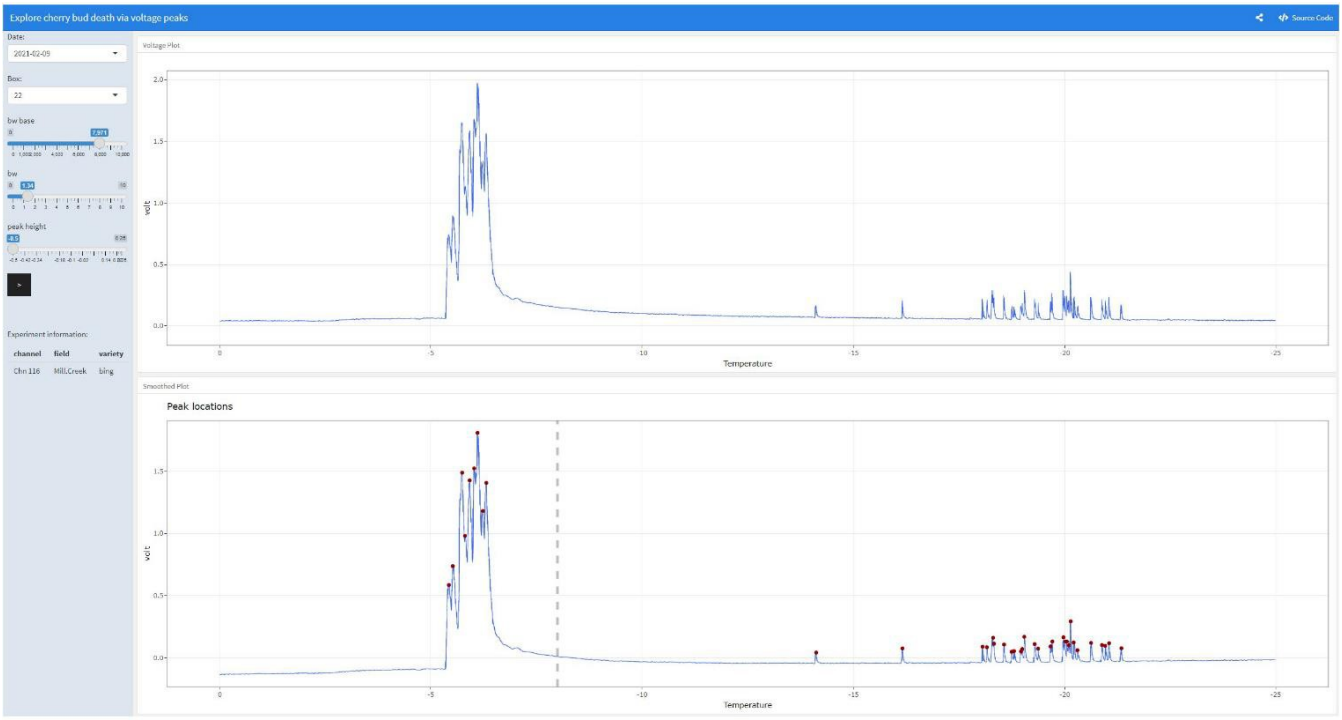


Figure 1. Example of the R program designed to call LTE and HTE peaks, for one sample of Bing collected on February 9th, 2021. Top panel shows the smoothed voltage curve. Bottom panel shows the peaks that are designated either LTEs (lower than -8°C (17.6°F)) or HTEs (higher than -8°C). User inputs include sliders to adjust the degree to which the curve is smoothed and the threshold for voltage height that is considered a peak.

freezes. The previous excel program performed a number of functions, including LTE and HTE peak calling, but it had a complicated user interface and the calculations it used were complex and in some cases, too cryptic for us to be confident in the results. For this reason, we developed a new, R-based GUI with adjustable inputs to take raw voltage data, apply baseline subtraction, smoothing and peak detection, and extract values for LTEs and HTEs (Fig. 1).

One interesting pattern we noticed when collecting LTE peak data in this way, was the surprisingly early shift out of super-cooling (Fig. 2., Fig 3.), indicating that the buds were leaving dormancy and initiating development earlier than we had anticipated. This was evident in both a decrease in the number of LTEs we obtained (Fig. 2) and in shifts in the distribution of the LTEs (Fig. 3). While this phenomenon has been reported to occur in early March[1,2], it occurred in our collections prior to any obvious changes in outward bud appearance. For example, on February 23rd, Sweethearts were at stage 1=20% and 2=80%, while all other cultivars were at 100% stage 1, but all cultivars show some evidence of leaving dormancy.

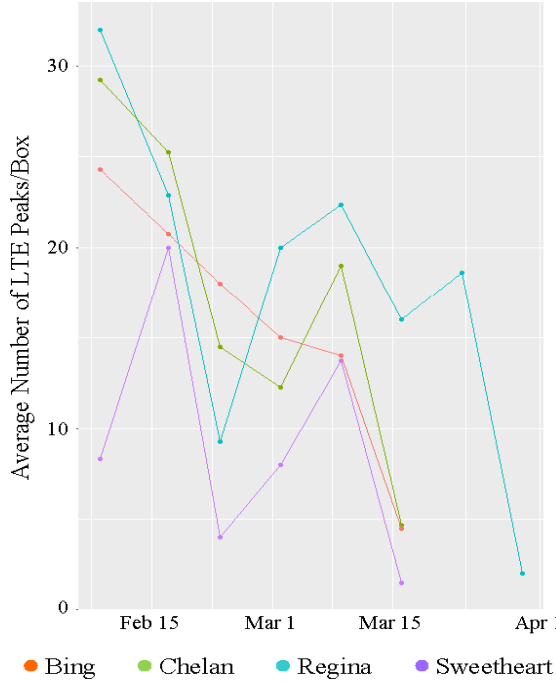


Figure 2. Average number of LTEs obtained from each sample for each cultivar throughout the season. Averages decrease as floral initials leave dormancy because they do not produce an LTE upon freezing. Apparent increase in mid-March are possibly caused by re-acclimation following cold temperatures at that time.

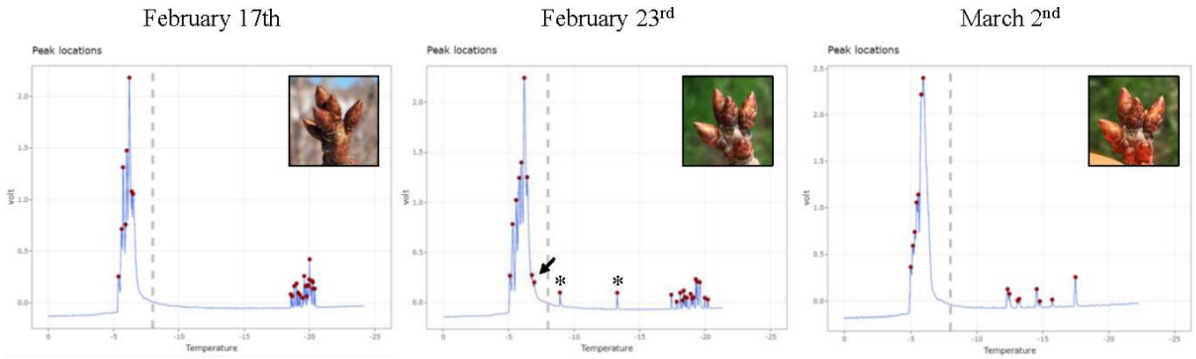


Figure 3. Comparison of LTE peak distribution on three dates, for Bing. On February 17th, LTEs are tightly clustered at a low temperature (~ -20°C (-4°F)), by February 23rd, some LTEs are shifted to higher temperature (asterisks), and are beginning to combine with HTE peaks (arrow). This indicates a general progression out of dormancy. By March 2nd, there are far fewer LTEs, indicating that many buds have already lost the ability to super-cool, even though bud phenology stage appears to be in between 0-1.

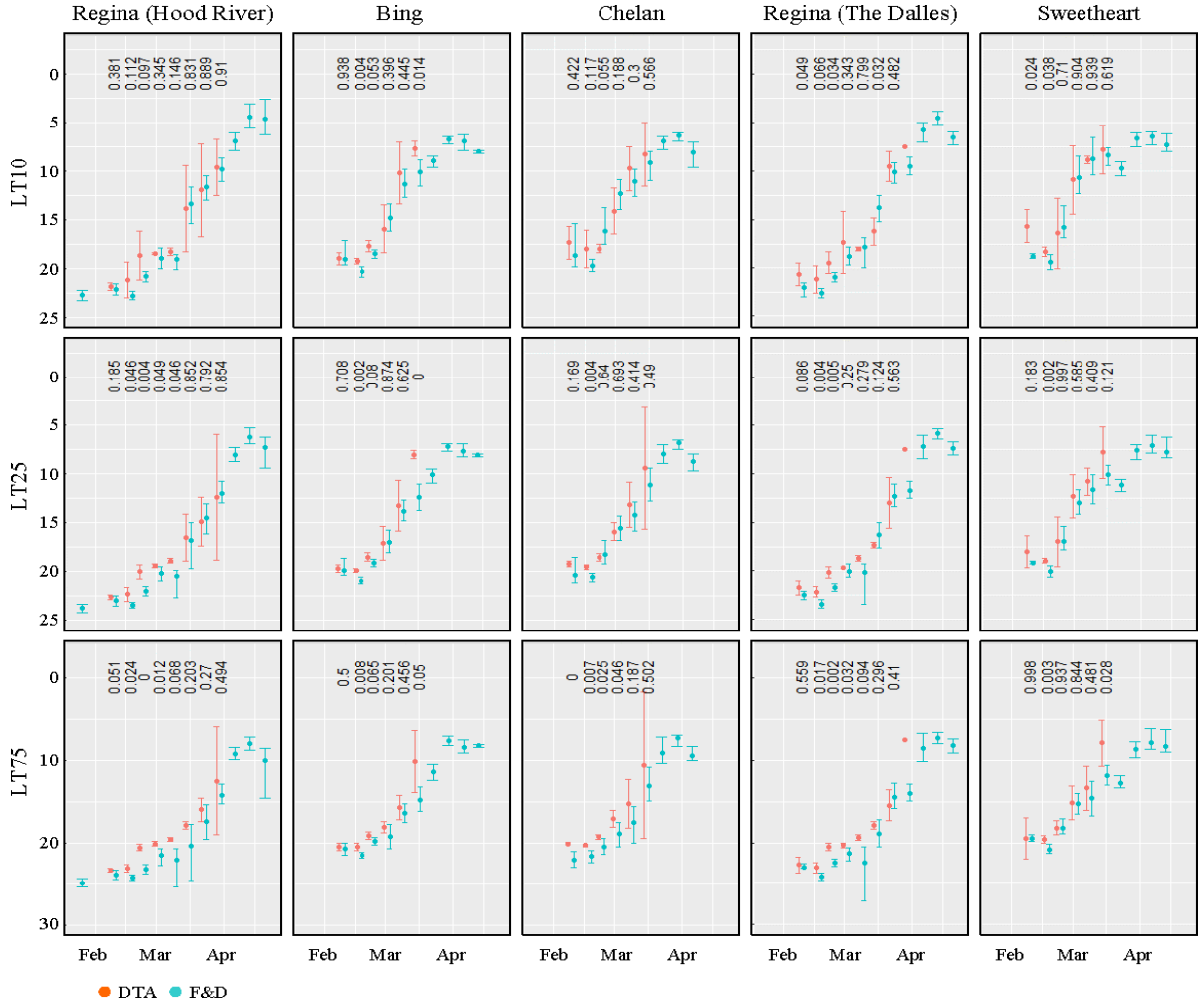


Figure 4. Graphs showing estimated LT25, LT50 and LT75 values for each method, for each cultivar collected in OR. DTA = orange, F&D = blue. Error bars = 95% confidence intervals. The absence of confidence intervals for DTA indicate that only a single box had usable data. DTA and F&D collections occurred on the same day, data points are slightly offset for better visibility. p-values associated with the comparison of mean LT between methods are listed above each date. Given the assumptions for both methods hold, small p-values can suggest a lack of correspondence between DTA and F&D. However, small p-values do not necessarily indicate that the discrepancy between methods is large, only that it is less plausibly zero. LT values for the last three dates (four for Chelan) are absent for DTA, as LTEs were no longer present in any of the voltage graphs as all buds had lost the ability to super-cool.

In order to compare the performance of the DTA and F&D methods, average lethal temperature values and their respective errors were calculated for each method. For the F&D method, average LTs were estimated by logistic regression and confidence intervals were obtained with cluster bootstrapping on the spur level using the quantile method. For the DTA method, average LTs were estimated by extracting 0.25, 0.5 and 0.75 quantiles for each box and then taking the mean across boxes. Confidence intervals were obtained assuming normality of the quantiles across boxes. Average LT25, LT50 and LT75 for each method were then plotted by date (Fig. 4), and correlation between the LT values was calculated across dates and cultivars. Overall, we observed a strong correlation between the average LTs estimated by each method (0.97 for LT25, 0.96 for LT50, 0.97 for LT25) (Fig. 4). However, it was impossible to obtain LTs for the DTA method past the end of March, because there were no longer any LTEs being produced. We also noticed an apparent increase in the error surrounding the DTA-predicted average LTs as the season progressed, which was in large, a result of a reduction in the number of boxes with any LTEs near the beginning of April. When error is averaged for each method and compared, this general pattern holds true (Fig. 5).

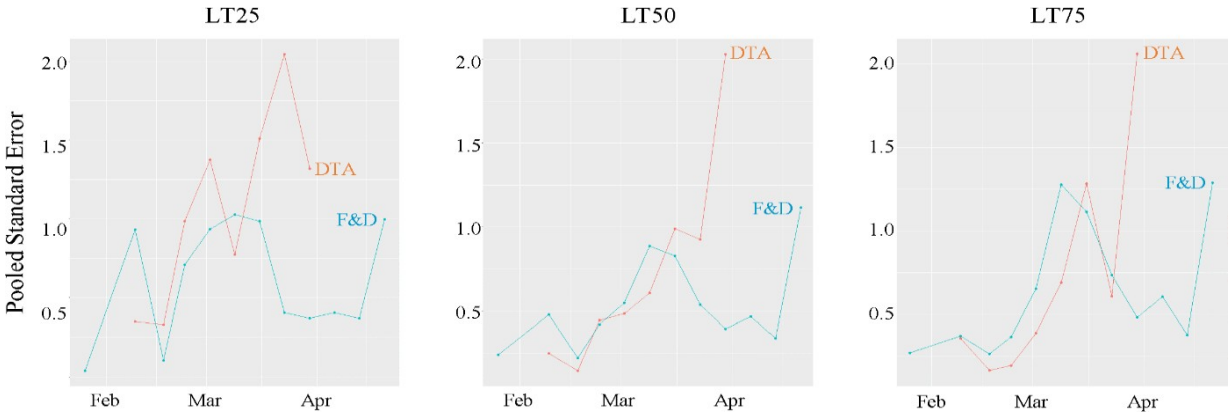


Figure 5. Average standard error values for all cultivars, for each method, compared throughout the season. DTA = orange, F&D = blue.

One additional important question that we wished to answer with this data analysis was whether HTEs could be used, later in the season, to estimate LT. This has been proposed recently for a number of stone fruits, including sweet cherry[3–5], with the premise that once floral initials lose the ability to supercool, all water within a bud sample freezes at the same time, which simultaneously kills the bud. We hypothesized that if we could show that LTs predicted from HTEs correlate well with the F&D method, that it would be possible to use DTA throughout the entire season. However, we did not find a strong correlation ($\rho_{25} = 0.13$, $\rho_{50} = 0.19$, $\rho_{75} = 0.28$) between LTs generated from the HTEs and LTs generated from the F&D method on the last three dates of sample collection (Fig. 6). Furthermore, we noted large mean absolute errors between the methods ($MAE_{25} = 2.22°C$, $MAE_{50} = 3.27°C$, $MAE_{75} = 4.29°C$). This indicates that this will not be a useful method for later collections in the future.

Conclusions

Overall, this project supported the ultimate goal of the development of a sweet cherry cold hardiness model in a number of ways. The additional five datasets we collected in Oregon made it possible to construct models for Sweetheart, Regina, Chelan and Bing. In comparing the performance of the two methods, we found a large amount of congruity in the predicted LTs overall, which was reassuring that both methods are legitimate and capable of providing quality data. We were surprised to see evidence of buds leaving dormancy so early – within bud stage 1 and 2. This observation confirms the idea that a large amount of development is occurring inside the bud, without visible changes to the

outside, supporting the value of a model based on GDD and not strictly on bud phenology. We did not observe a strong correlation between HTE-derived LTs and F&D-derived LTs, which discourages us from using this method later in the season. All-together, the early loss of LTEs in the DTA method, the increasing error throughout the season for DTA, and the fact that it remains unusable in later stages, confirms our plans to use the F&D method for any and all future data collection.

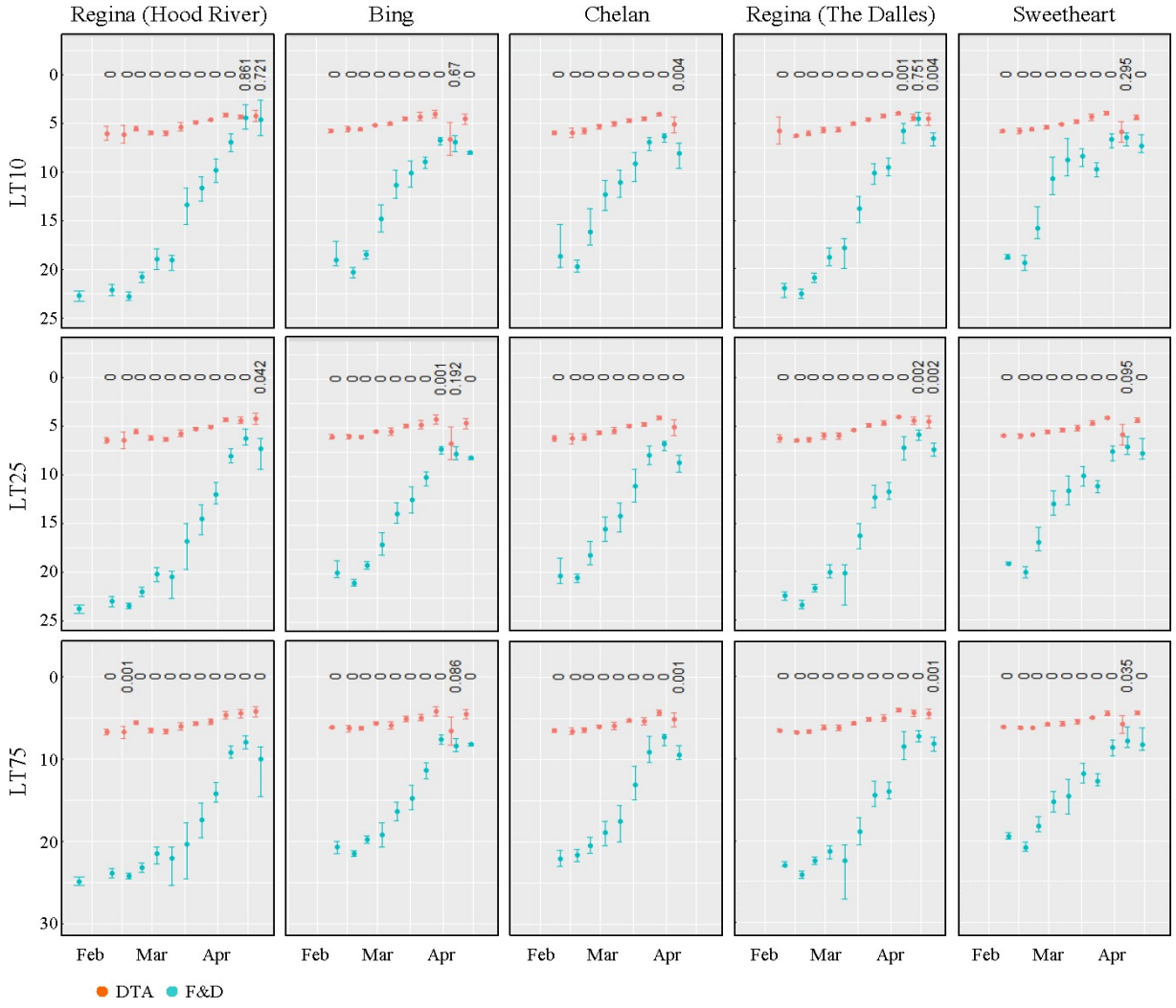


Figure 6. Graphs showing estimated LT25, LT50 and LT75 values for each method, for each cultivar collected in OR, except LTs for the DTA method are calculated from HTEs instead of LTEs. DTA = orange, F&D = blue. Error bars = 95% confidence intervals. The absence of confidence intervals for DTA indicate that only a single box had usable data. DTA and F&D collections occurred on the same day, data points are slightly offset for better visibility. p-values associated with the comparison of mean LT between methods are listed above each date. Given the assumptions for both methods hold, small p-values can suggest a lack of correspondence between DTA and F&D. However, small p-values do not necessarily indicate that the discrepancy between methods is large, only that it is less plausibly zero.

Executive Summary

Project Title: Increased Sampling for the PNW Sweet Cherry Bud Phenology and Cold Hardiness Model.

Key Words: *Prunus avium*, cold hardiness, model, dormancy, differential thermal analysis, DTA, frost, HTE, LTE.

Abstract:

In order to provide a weather-related decision-support tool to guide cherry growers in their response to cold weather events, we aim to develop a sweet cherry cold hardiness model capable of predicting lethal temperature (LT) based on growing degree days (GDD). This project was a sub-element of that larger goal, with two major objectives: increasing the amount of sampling done in the 2020-2021 season and directly comparing the two methods that had been used to determine LT. We successfully increased the sampling, from three datasets to eight, which were then used for model construction. These datasets showed high correlation across both states (OR and WA) when plotted against GDD. In comparing the two methods, we determined that differential thermal analysis (DTA) method and the freezing and dissecting (F&D) method had high correlation overall. However, we also determined that DTA error increases to a greater degree during the spring season, compared to F&D. This is likely related to the buds beginning to lose their ability to supercool in stages 1 and 2. When we tested the potential of using HTEs to estimate LTs late in the season, we did not see a strong enough correlation to the F&D-derived LTs to warrant using DTA in this way. Overall, we determined that for these reasons, F&D will be the best method moving forward as we gather additional cold hardiness data.

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

Primary PI: Bernardita Sallato
Organization: WSU- ANR
Telephone: 509-786-9205
Email: b.sallato@wsu.edu
Address: 24106 N Bunn Rd
City/State/Zip: Prosser, WA, 99350

Co-PI 2: Matthew Whiting
Organization: WSU- Horticulture
Telephone: 509-786-9205
Email: mdwhiting@wsu.edu
Address: 24106 N Bunn Rd
City/State/Zip: Prosser, WA, 99350

Co-PI 3: Carolina Torres
Organization: WSU- Horticulture
Telephone: 509-293-8808
Email: ctorres@wsu.edu
Address: 1100 North Western Ave.
City/State/Zip: Wenatchee, WA, 98801

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 propose to undertake a prospective analysis of orchard growing conditions, tree and soil health, irrigation management and nutrient composition and its relationship with the quality parameters: size, firmness, and storability. This research approach will permit an in-depth analysis of fruit nutritional content and fruit quality, identify predictors, determine nutrient extraction/demand, and begin 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 size varied between 15.2 and 31.5 mm, equivalent 14 and 8.5 row size. On average, Skeena (24 mm) was 4% larger than Chelan and Coral Champagne (23 mm).
- Fruit firmness varied between 123 $g \cdot mm^{-1}$ and 474 $g \cdot mm^{-1}$. Skeena (286 ± 2.8 $g \cdot mm^{-1}$) was firmer than Chelan (277 ± 3.6 $g \cdot mm^{-1}$) and both were firmer than Coral (245 ± 3.6 $g \cdot mm^{-1}$).
- Growing site (location) influenced fruit quality and nutrient concentration in different ways for each cultivar, thus analysis should be carried out independently for each growing location.
- Firmness had significant but weak correlation with N and K concentration.

- Fruit size was positively and strongly correlated with fruit weight, while positively but weakly correlated with N:Ca, K:Ca and (K + Mg):Ca ratios, and negatively correlated with Ca and K concentration in the fruit.

METHODS

This project is taken 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 and Skeena from at least three different commercial orchards. At commercial harvest, for each cultivar and orchard, we obtained four replicate bulk fruit samples of at least 5 lbs of the largest and smallest fruit 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 was divided in half (ca. 2.5 lbs), from each size category. 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 10^{th} and 90^{th} percentile ranking of firmness testing were selected 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 analyzes. 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.

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.

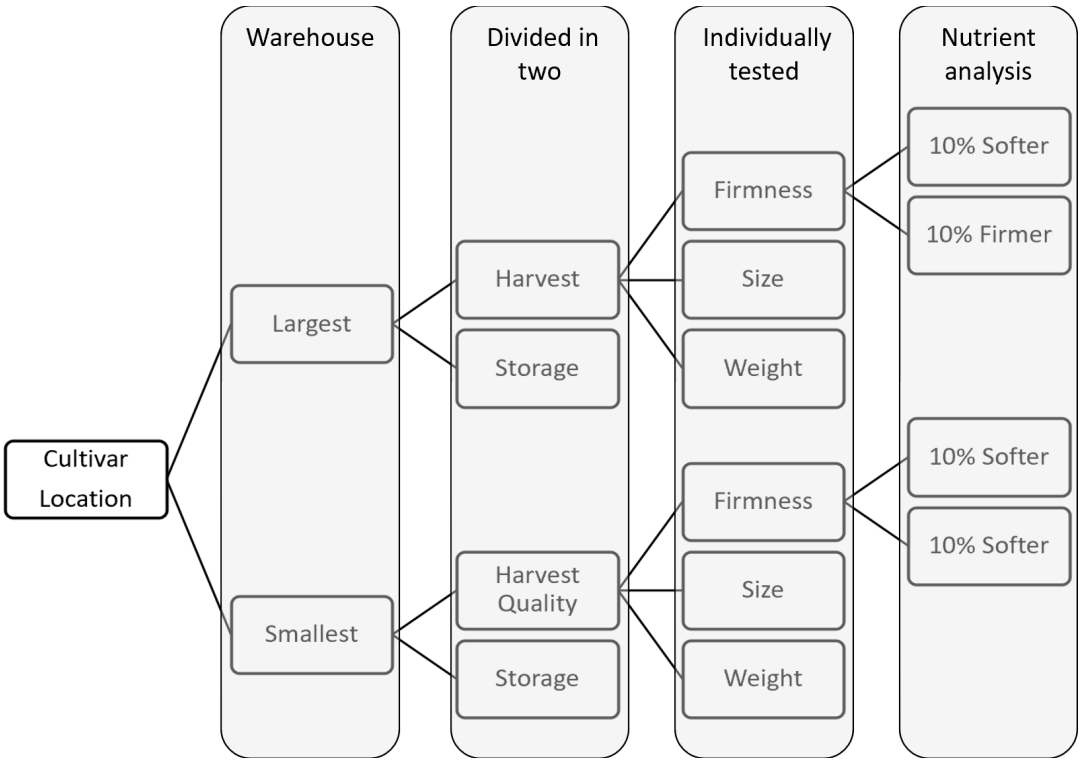


Figure 1. Fruit sampling scheme for nutrient and storability analyses.

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.

New findings will be shared with Washington and Oregon tree fruit industry throughout our several extension tree fruit methods, such as industry meetings, newsletter (e.g. “Fruit Matters” and Good Fruit Grower), and extension articles and fact sheets. Detailed preliminary results will be shared by Sallato during WTFRC review and Cherry Institute Meeting.

RESULTS AND DISCUSSION

Fruit size distribution by cultivar and size categories, including all growing locations is represented in Figure 2. Fruit diameter varied between 15.2 and 31.5 mm, equivalent to 14 and 8.5 row size, and between 5.6 and 12.4 grams. The size categories were significantly different between cultivars and warehouse size categories ($p < 0.001$), confirming adequate size sorting in the warehouse. On average, Skeena (24 mm) was 4% larger than Chelan and Coral Champagne (23 mm) (data not shown).

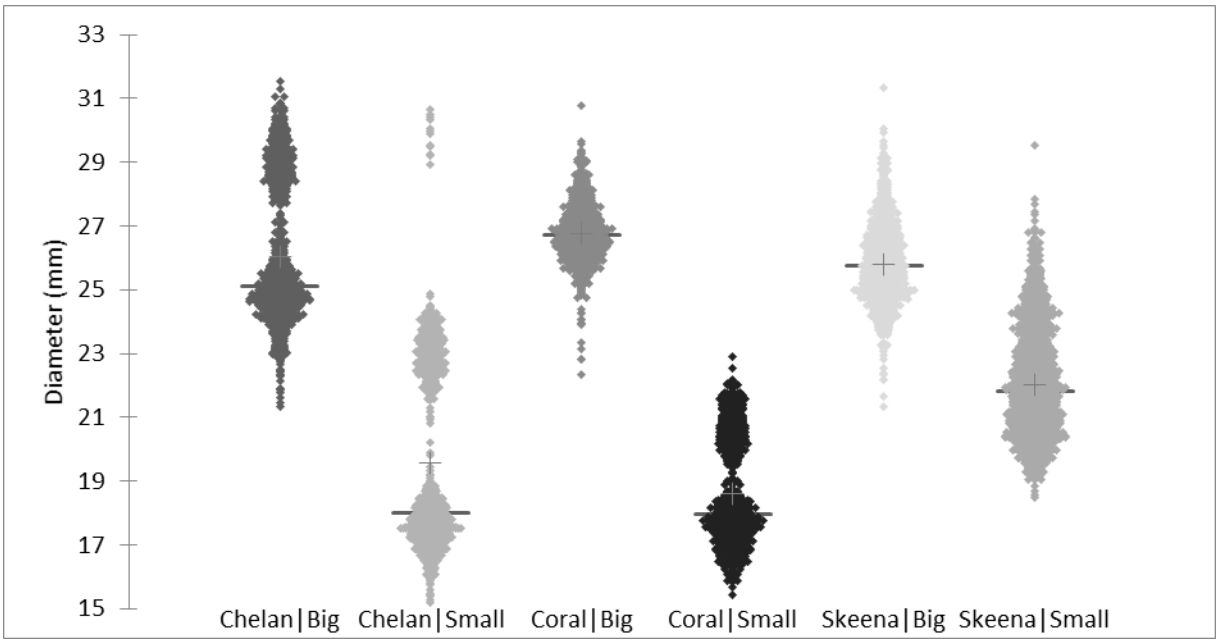


Figure 2. Scattergram plot for diameter of sweet cherry fruit by cultivar and size category. Dots represent each fruit measurement; the line represents the median and the cross represents the mean of the sample.

Fruit firmness varied between 123 g · mm^{-1} and 474 g · mm^{-1}. The distribution in fruit firmness, considering all fruit from each cultivar and size category, is presented in Figure 3. This analysis reveals the high variability in firmness within each cultivar. When comparing between cultivars, regardless of the size category, Skeena (286 ± 2.8 g · mm^{-1}) was firmer than Chelan (277 ± 3.6 g · mm^{-1}) and both were firmer than Coral (245 ± 3.6 g · mm^{-1}) (data not shown). When factoring the size category, smaller size fruit were always firmer than the bigger size for each cultivar ($p < 0.001$).

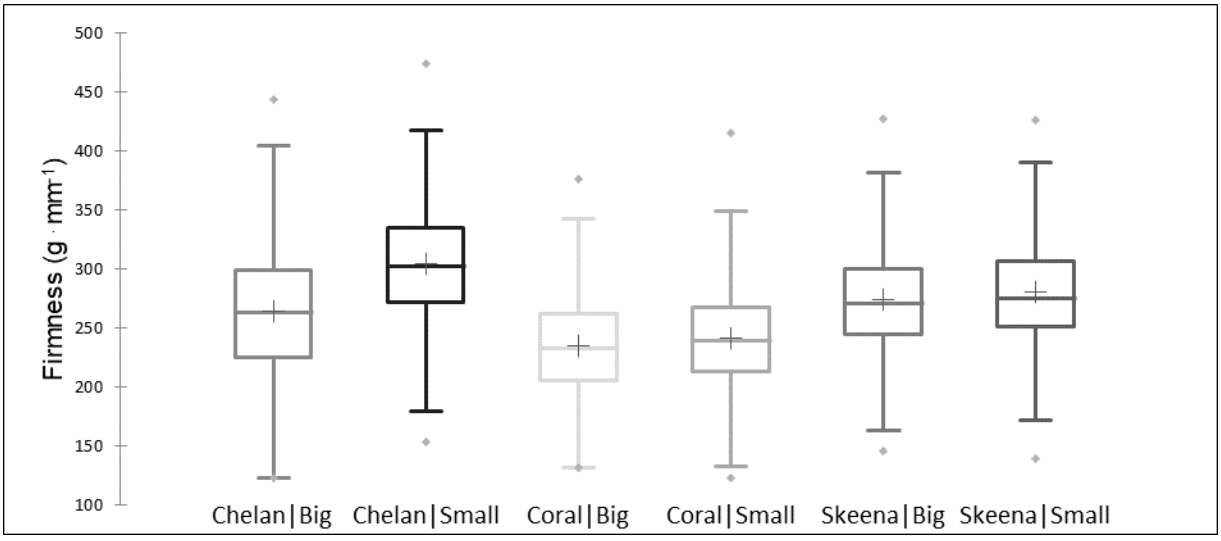


Figure 3. Box and whisker diagram for firmness, by cultivar and size category, representing minimum, maximum (♦), lower and upper quartile (end of whiskers), box represents 50% of the data. The vertical line that split the box in two is the median and the mean is indicated by the cross on the box plot.

For each size category and cultivar, the softest and firmest fruit were selected for nutrient analysis. For the firmest category of fruit, mean firmness ranged between 292 and 347 g · mm^{-1} among cultivars and fruit size, while softest fruit ranged between 187 and 232 g · mm^{-1} (Figure 4a). Among the firm fruit, Skeena and Chelan were firmer than Coral Champagne. Among the softest fruit, Skeena was the firmest and Coral Champagne the softest. Mean fruit diameter by cultivar and size category, regardless of firmness, ranged between 26 and 27 mm for the big category and 18.5 and 22 mm for the small category. Among the large fruit size category, Coral Champagne were larger than Skeena, with no differences with Chelan, while Coral Champagne had the smallest fruit mean among the smallest category (Figure 4b)

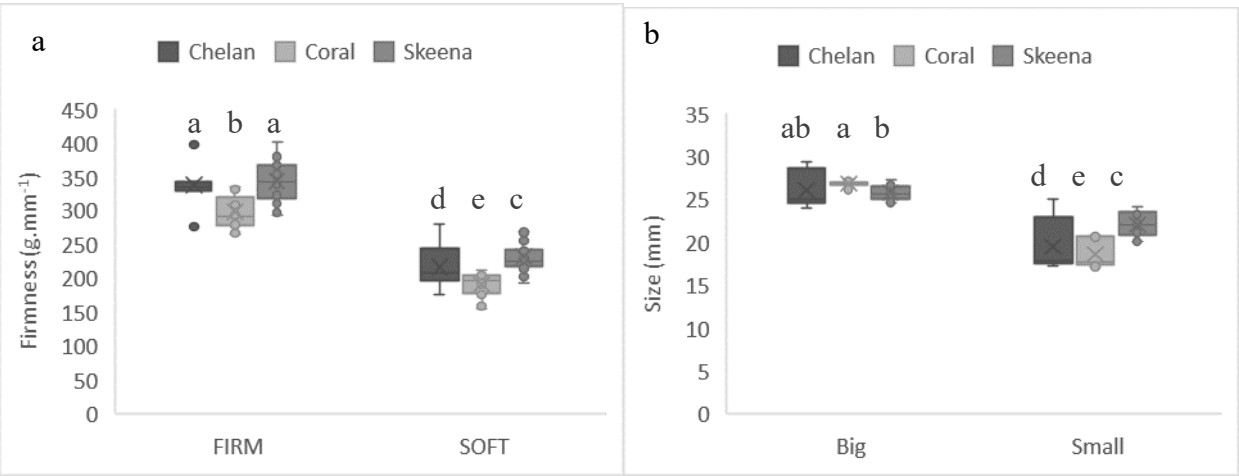


Figure 4. Box and whisker distribution of fruit firmness (a) and fruit size (b) by cultivar and subgrouping category. Different letters within each chart indicate significance at $p < 0.001$.

Fruit firmness had a negative correlation with fruit weight, however weak (R = -0.16), similarly with N and K concentration. Fruit weight and diameter were strongly correlated (R = 0.916), and both parameters were positively correlated with nutrient ratios N:Ca, K:Ca, and (K+Mg):Ca (R = 0.50, 0.60 and 0.60 respectively), and negatively correlated to fruit Ca and K concentration (R = -0.78 and -0.53, respectively). Figure 5.

Within cultivars, regardless of the size category, growing location influenced fruit quality and nutrient concentration differently. For example, in Chelan, growing location had an influence on fruit firmness, fruit diameter, proportion of fresh stems (attributed to thickness or length), flesh and pedicel dry matter and macronutrient concentration in the flesh. In Coral Champagne, growing location only influenced stem dry matter and macronutrient concentration in the flesh, with no differences in firmness or fruit size among the different orchards. In Skeena, growing location (5 sites) influenced fruit firmness, fruit diameter and weight, proportion of flesh and stems, flesh dry matter and all macronutrients (Table 1).

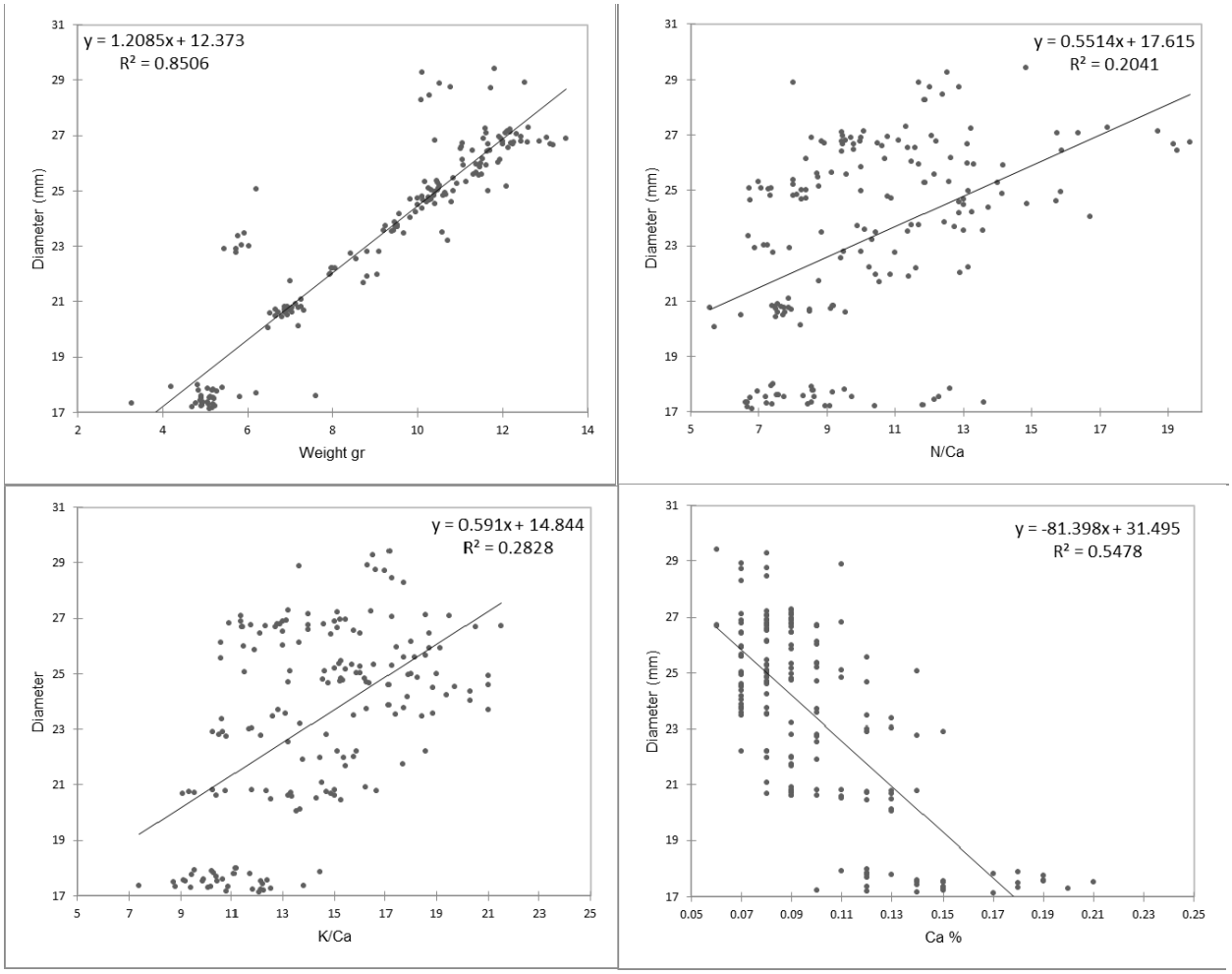


Figure 5. Relationship between fruit diameter (mm) and (a) fruit weight (g), (b) N:Ca ratio, (c) K:Ca ratio and (d) Ca concentration. Each plot includes all cultivars and firmness and size categories. R^2 indicates coefficient of determination.

For Chelan, firmer fruit had higher N, P, K concentration while Ca and Mg were not related to firmness. In Coral Champagne, there were no differences between firmness and fruit size, thus nutrient concentration differences were not attributed to either attribute. In Skeena, the firmest fruit had higher N concentration, but other nutrients were not related to fruit firmness.

Table 1. Fruit firmness, diameter, flesh and pedicel dry matter, and macronutrient concentration of sweet cherry fruit from different cultivars and growing locations. Different letters within a column and cultivar indicates significant differences at $p < 0.1$.

Cultivar	Location	Firmness	Diameter	Flesh % DM	Stem % DM	N %	P %	K %	Ca %	Mg %
Chelan	1	277 ab	21 b	18% b	30 % b	1.23 a	0.20 a	1.66 a	0.14 a	0.10
	2	303 a	21 b	22% a	43% a	1.34 a	0.20 a	1.62 a	0.11 b	0.10
	3	253 b	26 a	20% ab	34 % ab	0.94 b	0.15 b	1.38 b	0.10 b	0.10
	Pr > F(Model)	*0.110*	*0.000*	*0.025*	*0.048*	*0.000*	*<0.0001*	*0.001*	*0.056*	*0.845*
Coral	1	240	22	19%	38% b	1.01 a	0.18 a	1.36 a	0.12 a	0.09 a
	2	251	24	18%	37% b	0.91 b	0.14 b	1.11 b	0.10 b	0.08 b
	3	244	22	19%	48% a	0.88 b	0.15 b	1.20 b	0.10 ab	0.08 ab
	Pr > F(Model)	*0.864*	*0.482*	*0.735*	*0.003*	*0.018*	*<0.0001*	*0.002*	*0.092*	*0.059*
Skeena	1	317 a	24 a	24% ab	49%	1.14 a	0.16 c	1.34 b	0.08 c	0.09 a
	2	283 abc	25 a	21% c	44%	1.01 b	0.17 b	1.19 c	0.10 ab	0.08 b
	3	300 ab	23 b	23% b	42%	0.72 c	0.17 b	1.39 ab	0.09 b	0.08 b
	4	266 c	23 b	25% a	49%	0.75 c	0.18 a	1.47 a	0.10 a	0.09 a
	5	269 bc	25 a	23% b	47%	0.93 b	0.16 bc	1.32 b	0.07 c	0.08 b
	Pr > F(Model)	*0.081*	*0.017*	*<0.0001*	*0.473*	*<0.0001*	*0.006*	*<0.0001*	*<0.0001*	*0.000*

This project has generated an abundance of data that remain to be analyzed. We will continue to evaluate the relationships among fruit quality attributes and the role of nutrient concentration, as well as the shelf life of each firmness category of fruit.

FINAL PROJECT REPORT **YEAR**: 3 of 3 **(NCE)**

PROJECT TITLE: Fungicide Resistance: A Vital Need To Protect PNW Cherries From Mildew

PI: Gary Grove, PhD
Organization: Washington State University
Telephone: 509-786-9283
Email: grove@wsu.edu
Address: 24106 N Bunn Road
Address 2: IAREC
City/State/Zip: Prosser, WA 99350

Co-PI (2): Prashant Swamy, PhD
Organization: Washington State University
Telephone: 509-786-9284
Email: prashant.swamy@wsu.edu
Address: 24106 N Bunn Road
Address 2: IAREC
City/State/Zip: Prosser, WA 99350

Cooperators: Tianna DuPont, Bernardita Sallato, Neusa Guerra

TOTAL PROJECT REQUEST: **Year 1:** $60,175 **Year 2:** $71,276 **Year 3**: $0 (no cost extension)

Other funding sources:

Agency Name: Washington State Commission on Pesticide Registration
Amt. awarded: $28,801 (2020; no cost extension 2021)
Notes: "Group 11 fungicide resistance of cherry powdery mildew in orchards and nurseries".
CO-PIs: Gary Grove and Prashant Swamy

Agency Name: Northwest Nursery Improvement Institute
Amount Awarded: $14,180 (2021)
Notes: "Identification of FRAC 3 and FRAC 11 fungicide resistance of cherry powdery mildew in the Washington nurseries"
CO-PIs: Gary Grove and Prashant Swamy

WTFRC Budget

Organization Name: WSU-IAREC **Contract Administrator: Samantha Bridger**
Telephone: 509-786-2226 **Email address:** prosser.grants@wsu.edu

Item	2019	2020	2021 (NCE)
Salaries[a]	$18,405	$19,141	
Benefits	$8,958	$9,316	
Wages[b]	$11,520	$11,981	
Benefits	$1,152	$1,198	
Supplies[c]	$18,250	$27,750	
Travel[d]	$1,890	$1,890	
Plot Fees			
Total	$60,175	$71,276	**0**[c]

Footnotes:
[a] 0.5 FTE for an associate in research
[b] Time slip field and laboratory workers
[c] No cost extension

Recap of Objectives

1. **Investigate the presence and extent of fungicide resistance in commercial orchards in the Pacific Northwest.** A total of 20 unique orchard sites (WA and OR) in 2019 and a collection of 11 orchard sites (WA and OR, 192 individual single colony isolates) and 5 nursery sites (WA alone, 80 single colony isolates) were used in the 2020 study, while 100 were collected in 2021. We have made extensive progress in both years and have conclusively 2019-2020 identified the presence of fungicide resistance to FRAC 3, FRAC 7 and, FRAC 11 group of fungicides. Efforts in 2021 focused on the *extent* of Group 11 resistance.

2. **Identify and develop specific genetic markers for better identification of fungicide resistance.** We have been successful in designing, amplification, and sequencing of fungicide target genes of FRAC Groups 3, 7, and 11. The robustness of the assays was tested on several isolates and resistant colonies in 2019. In 2020 we identified genetic mutations in the FRAC group 7 target genes. This objective was successfully accomplished and at present, we have genetic information on three fungicide target genes.

3. **Develop alternative programs for disease management, if significant fungicide resistance is documented in this study (Conditional).** As we discuss the results below, insensitivity to some FRAC groups is present in both states. Our experimental data underscored the very problem of fungicide resistance in commercial orchard and nurseries. Moreover, given the extent of fungicide resistance to multiple groups of chemicals, there is an urgent need for alternative strategies to be implemented for managing powdery mildew. We initiated research in this direction, thanks to the additional collaborative funding from WSCPR. Results were documented in the 2020 Progress Report.

Significant Findings

Note that the causal pathogen, *Podosphaera clandestina* is now identified as *Podosphaera cerasi* (Moparthi, S., Pandey, B., Bradshaw, M., Rooney Latham, S., Braun, E., Meeboon, M., and Romberg, M.,and Grove, G.G., 2019. Phylogeny and taxonomy of *Podosphaera cerasi*, sp. nov., ad *Podosphaera prunicola sensu lato.* Mycologia 111: 647-659.)

The new nomenclature *Podosphaera cerasi* (*P. cerasi*) is used in this report.

- Leaf-disc bioassays confirmed our molecular data for FRAC Group 3 and 11 fungicides. Alternative mechanisms, other than genetic modification, may be responsible for the higher incidence of insensitive colonies in 2019 bioassay experiments.
- Several other FRAC Groups were also tested using the 2019 bioassays. In some cases, *P. cerasi* isolates were insensitive to fungicides from several FRAC Groups indicating that there is a good chance of fungicide resistance in FRAC Groups in addition to FRAC 3 and FRAC11. We complemented the bioassay results with molecular confirmation on isolates resistant to DMI and QoI fungicides. Focus was on molecular assays in 2020 and 2021.
- In order to determine the *prevalence* of the Group 11 and Group 7 resistance problem an additional 100 isolates were collected from the production areas of Eastern Washington in 2021. Sampling began 1 June in Benton, Franklin, and Yakima Counties and 15 June in Chelan, Douglas, Grant, and Okanogan counties. A total of 151 orchards were sampled for powdery mildew during as of October 15. Preharvest powdery mildew was rare and (despite several collection trips) significant orchard infestations were not found until late July.

Therefore 98 of the 100 isolates were collected July 21 through October 15. PCR evaluations of 2021 isolates for Groups 11 and 7 resistance will be conducted during fall 2021 and winter 2022. Foliar mildew was not found in 34% of the orchards sampled in 2021. Potential reasons for the significant delay in 2021 epidemics are discussed in the "Results and Discussion" section below. In our Roza experimental orchard (where no fungicides were applied) initial detection of foliar mildew occurred 8-9 weeks later than they appeared in 2008-2020.
DNA was extracted from each isolate upon return to the lab. Quantitative PCR reactions will be processed to determine Group 11 and Group 7 resistance status in October-December 2021

- DMI target gene was identified. Full-length gene was sequenced and assays to distinguish mutations corresponding to DMI -resistant and DMI-susceptible isolates were developed. The assay was used to identify the presence and extent of DMI resistance in nursery and orchard *P. cerasi* isolates. Overall, we found 28% isolates resistant to DMI fungicides (molecular-based detections).
- Major SDHI target gene, SDHB was identified using data from next-gen sequencing in 2018, and mutations corresponding to SDHI resistance were identified. Of the three fungicide groups (DMI, SDHI, and QoI), very few *P. cerasi* isolates were SDHI-resistant (10%).
- QoI target gene was tested in all cases and we found molecular evidence of fungicide resistance in 43% of isolates in 2019. Although there is no discernible pattern in the geographic distribution of isolates resistant to FRAC Group 11, we found a dramatic increase (87%) in QoI resistance in one of the orchards as a follow-up study.
- Fungicide programs without Group 11 fungicide components successfully managed powdery mildew.

Methods Used

- The methods for handling of *P. cerasi* isolates were followed as proposed in the project. In addition to the isolates from commercial orchards, several *samples* were collected from four independent nurseries and tested for the presence of DMI fungicide resistance in 2020. In 2019 all *P. cerasi* isolates were collected as composite isolates from each location. In 2020, individual colonies growing as foliar infections were collected directly in separate tubes and treated as single isolate (Table 1). Composite isolates were used in 2021 (Table 2).

Table 1. List of 2019-2020 *P. cerasi* isolates from orchard and nursery sites in Washington and Eastern Oregon.

						Collection method	
No.	**Code**	**Production Area**	**County**	**Variety**	**Management**	**2019**	**2020**
1	DH	Columbia Basin	Franklin	Bing	Conventional	Composite	Individual
2	CS	Columbia Basin	Franklin	Rainier	Conventional	Composite	
3	MH	Yakima Valley	Yakima	Bing	Conventional	Composite	Individual
4	HL	Columbia Basin	Grant	Rainier	Conventional	Composite	Individual
5	SC	Columbia Gorge	Wasco, OR	Rainier	Conventional	Composite	Individual

AR	Columbia Gorge	Hood River		Conventional	Composite	Individual
Roza	Yakima Valley	Benton	Bing	No fungicides	Composite	Individual
TP	Yakima Valley	Klickitat	Sweetheart	Conventional	Composite	Individual
RS	Wenatchee	Okanogan	Rainier	Organic	Composite	
BR	Wenatchee	Okanogan	Sweetheart	Conventional	Composite	
BO	Wenatchee	Chelan	Rainier	Conventional	Composite	
HF	Wenatchee	Chelan	Rainier	Conventional	Composite	Individual
ST-1	Wenatchee	Chelan	Sweetheart	Organic	Composite	Individual
ST-2	Wenatchee	Chelan	Bing	Organic	Composite	
BC	Yakima Valley	Benton	Bing	Conventional	Composite	Individual
BM	Columbia Basin	Grant	Bing	Conventional	Composite	
HT	Yakima Valley	Yakima	Rainier	Conventional	Composite	
OR	North Central WA	Okanogan	Lapins	Conventional		Individual
WD	Columbia Basin	Grant	Bing	Conventional	Nursery	Individual
ML	Columbia Basin	Grant	Bing	Conventional	Nursery	Individual
CO	Columbia Basin	Grant	Montmorency	Conventional	Nursery	Individual
CN	Columbia Basin	Franklin	Bing	Conventional	Nursery	Individual

- We found unexpectedly greater DNA sequence variability in the cytochrome b (cytb) sequence, the molecular target gene of Group 11 (QoI) fungicides (but the high similarity to deduced amino acid sequence). The approach to primer design for amplification of this gene from several samples is still a challenge. We used the data from the next-generation sequencing experiment to identify the full-length sequence but were unsuccessful to obtain (amplify) the complete sequence. Nonetheless, the partial cytb DNA sequence harboring mutations of interests were readily amplified using two sets of primer pairs.

- The CYP51 DNA sequences of myclobutanil and triflumizole- insensitive colonies (from bioassay experiments) were sequenced from several isolates. We found a single but less frequent mutation that correlated with bioassay results. The PCR analysis in 2020 was performed using an assay developed to identify the mutations of the target gene, CYP51. We used qPCR assays to distinguish between DMI-resistant- and susceptible isolate. Similarly, one of the major SDHI target genes, SDH-B was identified, and the information was used to obtain mutations corresponding to SDHI resistance in orchard isolates.

We have initiated efforts to provide recommendations based on our findings in 2019 and 2021. In 2021, we consulted with growers, WTFRC, pesticide companies, and extension specialists to prepare recommendations for resistance management. The resistance assays develop herein can be communicated to interested parties for the possible commercialization of the molecular diagnostic assays.

Table 2. 2021 powdery mildew isolate collection**.** Surveys were conducted 1 June-15 October, 2021. Note that 2021 epidemics were severely delayed due to weather factors.

County	Number of Isolates/Orchards with powdery mildew	Initial Positive Collection[1]	Total Orchards Visited
Benton	19	2 August	25
Chelan	22	30 July	30
Douglas	2	10 September	10
Franklin	8	24 August	20
Grant	4	15 July	10
Okanogan	21	10 September	32
Yakima	15	12 August	24

- We presented research results in written/online publications (Plant Disease, The Goodfruit Grower, Fruit Matters, and stone fruit pathology section of EB0419) and (due to COVID restraints) industry webinars (Table 3).

Results and Discussion

A series of industry webinars on fungicide resistance were held in January-February 2021 (Table 3). These included mitigation strategies for Group 11 and Group 3 fungicide resistance in cherry. Over > 1,000 individuals were reached.

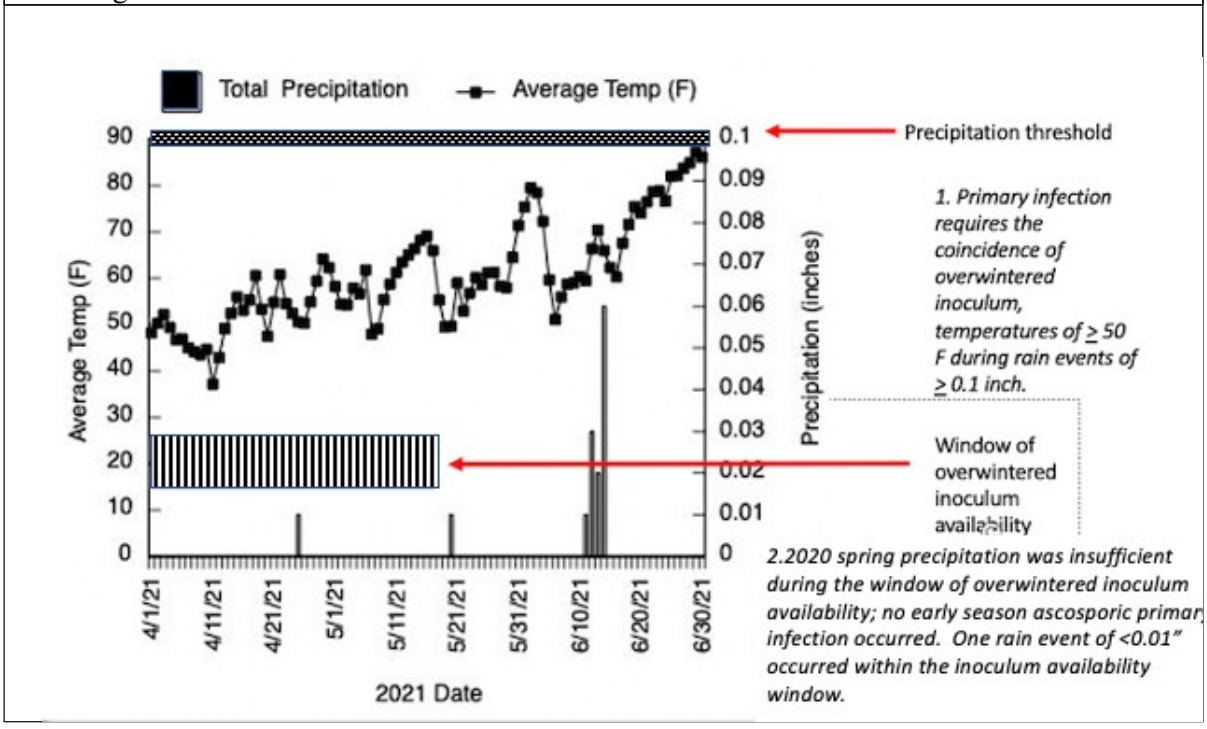
Table 3. 2021 Industry presentations on the fungicide resistance issue in sweet cherries.

Events	Type	Date	Title
Cherry Institute	Webinar	January 20	*Cherry Powdery Mildew Management and Fungicide Resistance*
Chelan Hort Day	Webinar	January 20	*Achieving Successful Cherry Powdery Mildew Control in the Face of Resistance*
WSU Tree Fruit Days	Webinar	January 21	*Cherry Powdery Mildew Product Resistance*
Chamberlain Distributors	Webinar	February 2	*Cherry Powdery Mildew Product Resistance*
WSU-OSU Tree Fruit Webinar Series	Webinar	February 11	*Sweet Cherry Powdery Mildew Update*
WSU Fruit Matters	Online video	April	*Sweet Cherry Powdery Mildew Updates*
WSU Fruit Matters	Online video	April	*Q&A with Dr. Gary Grove on Sweet Cherry Powdery Mildew*

In the 2021 growing season orchard sampling commenced on 1 June in Benton, Franklin, Grant and Yakima counties and 15 June in Chelan, Douglas, and Okanogan counties. A total of 151 orchard sites were visited over the course of the growing season. One hundred isolates (from 100 different orchards) of *P. cerasi* were collected beginning in mid-July (note that mildew was absent prior to the "collection" date) in Grant county and 2 August, 30 July, 10 September, 17 August, and 10 September in Benton, Chelan, Douglas, Franklin, and Okanogan counties, respectively. Due to the epidemiological "delay" in 2021, quantitative PCR reactions to detect resistance will be applied in October-December 2021 on the 100 isolates and the results distributed in a supplemental report.

The emergence of powdery mildew 4-6 weeks later than normal complicated our collection strategies but perhaps taught us a valuable lesion re: weather conditions and mildew epidemiology. For example, the initial observance of mildew symptoms and signs in our experimental orchard at WSU-Roza occurred fully two months later than in the 2008-2020 growing seasons. No fungicides have been applied in this orchard since establishment which ensures ample amounts of overwintered inoculum and high disease pressure. The delayed epidemic onset was probably due to lack of spring moisture and excessively high temperatures in late June (Figure 1). Primary infection requires at least 0.1" precipitation at ≥ 50 F during the window of overwintered inoculum availability while temperatures ≥ 85 F in inhibit the rate of establishment and reproduction of existing and new powdery mildew colonies. The moisture requirements were not met during the window of inoculum availability in the Roza orchard. The average temperatures reached nearly 90 degrees in late June. The moisture and temperature factors probably delayed the establishment and spread of powdery mildew, respectively.

Figure 1. Temperature and moisture conditions at WSU-Roza April-June, 2021. Data courtesy of WSU AgWeatherNet.



While the 2021 spring-summer weather complicated our sampling and delayed our PCR experiments they also may have offered the industry a respite from fungicide resistance selection pressure. The majority of fungicide applications are made earlier than mid-July in most low-elevation orchard

locations so it is possible that many populations of *P. cerasi* were not established during this time frame not exposed to fungicide selection pressure during the 2021 growing season.

In 2020 cherry growing season, several individual isolates of *P. cerasi* were collected from nursery and commercial orchards (including organic orchards) in all cherry growing regions of Oregon and Washington (Table 1). All isolates collected in 2019 were tested in mildew-susceptible leaf discs treated with candidate fungicides with an application rate equivalent to 200 gallons spray material per acre (please see 2019 continuing report). In 2020, all isolates were tested using molecular methods. Mutations corresponding to DMI, SDHI and QoI were identified using qPCR (DMI) and cloning and sequencing (SDHI and QoI) approach. In 2019, leaf discs treated with fungicides pointed out a potential problem of fungicide resistance in the PNW cherry orchards. Follow up with molecular targets and their underlying mutations corresponding to fungicide resistance confirmed that *P. cerasi* isolates were indeed resistant to either DMI or QoI fungicides. All isolates (composite, individual, and nursery) were subjected to qPCR assays to distinguish between DMI- resistant and DMI- sensitive *P. cerasi* isolates using a probe-based qPCR assay (Figure 1). We found fungicide resistance as high as 45% in commercial orchards while 75% isolates were resistant to DMI fungicides in two nursery locations. We did not find resistance specific mutations in some orchard locations (Table 4). Our experiments indicated a potentially severe DMI resistance problem than originally anticipated. It should be noted however that, DMI fungicides belong to a broad class of antifungal agents with multiple modes of action on target pathogen. Additionally, pathogen resistance to one DMI class (e.g. triazoles) does not guarantee resistance to another DMI class (e.g. pyrimidines). For these reasons, each spray application involving DMI should be carefully evaluated. The fungicides should be removed and replaced with other effective groups from the mildew management in case of poor disease management.

Three target genes are targets of SDHI fungicides and mutations within these genes affect fungicide efficacy. Among those, mutations linked to SDH-B gene are of major significance. Using data from next- , we identified partial SDH-B gene and used this information to design and amplify
the gene target from several individual isolates collected in 2020. The SDH-B gene from a total of 58 isolates was sequenced which harbored region of interest potentially carrying all mutations correlated with SDHI resistance in several species. Of these isolates, 4 isolates contained H272R mutation while 3 independent isolates contained N230I/H mutation, both corresponding to SDHI resistance in other pathogen species. The percentage of mutation (or potential resistance) against SDHI fungicides appeared relatively lower in the cherry orchards suggesting a minimal risk of developing widespread resistance within a short period of time.

Analysis of QoI target gene, cytb was particularly challenging due to high heterogeneity in the DNA sequence. Partial gene sequencing using two independent sets of primer pairs revealed 40% resistance (G143A) in 2019. All resistance isolates from the bioassay experiments were confirmed to contain G143A mutation. We followed up the mutation rate in one of the orchard sites (Roza experimental orchard) in 2020 and the analysis revealed that as much as 87% (13 of 15) isolates contained G143A mutation (Table 5). Based on this study, QoI fungicides are clearly at risk of losing its efficacy either as single or premixed formulation.

Table 4. Analysis of DMI resistant in individual *P. cerasi* isolates from commercial orchard and nursery locations collected in 2020.

	CYP 51 alleles						
Isolates	**G**	**G+S**	**S**	**ND**	**Total**	**Resistant colonies**	**% Resistance**
Roza	14	2	0	4	20	2	10
MH	9	8	0	5	22	8	36
BC	7	0	0	1	8	0	0
DH	17	0	0	3	20	0	0
HL	2	0	0	0	2	0	0
HF	12	0	0	8	20	0	0
OR	12	2	4	2	20	6	30
ST	13	0	7	0	20	7	35
TP	12	0	3	5	20	3	15
SC	12	1	6	1	20	7	35
AR	8	5	4	3	20	9	45
WD	13	3	0	4	20	3	15
ML	19	1	0	0	20	1	5
CO	5	15	0	0	20	15	75
CN	5	15	0	0	20	15	75

Table 5. Summary of QoI resistant *P. cerasi* isolates in PNW.

Season	Isolate	Mutation	# resistant isolates	Percent QoI resistance
2019	Composite	G143A	6 of 15	40
2019	QoI resistant (bioassay)	G143A	9 of 9	100
2020	Individual	G143A	13 of 15	87

In anticipation of the widespread QoI resistance in PNW, we initiated a response program to identify efficacies of fungicide programs with or without QoI fungicides in a nursery trial. A nursery was chosen for the trial because of expansive planting, high powdery mildew disease pressure in every growing season, and ease of spray applications in nursery settings. We included three applications of QoI fungicides (single and premixed formulation) in regular intervals while non-QoI fungicide program included in another non-QoI treatment. At the end of nursery trials, the foliar disease incidence and severity were measured which indicated better disease management (less disease severity) in trees sprayed with non QoI fungicides (Table 6).

Table 6. Powdery mildew disease incidence and severity in the nursery spray trial involving QoI and non-QoI fungicides. 2020* severity is based on surface area colonized. ** significant compared to QoI applications. *** significantly higher incidence and severity in control, untreated trees.

		Incidence (of 25 trees)			Severity	
	Plot	22-Jun	6-Jul	24-Aug	6-Jul	24-Aug
Non-QoI	1	12	16	12	14	13**
	2	11	10	15	12	13**
QoI	1	8	11	17	11	18
	2	18	13	12	12	15
Control	-	16	19	25	15	33***

* severity is based on surface area colonized. ** significant compared to QoI applications. *** significantly higher incidence and severity in control, untreated trees.

EXECUTIVE SUMMARY

Fungicide Resistance: A Vital Need To Protect PNW Cherries From Mildew

Keywords: fungicide resistance, FRAC Group 11, FRAC Group 3, FRAC Group 7, DMI, QoI, SDHI

Abstract

Management of the powdery mildew of cherry, caused by *Podosphaera cerasi*, is due to its explosive epidemiological nature, expense, and MRL complications a complex issue for the PNW cherry industry. Fungicide resistance was studied 2019-2021. The project was originally focused on FRAC Group 11 fungicides but was expanded to include FRAC Groups 3 and 7. Resistance was documented in Group 3, Group 11, and Group 7 compounds, respectively, in 2019 and 2020. *Resistance was far less prevalent to Group 7 compounds*. Powdery mildew was "epidemiologically late" in 2021 and the foliar phase of the disease did not appear until mid-July, far later than during previous growing seasons. The 100 isolates collected in late July-October of 2021 are currently being processed for detection Group 11 and 7 resistance. Revised management guidelines were communicated to the industry using webinars and online articles during the winter of 2021. Management guidelines included the adoption of an "areawide" approach to disease management, added reliance on fungicides not in FRAC Groups 3 and 11 with cognizance of potential MRL issues, limiting Group 3 and 11 usage to 1 application each per growing season, the use of tank mixes of contact fungicides (e.g. sulfur) with Group 3 and 11 compounds, and the avoidance of sequential applications of Group 3 and 11 compounds. Industry feedback indicated the MRL issues preclude the complete adoption of the recommended strategies. Fungicide programs free of Group 11 compounds provided somewhat better control of PM in nurseries than did those containing them. However, comparing multiple fungicide programs minus Group 7 and 3 compounds was beyond the scope of this study. The reasons for the extended delay in the development of foliar mildew in 2021 was probably due to meteorological conditions. First, the moisture needed for primary infection (0.1" of moisture at $\geq$50 F) were not met during the window of available overwintered inoculum (produced during late spring and summer of 2020). The, average temperatures during the last week of June was nearly 90 F, well above conditions that promote spread and intensification of the disease. These two factors served to delay the exponential phase of 2021 foliar cherry mildew epidemics from June to late July/August, well after harvest. This phenomenon served to significantly lower the risk of fruit infection. Therefore, the industry experienced a weather-related "respite" from resistance selection pressure because in many cases fungicides were applied when the disease was not yet present in orchards. The 2021 mildew scenario has serious future (and in some cases positive) implications if the temperature and moisture conditions of 2021 become a permanent pattern.

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

PROJECT TITLE: Durable genetic solutions to powdery mildew infection in sweet cherry

PI:	Cameron Peace	**Co-PI (2):**	Per McCord
Organization:	WSU - Horticulture	**Organization**:	WSU - IAREC
Telephone:	509-335-6899	**Telephone**:	509-786-9254
Email:	cpeace@wsu.edu	**Email**:	phmccord@wsu.edu
Address:	Johnson 39	**Address**:	WSU - IAREC
Address 2:	PO Box 646414	**Address 2**:	24106 N Bunn Rd.
City/State/Zip:	Pullman/WA/99164	**City/State/Zip**:	Prosser/WA/99350

Cooperators: Alexandra Johnson (WSU graduate student – Horticulture, Pullman); Gary Grove (WSU – IAREC Plant Pathology, Prosser)

TOTAL PROJECT REQUEST: **Year 1:** $44,000 **Year 2:** $44,000 **Year 3**: $0

Other funding sources:

Agency Name: USDA Germplasm Evaluation Funds, Prunus
Amt. awarded: $28,000 (2020-2021)
Notes: "Germplasm evaluation for sweet cherry genetic diversity and disease resistance" – a project involving DNA profiling and leaf disk assay phenotyping for powdery mildew, bacterial canker, and X-disease infections of all cherry accessions in the National Plant Germplasm System growing at the National Clonal Germplasm repository in Davis, CA.
PI: Cameron Peace. Co-PIs: John Preece, Stijn Vanderzande, Alexandra Johnson.

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 NIFA – SCRI
Amt. awarded: $10 million (Sep 2014 – Aug 2019)
Notes: "RosBREED: Combining disease resistance with horticultural quality in new rosaceous cultivars" for expanding DNA-informed breeding strategies, tools, and knowledge for rosaceous crops. PI: Amy Iezzoni. Co-PIs include Cameron Peace and Per McCord.

BUDGET

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

Item	2019	2020	2021
Salaries[a]	$26,236	$27,285	
Benefits	$2443	$2541	
Wages	$5330	$5543	
Benefits			
Equipment			
Supplies[b]	$3516	$2156	
Travel[c]	$2000	$2000	
Plot Fees	$4475	$4475	
Miscellaneous			
Total	$44,000	$44,000	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

RECAP OF OBJECTIVES

1. Determine the long-term durability of Pmr1 for providing mildew resistance to the PNW industry
   a. Ascertain the pathogen's ability to overcome Pmr1 resistance
   b. Update knowledge about which selections and other PNWSCBP germplasm have Pmr1

2. Determine usability of alternative genetic sources for powdery mildew resistance
   a. Evaluate a diverse set of germplasm for degree of fruit powdery mildew resistance
   b. Identify other genetic factors capable of conferring mildew resistance in PNWSCBP germplasm
   c. Refine the DNA test for resistance to encompass new sources if they exist
   d. Identify which alternative genetic resistance factors are present in important germplasm individuals

SIGNIFICANT FINDINGS

- Four genetic variants (alleles) at the *Pmr1* gene were identified, tested, and defined for their influence on imparting resistance or susceptibility to the devastating foliar and fruit disease, powdery mildew (PM) (Obj 1 & 2)
- The four *Pmr1* alleles explain the major genetic differences in PM incidence observed among cultivars and breeding material; no further genomic regions were detected (Obj 2b). Three of these alleles represent "genetic resistance factors":
    - Pmr1a: Confers complete resistance to PM infection, regardless of mildew pressure, as concluded in previous years and confirmed over the past three years as the positive control
    - Pmr1b: Confers complete resistance to PM infection, regardless of mildew pressure, as concluded by no infection observed over three years in detached leaf disk assay
    - Pmr1c: Sometimes confers field resistance to PM but not reliable, as infection was repeatedly observed in detached leaf disk assay
- The current DNA test can determine the *Pmr1* genotype and thus predicted resistance vs. susceptibility of most breeding individuals. But genotypic similarity of Pmr1b and c sometimes will necessitate use of the detached leaf disk assay for new greenhouse seedlings until we complete the DNA test refinement (Obj 2c). For parents and selections, DNA profiling with the cherry SNP array readily distinguishes all allelic combinations
- PNWSCBP parents, selections, and seedlings were identified as carriers of Pmr1a, Pmr1b, Pmr1c, and the common susceptibility allele, pmr1, and such DNA information is available to inform breeding decisions (Obj 1b, 2d)

METHODS

Three-year plan: **2019** – Use previously optimized foliar infection protocols (field and in vitro) for collection of a first season of data. Begin genetic dissection of fruit resistance. **2020** – Continue use of foliar infection protocols (field and lab) to collect a second season of data to validate 2019 results. **2021** – Develop a refined DNA test using data gathered over three years.

Germplasm use and tree management: Trees used for evaluation were growing at the Roza experimental orchard, part of Washington State University-Irrigated Agriculture Research and Extension Center (WSU-IAREC). Individuals selected for this study came from genetic stock trees in the RosBREED block (C53), breeding program mother block (B53), and the Toyama selection block

(A37); all of which represent the diversity of the WSU sweet cherry breeding program (about 510 genetically distinct trees total). High-resolution, DNA-profiles of trees in the germplasm from the RosBREED project included those individuals thought to harbor PM-resistance factors.

Germplasm used in 2019, 2020, and 2021 included several offspring from resistance sources (15 individuals). These descendants along with their parental sources (40 trees, 25 individuals) included trees expected to be PM-resistant and others expected to be susceptible based on the genotypic presence/absence of Pmr1 and Pmr1-like genetic factors. Pedigree-connected cultivars known to be susceptible were included as positive controls, including 'Bing,' 'Rainier,' and 'Sweetheart.' Management of orchard trees was conducted in accordance with standard practices of the WSU breeding program with the exception of a misapplication of fungicides mid-season in 2019 in which the original intention for the project was no fungicides to be used at all to keep the pathogen pressure high in the research orchard block.

Foliar PM-resistance evaluation – orchard: To assess initial infection within the orchard, chosen trees were observed for signs of mildew infection beginning with leaf emergence. Infection assessment was halted upon discovery of fungicidal applications in 2019. In 2020 and 2021, chosen trees at the Roza were observed weekly from leaf emergence to early senescence for signs of mildew infection.

Foliar PM-resistance evaluation – lab: A lab-based detached leaf disk assay was performed on the chosen germplasm set for a second year. Briefly, this previously optimized assay began with collecting the first fully expanded leaf from a terminal shoot and transporting it to the lab for surface-disinfestation (10% bleach solution for 3 minutes followed by a quadruple rinse in sterile distilled water). From each leaf, a circular disk (12 mm in diameter) was excised and placed abaxial side up on a new well containing 500 µl water agar of a 24-well plate. Assays conducted for PM resistance/susceptibility consisted of two leaf disks from two independent leaves sampled from each germplasm individual. Conidial suspensions of *P. clandestina* were generated by gathering infected leaves from the mildew block of 'Bing' and 'Sweetheart' trees at the Roza as well as mildew-infected cherry trees growing in a greenhouse in Pullman, submerging them in a 0.01% TWEEN solution, and agitating the mixture until conidia were present in solution at sufficient numbers. A 10 µl conidial suspension of 15,000 conidia per mL (quantified through manual count using a hemocytometer) was administered to each leaf. Upon deposition on the leaf disks, conidia were allowed to settle for 5 minutes before residual moisture was wicked away using a sterile cotton swab. Settling time maximizes number of infectious propagules achieving contact with leaf surface, which in turn maximizes likelihood for infection establishment. Plates were subsequently sealed with parafilm to prevent contamination as well as moisture loss, and leaves with conidia were co-cultivated for 14 days in a plant growth chamber at 20°C and a 14 h light period. Plates were then viewed using a stereoscope and mildew presence/absence was assessed. A result was noted as positive if any signs of infection were observed, and negative only if zero mildew was found.

Foliar PM-resistance evaluation – durability under high pathogen pressure – lab: Testing of individuals harboring Pmr1 and Pmr1-like resistance factors for breakdown to resistance under high pathogen pressure was conducted using the standard detached leaf disk assay with different conidial concentrations. Conidia were collected from the mildew block of 'Bing' and 'Sweetheart' trees at the Roza as well as from trees infected with mildew growing in a greenhouse in Pullman, and three concentrations of mildew suspension containing low (140 conidia per mL), medium (1,400 conidia per mL), and high (15,000 conidia per mL) levels of conidia were generated. These three concentrations of pathogen were applied to leaf disks according to the standard detached leaf disk assay protocol and infection presence/absence was assessed after 14 days. Any mildew growth was noted as positive for infection, and negative was recorded only if zero mildew was found.

Genetic dissection of resistance/susceptibility: Comparison of DNA profile information from use of the 6K and 6+9K SNP arrays gathered previously from the RosBREED project was used to facilitate the discovery of genetic differences among alleles of the *Pmr1* locus.

RESULTS & DISCUSSION

Summary: The two identified and verified genetic sources for PM resistance in the PNWSCBP are Pmr1a and Pmr1b. Both genetic factors appear to be durable to high pathogen pressures. Resistance versus susceptibility to PM infection was discernable and repeatable for all individuals tested over three years. Evaluations during the 2021 season verified findings from the 2019 and 2020 seasons, and further substantiated the presence of a second mildew resistance factor. Beyond the previously known Pmr1 resistance factor from 'Moreau' (Pmr1a), evidence was obtained for an additional mildew resistance factor (Pmr1b) from 'Hedelfingen' and Mildew Immune Mazzards. A third resistance factor, Pmr1c, from 'Schneiders' and its offspring 'Regina', was confirmed to be associated with mildew susceptibility (Figure 1). Genetic dissection could not elucidate differences between Pmr1b and Pmr1c at the current level of resolution available; however, work is ongoing to update the DNA-based test. Therefore, PM genetic resistance status of any cultivar, parent, selection, or seedling can be efficiently determined at present by utilizing the available DNA test and followed in some cases with the detached leaf-disk assay, or via the 6+9K sweet cherry SNP array except for seedlings.

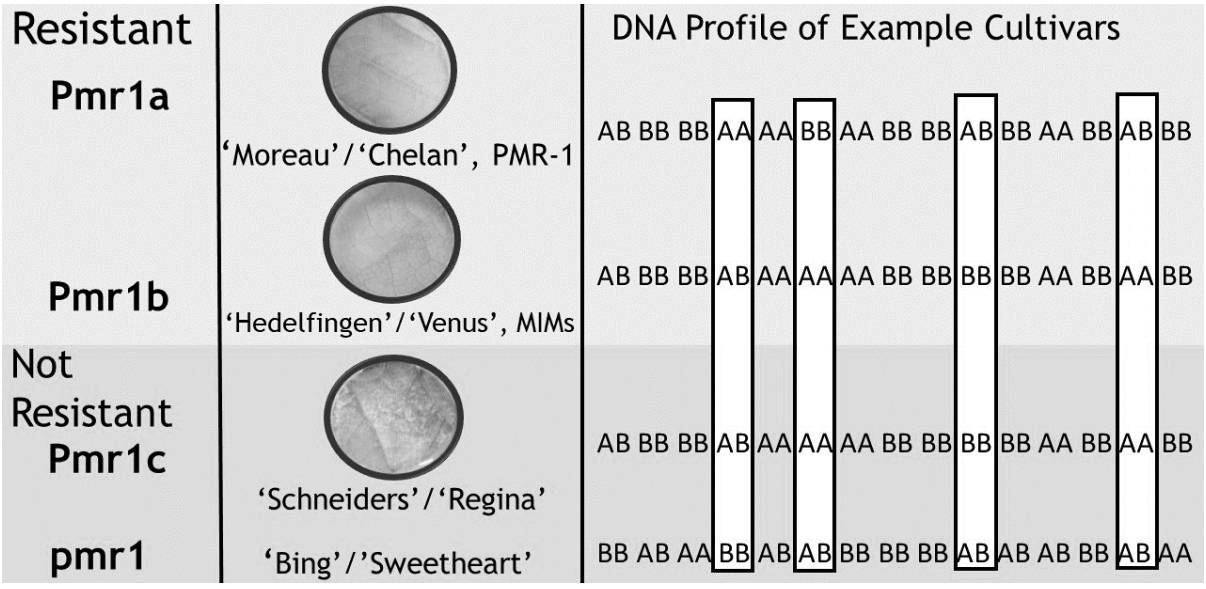


Figure 1. Grouping of mildew resistance determined by leaf disk assay results and DNA profiles (a portion of the DNA profile at the Pmr1 *gene is shown). At the examined level of resolution (DNA profiling by the 6+9K cherry SNP array), Pmr1b and Pmr1c are unfortunately identical.*

Foliar PM infection – in-lab evaluation: Resistance vs. susceptibility results from 2019 and 2020 were confirmed again in 2021 using the detached leaf disk assay. Consistent with previous findings, individuals containing one or two copies of Pmr1a (from 'Moreau' and its offspring) were observed to have no forms of mildew infection. Individuals harboring one or two copies of the Pmr1b resistance factor (from 'Hedelfingen' and MIMs lineages) were also observed to be associated with complete freedom from mildew infection, substantiating the strong evidence for the presence of this

second resistance factor in the PNWSCBP germplasm. However, individuals within the 'Schneiders' family (including its offspring 'Regina') with one or two copies of Pmr1c consistently developed PM infection in detached leaf disk assays, confirming the previous results that Pmr1c is not effective for conferring complete mildew resistance. However, in-orchard evaluations have determined that individuals harboring the Pmr1c resistance factor are less susceptible to mildew infection, indicating this factor might offer some protection in some orchard conditions and that it is not the same as the allele for full susceptibility, pmr1.

Foliar PM infection – in-orchard evaluation: While orchard observations were conducted weekly in 2020 and 2021, cool weather unfortunately (for this research) delayed mildew development in the orchard until leaves were mature and therefore physiologically resistant to mildew infection. Infection was observed in some susceptible 'Bing' and 'Sweetheart' cultivars at the Roza orchard during the 2020 season and late in the 2021 season, but mildew infections were not identified in other cultivars, selections, and seedlings.

Durability of genetic resistance for Pmr1 variants: Experiments conducted in 2020 using increasing levels of lab-standardized pathogen pressure – low (140 conidia per mL), medium (1400 conidia per mL), and high (14,000 conidia per mL) – demonstrated the effectiveness of Pmr1a and Pmr1b in resisting mildew infection regardless of the quantity of conidia present. In contrast, individuals harboring Pmr1c were consistently susceptible to mildew infection regardless of conidial concentration. These results indicate that the newly defined Pmr1b resistance factor is likely just as robust as the previously identified mildew resistance factor Pmr1a in inhibiting mildew infection. Additional testing during the 2021 season provided similar results, with Pmr1a- and Pmr1b-containing individuals never succumbing to mildew infection while those with only Pmr1c or pmr1 developed infection. Mildew pressure over 15,000 conidia per mL was attempted but not achievable, even with additional mildew gathered from infected cherry seedlings maintained in a greenhouse in Pullman.

Genetic dissection of Pmr1b resistance: Further genetic dissection in 2020 revealed genotypic similarity between the resistance-conferring Pmr1b from 'Hedelfingen' and MIM lineages and the ineffective Pmr1c from the 'Schneiders' lineage. Additional genetic examination in 2021 could not reveal differences between the effective Pmr1b and the ineffective Pmr1c. Work is ongoing through the end of 2021 to identify DNA-based differences that could be used to update the DNA test for efficient screening of seedlings in the PNWSCBP via routine marker-assisted seedling selection. While the currently available PM-resistance DNA test generates the same outcome for presence of Pmr1a, Pmr1b, and Pmr1c resistance factors, due to each group's phenotypic differences, incorporation of a detached leaf-disk assay could be used to confirm resistance status in some cases. This approach would involve first using the DNA tests to greenhouse-raised seedlings as usual, and then applying the leaf disk assay to those seedlings known to have one or two parents having both Pmr1a or b and Pmr1c. This second step of the leaf disk assay should readily identify those seedlings that have not inherited Pmr1a or b.

Phenotypic results from field and lab evaluation combined with genotypic results distinguished four groups of sweet cherry individuals (Table 1). Groups 1 and 2 both represented resistance to powdery mildew, and can be genotypically distinguished from each other (if desired) and from Group 4. Groups 3 and 4 both represented susceptibility to powdery mildew. Groups 2 and 3 cannot yet be genotypically separated – resolving this is the target for the final few months of the project in late 2021. No individuals included in Table 1 carried two Pmr1 resistance factors – all carried at least one pmr1 allele. Some other cultivars, RosBREED germplasm individuals, National Plant Germplasm System collection accessions, and PNWSCBP seedlings have been detected that carry two copies of Pmr1a (Group 1), one Pmr1a and one Pmr1b (Group 1), one Pmr1a and one Pmr1c (Group 1), or one Pmr1b and one Pmr1c (Group 2). None were identified with two Pmr1c

copies (Group 3). Of the ten R selections currently in PNWSCBP Phase 2 and Phase 3 trials, none carry the resistance factors Pmr1a or Pmr1b. Nine carry two copies of pmr1 for susceptibility (thus Group 4), while one carries pmr1 and the other allele associated with some susceptibility, Pmr1c (Group 3).

Resistance factor group	Alleles carried	Germplasm source of resistance factor	Field observations and lab evaluations			Individuals exemplifying and examined
			2019	2020	2021	
1 – Resistant	≥ one Pmr1a	Moreau	resistant	resistant	resistant	Moreau, PMR-1, Chelan, DD, GG
2 – Resistant	Not above and ≥ one Pmr1b	Hedelfingen, MIMs	resistant	resistant	resistant	Hedelfingen, Venus, MIM 17, MIM 23
3 – Susceptible	Not above and ≥ one Pmr1c	Schneiders	susceptible	susceptible	susceptible	Schneiders, Regina
4 – Susceptible	Two pmr1	na	susceptible	susceptible	susceptible	Bing, Sweetheart, Rainier

Table 1: Four genetic groups determined in this project for powdery mildew resistance in sweet cherry cultivars and other germplasm

Pmr1a and Pmr1b are capable of providing "singe-gene" resistance to PM infection, and this resistance appears to be durable to high pathogen pressure. The complete resistance effect associated with the presence of either of these resistance factors masked any additional effect of having one of each factor in the same individual. If somehow one of the resistance factors could be overcome by the pathogen in the future, perhaps the other resistance factors would not succumb simultaneously. Additionally, it is not known if two copies of Pmr1c in an individual would improve resistance, or if Pmr1c is capable of providing weak protection from mildew infection in orchard settings that have reduced pathogen pressure. Field experiments designed to determine if Pmr1c is mildly effective in reducing PM infection would help elucidate if this factor confers any desirable degree of mildew-avoidance in 'Schneiders' and its descendants. Updating the DNA test to detect all functional genotypic differences among the alleles will be useful in developing superior new cultivars with combined tree and fruit qualities that meet the needs of the PNW cherry industry.

EXECUTIVE SUMMARY

PROJECT TITLE: Durable genetic solutions to powdery mildew infection in sweet cherry

Key words: infection, resistance, breeding, germplasm

The objectives of this project were to push the limits of durability of the previously described powdery mildew (PM) resistance factor, Pmr1, and to elucidate if other mildew resistance factors were present in the PNW sweet cherry breeding program (PNWSCBP) to inform refining of the available DNA test and determining the genotype any breeding individual. These objectives were met by evaluating a large, representative germplasm set that encompassed the diversity of the PNWSCBP. Multiple Pmr1 resistance factors were identified and characterized in this project.

Resistant and susceptible cultivars were evaluated over three seasons for PM infection in the field as well as in the laboratory using detached leaf disk assays. In the orchard, mildew pressure was significantly reduced by misapplication of fungicide in the first year of this project. Subsequent years had no mildew in the treated orchards and very low mildew pressure in surrounding, untreated trees. Leaf disk assay results of 2019 indicated the presence of further resistance gene; however, fungicides rendered results inconclusive. Leaf disk assays were again performed in 2020 and 2021. All leaf assays indicated strong resistance in 'Moreau' and some descendants as well as in Mildew Immune Mazzards, 'Hedelfingen', and some descendants. All other cultivars, including 'Regina', were observed to be susceptible to infection. Genotypic differences were detected among Pmr1a, Pmr1b, and pmr. However, there was no discernable difference between Pmr1b and Pmr1c, requiring increased genomic resolution to refine the DNA test. Genotyping used the available PM DNA test and genome-wide DNA profiles. Comparison of phenotypic and genotypic information has revealed:

1) The original factor described for PM resistance from 'Moreau', its offspring 'Chelan', and its grandchild PMR-1 is not the only one at the *Pmr1* genomic region and thus is now distinguished as "Pmr1a". Pmr1a remained durable to very high mildew pressure in leaf disk assays.
2) An additional factor, "Pmr1b", was also associated with complete resistance to PM infection and, like Pmr1a, only needs to be present in one copy to be as durable when presented with high pathogen pressure in leaf disk assays. The pedigree origins of Pmr1b are 'Hedelfingen' and its offspring 'Venus'.
3) A third factor from 'Schneiders' and its offspring 'Regina', "Pmr1c", was identified as genotypically identical to Pmr1b but was associated with susceptibility to PM infection at low through to high pathogen pressures in leaf disk assays.
4) All other factors present at the same genomic region were associated with susceptibility to PM infection when present as two copies (such as in 'Bing', 'Sweetheart', and most others cultivars) and are thus are all termed "pmr".
5) Screening for mildew resistance vs. susceptibility can be done quickly and efficiently, particularly of seedlings, utilizing the already available DNA assay in conjunction with a detached leaf disk assay where needed. Work is ongoing in 2021 to elucidate DNA-level differences between Pmr1b and Pmr1c that can be used to update the DNA test for distinguishing the presence of each factor.

Information gained from this project will be useful in establishing the PM genetic resistance status of any selection and cultivar as well as in developing new PM resistant cultivars. Evidence from this project indicates that cultivars with either the Pmr1a or Pmr1b genetic resistance factors will be durably resistant to powdery mildew infection. Having both genetic factors might provide increased durability. The tools and information developed in this project facilitate breeding for PM resistance, supporting the needs of the PNW sweet cherry industry for superior new cultivars.