2025 NW Cherry and Stone Fruit Research Review

Gerardo Garcia, internal member at the WTFRC, is spraying an orchard in East Wenatchee, WA for the Cherry Residue research project.

Photo credit: David Gonzalez

November 5, 2024

Hybrid

Yakima, WA

Northwest Cherry and Stone Fruit Research Review

Yakima Valley College Conference Center, Room C

November 5, 2024

Hosts: WA Tree Fruit Research Commission (WTFRC) & Oregon Sweet Cherry Commission (OSCC)

Guests: California Cherry Board (CCB), Northwest Nursery Improvement Institute (NNII)

Chairs: Craig Harris (WTFRC), Ian Chandler (OSCC)

Pesticide Credits: 3 Oregon and 4 Washington credits available

Time	Presenter	Title^	Status*	Year(s)
8:30		Registration		
9:00	Chairs	Welcome		
	Hanrahan	Meeting Etiquette & Housekeeping **(LIVE)**		
Project Reports				
		Little Cherry Disease		
9:15	Northfield	Overview of LCD activities + Taskforce updates **(LIVE)**		
9:25	Harper	Understanding little cherry disease pathogenicity	NCE/FINAL	20-22
9:35	Harper	Studying the infection progression of LCD in young trees	NCE/FINAL	22-23
9:45	Galimba	Determining Sweet Cherry Rootstock Sensitivity to X-Disease	CONT	23-25
9:55	Nottingham	Developing a Leafhopper DD Spray Program for Cherry IPM	NCE/CONT	22-24
10:05	Adams	Dispersive distance of X-disease vector leafhoppers in cherry	FINAL	22-24
Break 10:20 – 11:00				
11:05	Galimba	Physiology-based identification of X-disease infected trees	NCE/CONT	22-24
11:15	Zhao	Cas12a-Based Rapid Method for Early Detection of X-Disease Phytoplasma	FINAL	23-24
11:30	Khot	Towards Identification of LCD linked volatile biomarkers	NCE/FINAL	22-23
11:40	Kohntopp	Real-Time Detection of LCD using Detector Canines *(out of cycle)*	CONT	23-24
11:50	Harper	Evaluation of Simple, Cheap Tests for X-Disease in Bare Root Trees	FINAL	24
12:05	Pitino	Precision Agriculture: Innovating Cherry Disease Management with DPI	CONT	24-26
12:15	Pitino	Sustainable Cherry Protection with Symbiont	CONT	24-26
Lunch Break 12:25 – 2:00				
2:00	Schmidt	Resources for stakeholders **(LIVE)**		
		Spotted Winged Drosophila		
2:10	Beers	Coordinating SWD and X disease management	NCE/FINAL	21-23
2:20	Adams	Ganapsis Brasiliensis for Biological Control of SWD	CONT	23-25
2:30	Adams	SWD in-orchard movement + overwintering dynamics	NCE/CONT	22-24
		Powdery mildew/bacterial diseases/Food Safety		
2:40	Grove	Etiology of Wood Dieback of PNW Cherries *(out of cycle)*	NCE/FINAL	23
2:50	Zhao	Investigating Bacterial Canker Disease of Cherry in Young Orchards	CONT	24-26
3:00	Collum	Evaluation of UV-C for Management of Cherry Diseases and Pests	CONT	24-26
3:10	Murphy	Understanding Food Safety Risks During Post Harvest Cherry Production	CONT	24-26
Afternoon Break 3:10 – 3:40				
		Horticulture/Nutrition/Breeding/Misc.		
3:40	Torres	Improving grading methods to infer eating quality in sweet cherries **(LIVE)**		
3:50	Schmidt	Pesticide residues on WA cherries	CONT	23-25
4:00	McCord	A Robust PNW sweet cherry breeding and genetics program	FINAL	22-24
4:15	Sallato	Nutrient management for high quality sweet cherries	NCE/FINAL	21-23
4:25	Sallato	Precision Nutrient Management for Sweet Cherry Orchards	CONT	23-25
4:35	Whiting	Targets and Tools for Post-Bloom Thinning	FINAL	23-24
4:50	Leisso	Sweet Cherry Cultivar-Specific Export Suitability	FINAL	23-24
Social hour at Yakima Valley Vintners Teaching Winery 5:00-6:00 pm				

^Titles: some titles have been abbreviated to fit into table, see project reports for full titles *CONT = continuing project report (10 min), FINAL = final project report (15 min), **NCE: no-cost-extension (10 min)** All reports are available for download approximately two weeks prior to the research review from www.treefruitresearch.org. Final reports will be permanently added to the searchable database (https://treefruitresearch.org/advanced-search/) within three months of the research review. Additional inquiries may be addressed to Mackenzie Perrault (Mackenziep@treefruitresearch.com).

Project Title: Understanding little cherry disease pathogenicity.

Report Type: Final Project Report

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Cooperators: Washington cherry growers and extension agents.

Report Type: Continuing Project Report

Project Duration: 3 Years + No-Cost Extension

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 related/associated funding sources: Awarded
Funding Duration: 2021 – 2023
Amount: $249,000
Agency Name: WSDA Specialty Crop Block Grant Program
Notes: SCBG project 'Epidemiology of the X-disease phytoplasma' data aided in understanding biological and genetic evolution of the phytoplasma.

WTFRC Collaborative Costs: 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

Item	2020	2021	2022
Salaries	60,528	62,950	65,468
Benefits	23,034	23,956	24,915
Wages	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

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 interacting genes/proteins at the host level to develop a method to screen germplasm for tolerance/susceptibility.

Significant Findings:
1. The X-disease phytoplasma in the current outbreak isn't the same strain that caused the outbreaks in the 1930s-1950s. The current strain is milder, producing no obvious foliar or dieback symptoms, though still affects cherry quality.
2. All currently grown commercial cultivars of sweet cherry, sour cherry, and peaches/nectarines are susceptible to X-disease, with minor or subjective differences in severity. Environment, plant age, and general plant health are also significant determinants of disease progression. Plums and apricots show only minor symptoms. Furthermore, even in susceptible species, symptom onset and severity are virus or phytoplasma concentration dependent.
3. Fruit symptoms are the result of pathogen effector activity and plant response across the fruit maturation process, particularly from pit hardening to harvest. This coincides with the second sigmoidal phase of fruit development and highest levels of pathogen accumulation, and is across multiple pathways, prioritizing stress response over normal development, ultimately reducing fruit maturation.

Methods:

Objective 1. We established a 1-acre test block at WSU-IAREC consisting of 30 different cherry varieties, including commercially grown varieties, as well as cherries reported to have some level of tolerance or resistance to LChV2 or X-disease, and several accessions that represent more unique genetic backgrounds. To promote early fruiting, scions were budded on the precocious rootstock Gisela-6. In mid-late spring of 2020 (and 2021 for replacements), the budded trees were transplanted to the field. Inoculation with LChV2 or the X-disease phytoplasma was performed by chip budding of highly infected material in late summer of 2021. Orchard maintenance, including pruning, fertilization, pesticide application, and weed control, was conducted according to current horticultural practices.

Objective 2. Knowing how different cultivars respond to both LChV2 and X-disease phytoplasma is essential to developing an accurate field guide for growers. Therefore, we collected symptom development observations and physiological data from grower fields throughout the state, focusing on documenting symptom progression from fruit set to harvest on known infected trees, collecting data on fruit size, color (both skin and pulp) and shape, and correlated this data with cultivar type. We also examined 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 compared these to fruit from healthy, uninfected trees to determine infection effects on fruit quality.

Objective 3. The underlying genetic basis of X-disease development was examined in parallel with physiological studies. Samples of fruit, pedicel, and leaf tissue were collected from commercially grown Bing from bloom to harvest, depending on availability; due to removal of key orchards during this study, samples were collected from multiple sites. Tissue was macerated and total RNA extracted for library preparation and RNAseq. The resulting data was analyzed to identify genes that were differentially expressed to determine which pathways may be altered in cherry when infected with '*Ca*. P. pruni' and associated with the expression of the X-disease phenotype. LChV2-infected sweet cherry gene expression was discarded as few infected trees could be followed (due to removals and/or infection with other pathogens) for analysis.

Results and Discussion:

Objective 1. The test plot was established in May 2021 at the WSU Pear Acres field site. Scions were grafted onto Gisela-6 rootstock in the greenhouse during 2020 and early 2021, with failures re-grafted in the field after planting in August 2021 and bark-chip grafted with Little cherry virus-2 genotype Rube-74 or '*Ca*. P. pruni' genotype 3 in September of 2021. Replacement scion grafts were made in late spring of 2022. Between 2021 through 2023 there was significant mortality in the block due to winter injury and vole and deer damage, reducing the total number of trees and/or scion combinations. A total of 31 trees were lost in 2022, and 37 in 2023, leaving 198 trees from the initial 276. Of these 119 have the grafted scion/rootstock combination, the remainder are Gisela 6 rootstocks without scions, and only 4 infected plants remain after the attrition of the past two years. In 2023 12-foot-high netting was erected around the block to exclude leafhoppers from entry, because this has been a problem in the past, and to prevent escape phytoplasma from inoculated plants.

As so many of the inoculated scion/rootstock combinations have been lost, we reduced the scope of this experiment and will maintain the orchard with other funds. It should be noted that we have observed LChV2 and/or '*Ca*. P. pruni' on a range of different cultivars in the field, with variance in symptoms being attributed to variance in infecting strain and environmental conditions rather than the host cultivar (see objective 2). In addition, commercially grown cultivars are highly interbred and thus likely lack the genetic diversity to be resistant to '*Ca*. P. pruni'. The only tolerant, let alone resistant species of *Prunus* identified either by our research or others are *P. domestica, P. armeniaca*, and wild ornamentals such as *P. serotina*. A search for resistance will require an experiment of different scope.

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, scattered symptoms, and 3. Established, which correlated with severe symptoms across the entire tree. As can be seen in Table 1 and Figure 1, 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.

Table 1. Effects of different stages of X-disease phytoplasma infection on fruit color and size (1: normal range for the cultivar, 2: >25% size reduction, 3: >50% size reduction) for each cultivar examined.

Cultivar	Type	Site	'*Ca*. P. pruni' titer	Fruit Size (Avg.)	Fruit Color Range	Abnormalities Observed
'Benton'	Dark Cherry	A	Uninfected	N/A		
			Early	2	Bright Red to Dark Red	Some flattened fruit
			Established	2	Yellow-pink to Dark Red	Flattened fruit, pointed fruit, scattered flower development at harvest
'Bing'	Dark Cherry	B	Uninfected	2	Dark Red	
			Early	1.86 ± 0.12	Medium to Dark Red	
			Established	2.17 ± 0.17	Yellow to Pink	
'Cristalina'	Dark Cherry	C	Uninfected	2.33 ± 0.17	Bright Red to Dark Red	
			Early	1.5	Medium Red	
			Established	2.42 ± 0.083	Yellow-pink to Bright Red	Pointed fruit
		D	Uninfected	N/A		
			Early	1	Medium to Dark Red	
			Established	2.25 ± 0.14	Green to Dark Red	Pointed fruit, Scattered flower development at harvest
'Santina'	Dark Cherry	E	Uninfected	1		
			Early	2.13 ± 0.28	Medium Red to Dark Red	
			Established	2.33 ± 0.17	White-pink to Medium Red	Pointed fruit, Scattered flower development at harvest
'Rainier'	Yellow Cherry	F	Uninfected	1		
			Early	1.86 ± 0.09	Yellow to Blush	
			Established	2.33 ± 0.17	Yellow-white to Blush	
		G	Uninfected	1.5	Blush	
			Early	2 ± 0.14	Medium Blush	
			Established	2.75 ± 0.25	Yellow	
		H	Uninfected	1	Blush	
			Early	1.4 ± 0.24	Yellow to Blush	
			Established	2.33 ± 0.17	Green to Blush	
'Early Robin'	Yellow Cherry	I	Uninfected	1.6 ± 0.1	Yellow to Blush	
			Early	1.4 ± 0.19	Green to Blush	
			Established	N/A		

Figure 1. The effect of infection progression of fruit symptoms from asymptomatic (1) to severe (5) in dark and blush cherries.

The infection stages were also found to correlate with the amount of phytoplasma present in the tree (Figure 2), 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.

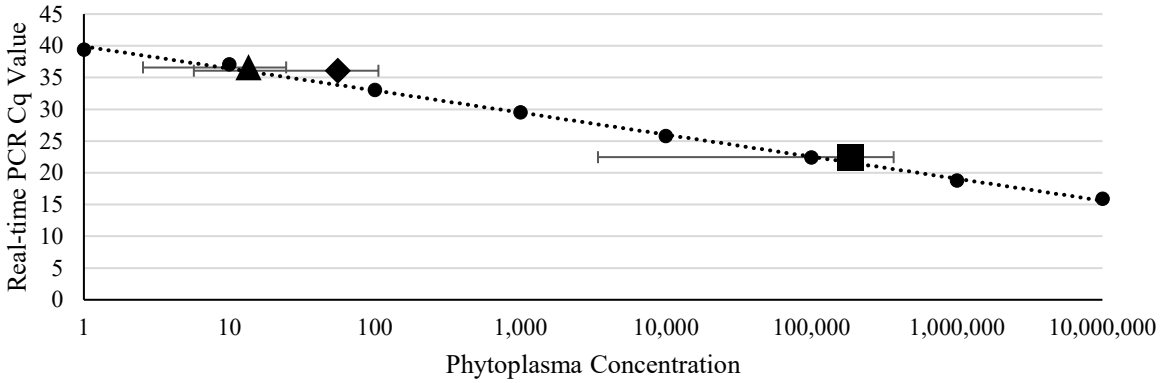

Figure 2. Concentration of X-disease phytoplasma present in asymptomatic (diamond), mild (triangle), and severely (square) symptomatic plants.

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. For trees with established X-disease infections, all three sugars showed a decrease in concentration across most cultivars and locations (Figure 3). 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 LChV2 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 LChV2 infected cherries was fruit citric acid content concentration was lower in trees with high LChV2 concentrations which correlates with the bitter taste of LCD-afflicted cherries.

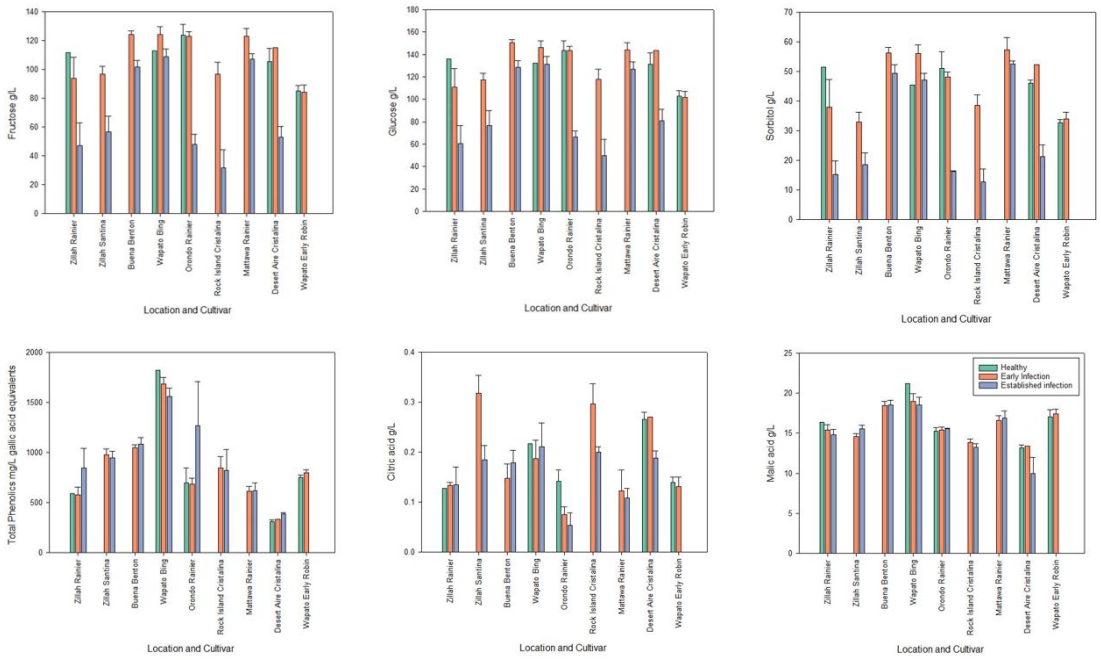

Figure 3. Fructose, glucose, sorbitol, total phenolics, citric acid, and malic acid content across cultivars and locations for healthy, early infections, and established infections.

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 4). 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. Over the course of this project, we have seen that even heavily infected trees vary in their symptom severity from year-to-year, largely in response to location, management and environmental factors.

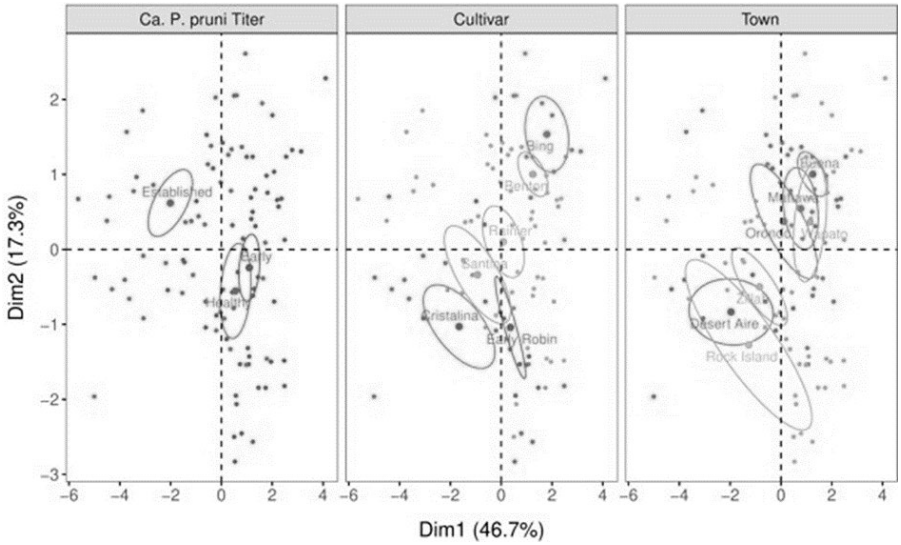

Figure 4. Principal components analysis (PCA) analysis of individual plots comparing pathogen titer, cultivar and location on disease severity.

The discovery of multiple distinct genotypes of '*Ca*. P. pruni' present in WA during the SCBG project '*Epidemiology of the X-disease phytoplasma*' caused us to re-examine this data and put the disease development timeframe and symptoms patterns into a new context. The previous outbreaks of X-disease occurred prior to the advent of DNA sequencing technologies, so what strains were which, and how they differed remains unknown. In the past 20 years, core genes such as the 16S rRNA, secY and secA translocases, and ribosomal proteins of different X-disease inducing strains have been sequenced. However, in most cases, there is little biological information about the strain itself, with the exception of descriptive names such as 'Green Valley', 'Peach-X', or 'little peach', 'red suture', or 'peach yellows'. Even studies that looked at multiple strains within a single outbreak, and/or found multiple, diverged strains, such as Villamor and Eastwell (2019), did not correlate symptom expression with genotype.

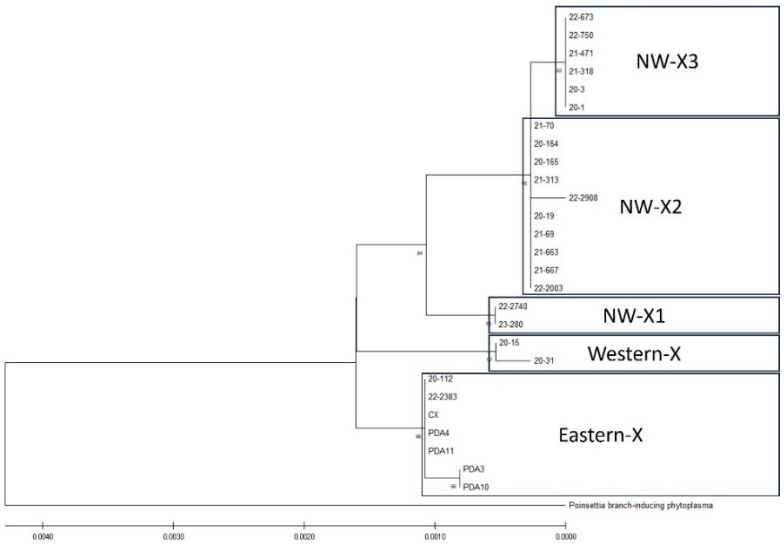

Figure 5. A phylogeny of sequences of the five strain groupings of X-disease-inducing '*Ca*. P. pruni' that have been identified to date.

We have identified and characterized five genetically distinct strains of '*Ca*. P. pruni' that induce X-disease, named, in order of where they were first reported and/or where they are most widely distributed as 'Eastern-X', 'Western-X', 'Northwestern-X1', 'Northwestern-X2', and 'Northwestern-X3' (Figure 5). 'Northwestern-X3' is the dominant strain in Washington state, where it has largely replaced the closely related 'Northwestern-X2' strain that was more common pre-2018. Oregon has primarily 'Northwestern-X2' while in both states what we are terming 'Eastern-X' and 'Western-X' are rare. For reference 'Western-X' grouping includes the 'Green Valley' strain that was a major problem in California in the 1980s.

From the beginning of this epidemic there has been confusion between Little Cherry and X-disease symptoms because both caused a reduction in fruit size, pale skin and pulp color, and lower sugar content. Many of the symptoms reported in previous epidemics, such as in California in the 1970s, and earlier (1930s on the east and west coasts), which included 'buckskin' mottle on fruit skin, along with fruit distortion, enlarged stipules, foliar chlorosis and/or anthocyanosis, and tree decline have not been observed recently. We now know that this was because the dominant strains in the PNW (Northwestern-X2 and Northwestern X3) do not cause these symptoms during the course of a normal in-field infection (Table 2) and indeed, look far more similar to little cherry virus 2-induced disease. Below is a table describing what symptom patterns the different strains produce in major *Prunus* hosts.

Table 2. Symptom patterns of different '*Ca* P. pruni' strains on commercially grown *Prunus* species.

Genotype	Symptoms Observed		
	Sweet cherry	*Sour cherry*	*Peach/Nectarine*
Eastern-X	• Fruit: Small, pale • Leaves: Witches' broom, enlarged stipules • Other: Stunted growth, dieback, abnormal flowering	• Not observed	• Fruit: Small, distorted delayed maturation • Leaves: Epinasty, chlorosis, shot holing • Other: Decline & dieback.
Western-X	• Fruit: Small, pale • Leaves: Normal	• Fruit: Small, pale • Leaves: Enlarged stipules	• Not observed
Northwestern-X1	• Fruit: Small, pale • Leaves: Witches' broom & enlarged stipules • Other: Normal	• Not observed	• Not observed
Northwestern-X2 Northwestern-X3	• Fruit: Small, pale • Leaves: Normal • Other: N/A	• Fruit: Small, distorted, delayed maturation	• Fruit: Small, distorted, delayed maturation • Leaves: Epinasty, chlorosis, shot holing • Other: Decline and dieback

Northwestern-X3 is the dominant strain in Washington state, while Northwestern-X2 is in Oregon. These strains are largely biologically similar in terms of symptom expression, with symptoms being expressed only on the fruit of cherry. Both impact peach and nectarine more severely (Table 2). Fortunately, there are very few sites in Washington or Oregon that have the Eastern- or Western-X strains, and they do not appear to be spreading naturally by leafhopper transmission; it is likely that they are adapted to other leafhopper species that are rare or absent in the Pacific Northwest but common in the Eastern/Midwestern states or California, such as *Scaphytopius acutus, Fieberellia florii, C. clitellarius*, and *Paraphlepsus irroratus*.

Objective 3. To understand 'how' the phytoplasma is causing X-disease in infected sweet cherry we followed *P. avium* cv. 'Bing' trees at multiple sites in central Washington from 2019 through 2023, collecting samples at the major fruit development time points from bloom though to harvest. RNAseq was performed on the samples to study the host gene expression and identify differential expression in genes/pathways that correlated with the observed diseased phenotype i.e. small, pale, fruit with low sugar content.

Comparison of genomic expression profiles from symptomatic and asymptomatic fruit gathered at shuck fall, pit hardening, straw, and harvest timepoints revealed the differential impact of the pathogen on the fruit developmental process. In symptomatic fruit across all timepoints, and after removal of

low-quality data, a total of 1,287 genes were identified (using the parameters of ($p \leq 0.01$, ±0.5 log2 fold-change) as being upregulated and 765 genes downregulated in comparison with asymptomatic fruit. The number of differentially expressed genes (DEGs) was found to differ between timepoints, with more DEGs found later in the developmental cycle, at straw and harvest (Table 3). Interestingly, DEGs could be also grouped into two major classes, those with differential expression at a single timepoint and thus transient, or differential expression across multiple timepoints.

Table 3. Number of differentially expressed genes (DEGs) ($p \leq 0.01$, ±0.5 log2 fold change) in X-disease symptomatic vs. asymptomatic sweet cherry fruit over the course of the fruit developmental cycle.

Timepoint	Upregulated	Downregulated	Total DEGs
Bloom	6	11	17
Shuck fall	43	15	58
Pit Hardening	437	271	708
Straw	555	254	809
Harvest	246	214	503

Of these, totals of 867 upregulated and 660 downregulated genes were characterized, with a known function or one inferred from *in silico* analysis. Ontological analysis, which groups genes based on function or pathways, revealed wide-scale biomolecular processes involved, from protein and polysaccharide binding to intracellular trafficking, and translation regulation in association with the expression of the disease phenotype. At bloom no functionally characterized genes were determined to be differentially expressed (Figure 3); however, post-bloom, during the first phase of fruit maturation at shuck fall two genes, involved in jasmonic acid precursor production (cellular signaling) and energy transfer were upregulated in symptomatic fruit.

The next three timepoints, pit hardening, straw, and bloom saw the greatest numbers of DEGs. At pit hardening, upregulated genes included those involved in biomolecular processes such as transcription initiation, post-translational protein processing, organellar synthesis, and hormone regulation, while photosystem I and II components were downregulated. Interestingly, some of the upregulated genes, such as pectin methylesterase inhibitors are reported to be involved in plant defense against bacterial and fungal pathogens, while downregulation of the photosystems and resulting loss of carbohydrate synthesis is a common plant response to systemic pathogen infection. Straw, which coincides with the second phase of fruit size expansion and maturation as well as significant rates of '*Ca*. P. pruni' accumulation in plant tissues, saw upregulation of genes involved in gene expression regulation, and stress responses across a wide range of pathways, and downregulation of oxidoreductase activity. Towards harvest when observable characteristics of fruit maturation such as anthocyanin accumulation are expressed, upregulated genes included those associated with energy-mediated protein modification while the third was determined to be associated with vitamin B6 (pyridoxine) modulated stress responses (Figure 3). Downregulated genes in symptomatic fruit at this final transitional timepoint include those involved in general protein binding and oxidoreductase activity.

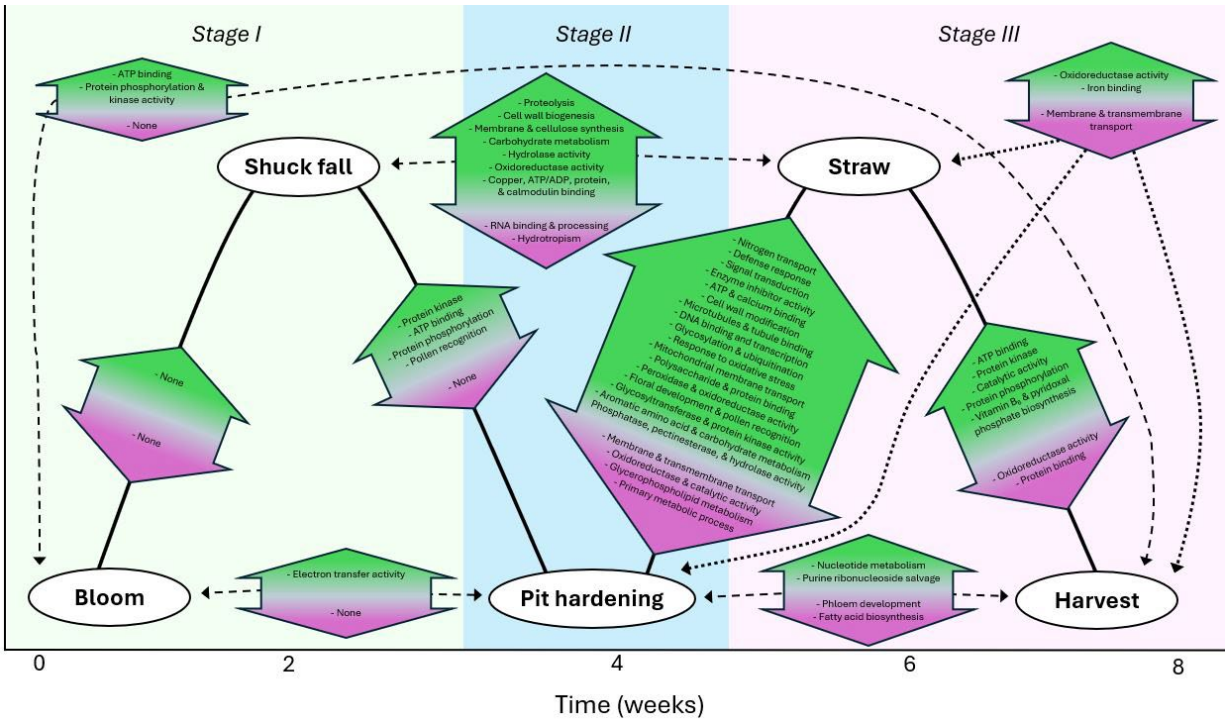

Figure 3. Differential expression of genes, grouped by gene ontology at the different stages of cherry fruit development. Green highlights represent groups of upregulated genes, and pink, groups of downregulated genes ($p \leq 0.01$, ±0.5 log2 fold change).

Some DEGs were found to be expressed at non-sequential timepoints and more closely aligned with the double sigmoidal growth curve used to describe fruit maturation. For example, genes involved in increased cell wall biosynthesis, energy utilization, Ca^{2+}-mediated cellular signaling, and protein degradation were upregulated, while transcription decreased. Involvement of these pathways at the two peak expansion points might indicate a biomolecular shift towards pathogen defense at the expense of normal, cellular proliferative and expansive growth. Similarly, at pit hardening and harvest, which correspond to minima in the fruit growth curve, one gene associated with the purine salvage pathway was identified as upregulated in symptomatic fruit while two genes associated with energy-mediated fatty acid biosynthesis and phloem development had reduced expression. Both time points coincide with troughs in the fruit growth curve and because cellular energy turnover is a closely regulated process, this might indicate a biomolecular shift in symptomatic, non-expanding fruit toward nucleotide production at the expense of cuticle formation and generation of secondary metabolites responsible for flavor, fragrance, and texture. Finally, across the pit hardening, straw, and harvest timepoints differential expression of genes oxidoreductase activity and membrane trafficking, both of which are reported to be associated with plant defense responses in other plant taxa, and downregulation of the latter in particular has been proposed as a response to pathogen-secreted effectors.

Infection also results in plant-wide changes in gene expression, including the systemic signaling, expression regulation and notably, stress-related responses including oxidoreductase activity (data not shown). Metabolic, catabolic and energy transfer processes are also affected. Cumulatively, these data (Figure 3) indicates that changes in gene expression between symptomatic vs. asymptomatic sweet cherry fruit occur across the reproductive continuum, from flower to harvest, and correspond with the physical maturation states, such as maxima and minima of the fruit growth curve as well as response to phytoplasma accumulation over the developmental process. These results indicate that the symptoms of X-disease in cherry fruit are due to phytoplasma effector-induced changes in the maturation process,

with the plant upregulating pathogen response and resistance over normal physiology and maturation, and that this response is skewed towards the second half of the maturation cycle.

Conclusions
Through this study we have identified first, that X-disease is the major pathogen causing small, pale, and immature cherries in the Pacific Northwest, and most significantly that its patterns of pathogenicity and virulence are not the same as what was described in previous outbreaks. Second, we have found that it harms all common commercial sweet and sour cherry varieties in production, and that based on their genetic background, and how the disease is expressed, any sources of resistance or tolerance would have to come from genetically distant species, such as apricot, plum, or wild *Prunus* relatives. Third, we have found that the pathogen induces gene expression changes across the fruit maturation process; this is not a single-gene or single pathway response but a combination of pathogen-induced and plant stress/pathogen defense responses that inhibits normal fruit development.

The impact of this work is twofold. 1) An understanding of the pathogen that we are dealing with. The old assumptions about what the pathogen does, how disease progresses, need to be understood in the context of the strain we have, which is slow to accumulate unless the plant is overloaded with inoculum, and expresses clear symptoms only on cherry fruit. 2) The pathogenicity of this strain, and the differential expression of host genes across the fruit maturation pathway implies that, unlike other phytoplasmas, both effector target action and plant response need to be considered. This complicates breeding efforts of resistant or tolerant varieties but is not insurmountable – tolerance exists but it genetically further out (i.e. apricots & wild *Prunus* spp.).

This work lays the foundation for studies on the identification of the effectors of X-disease, and breeding for resistance/tolerance. It is also of regulatory concern, raising awareness that pathogens evolve making detection and diagnosis more difficult, and that older control measures, like planting on *P. mahaleb*, may no longer be effective.

Publications:

- Wright AA, Shires M, Beaver C, Bishop GM, DuPont ST, Naranjo R, Harper SJ (2021) The effect of '*Candidatus* Phytoplasma pruni' infection on sweet cherry fruit. *Phytopathology*, 111: 2195-2202.
- Wright AA, Shires M, Molnar C, Bishop G, Johnson A, Frias C, Harper SJ. (2022) Titer and Distribution of '*Candidatus* Phytoplasma pruni' in *Prunus avium*. *Phytopathology*, 112: 1406-1412.
- Harper SJ, Northfield TD, Nottingham LR, DuPont ST, Thompson AA, Sallato BV, Serban CF, Shires MK, Wright AA, Catron KA, Marshall AT, Molnar C, Cooper WR. (2023) Recovery plan for X-disease in stone fruit caused by '*Candidatus* Phytoplasma pruni'. *Plant Health Progress* 24: 258–295.
- Shires MK, Molnar C, Wright AA, Bishop G, Harper SJ (2024) Distribution and frequency of little cherry virus 2 genotypes in both production and ornamental fruit trees in the Pacific Northwest. *Plant Health Progress*. 25: 201-206.
- Molnar C, Shires MK, Wright AA, Hoskins MC, Cowell SJ, Nikolaeva EV, Knier R, Harper SJ. (2024) Putting 'X' into context: the diversity of '*Candidatus* Phytoplasma pruni' strains associated with the induction of X-disease. *Plant Disease*, 108: 2677–2687.

Executive summary

At the beginning of this project there had been a forty-year research gap on X-disease, which led to significant confusion between X-disease, caused by '*Candidatus* Phytoplasma pruni' and little cherry disease, caused by little cherry viruses 1 & 2. In this study we identified differences in the symptoms induced by little cherry virus and the X-disease phytoplasma, and most notably, found that the reason that the diseases caused by the two pathogens were being confused was that the phytoplasma strain present in the PNW is not the same as described from previous outbreaks. What we have is a strain that is both genetically and biologically different from the 'older' strains, and most importantly, induces clear symptoms only on fruit, and does not cause appreciable foliar or dieback symptoms.

We found that all common commercial sweet cherry cultivars in production are susceptible to these pathogens, and that any differences in symptom severity were subjective and influenced by infection progression state and pathogen concentration, as well as environmental/management influences. No sources of tolerance or resistance were observed in sweet or sour cherry, and from this and other work we have performed, any tolerance would have to be introduced from distantly related *Prunus* species.

Next, we found that the development of fruit symptoms is the result of changes across a broad range of gene and genetic pathways. These were most notable from pit hardening to harvest, but stress-related responses by the plant were observed throughout the fruit development cycle. These produce a picture of the plant upregulating plant defense and stress pathways at the expense of normal fruit development. The degree to which this is the result of phytoplasma effector activity versus plant response to infection requires further research, and cumulatively this data will be used by pathology researchers at WSU and USDA-ARS to study disease expression and potential control measures, and by the WSU cherry breeding program to screen lines for disease susceptibility.

Project Title: Studying the infection progression of LCD pathogens in young trees

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

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

Budget 1
Primary PI: Scott Harper
Organization Name: Washington State University
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Item	2022	2023
Salaries	24,916	25,913
Benefits	9,079	9,443
Wages		
Benefits		
Equipment		
Supplies	31,661	26,661
Travel		
Miscellaneous		
Plot Fees		
Total	65,656	62,017

Objectives:
1. Determine how rapidly LChV-2 and/or the X-disease phytoplasma can infect young trees and establish a systemic infection after inoculation.
2. Examine potential routes of entry of the pathogens into orchard systems.
3. Examine the effect of extraction and PCR methodology on detection of the X-disease phytoplasma.

Significant Findings:
- Early after infection the pathogen distribution *in planta* is scattered, and while there is general movement towards and accumulation up from the roots, it can take several years before becoming consistently detectable.
- Infection rate of the phytoplasma is very slow and depends on the amount of inoculum delivered to the plant as well as plant size, growth rate, environmental conditions and the presence of other

vascular damaging pathogens (*Pseudomonas*, *Cytospora*, etc.). Virus infection rate is faster, but in either case positive results should not be ignored.

Methods:

Objective 1

Infection progression after graft-inoculation

Tissue from potential inoculum sources were collected from commercial orchards in Washington state in late 2021 and early 2022, total nucleic acids extracted as per established protocols, and the samples tested for LChV1, LChV2, and '*Ca*. P. pruni' using validated assays (Katsiani et al. 2018; Kogej et al. 2020; Shires et al. 2022). Budwood from selected inoculum sources was collected in July 2021, and inoculation of 12-month-old *P. avium* cv. 'Mazzard' seedlings performed via t-grafting two buds at approximately halfway up the stem of the rootstock. Graft survival was assessed at 4- and 12-weeks post-grafting. Trees were sampled at three months post-inoculation, then dissected and tested sequentially to map the progression of the inoculated pathogens at 14 months post-inoculation.

Infection progression after leafhopper-inoculation

Colony-reared leafhoppers were placed onto detached cuttings taken from heavily '*Ca*. P. pruni' infected trees for an acquisition period of three days, then individually transferred to year-old *P. avium* cv. 'Mazzard' seedlings for an inoculation period of three days using clip-cages. Leafhoppers were then killed with insecticide, and the insects, as well as a midrib sample from the leaf they were feeding on, were collected, DNA extracted and tested for the presence of '*Ca*. P. pruni'. Plants were maintained in greenhouse conditions and sequentially dissected at three months (fall, still active), and six months (winter, dormant), post inoculation. Nucleic acids were extracted and tested for pathogen presence as above.

Objective 2

Testing of new planting stock

Combined root and cuttings from the top of the main stem/trunk of tree were collected from between 50-72 individual trees from 3 new cherry and 4 new peach orchards before the trees were planted in the spring of 2022. In 2023, samples were collected from 20 new cherry plantings, although sample size per-site was reduced. Samples were extracted and tested by qPCR for the presence of '*Ca*. P. pruni' as above. In addition, with the grower cooperation, a series of trees that were positive at planting were maintained in an orchard and dissected and sequentially tested the following fall to map infection progression under field conditions.

Risk of seed transmission of pathogens into planting stock

Seeds were collected from known '*Ca*. P. pruni' or LChV2 positives trees showing different degrees of symptom severity and pathogen titer during the 2021 field season, were surface sterilized in 10% bleach, dried and processed. First, subsets from each seed lot were dissected, separating the seed coat and embryo, and total nucleic acids were extracted from each and tested separately. The remaining seeds were cold stratified and germinated in moist vermiculite for a period of six months, survival assessed, and viable seeds planted in soil. Seedlings were grown on a mist-bench for three months, with gradual reduction in watering, then transferred to larger pots and moved to a greenhouse environment. Plants were tested for pathogen presence at three- and six-months post-germination.

Objective 3

The effect of differences in laboratory diagnostic methodology on the successful detection of the LCD/X-disease pathogens was examined. Tissue samples of high vs. low phytoplasma or virus concentration, as well as negative controls, were obtained from plants inoculated for other and maintained under controlled conditions. We compared three tissue disruption methods (mortar &

pestles with liquid nitrogen, rotary bearing-head with Bioreba mesh sample bags, and bead beating in tubes), three extraction methods (Qiagen columns, MagMax magnetic bead extraction, and CTAB), and three PCR chemistries (Invitrogen SSIII/Platinum Taq, ABI Taqman Fast/Fast Virus, & Quanta Perfecta/Toughmix) with all relevant permutations. LChV 2 detection was performed using the Shires et al. (2023) assay, while X-disease phytoplasma was detected using the Wright et al. (2021) assay. Data was compiled to compare their effects on detection of strong and weak positives.

Results and Discussion

Objective 1

Infection progression after graft-inoculation

A total of 67 *P. avium* cv. 'Mazzard' seedlings were grafted with buds from a high concentration LChV2 / low XDP-positive source, and 75 from a high concentration XDP / low LchV2-positive source, in July of 2022. Graft survival was poor, with 52% of LChV2 grafts taking, but only 13% of X-disease grafts (Table 1). When tested at three months post inoculation, only one LChV-2 inoculated plant was positive, therefore the plants were allowed to become dormant through the following winter and maintained in greenhouse conditions throughout the following season before dissecting the plants and the beginning of fall (early September 2023).

Table 1. Graft inoculation success and outcomes of inoculation after 14 months.

Source Inoculum	No. Graft Inoculated	No. Grafts survived	No. Positive after 3 months		No. Positive after 14 months	
			LChV2	XDP	LChV2	XDP
High LChV2 / low XDP	67	35	1	0	19	17
High XDP / low LChV2	75	10	0	0	7	9

It can be seen in Table 1 that while only one LChV2 positive was detected three months after grafting, this changed by 14 months, with most of the residual surviving grafts successfully inoculating the plants with both pathogens. Interestingly, the resulting titer of the pathogen in the plants after 14 months correlated with the relative titer found in the initial inoculum (Figure 1). For example, plants inoculated with the high LChV2 / low XDP combination had average titers of the two pathogens that reflected this, although the virus accumulated more rapidly vis-à-vis the inoculum amount delivered which is to be expected as it is a similar organism less affected by the environmental conditions.

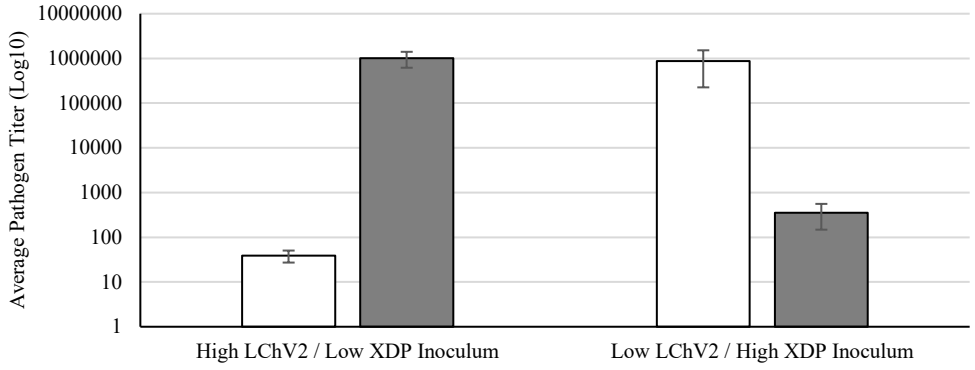

Figure 1. Average titer of populations of the X-disease phytoplasma (white) and little cherry virus 2 (grey) in *P. avium* cv. 'Mazzard' 14 months after infection from two different sources of inoculum.

Dissection of the infected plants to determine where the pathogens were accumulating 14-months post-inoculation revealed that while both pathogens had established a systemic infection in the young trees,

there were different patterns in the pathogen distribution and concentration (Figure 2). For LChV2 inoculations, high-titer inoculum produced a high titer systemic infection throughout the plant, although the highest concentrations were above and just below the graft, suggesting in-season acropetal movement and accumulation. This is to be expected as the virus readily infects newer tissues, while older tissues are less accessible due to fewer plasmodesmata connections between the sieve elements and companion cells where the virus replicates. The virus was at markedly lower concentration in the root tissues. No low-titer LChV2 infected trees were dissected due to low sample size. The phytoplasma inoculations showed a similar low-titer vs. high-titer inoculation pattern, though in both cases a systemic infection occurred. In contrast to the virus, the phytoplasma was concentrated in the lower tissues of the plant, below the graft, and not in the upper sections of the stem of side branches. For both high- and low-titer inoculum the resulting pattern was the same, differing only in concentration (Table 2).

Table 2. Distribution and concentration of '*Ca*. P. pruni' and LChV2 in dissected cherry seedlings at inoculated with high and low titers of the pathogen, at 14 months post-inoculation.

Timepoint	Initial inoculum titer	Average titer in young trees					
		Shoot Tip	Stem at 75% height	Graft	Stem at 25% height	Soil level	Root
LChV2	High	1.1M ± 509K	1.7M ± 1.4M	1.1M ± 760K	1.9 ± 1.2M	892K ± 799K	128K ± 76K
	Low	N/A	N/A	N/A	N/A	N/A	N/A
Ca. P. pruni'	High	N/A	994 ± 0	63K ± 0	4.4M ± 0	2.8M ± 0	1.2M ± 0
	Low	99 ± 10	179 ± 10	105 ± 7	268 ± 48	235 ± 34	235 ± 22

Infection progression after leafhopper-inoculation

To compare-and-contrast with graft inoculations, leafhopper transmissions of '*Ca*. P. pruni' were performed in late 2023 and 2024; virus transmissions were not attempted as they were the focus of previous WTFRC-funded studies. Both plants and leafhoppers were tested after the three day-inoculation period, and then batches of the inoculated plants dissected and sequentially tested at 3- and 6-months post-transmission (Table 3). This resulting data indicated that a) the leafhoppers on average transmitted relatively low concentrations (approximately 10-100 cells) of phytoplasma to the plant during the short feeding period allowed, and that b) this resulted in slow accumulation and systemic movement throughout the plant.

Table 3. The in-planta infection rates and distribution patterns of '*Ca*. P. pruni' after leafhopper transmission to *P. avium* cv. 'Mazzard' seedlings at three- and six-months post inoculation.

Timepoint	Type (n)	No. leafhoppers positive after inoculation period	No. feeding leaves positive after inoculation period	No. trees with ≥1 positive section	No. positive per tissue sampling location site					
					Shoot Tip	Stem at 75% height	Middle of stem	Stem at 25% height	Soil level	Root
3 Months	Inoculated (18)	18 / 18	15 / 18	3 / 18	0 / 18	1 / 18	1 / 18	0 / 18	1 / 18	0 / 18
	Control (4)	N/A	N/A	0 / 5	0 / 5	0 / 5	0 / 5	0 / 5	0 / 5	0 / 5
6 Months	Inoculated (23)	15 / 23	11 / 23	12 / 23	3 / 23	4 / 23	3 / 23	5 / 23	4 / 23	6 / 23
	Control (2)	N/A	N/A	0 / 2	0 / 2	0 / 2	0 / 2	0 / 2	0 / 2	0 / 2

At three months post-inoculation only 3 plants out of 18 had any positive sections, and those were very weak (<10 cells/section). By six months post-inoculation, the phytoplasma had accumulated to detectable levels in 12 out of 23 plants sectioned, however, phytoplasma titer remained low in all plant sections (between approximately 10-100 cells). In both cases, there was no consistent pattern of

distribution in planta. For three months this may be due to simply being too early after infection for the pathogen to accumulate, whereas at six months, while there was some accumulation and movement, complete, systemic infections were not observed. We cannot discount that the six-month post-inoculation plants were dormant, with little active phloem transport, in which case the results likely reflect where the pathogen stopped moving when the plants entered dormancy. These results do, however, agree with graft inoculation results, where detection in the year of infection or too close to dormancy is difficult due to uneven and unpredictable distribution, even in small trees (and these were approximately 24-30" in height).

Objective 2

Testing of new planting stock

In spring of 2022 and 2023 young trees being planted in new cherry and/or peach orchards in Washington and Oregon were tested for the presence of the X-disease phytoplasma ('Ca. P. pruni') and little cherry virus 2 (2023 only). No LChV2 positives were found, while X-disease phytoplasma incidence ranges from 5-12% in 2022, and from 2-60% in 2023; the 60% was from a lot with small sample size (n=10) so is an outlier (Table 4). X-disease positives were tested and confirmed by an assay developed to determine whether the '*Ca*. P. pruni' pathogen was replicating (Harper, *unpublished*). Positives were concentrated in specific varieties from multiple propagators which suggests that there may be problems with specific source material, and or from specific geographic areas. These will not be named to preserve confidentiality. Whether these plants were certified by a state regulatory agency-managed program or not is unknown.

Table 4. Results of randomized testing of young cherry and peach trees at planting for the X-disease phytoplasma in 2021 and 2022. Asterisk indicates result with small sample size (n=10).

Year	No. lots tested	No. positive lots	Sample size range	Percentage positives per lot
2022	8	7	48-72 plants per lot	5% to 12%
2023	20	10	10 to 48 plants per lot	2% to 60%*

While there were outliers with higher pathogen titer that may have been indicative of the use of heavily infected budwood, most positives were at low concentration which suggests low concentration budwood or leafhopper transmission into finished plants prior to dormancy. With the kind cooperation of a grower, a set of low titer peach trees from the 2022 planting period were maintained for the purposes of tracking pathogen accumulation over time (Table 5).

Table 5. The in-planta infection rates and distribution patterns of '*Ca*. P. pruni' in *P. persica* trees that were found to be positive at planting, after six and eighteen months of field growth.

Timepoint	Type and No. Trees Examined	East Leader			Central Leader			West Leader			Trunk	Graft Union	Roots
		Tip	Side Branches	Base	Tip	Side Branches	Base	Tip	Side Branches	Base			
6 Months	Infected (17)	N/A	N/A	3 / 17	N/A	N/A	4 / 17	N/A	N/A	3 / 17	3 / 17	3 / 17	5 / 17
	Control (2)	0 / 2	0 / 2	0 / 2	0 / 2	0 / 2	0 / 2	0 / 2	0 / 2	0 / 2	0 / 2	0 / 2	0 / 2
18 Months	Infected (15)	3 / 15	5 / 15	3 / 15	3 / 15	4 / 15	5 / 15	3 / 15	4 / 15	3 / 15	5 / 15	4 / 15	8 / 15
	Control (5)	0 / 5	0 / 5	0 / 5	0 / 5	0 / 5	0 / 5	0 / 5	0 / 5	0 / 5	0 / 5	0 / 5	0 / 5

At 6-months post planting, the infected trees had scattered low-concentration (<100 cells/section) distribution in one limb or leader, or the roots, while the rest of the tree remained negative. There was no pattern to the limbs infected. Two trees were systemically infected from the roots and into the leaders, though concentration was low. At 18-months post-planting, two trees contained systemic

infections and had started to produce classic X-disease foliar symptoms with phytoplasma concentrations in the range of >10K cells in affected sections. A further eight trees sampled had scattered >10K cells/section infections in one limb or the trunk without systemic movement. This is likely the result of heavy bacterial canker (*Pseudomonas syringae* pv. *syringae*) and wood-rot fungal infections (*Cytospora leucostoma* or *Eutypa lata*) that appear to have entered these trees the graft union and spread systemically up the tree, blocking phytoplasma movement. These secondary pathogens also appear to have hindered the systemic infection of two trees in which infection had become established, damaging the phloem system or killing limbs such that the pathogen could not move. All control trees that tested negative at planting remained negative at both 6-and 18-months post planting (Table 5)

Risk of seed transmission of pathogens into planting stock
It has been assumed, but not confirmed that neither LChV2 or '*Ca*. P. pruni' are seed transmissible. '*Ca*. P. prunorum' (a *Prunus*-infecting phytoplasma present in Europe) has been reported to be seed transmissible, and therefore the risk of seed grown trees and/or volunteer trees grown from dropped fruit was assessed. We obtained cherry fruit from a range of infected trees, removed and cleaned the seeds, then either a) dissected and tested the seeds, or b) stratified and geminated the seeds for subsequent testing.

LChV2 was detected in both the seed coat and embryo, although frequency and virus concentration were very low (Table 6). There were 3 very weak seedling positives, but these could not be confirmed months later, and we concluded that the virus did not transfer across to the seedlings produced from these pools. The phytoplasma was found in both the seed coat and embryo of seeds from all levels of infected trees, and after DNAse treatment and testing of RNA, was confirmed to be alive and replicating. Interestingly, '*Ca*. P. pruni' DNA was also found in a handful of seedlings and 3- and 6-months post-germination, but RNA testing suggested that it was carryover from the seeds and not actually live, viable phytoplasma cells (Table 6).

Table 6. Incidence of LChV2 and the X-disease phytoplasma ('*Ca*. P. pruni') in seed samples collected from infected trees, and in resulting seedlings produced from seed pools. '-' represents pools where samples were not tested.

Pathogen	Source Pool	Disease Severity	Seed Coat Positives		Embryo Positives		Seedling Positives	
			DNA	RNA	DNA	RNA	DNA	RNA
LChV2	1	Severe	N/A	1 / 20	N/A	4 / 20	N/A	-
	2	Mild	N/A	0 / 20	N/A	2 / 20	N/A	-
	3	Severe	N/A	2 / 20	N/A	3 / 20	N/A	0 / 42
Ca. P. pruni'	5	Asymptomatic	12 / 15	-	8 / 15	-	1 / 22	0 / 1
	6	Mild	15 / 15	-	15 / 15	-	4 / 92	0 / 3
	7	Severe	9 / 15	-	15 / 15	-	2 / 16	0 / 2
	8	Severe	-	16 / 20	-	19 / 20	-	-
	9	Severe	-	19 / 20	-	20 / 20	-	-
	10	Severe	-	13 / 20	-	12 / 20	-	-

Objective 3
Differences in diagnostic approach, including differences in plant tissue disruption to release nuclic acids from cells, in the extraction method to recover those nucleic acids, and in PCR chemistry, can have a major impact on diagnostic sensitivity and reproducibility. Therefore, using three biological and two technical replicates from strong (Ct values 20-30 cycles), weak (Ct > 35 cycles & X-disease only due to plant availability), or pathogen-negative plants we analyzed the effect of three different tissue disruption methods, three nucleic acid recovery methods, and three PCR chemistries on LChV2 and X-disease phytoplasma detection.

For both pathogens, disruption method mattered in terms of overall recovery and detection of pathogens, but also carryover of PCR inhibitors like polysaccharides or phenolics present in the plants. Bead beating frozen tissue using a Qiagen Tissuelyser or using a mortar & pestle with liquid nitrogen performed better in terms of raw recovery and detection of both strong and weak positives, but at a potential cost in PCR inhibitor carryover (Tables 7 & 8). The BioReba mesh bags and Homex-6 bearing head homogenizer gave less uniform disruption of frozen woody or tough tissues, so detection of even strong positives was less consistent, though with the likely benefit of less inhibitor carryover.

Table 7. Number of samples amplified and average Ct values of X-disease phytoplasma using combinations of three different issue disruption methods, three different nucleic extraction methods, and three different PCR chemistries.

Disruption Method	Extraction Method	Target	Quanta Perfecta		Invitrogen Platinum Taq		ABI Taqman Fast	
			No. Amplified	Ct Avg & SE	No. Amplified	Ct Avg & SE	No. Amplified	Ct Avg & SE
Bioreba Mesh Bags with Homex-6 Homogenizer	CTAB	Strong XDP Positives	6 / 6	25.6 ± 2.7	6 / 6	26.7 ± 2.7	5 / 6	23.4 ± 3.2
		Weak XDP Positives	3 / 6	36.6 ± 0.8	3 / 6	37.9 ± 0.4	3 / 6	36.2 ± 1.3
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.
	MagMax	Strong XDP Positives	6 / 6	22.2 ± 0.7	4 / 6	22.7 ± 0.9	6 / 6	21.7 ± 0.7
		Weak XDP Positives	1 / 6	38.9 ± 0.0	0 / 6	No Amp.	1 / 6	38.5 ± 0.0
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.
	RNEasy	Strong XDP Positives	6 / 6	23.5 ± 0.6	6 / 6	27.2 ± 0.9	6 / 6	23.5 ± 0.6
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.
	DNEasy	Strong XDP Positives	6 / 6	22.7 ± 0.4	6 / 6	22.9 ± 0.7	6 / 6	21.8 ± 0.5
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.
Bead Beating with Tissuelyser	CTAB	Strong XDP Positives	6 / 6	21.7 ± 1.1	6 / 6	22.2 ± 1.1	6 / 6	21.8 ± 1.2
		Weak XDP Positives	6 / 6	37.2 ± 0.2	0 / 6	No Amp.	6 / 6	37.0 ± 0.4
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.
	MagMax	Strong XDP Positives	6 / 6	21.3 ± 0.4	6 / 6	22.7 ± 1.6	6 / 6	20.7 ± 0.4
		Weak XDP Positives	2 / 6	37.7 ± 0.3	0 / 6	No Amp.	1 / 6	36.2 ± 0.0
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.
	RNEasy	Strong XDP Positives	6 / 6	25.6 ± 2.6	5 / 6	23.1 ± 0.2	6 / 6	25.6 ± 2.7
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.
	DNEasy	Strong XDP Positives	6 / 6	19.2 ± 0.3	6 / 6	23.1 ± 0.8	6 / 6	18.6 ± 0.3
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.
Mortar & Pestle	CTAB	Strong XDP Positives	6 / 6	23.4 ± 0.8	6 / 6	23.7 ± 0.8	6 / 6	23.4 ± .07
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.
	MagMax	Strong XDP Positives	6 / 6	22.4 ± 0.4	1 / 6	24.7 ± 0.0	6 / 6	21.7 ± 0.4
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.
	RNEasy	Strong XDP Positives	6 / 6	23.1 ± 0.4	6 / 6	24.7 ± 0.6	6 / 6	23.1 ± 0.4
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.
	DNEasy	Strong XDP Positives	6 / 6	22.8 ± 0.6	6 / 6	27.2 ± 1.5	6 / 6	22.1 ± 0.6
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.

Nucleic acid extraction methodology also showed differences, with recovery of more weak positives with CTAB rather than the magnetic bead-based MagMax method (Table 7), though both carried over

PCR inhibitors that affected one of the PCR chemistries. As seen in previous studies, the Superscript III and/or Platinum Taq combination fared poorly with inhibitor heavy samples described above, reducing detection of both strong and weak pathogen positives, whereas Quanta Perfecta/Toughmix and the ABI Taqman Fast/Fast Virus kits worked well. It should also be noted that no false positives were detected using any of the combinations.

Table 8. Number of samples amplified and average Ct values of little cherry virus 2 using combinations of three different issue disruption methods, three different nucleic extraction methods, and three different PCR chemistries.

Disruption Method	Extraction Method	Target	Quanta Toughmix		Invitrogen SSIII/Ptaq		ABI Taqman Fast Virus	
			No. Amplified	Ct Avg & SE	No. Amplified	Ct Avg & SE	No. Amplified	Ct Avg & SE
Bioreba Mesh Bags with Homex-6 Homogenizer	CTAB	Strong LChV2 Pos.	6 / 6	21.2 ± 0.5	6 / 6	22.7 ± 0.5	6 / 6	21.1 ± 0.5
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.
	MagMax	Strong LChV2 Pos.	6 / 6	20.0 ± 0.6	4 / 6	25.6 ± 0.8	6 / 6	19.1 ± 0.6
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.
	Rneasy	Strong LChV2 Pos.	6 / 6	20.4 ± 0.6	6 / 6	20.4 ± 0.5	6 / 6	19.5 ± 0.6
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.
Bead Beating with Tissuelyser	CTAB	Strong LChV2 Pos.	6 / 6	20.5 ± 0.3	6 / 6	21.4 ± 0.4	6 / 6	18.7 ± 0.4
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.
	MagMax	Strong LChV2 Pos.	6 / 6	22.0 ± 0.9	1 / 6	23.6 ± 0.0	6 / 6	19.3 ± 0.9
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.
	Rneasy	Strong LChV2 Pos.	6 / 6	21.5 ± 0.3	3 / 6	20.5 ± 3.9	6 / 6	20.6 ± 0.3
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.
Mortar & Pestle	CTAB	Strong LChV2 Pos.	6 / 6	23.9 ± 0.7	6 / 6	24.9 ± 0.7	6 / 6	22.5 ± 0.8
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.
	MagMax	Strong LChV2 Pos.	6 / 6	22.4 ± 0.3	2 / 6	29.2 ± 4.4	6 / 6	19.6 ± 0.2
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.
	Rneasy	Strong LChV2 Pos.	6 / 6	22.1 ± 0.3	4 / 6	23.6 ± 1.9	6 / 6	20.9 ± 0.3
		Negative	0 / 6	No Amp.	0 / 6	No Amp.	0 / 6	No Amp.

In summary, tissue disruption method and PCR chemistry selection are key factors in the detection of both little cherry virus 2 and the X-disease phytoplasma using extant PCR based methods, particularly of weak positives where the effects of less tissue disruption and/or inhibitor carryover are more pronounced and result in higher numbers of false negatives.

Conclusions

Cumulatively these data suggest that early in the infection cycle the X-disease phytoplasma is difficult to detect by any diagnostic method that requires the extraction and PCR from plant tissues. The concentration remains very low during the year of infection, and only begins to accumulate after dormancy and well into the following season. The rate at which accumulation occurs is heavily influenced by the amount of inoculum initially delivered to the plant. Leafhoppers, even heavily infected leafhoppers, appear to deliver low amounts of inoculum even when forced to feed on *Prunus* species, therefore the initial level of inoculum delivered to a plant is low. The other point that needs to be made here is trees that are graft-inoculated or made from infected propagative material will likely have a higher and more detectable titer earlier in the infection cycle, or become systemically infected faster because unlike leafhoppers which are a one-time inoculation, the grafted tissue acts as a longer-

term source of phytoplasma because not only can it unload into the attached phloem, but also replicate in the graft itself.

Once in a tree, systemic infection requires basipetal movement of '*Ca*. P. pruni' to the roots, and after overwintering there, root-upwards recolonization of the tree limbs and colonization of the emerging leaves in the following season. Within-season movement in a tree is largely local, which correlates with the general patterns of source-sink photoassimilate flow in the phloem. This also agrees with our previous work mapping infections in heavily infected cherry (Wright et al. 2021). Other factors, including the environmental conditions and other pathogens (i.e., bacterial canker) can determine the extent or rate at which a systemic infection occurs, or why in some cases, it doesn't for several years. These factors can make consistent detection of the pathogen in an individual tree difficult early in the infection cycle prior to the pathogen establishing a full, systemic, infection and accumulating to a level that makes detection easy. Adding to this, sample extraction and PCR chemistries matter significantly, and fail to detect a weak positive.

What does this mean for detection and diagnosis? Yes, infections in young trees can be detected, but there will be a significant undercounting of positives and infections may take several years t o accumulate to consistently detectable levels. This makes acting when positives are found important. The tree it may not become symptomatic immediately, or even within 3-5 years (because symptom expression and severity is concentration, cultivar, and environmentally determined) but fruit quality and yield will eventually be affected.

Executive Summary
In this study the rate of infection progression of the X-disease phytoplasma (‘*Candidatus* Phytoplasma pruni’) and little cherry virus 2 in young plants was assessed, asking the questions of where and how rapidly do these pathogens move though infected plants. Different inoculation methods were assessed, including grafting versus vector transmission, as was the potential for the pathogens to be transmitted through seed or planting stock. Finally, the sensitivity of different extraction methods was assessed to show what factors can produce false negatives in diagnostics.

The rate of infection progression by the phytoplasma is very slow and depends on the amount of inoculum delivered to the plant as well as plant size, particularly when low concentrations are delivered by leafhoppers or grafting from low-concentration sources. Distribution in the infected plants was generally scattered, and no pattern was observed early (up to six months) after infection. As infections progress there is general movement towards and accumulation up from the roots, it can take several years before becoming consistently detectable. Other factors identified that affect distribution and accumulation include growth rate, environmental conditions and the presence of other vascular damaging pathogens (bacterial canker and fungi such as *Cytospora* sp.). While not examined using as many permutations, the infection progression of little cherry virus 2 is much faster, with systemic infection of young plants occurring within a year. We did not find evidence of seed transmission of either little cherry virus 2 or the X-disease phytoplasma but did observe that the potential for spread in planting stock exists. Finally, differences in diagnostic methodology can significantly influence detection of these pathogens.

Cumulatively, these data show that the X-disease phytoplasma can be very slow to accumulate and spread in young plants, making early detection difficult using direct-sampling methods. This goes some way to explaining why disease progression and the onset of symptoms in orchard trees can take many years. It also underlines that positive detections of these pathogens should be taken seriously and managed before they impact tree productivity and fruit quality.

Proposal Title: Determining Sweet Cherry Rootstock Sensitivity to X-Disease
Report Type: Continuing Project Report

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Cooperators: Scott Harper (WSU), Melodie Putnum (OSU Plant Clinic)

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 37,323
Total Project Request for Year 2 Funding: $ 38,207
Total Project Request for Year 3 Funding: $ 39,119

Other related/associated funding sources: Awarded
Funding Duration: 2020 - 2023
Amount: $8,000
Agency Name: Oregon State University
Notes: Start-up equipment funding provided by OSU will be used to purchase a hoop house to contain/quarantine X-Disease inoculated potted cherry trees.

Budget 1
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Item	2023	2024	2025
Salaries1	$ 23,111.00	$23,804.00	$24,518.00
Benefits	$5,609.00	$5,777.00	$5,951.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies2	$4,000.00	$4,000.00	$4,000.00
Travel			
Plot Fees			
Miscellaneous			
Total	$32,720.00	$33,581.00	$34,469.00

Footnotes: [1] Partial summer salary for Galimba and salary for 0.75 FTE Master's student.
[2] Rootstocks and potting, grafting, and inoculation supplies, qPCR testing.

Budget 2
Co PI 2: Ashley Thompson
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Item	2023	2024	2025
Salaries	$3,836.00	$3,836.00	$3,836.00
Benefits	$767.00	$790.00	$814.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies			
Travel			
Plot Fees			
Miscellaneous			
Total	$4,603.00	$4,626.00	$4,650.00

Footnotes: [1] Partial summer salary for Thompson.

Objectives

Objective 1: Collect and analyze data regarding scion and rootstock type from known X-Disease infections across OR and WA to determine what germplasm has evidence of susceptibility.

Objective 2: Evaluate the susceptibility of rootstocks that are never, or infrequently, represented in the infected dataset.

Significant Findings

1. X-disease infections without hypersensitive response symptoms have been verified in Gisela 6, Gisela 12, Krymsk 5, Krymsk 6, and Mazzard, indicating these rootstocks are susceptible.

2. Rootstocks that were acquired in winter 2023/spring 2024 include: Mahaleb, Mazzard, Gisela 5, MaxMa 14, Cass, Clinton, Crawford, Weigi 2, Weigi 3, Colt and Krymsk 7.

3. A high tunnel was purchased and constructed in 2024 to serve as an insect-free location to house X-Disease-infected rootstocks.

4. Rootstocks vary in size based on size-received and growth over the 2024 season. There are currently seven that have reached an appropriate size to bud on to. We are exploring alternate methods of inoculation for these and especially for the four which have not achieved an appropriate diameter.

Methods

<u>Objective 1: Collect and analyze data regarding scion and rootstock type from known X-Disease infections across OR and WA to determine what germplasm has evidence of susceptibility.</u>

Records related to X-disease testing performed in OR and WA were obtained and used to determine what scion and rootstock varieties have tested positive for X-Disease in the past. Unfortunately, many tests are performed without recording scion data, and very few have records regarding rootstock. Data for the following rootstocks and scion cultivars were acquired:

Rootstock	**Scion**
Mazzard	Ann
Gisela 6	Attika
Gisela 12	Bing
Krymsk 5	Black Pearl
Krymsk 6	Burgundy Pearl
	Chelan
	Coral Champagne
	Lapins
	Rainier

	Regina
	Skeena
	Starletta
	Suite Note
	Van

Cherry rootstocks and scions recorded in X-Disease testing data from OR and WA.

Objective 2: Evaluate the susceptibility of rootstocks that are never, or infrequently, represented in the infected dataset.

Rootstocks were ordered from multiple nurseries/ tissue culture facilities following notification of funding in December, 2022. Rootstocks arrived winter 2023/spring 2024 and were first potted in one-gallon pots and grown in a greenhouse. Later in the season, rootstocks were transferred to 3.5-gallon pots and moved outside, to a weed-cloth covered area. Weigi 2 and Weigi 3 were the only rootstocks available with a grafted scion (Chelan), these were potted in 3.5-gallon pots on arrival and root suckers were used to propagate additional, non-grafted plants.

Results and Discussion

Objective 1

Of the five rootstocks we identified in X-disease testing records, none had 0% positives. These tests came from a number of sources, including tests requested by growers, done on trees with fruit symptoms as well as from research projects looking at X-disease spread. Because none of this testing was originally done to identify susceptibility, it is important not to give too much weight to the numbers, since they are almost certainly biased. We are considering any positives to be evidence that a particular rootstock is susceptible. We will focus on obtaining as many more X-disease records as we can throughout the project, but for now, Gisela 6, Gisela 12, Krymsk 5 and Krymsk 6 will be omitted from Objective 2.

Rootstock	Notes	Number Tested	% Positive
Mazzard	*P. avium*. Negative Control: susceptible to X-Disease.	58	22%
Gisela 6	*P. cerasus* x *P. canescens*	40	8%
Gisela 12	*P. cerasus* x *P. canescens*	100	60%
Krymsk 5	*P. fruticosa* x *P. serrulata.* Higher chance of hypersensitive response to X-Disease based on parentage.	31	3%
Krymsk 6	*P. cerasus* x *P. maackii*	61	64%

A number of scions were also present in testing records, and the results are listed here. Again, testing purposes varied, so numbers are biased. If records can be increased, this information may eventually help inform breeding for X-disease resistance.

Scion	Number Tested	% Positive
Ann	4	50%
Attika	10	10%
Bing	18	33%
Black Pearl	71	13%
Burgundy Pearl	30	0%
Chelan	3	33%
Coral Champagne	27	4%
Lapins	1	100%
Rainier	1	100%
Regina	2	50%
Skeena	12	33%
Starletta	2	0%
Suite Note	10	30%
Van	2	100%

Objective 2

Eleven rootstocks were obtained for this trial. From the original list, nine were obtained, with an additional two (Colt and K7) added because they were available. Both negative and positive controls were acquired. Rootstocks acquired are highlighted in green below.

Rootstock	Notes
Mahaleb	*P. mahaleb*. Positive Control: exhibits hypersensitive response to X-Disease.
Mazzard	*P. avium*. Negative Control: susceptible to X-Disease.
Gisela 5	*P. cerasus* x *P. canescens*
Maxma 14	*P. avium* x *P. mahaleb.* Higher chance of hypersensitive response to X-Disease based on parentage.
Clinton	*P. cerasus* x *P. canescens*
Lake	*P. avium* x *P. fruticosa*
Crawford	*P. cerasus* x *P. canescens*
Cass	*P. avium* x *P. cerasus* x *P. fruticosa*
Clare	*P. avium* x *P. cerasus* x *P. fruticosa*
Weigi 1	*P. cerasus*
Weigi 2	*P. avium* x *P. canescens*
Weigi 3	*P. avium* x *P. cerasus* x *P. canescens*
Weiroot 720	*P. cerasus*
Colt	*P. avium* x *P. pseudocerasus*
Krymsk 7	*P. lannesiana*

In fall, 2024 roostocks were moved to a 20’ x 60’ high tunnel that was purchased and erected in summer, 2024. This high tunnel will provide an insect-free location to inoculate and house infected plant material.

High tunnel with four of the eleven rootstock selections moved in.

The rootstocks vary greatly in size and health after the first season of growth at MCAREC, and the variability is caused both by the size of the rootstocks when they arrived and the amount of growth each rootstock put on in the first year. Notes are listed below.

We are interested in exploring other methods of inoculation for many of these rootstock selections, given that most of them were only available as small, un-grafted trees that need multiple growing seasons before they can be inoculated by grafting. We currently have enough of each selection (at least 15 each) that we can move forward with chip budding like we outlined in the original proposal, and also explore other methods. The most promising is using infected leafhopper to inoculate the leaves of rootstocks. Multiple labs (Harper, Marshall, Adams) either have colonies of infected leafhoppers or are working towards establishing colonies. Using leafhoppers would be a good solution to avoid grafting and discussions are underway with all of these labs.

Rootstock	**Notes**
Mahaleb	Positive Control. Vigorous growth in 2024. Will bud easily in 2025. Many leaves for potential leafhopper inoculation.
Mazzard	Negative Control. Moderate growth in 2024. Trunk diameter is still small – will be possible to bud in 2025.
Gisela 5	Very weak growth in 2024. Budding will be challenging. Some leaves are present – leafhopper inoculations would be possible.
Maxma 14	Vigorous growth in 2024. Will bud easily in 2025. Many leaves for potential leafhopper inoculation.
Clinton	Received very small (thin diameter) plants. Very weak growth in 2024. Budding will be challenging. Some leaves are present – leafhopper inoculations would be possible.
Crawford	Received very small (thin diameter) plants. Very weak growth in 2024. Budding will be challenging. Some leaves are present – leafhopper inoculations would be possible.
Cass	Received very small (thin diameter) plants. Very weak growth in 2024. Budding will be challenging. Some leaves are present – leafhopper inoculations would be possible.
Weigi 2	Received grafted with Chelan – will easily bud in 2025. Also propagated without scion (from sucker cuttings) – these have small diameter but many leaves.
Weigi 3	Received grafted with Chelan – will easily bud in 2025. Also propagated without scion (from sucker cuttings) – these have small diameter but many leaves.
Colt	Vigorous growth in 2024 but trunk diameter is still small – will be possible to bud in 2025.
Krymsk 7	Moderate growth in 2024. Trunk diameter is still small – will be possible to bud in 2025. Many leaves for potential leafhopper inoculation.

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

Report Type: Continuing Project Report

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Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 77,930
Total Project Request for Year 2 Funding: $ 80,918
Total Project Request for Year 3 Funding: $ 84,025
Total Request: $242,873

Other related/associated funding sources: Awarded
Funding Duration: 2020–2023
Amount: $36,000

Agency Name: Corteva Agriscience, Gowan Co, Brandt Co., Nichino America, Kemin Industries**,** GroPro
Notes: Funds from chemical industry contribute to salaries and research in Obj. 3.

Other related/associated funding sources: Awarded
Funding Duration: 2023-2025
Amount: $249,813
Agency Name: WSDA Specialty Crop Block Grant
Notes: Project "Leafhopper phenology model development and habitat assessment to improve cherry X-disease management" was awarded to PI Orpet and co-PI Northfield to support leafhopper sampling and expand monitoring to habitat bordering orchards.

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Item	2022	2023	2024
Salaries	$47,727.00	$49,636.00	$51,621.00
Benefits	$17,498.00	$18,198.00	$18,926.00
Wages	$5,760.00	$5,990.00	$6,230.00
Benefits	$551.00	$573.00	$596.00
RCA Room Rental	$0.00	$0.00	$0.00
Shipping	$0.00	$0.00	$0.00
Supplies	$0.00	$0.00	$0.00
Travel	$0.00	$0.00	$0.00
Plot Fees	$0.00	$0.00	$0.00
Miscellaneous	$0.00	$0.00	$0.00
Total	$71,536.00	$74,397.00	$77,373.00

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

Budget 2
Co PI 4: Dr. Christopher Adams
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Item	2022	2023	2024
Salaries	$0.00	$0.00	$0.00
Benefits	$0.00	$0.00	$0.00
Wages	$5,760.00	$5,875.00	$5,993.00
Benefits	$634.00	$646.00	$659.00
RCA Room Rental	$0.00	$0.00	$0.00
Shipping	$0.00	$0.00	$0.00
Supplies	$0.00	$0.00	$0.00
Travel	$0.00	$0.00	$0.00
Plot Fees	$0.00	$0.00	$0.00
Miscellaneous	$0.00	$0.00	$0.00
Total	$6,394.00	$6,521.00	$6,652.00

Footnotes:
[1]Non-student temporary employee: $15/hr x 16 hrs/wk x 24 wks = $5,760 for year 1 x 1.04 each additional year. Benefits at 11%.
[2]Funded with industry gifts and leveraged matching funds.

Objectives

1. Collect model development and validation data: scout selected WA and OR orchards containing high populations of leafhoppers twice per week while compiling weather data.

Deviations: Scouting occurred weekly in 2022, 2023, and 2024.

2. Modeling phenology curves: Use leafhopper abundance data to parameterize and validate the model.

Deviations: None / the objective is *in progress.*

3. Insecticide trials: Screen conventional and organic insecticides for efficacy against leafhoppers and partner with growers to test spray timings.

Deviations: Some new laboratory tests were conducted, but since phenology models will be drafted and validated in 2025, commercial field timing tests are unlikely to occur within the realized scope of this project.

Significant Findings

- Relative abundance of leafhoppers of concern in Washington and Oregon orchards have been defined. From 2022–2023, a total of 14,496 adult leafhoppers were counted on sticky cards and 19,765 from vacuum samples. Most of the leafhoppers were species we can assume are innocuous to tree fruit production (61% and 77% of total leafhopper catch from sticky card and vacuum sampling, respectively). Among the three main assumed potential vectors of X-disease, the most common were *Colladonus reductus* (26%, 7%) and *Euscelidius variegatus* (11%, 15%). Oregon sites tended to have relatively more *E. variegatus.* The third species, *Colladonus geminatus,* was least common (2%, 0.4%).
- *Colladonus montanus reductus* adults have three seasonal peaks in orchards. Adults first appear as early as May, peaking in June–July. A summer peak usually occurs in August. A final peak occurs in fall, with adults persisting through November. The size of the peaks can vary greatly between peaks, locations, and years.
- Visual inspection, dissection, molecular diagnosis, and literature review conducted among the laboratories of co-PIs and collaborators indicated *E. variegatus* adults vary greatly in pigmentation. This results in a gradient of light and dark forms. Due to earlier confusion, adults of this species were previously undercounted on this project in Washington. They were re-counted for the current report.

Methods

Site selection. In 2022, 10 Washington and three Oregon orchards were selected for vacuum and sticky card sampling (Table 1) in consultation with industry collaborators Bishop, Voelker, and Smith. Orchards were chosen where large numbers of leafhoppers were expected. The orchards were either apricot, cherry, nectarine, apple, or pear. In 2023, the same sites were used, except Richland 1 was replaced with Richland 2 because few leafhoppers were found at Richland 1 in 2022. In 2024, two sites were cut to improve sampling route efficiency and reliability of weekly coverage (Richland 2 and Prosser 1).

Leafhopper monitoring. In 2022–2024, leafhoppers were monitored from May through October using sticky card and vacuum methods. Variable methods were used in 2022 between Washington and Oregon, but methods were standardized among locations starting in 2023.

For sticky cards, in 2022, two yellow cards were collected and replaced from the lowest-height tree limbs for Washington sites, and one sticky card was collected and replaced from a wooden post

between two trees for Oregon sites. In 2023, all sites used two sticky cards placed on low limbs. In 2024, three sticky cards were placed per orchard, on wooden posts. The number of *C. reductus* adults, *C. geminatus* adults, *E. variegatus* adults, and other leafhopper adults on each card was recorded.

For vacuum samples, in 2022, Washington site sampling was conducted in a standardized area based on nine 0.81-m (32-inch) diameter circular areas per site whereas Oregon sites standardized sampling based on walking 56–82 m (180–270 ft). In 2023 and 2024, sampling was standardized to nine 0.81 m (32-inch) diameter circular areas per site. A 5-gallon paint strainer bag, held on the vacuum tube with rubber bands, collected the insects and prevented them from being sucked into the motor. After vacuuming, the contents of the paint strainer bag were emptied into a zip-top bag. Zip-top bags were returned to the lab and frozen for later quantification of leafhoppers.

All samples from 2022–2023 have been quantified. Samples from 2024 are stored in a freezer and will be counted this fall–winter.

Temperature monitoring. At sites not close to AgWeatherNet or AgriMet temperature sensors, METER Group ZL6 Advanced Cloud Data Loggers with ECT/RT temperature sensors were installed to record air temperature every 15 minutes. However, temperature data from loggers was found to be unreliable during 2024 and will not be used. Solar shading requirements of both sensors and metal poles were not adequately controlled, resulting in aberrant high morning or evening temperature readings according to site aspect and unreasonably high daily temperature maxima at some locations.

Table 1. Site names, fruit crops, and years sampled for leafhoppers in 2022–2023.

State	Site	Internal Name	Fruit Crop	Years sampled
Washington	Peshastin	McDevitt	Pear	2022, 2023, 2024
	Wenatchee	TFREC	Pear	2022, 2023, 2024
	Rock Island 1	CRO	Apple	2022, 2023, 2024
	Rock Island 2	5E	Apple	2022, 2023, 2024
	Royal City 1	DOR	Cherry	2022, 2023, 2024
	Wapato	LR	Apricot	2022, 2023, 2024
	Zillah	SMO	Cherry	2022, 2023, 2024
	Richland 1	GRAY	Nectarine	2022
	Richland 2	HAY	Apple	2023
	Prosser 1	ROZ	Cherry	2022, 2023
	Prosser 2	OB1	Cherry	2022, 2023, 2024
Oregon	Hood River	MCAREC	Cherry	2022, 2023, 2024
	Mosier 1	IDIOTS	Cherry	2022, 2023, 2024
	Mosier 2	ROOTS	Cherry	2022, 2023, 2025

Model development. An analytical algorithm is being evaluated and tested by co-PI Northfield to parameterize a predictive model for *C. reductus* sticky card counts using degree day accumulation. The algorithm estimates minimum and maximum temperature thresholds directly from the field data. The algorithm predicts cumulative leafhopper count data as a function of cumulative degree-days calculated from weather data and the estimated temperature thresholds. Then, the program determines parameters for a gamma cumulative density function for generations 1 and 2. Generation 3 is expected to be modeled too because we have been finding considerable amounts of leafhoppers during fall each year. The analysis is still being conducted to allow optimization of temperature developmental thresholds, comparison of alternative cumulative density functions and error distributions, and utilization of all data across sites for a single best-fit model. Additional analysis is planned to compare sticky card vs. vacuum data and the effect of sampling resolution on conclusions.

Insecticide trials. Bioassay methods for leafhoppers were developed on a related project entitled "Insecticidal control of leafhoppers in cherries" led by co-PI Nottingham funded by the Washington Tree Fruit Research Commission. The same methods were applied in 2023 to test two rates of a new chemical, Wrath (geraniol 30%, peppermint oil 1%, cottonseed oil 0.1%, and rosemary oil 0.01%; manufactured by GroPro) in comparison with PyGanic. Data were reported in last year's continuing report for this project. In 2024, another chemical was tested, Sefina 4.89% afidopyropen and cyclopropanecarboxylate). Results and analysis will be available during fall 2024.

Results and Discussion

Leafhopper counts from sticky cards (Figure 1) and vacuum sampling (Figure 2) show similar patterns during 2022–2023. The species *C. m. reductus* clearly had three distinct periods of adult peaks. Phenology of *E. variegatus* was more idiosyncratic, and *C. geminatus* were too uncommonly found to visualize clear patterns. For both methods, most of the leafhoppers collected did not belong to any of these species and were probably of no concern as potential vectors of X-disease.

There were some differences between the two sampling methods. Vacuum sampling seemed to have more variation in counts between samples than sticky cards, and vacuum samples tended to catch relatively more *E. variegatus* than sticky cards. With both methods, the three Oregon orchards tended to have more *E. variegatus.* In Washington orchards, *C. m. reductus* was clearly the dominant species with sticky card monitoring, whereas in Washington vacuum samples, *E. variegatus* were often found in similar or greater numbers than *C. m. reductus.*

The current data are expected to be sufficient for creating a degree-day phenology model for *C. m. reductus* and may be suitable for *E. variegatus.* Previous research suggests *E. variegatus* has a single generation in Italian vineyards (Bosco et al. 1997, Ann. Appl. Biol. 130:1), and this may also be the case in Pacific Northwest orchards. Until models can be completed, all summary raw population dynamics data are available on PI Orpet's laboratory website (https://cahnrs.wsu.edu/tfrec-orpet/leafhopper-phenology-study/).

Additional work in 2024–2025 will complete the project objectives. Technicians are recruited to complete quantification of 2024 samples during the fall–winter. We also seek pesticide records from grower cooperators to correlate with leafhopper abundance. The PI from 2023–2024, Dr. Orpet, is departing Washington State University but will remain as a co-PI on the project, and Dr. Northfield will lead starting 2025 to oversee the remaining work in leafhopper quantification and model development. The datasets will be augmented by additional data from 2024 and 2025 related to a Washington State Department of Agriculture Specialty Crop Block Grant project entitled "Leafhopper phenology model development and habitat assessment to improve cherry X-disease management" awarded to PI Orpet and co-PI Northfield. That grant supports technical assistance and leafhopper sampling in three-point transects within orchards: point 1 is adjacent vegetation, point 2 is the orchard edge, and point 3 is the orchard center. This expanded sampling will help us understand the role of orchard-adjacent habitat as a potential leafhopper source and its role in phenology of the leafhoppers. Additional pesticide tests are also being planned. Altogether, the project is on target for its intended outcomes of permitting better-timed and effective sprays within a leafhopper management program.

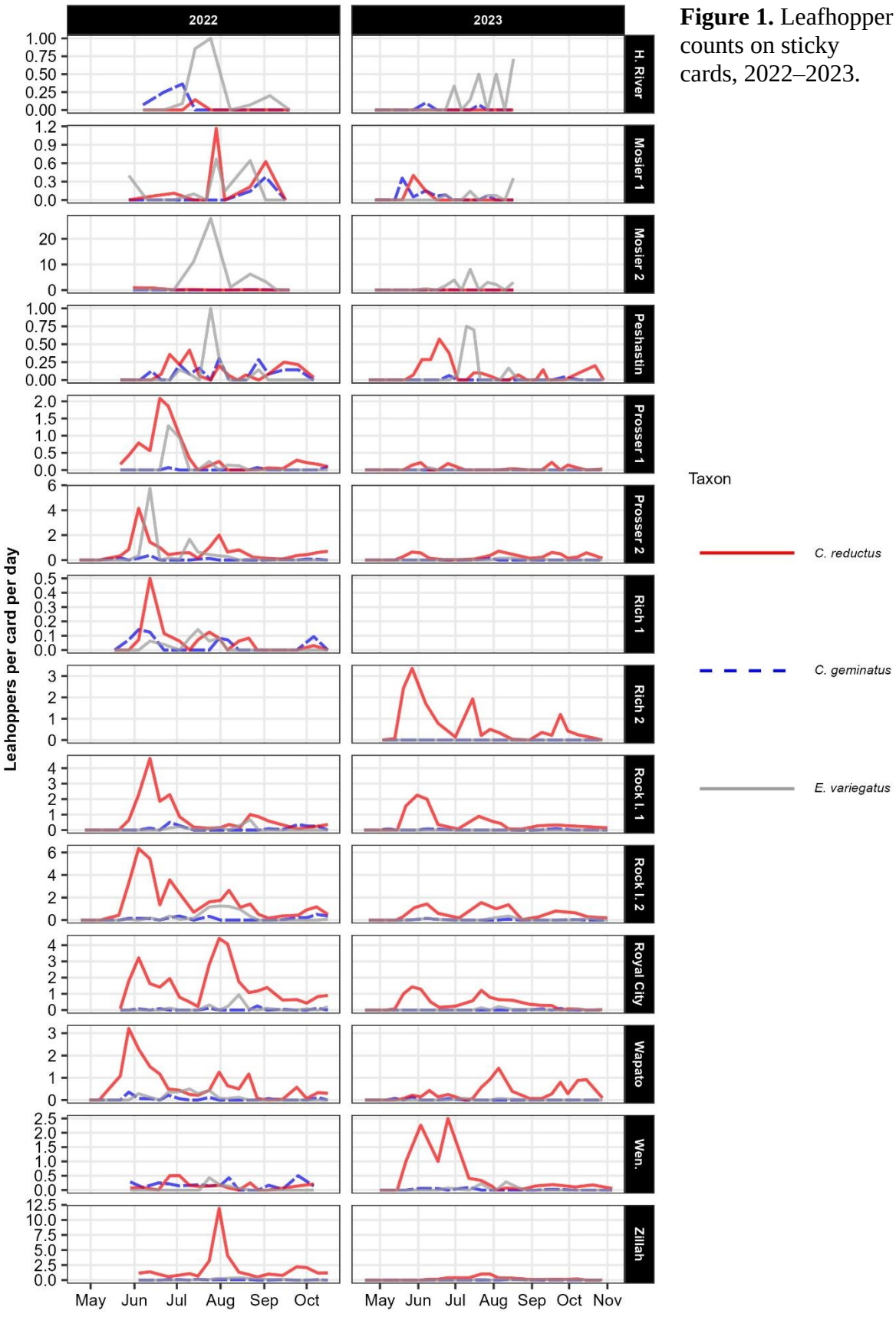

Figure 1. Leafhopper counts on sticky cards, 2022–2023.

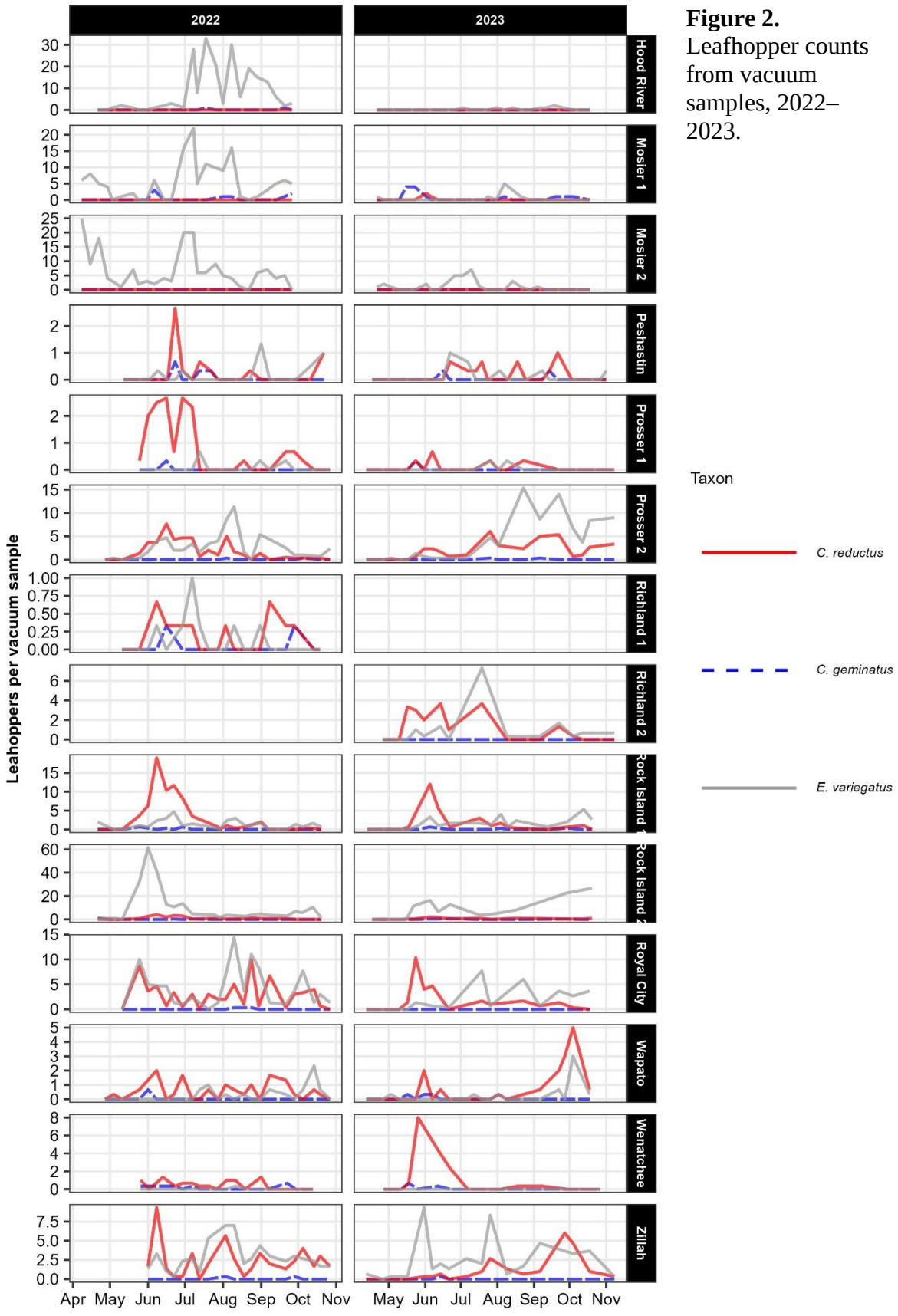

Figure 2.
Leafhopper counts from vacuum samples, 2022–2023.

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

Report Type: Final Project Report

Primary PI: Christopher Adams
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Cooperators: Orchard View Orchards

Project Duration: 3 Year

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

Other related/associated funding sources: None

WTFRC Collaborative Costs:

Budget 1
Primary PI: Christopher Adams
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Station Manager/Supervisor: Brian Pearson
Station manager/supervisor email address: brian.pearson@oregonstate.edu

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

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

Budget 2
Co PI 2: Kelsey Galimba
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Station Manager/Supervisor: Brian Pearson
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Item	2022	2023	2024
Salaries[1]	$1,000.00	$1,000.00	$1,000.00
Benefits			
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies[2]	$2,127.00	$2,453.00	$1,679.00
Travel[3]	$300.00	$300.00	$300.00
Plot Fees			
Miscellaneous			
Total	$3,427.00	$3,753.00	$2,979.00

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

Objectives

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

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

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

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

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

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

Significant Findings

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

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

Significant Findings for Objective 1

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

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

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

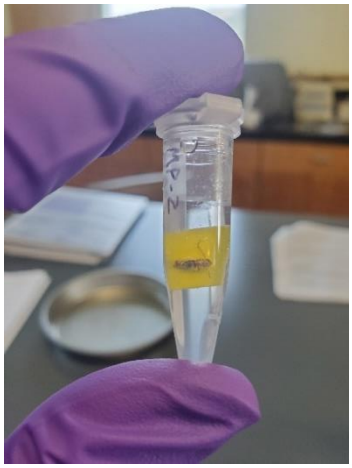

Figure 1. leafhopper + sticky card

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

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

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

Table1: ELISA Protein Marker Results

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

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

Methods for Objective 1

Protein marking

Set up: one replicate consisted of:
4 Cages

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

Figure 2. Grass sprayed with milk protein marker

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

Controls (for milk protein)

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

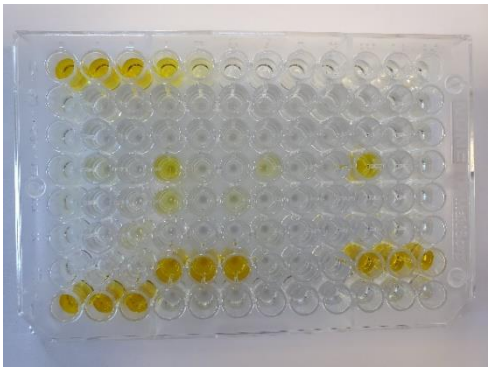

Figure 2. ELISA tray control results

Methods for Objective 1 - continued

Milk Samples

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

Egg Whites

Control (for egg protein)

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

Egg Whites

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

Significant Findings Objective 2

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

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

Significant Findings Objective 3

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

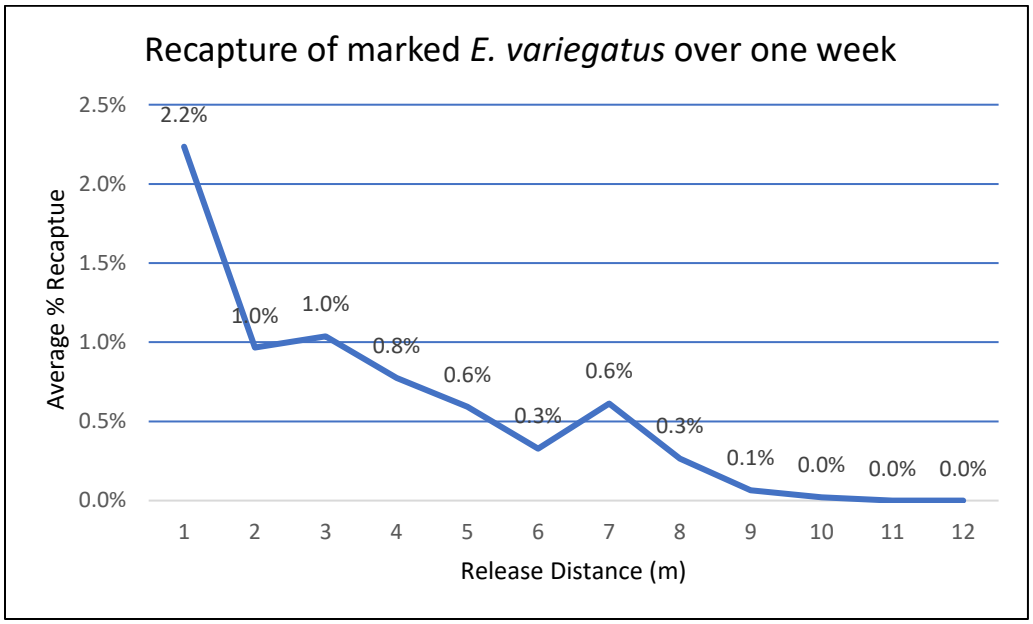

Figure 3. Proportion of released population recaptured over distance.

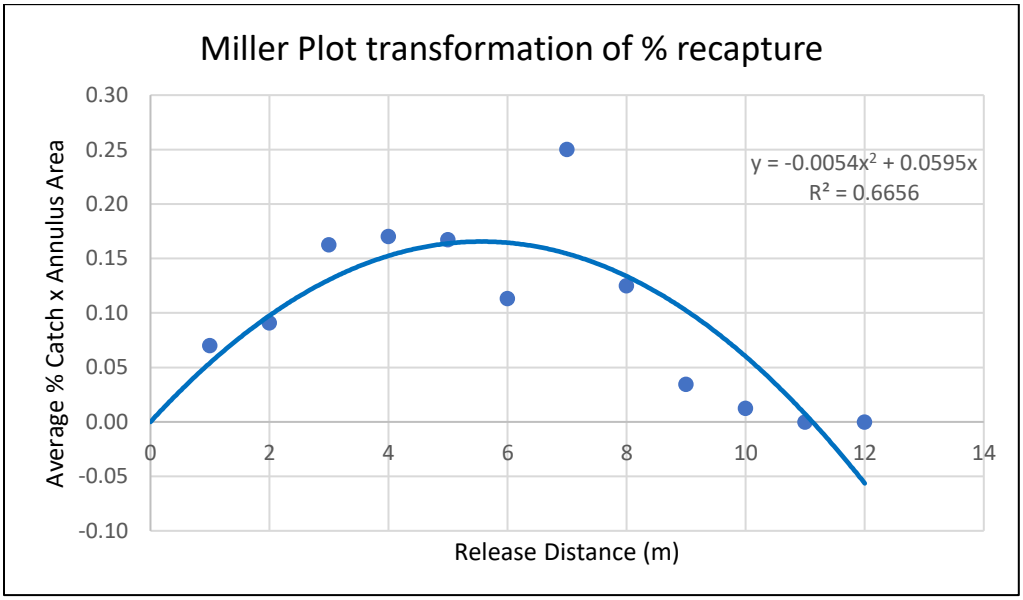

Figure 4. The Miller plot transformation of capture data deals with the low proportion recapture with increasing distance and reveals maximum dispersive distance of the released population. These data indicate that the maximum dispersive distance for *E variegatus* is just over 10 m.

Methods for Objective 2

DayGlow Powder Marking

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

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

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

Conclusions and Future Directions

Protein marking

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

DayGlow powder

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

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

EXECUTIVE SUMMARY

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

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

Abstract:

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

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

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

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

Report Type: Continuing Project Report (No-Cost Extension)

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Cooperators: John Byers (Grower), Tim Hudson (Grower), Ken Newman (Grower), Garret Bishop (G.S. Long)

Project Duration: 3 Year

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

Budget 1
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Station Manager/Supervisor: Steve Castagnoli
Station manager/supervisor email address: steve.castagnoli@oregonstate.edu

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

Footnotes:

[1] Estimated salary for one FRA to perform sample collection, testing and data analysis + 2 weeks of PI summer salary.

[2] Field testing equipment for NIR and Ca2+.

[3] Lab supplies and reagents.

Budget 2

Co PI 2: Ashley Thompson

Organization Name: Oregon State University

Contract Administrator: Charlene Wilkinson

Telephone: 541-737-3228

Contract administrator email address: charlene.wilkinson@oregonstate.edu

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

Budget 3

Co PI 2: Corina Serban

Organization Name: Washington State University

Contract Administrator: Stacy Mondy

Telephone: 916-897-1960

Contract administrator email address: arcgrants@wsu.edu

Item	2022	2023	2024
Salaries			
Benefits			
Wages			

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

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

Objectives

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

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

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

Significant Findings

Objective 1

2022

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

2023

- New infections were identified for testing after removal of trees used in 2022. Three sites with positive trees in The Dalles, OR that are being tested for phytoplasma titer dynamics (USDA-CPPM project led by T. Northfield) were collected from throughout the growing season.
- Infected trees were identified late in the season at MCAREC, Hood River, OR

Objective 2

2022

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

- Starch content of cherry leaves appears to by cyclical, with quantities and spatial distribution varying throughout the day.

2023

- All leaves sampled in Objective 1 were scanned with a hand-held spectrometer to gather spectral reflectance data for >2,000 individual wavelengths.

2024

- Spectrometer data from 2024 was analyzed. Some trees with verified infections appear to have spectral signatures, others do not. We are working on determining the cause for this.

Objective 3

2022

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

Methods

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

In 2022, X-disease infections were identified and verified using qPCR (OSU Plant Clinic, Corvallis OR) at three separate locations in The Dalles, OR. Cultivars included 'Bing', 'Benton', and 'Royal Ann'. Collections of leaves from 3-5 infected and 3-5 non-infected trees were made at five dates throughout July and August. Collections were taken preferentially from limbs that bore symptomatic fruit, and from lower on the tree. For each tree, samples from 10 leaves were weighed and flash frozen for further processing to analyze starch content. A colorimetric Starch Assay Kit was purchased from Cell Biolabs. Testing was initiated in spring, 2023, but was paused after key personnel resigned shortly after. Sampling continued in 2023, but in three new locations following the removal of trees (Fig. 1). These orchards are associated with a growing/packing organization that plans to leave infected trees in the ground for the foreseeable future. Trees that were collected from were originally identified using qPCR, and are currently being tested every three weeks for titer level (USDA-CPPM project led by T. Northfield).

Figure 1. Example of infected cherry tree included in 2023 sampling, exhibiting strong symptoms.

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

In 2022, simultaneous collections were made from the same trees to use for both iodine testing and for spectral analysis. For the spectrometer, a midpoint between the distal and

proximal ends and between the midvein and leaf margin was scanned for each leaf. Data is currently being organized for analysis. Communications with a statistician (Dr. Clark Kogan, statistician on the Cherry Cold Hardiness project) have been initiated to determine whether modeling will be necessary to develop spectral signatures for X-Disease.

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

The second method involved clearing leaves before staining with iodine. At first whole leaves were attempted, but the size of the leaves proved difficult to manipulate and limited the number of leaves we could process. To optimize, we switched to using 1x1 inch squares that were cut from the basal portion of each leaf blade, avoiding the midrib if possible (Fig. 2). Leaf squares were boiled for two minutes, then soaked in room temperature (RT) water to cool. They were then boiled in 90% EtOH until bleached, changing out EtOH if needed. Once squares were bleached, they were placed in RT water bath to rehydrate. Pictures were taken of bleached squares and then they were added to a glass dish with iodine solution. They were allowed to remain in contact with iodine for ~1 minute before being rinse in a water bath and photographed.

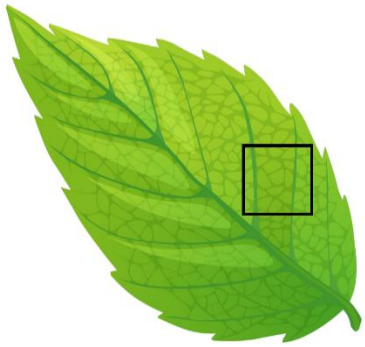

Figure 2. Leaf area that was used to clear and stain with iodine.

In 2023, iodine testing was paused because of key personnel resignation. Scanning in the field still occurred, in the trees described in Objective 1. Leaves from one branch with verified infections were made every three weeks.

In 2024, spectral data from the past two years was analyzed. In order to visualize the difference in the reflectance spectra of infected and uninfected trees, we plotted all values as points at each wavelength, from 144.7 nanometers (nm) to 1334.5 nm. Additionally, we calculated the mean reflectance at each wavelength for infected and control trees to summarize the difference between them, and plotted them as lines. We made separate line graphs for two data collection events from The Dalles, and three from the Phase 2 Trial associated with the WSU Breeding Trial, located at MCAREC.

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

In 2022, phloem sap was collected from trees used in Objective 1. ¾ inch sections of first- and second-year wood were cut, scored, and centrifuged as in Hijaz & Killiny 2014, then flash frozen for further analysis. Collected liquid should consist of both xylem and phloem contents.

In 2023, visual observations were made of leaf senescence in infected and non-infected limbs, with the goal of determining whether this can be used a field diagnostic marker. No clear pattern related to infection was established, indicating that while X-disease may alter leaf phenology in the fall, it is not consistent enough to be used as a reliable marker.

Problems/Limitations Encountered – Reasons for NCE

- Staffing was a major issue in 2022. We were able to hire a full-time research technician at the beginning of August, 2022 but she unfortunately resigned two months later. Out of five prospective summer students, only one followed through with applying and taking the position. The majority of all research is being performed by one full-time FRA and one part-time technician.
- Staffing was also a major issue in 2023, after the resignation of the lab's full-time FRA and a failed search to replace the full-time technician. Recruiting and retaining personnel at MCAREC has been challenging for all of the labs here, with three major resignations this spring/summer between the labs and ongoing empty director and office manager positions.
- Obtaining materials and supplies necessary for research has been challenging because of supply chain issues. One example: Potassium Iodide ordered in September didn't ship until January, 2022.
- The majority of infected trees we used in 2022 from growers' orchards were pushed out, requiring us to find all new sample sites in 2023. This highlights the need for infected trees at MCAREC that we can use for experimentation. We are currently designing and planning for a screen house that will be able to house and contain them. In the meantime, we are attempting to infect turnip and other herbaceous species used as model systems for X-Disease in the past, using cherry material from The Dalles and the leafhopper *E. variegatus*. Recent discovery of X-disease-positive trees in a current planting at MCAREC may lessen the need for containment and provide readily-available material for testing, but management of these trees is still being determined.
- Renovations occurred at MCAREC from November 2023 to September 2024.

Results and Discussion

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

Iodine Methods

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

In 2022, iodine testing was attempted in two different ways. The sandpaper + baggy method that was shown to work in HLB infections in citrus has yet to show significant visual differences between infected and non-infected leaves. When the method is used on potato tubers, black cells can be seen floating in the iodine solution (Fig. 3). While darker leaf material can be seen floating in solution containing sandpaper from both infected and non-infected samples, it is never as dark as the starch-filled cells from the potato. Additionally, the solution doesn't appear to differ between the infected and non-infected leaves. This testing was performed at five different dates and on different cultivars with similar results.

The clearing and iodine staining method using leaves or portions of leaves gave unanticipated results. Previous research done at WSU in the 1980's asserts that normal (i.e. uninfected) sweet cherry leaves contain no, or very little starch. This would mean that the extra

starch we hope is being accumulated in X-Disease infected leaves should be easy to distinguish. However, what we found after clearing leaves from X-Disease infected and non-infected trees is that both seem to contain starch, and in some cases uninfected leaves contain much more starch. This led us to expand our investigation. Additional testing suggests that starch does occur in cherry, and that its metabolism is dynamic, changing in response to circadian rhythms and possibly to light/solar exposure. Uninfected and infected eaves that had been kept in a dark refrigerator overnight showed no starch in mesophyll cells, but instead obvious starch granules in the guard cells surrounding the abaxial stomata. Leaves from uninfected (presumably) 'Bing' trees at MCAREC showed that while starch is present in both leaves exposed and shaded from the sun, it appears more uniform and more concentrated in shaded leaves. These results will be verified by starch assay. Altogether, the results from iodine testing in 2022 indicate a strong need for additional research into starch metabolism in sweet cherry leaves, with strong evidence that former conclusions need to be re-explored before starch can be used as a marker for X-Disease infection. Having infected trees present at MCAREC within walking distance of the lab space should allow for less complicated sampling and testing, if starch content is as dynamic as previous testing implies.

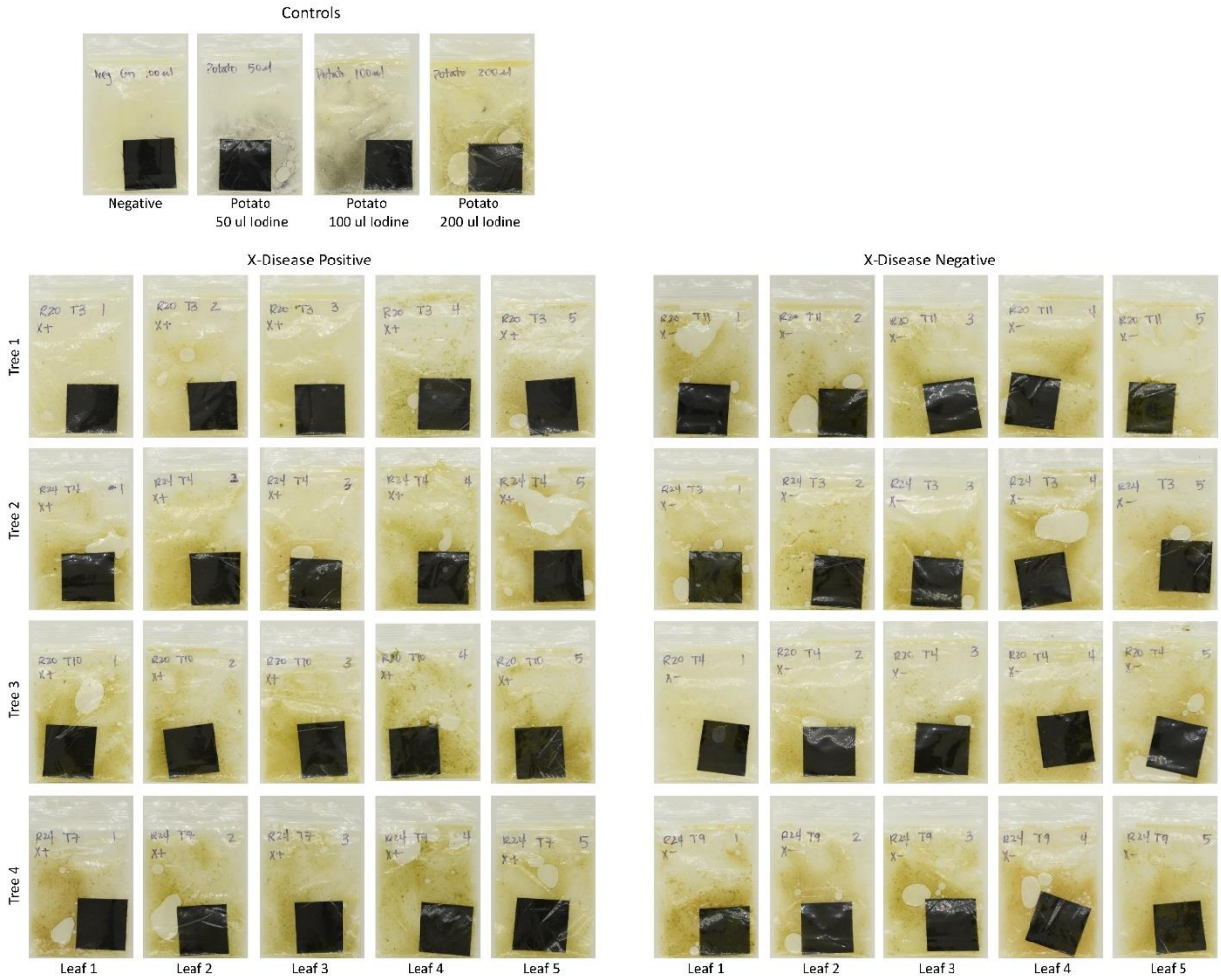

Figure 3. Baggy iodine method to detect starch in infected leaves. Sandpaper squares were used to accumulate leaf tissue from infected (left) and non-infected (right) leaves. 100 ul of iodine and 1 ml of water were added and color change was observed. No apparent difference between infected and non-infected leaves is present.

Spectroscopy Methods

As can be seen in the line graphs below, peaks of reflectance occur at the low end of the spectrum and at the high end of the spectrum (Fig. 4). To examine these peaks with more detail, we plotted the values again, between the ranges of approximately 144 – 152 nm (Fig. 5) and 1326 – 1334 nm (Fig. 6). We did not observe high-end peaks at CIB on 7.15.24 or 7.17.24, so for those days we show only the low-end of the spectrum. We can see that in The Dalles on 7.12.24, control trees had higher mean reflectance than infected trees at 149 and 152 nm, while infected trees had higher mean reflectance than control trees at 1330.2 nm. However, on 7.31.24 in The Dalles, control and infected trees did not seem to differ greatly from each other in the low-end range, and showed a smaller difference at the high-end range than had been seen on 7.12.24.

In contrast with our results from The Dalles, among the trees from the Phase 2 Trial at MCAREC in July, infected trees exhibited a higher mean reflectance than the control trees at the low-end range, and did not differ at the high-end range. In September at MCAREC, we once again observed low-end and high-end peaks, but there was little difference between the means of the infected and control trees.

The approach we described above is limited to comparing differences between control and infected trees at specific wavelengths. To better summarize the difference between the trees across all wavelengths, we performed principal component analysis (PCA) for the data from each day and location. PCA is often used with large, multi-dimensional datasets to create a 2D visualization of the differences or similarities between groups in the data. PCA decomposes data into axes called principal components (PCs). PCs are ordered such that the first PC explains most of the variation in the data, the second PC less, and so on. We have created biplots of the first two PCs for each day and location. In these biplots, points that are closer together are more similar to each other, and points that are further apart are more different. In the biplot for The Dalles on 7.12.24, we can see clear separation between the control and infected groups (Fig. 7). On the other days and locations, however, there is much less dissimilarity between the control and infected trees. The data from CIB on 7.15.24 shows some promise; infected trees are distinct from control trees, and trees of unknown infection status are scattered between the two other groups. This difference apparently did not persist until 9.26.24 (Fig. 8), where there is a large overlap between control and infected trees. The data from September is, however, a smaller sample and does not show every tree represented in the data from July.

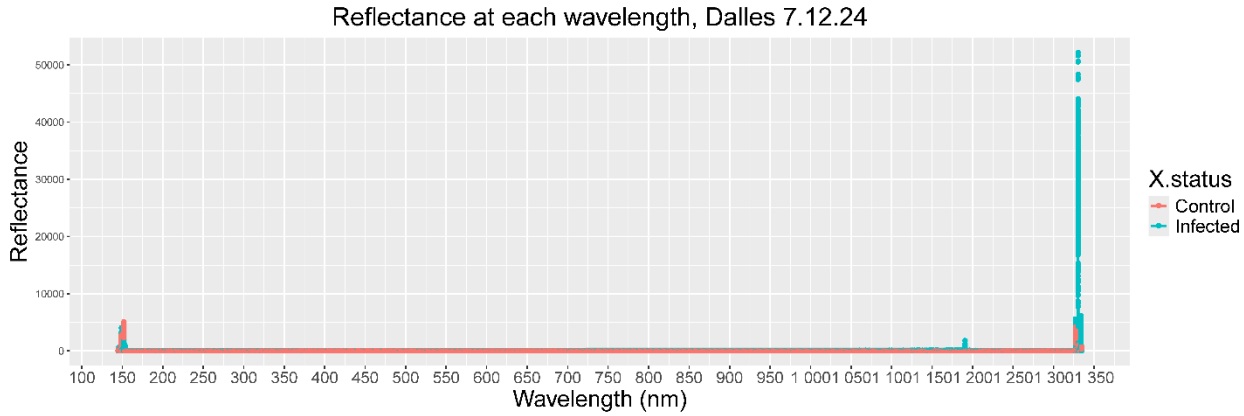

Figure 4. Peaks of reflectance occur at the low end of the spectrum and at the high end of the spectrum for data collected from the Dalles in July 12, 2024

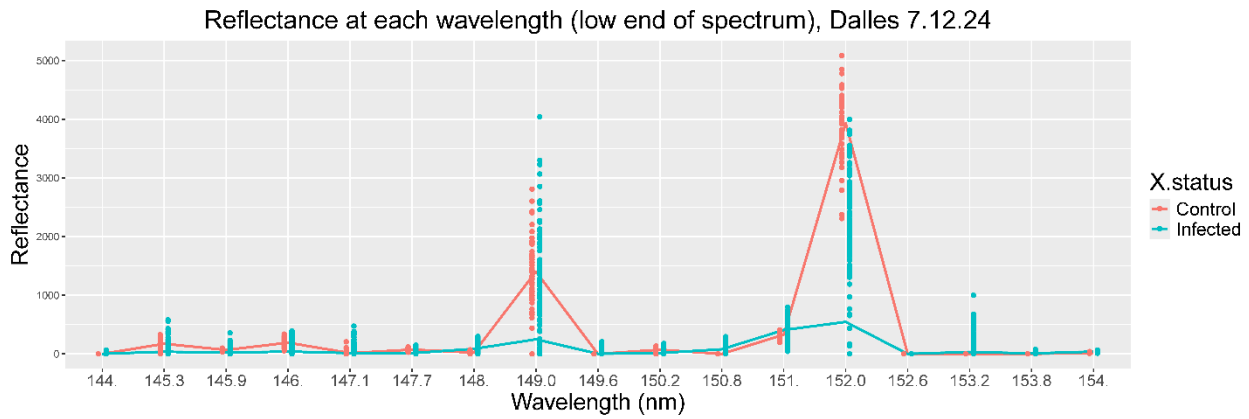

Figure 5. Peaks of reflectance occur at the low end of the spectrum for data collected from the Dalles in July 12, 2024

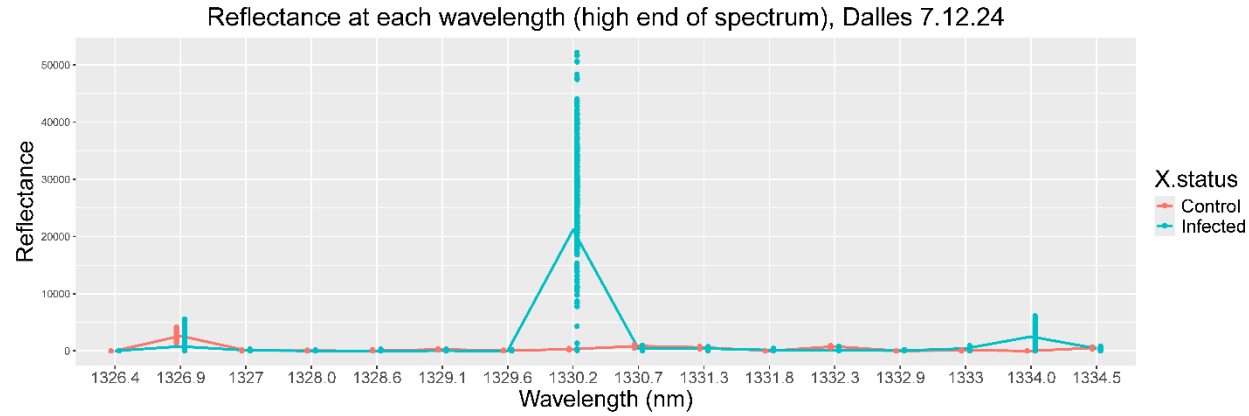

Figure 6. Peaks of reflectance occur at the high end of the spectrum for data collected from the Dalles in July 12, 2024

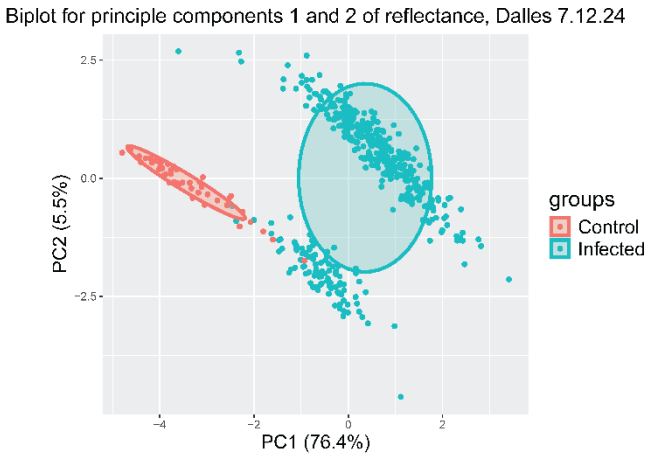

Figure 7. PCA for data collected from the Dalles on July 12, 2024

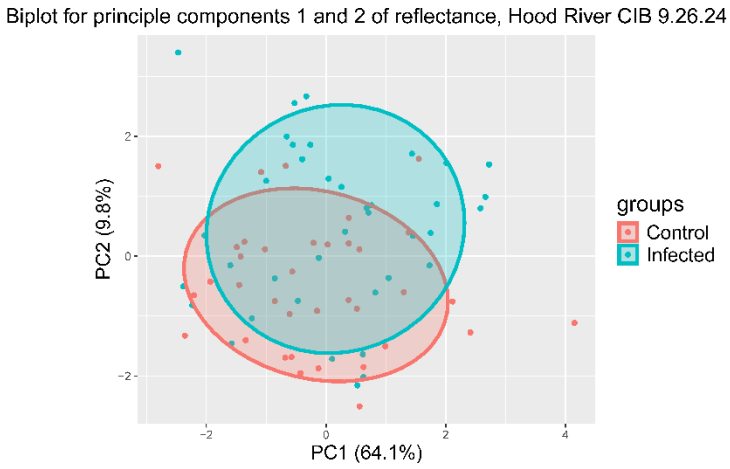

Figure 8. PCA for data collected from MCAREC on September 26, 2024

Project Title: Cas12a-based rapid method for early detection of X-disease phytoplasma

Report Type: Final

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Project Duration: 2-Year

Total Project Request for Year 1 Funding: $ 85,800
Total Project Request for Year 2 Funding: $ 89,232

Other related/associated funding sources: Awarded (Harper)
Funding Duration: 2022 - 2023
Amount: $79,740
Agency Name: USDA-ARS
Notes:

WTFRC Collaborative Costs: None

Budget 1

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Item	1/3/2023	1/3/2024
Salaries[1]	$20,925.00	$21,762.00
Benefits[1]	$7,421.00	$7,718.00
Wages		
Benefits		
RCA Room Rental		
Shipping[2]	$254.00	$316.00
Supplies[3]	$14,300.00	$14,940.00
Travel	$3,000.00	$3,000.00
Plot Fees		
Miscellaneous		
Total	$45,900.00	$47,736.00

Footnotes: 4% inflation for year 2. [1]Postdoc salary for 4.5 months and postdoc benefit rate at 35.5%.[2]Shipping materials to PSU. [3]Including budget for co-PI Scott Harper: $6000 for year 1 and $6240 for year 2.

Budget 2

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Item	1/3/2023	1/3/2024
Salaries	$24,311.00	$25,283.00
Benefits	$8,752.00	$9,102.00
Wages		
Benefits		
RCA Room Rental		
Shipping		
Supplies	$4,837.00	$5,111.00
Travel	$2,000.00	$2,000.00
Plot Fees		
Miscellaneous		
Total	$39,900.00	$41,496.00

Footnotes: 4% inflation for year 2.

Budget 3

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Footnotes: Scott's budget is included in PI Zhao's budget 1.

Objectives:

1. To establish and optimize a Cas12a-based method for early and rapid detection of cherry X-disease phytoplasma;

2. To apply the Cas12a-based method for field sample diagnosis (cherry, insects, weeds);

3. To train and promote the Cas12a-based method for diagnosis of X-disease phytoplasma.

Significant Findings

- XDP-specific RPA primers and crRNAs have been identified and synthesized based on the DNA sequence alignment and analysis of the *SecY* genes from the phytoplasma 16SrIII subgroups (Figures 1-2).
- An XDP-specific primer pair has been selected and validated for its high efficiency of RPA.
- A specific crRNA was selected for the XDP Cas12a detection assay.
- A two-pot RPA/Cas12a assay has been successfully developed for highly sensitive and specific detection of different XDP strains (Figures 3-4).
- A simple protocol of the RPA/Cas12a assay has been established for rapid detection of XDP.
- The RPA/Cas12a method has been demonstrated to be capable of specific and rapid detection of XDP-infected samples from the field (Figures 5-6).
- Double blind assay results from Cas12a-based tests for more than 100 samples of sweet cherries, weeds and insects correlated well with the qPCR assay results (Figures 7-10).
- Developed a 5-min genomic DNA extraction method for the RPA/Cas12a assay (Figure 11).
- One-pot RPA/Cas12a assay based on the cap/spin approach was recently established to facilitate the field-deployable detection of XDP in the near future.

Methods:

Procedures and Methodology. Since the initiation of this project in January 2023, we have successfully established an RPA/Cas12a method for highly specific, supersensitive, and rapid detection of cherry X-disease phytoplasma (XDP).

The DNA sequences of *SecY* genes from the XDP strains and other 16SrIII subgroups were obtained and aligned using sequence analysis tools. RPA primers and crRNAs were designed, chemically synthesized, and experimentally tested in RPA and Cas12a assays, respectively. They were evaluated for their specificity, amplification or cleavage efficiency, compatibility, and reproducibility. In the subsequent two-pot assay, both RPA and Cas12a reactions were performed under the isothermal condition at 102ºF (39ºC) (see **Fig. 1**). The fluorescent signal released from the oligo reporter due to Cas12a trans-cleavage was detected and quantified by a fluorescence microplate reader using following setting: excitation 485/20; emission 530/25nm. The RPA/Cas12a assay for XDP detection can be completed within one hour.

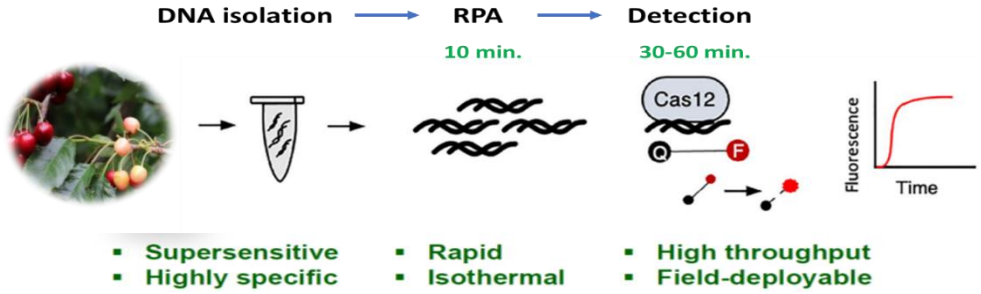


Fig. 1. Illustration of the RPA/Cas12a assay for XDP detection, which includes total DNA extraction, target DNA amplification by RPA and subsequent Cas12a detection with a fluorescence microplate reader based on trans-cleavage of oligonucleotide reporters.

Once successfully established our assay, we validated our assay specificity by using pure DNA fragment and DNA from infected cherry samples and determined the sensitivity of the assay by 10x

serial dilution of pure DNA samples and infected cherry DNA samples (see **Figs. 4-6**). To further confirm our method, double blind experiments were carried out using the Cas12a-based assay for more than 100 samples of sweet cherries, weeds and insects and compared with those of the qPCR assay results (**Figs. 7-10**) In addition, we adapted and optimized a 30-second rapid method to extract genomic DNAs from cherry plant samples based on cellulose dipsticks (**Fig. 11**).

Types and timing of anticipated results. The step-by-step protocol for the RPA/Cas12a-based diagnostic assay for cherry XDP was finalized, including RPA primer pairs, specific crRNA, and FAM reporter. We have also determined the detection limit and sensitivity of the assay. We optimized the times of RPA reaction, Cas12a detection procedure and signal detection by fluorescent microplate reader, which will significantly shorten the procedure and obtain results within an hour. The method was also verified by double blind assay and compared with qPCR results.

Potential problems or limitations. We did not encounter technical problems and limitations so far as the method is technically straightforward.

Results and Discussion:

Based on the DNA sequence alignment of *SecY* genes from the XDP strains and other 16SrIII subgroups, five RPA primer pairs were designed, synthesized, and experimentally tested for specificity, amplification efficiency and reproducibility. The best pair of primers (FP1/RP1) was selected for highly efficient and specific amplification of XDP (**Fig. 2**). Importantly, the FP1/RP1 primer pair did not amplify DNAs from cherry plant or other phytoplasmas such as Phytoplasma solani and Phytoplasma mali.

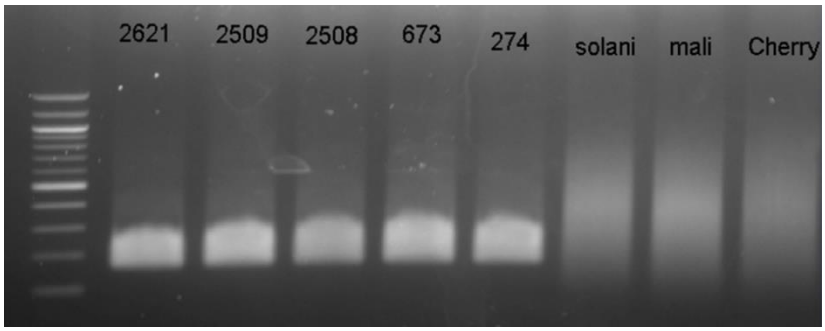


Fig. 2. Specificity and amplification efficiency of the FP1/RP1 primer pairs. Amplification of 185 bp fragment by the RPA assay only occurred with the XDP DNA samples (2621, 2509, 2508, 673, 274), but not with the DNA samples from Phytoplasma solani, Phytoplasma mali or cherry plant.

In addition, three crRNAs were designed, chemically synthesized, and tested for specificity, cleavage efficiency and compatibility with RPA. One specific crRNA was selected for the XDP Cas12a detection assay.

After the establishment of individual RPA and Cas12a detection assay, a two-pot RPA/Cas12a assay was successfully developed in Co-PI Dr. Yang's lab at Penn State for highly sensitive and specific detection of XDP DNA. Preliminary data showed that the RPA/Cas12a assay can detect XDP DNA at the sensitivity level of at least 100 aM (**Fig. 3**). Subsequently, we further optimized the assay reagents and conditions and achieved the attomolar detection sensitivity (~1 copy per microliter) in both Yang and Zhao labs (**Fig. 4**).

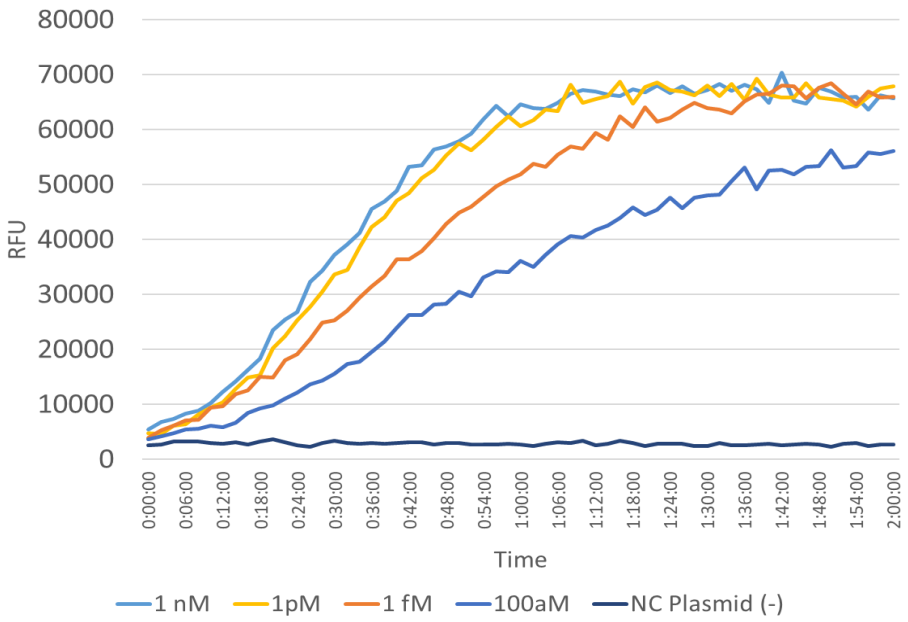
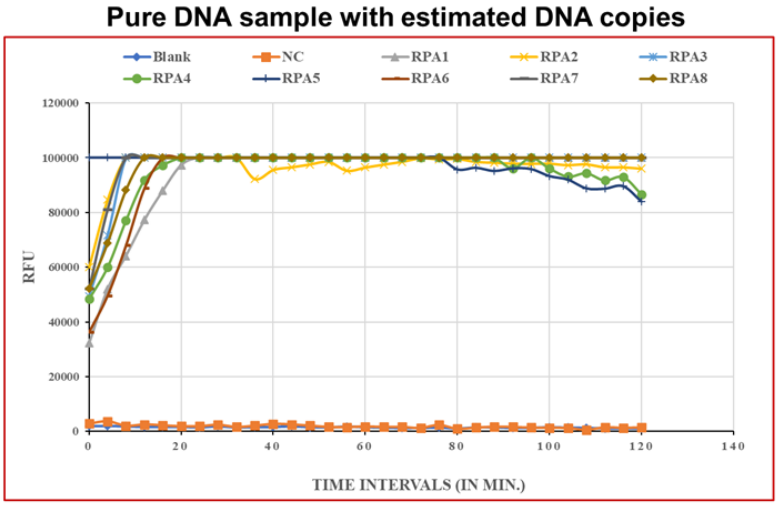


Fig. 3. The initial sensitivity test of the RPA/Cas12a assay for XDP DNA detection. A dilution series (1 nM, 1 pM, 1 fM, 100 aM) of plasmid DNA containing the *SecY* gene target region were used along with negative control plasmid. XDP DNA at 100 aM level could be readily detected by fluorescence microplate reader within 12 minutes of Cas12a reaction.



Sample	Copy no.
RPA1	1
RPA2	10
RPA3	100
RPA4	1000
RPA5	10000
RPA6	100000
RPA7	1000000
RPA8	10000000

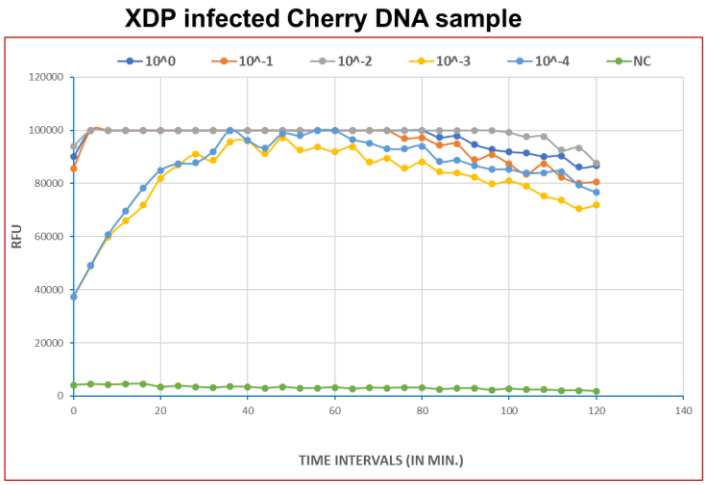
NC: Negative control (non-infected sample); **Blank:** Water.

The max fluorescence reading is 100000 RFU.

Fig. 4. Detection of XDP DNA by the RPA/Cas12a assay at the single copy sensitivity. The XDP *SecY* gene fragment was amplified by PCR, quantified for DNA concentration and used to generate serial dilutions for the detection sensitivity test.

To validate the RPA/Cas12a assay for XDP detection of field samples, we used the assay protocol provided by Co-PI Dr. Yang to detect XDP at Washington State University in Prosser. We were able to successfully replicate and validate the RPA/Cas12a assay for XDP detection (**Figs. 4-6**). We could detect one to two copies of DNA by the RPA/Cas12a assay within 10 min using purified DNA and cherry DNA samples, respectively (**Figs. 4-5**). Furthermore, the two-pot RPA/Cas12a assay was

demonstrated to be capable of specific and rapid detection of XDP-infected samples from the field with various concentration of XDP, ranging from 300 copies to 1 million copies of DNA (with Ct values 18 to 31) (**Fig. 6**). These results showed a very higher sensitivity of RPA/Cas12a assay for XDP detection in the field samples. Double blind experiment results from Cas12a-based assay for more than 100 samples of sweet cherries, weeds and insects correlated well with the qPCR assay results (**Figures 7-10**). These results suggest that this method is capable of detecting XDP at low titer and applicable to various samples from the field.

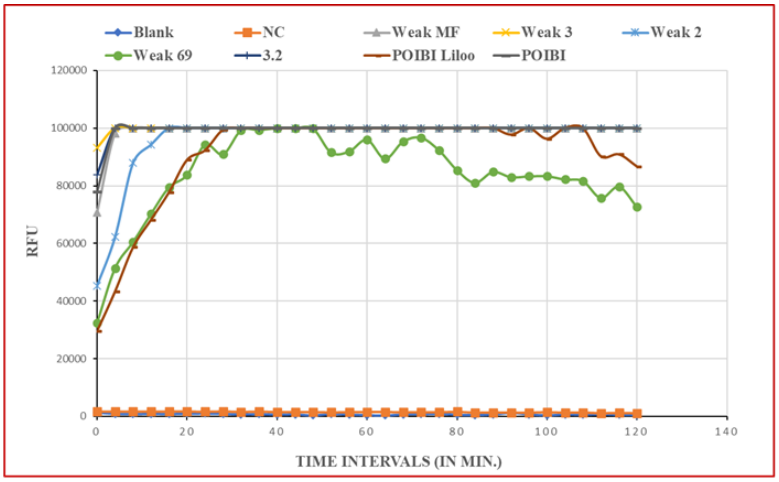


Template	Copy no.
10^{0}	20000
10^{-1}	2000
10^{-2}	200
10^{-3}	20
10^{-4}	2

NC: Negative control (non-infected sample)

The max fluorescence reading is 100000 RFU.

Fig. 5. Detection of XDP by RPA/Cas12a in a DNA extract from an infected cherry tree.



Cherry samples
i) Weak MF
ii) Weak 3
iii) Weak 2
iv) Weak 69
v) 3.2

Other phytoplasma

i) POIBI Liloo
ii) POIBI

NC: Negative control (non-infected sample); **Blank:** Water.

The max fluorescence reading is 100000 RFU.

Fig. 6. Detection of XDP from infected cherry samples and other phytoplasmas.

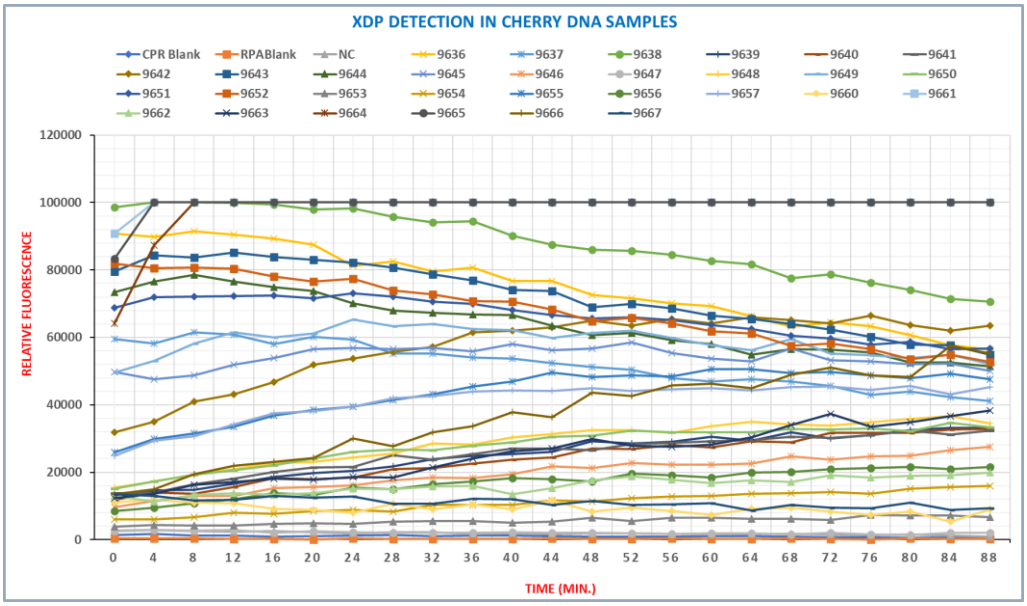


Fig. 7. Double blind tests of 30 cherry samples (all positive) with Ct values ranging from 21 to 39.

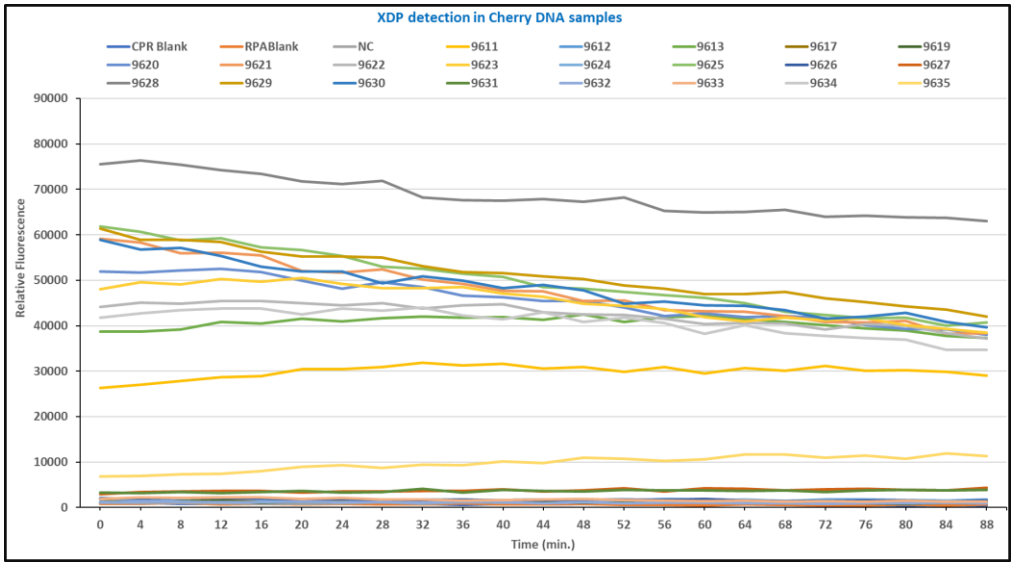


Fig. 8. Double blind tests of 21 cherry samples. 14 samples tested positive with CT values ranging from 21 to 36 and 7 samples tested negative.

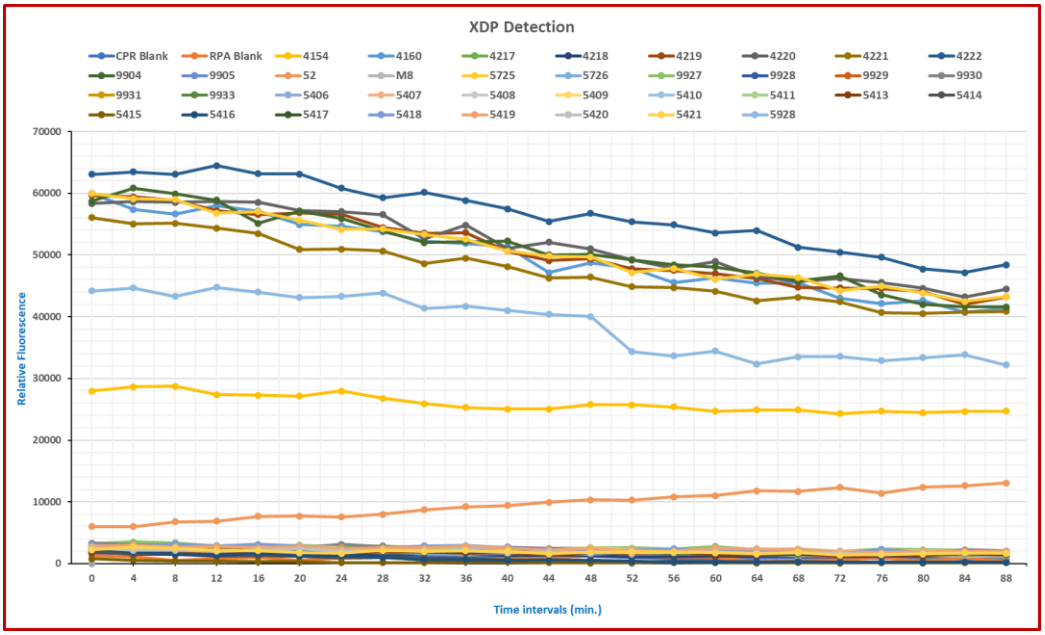


Fig. 9. Double blind tests of 36 samples from weed, leafhopper, cherry and celery. 10 samples tested positive with Ct values ranging from 26 to 38.

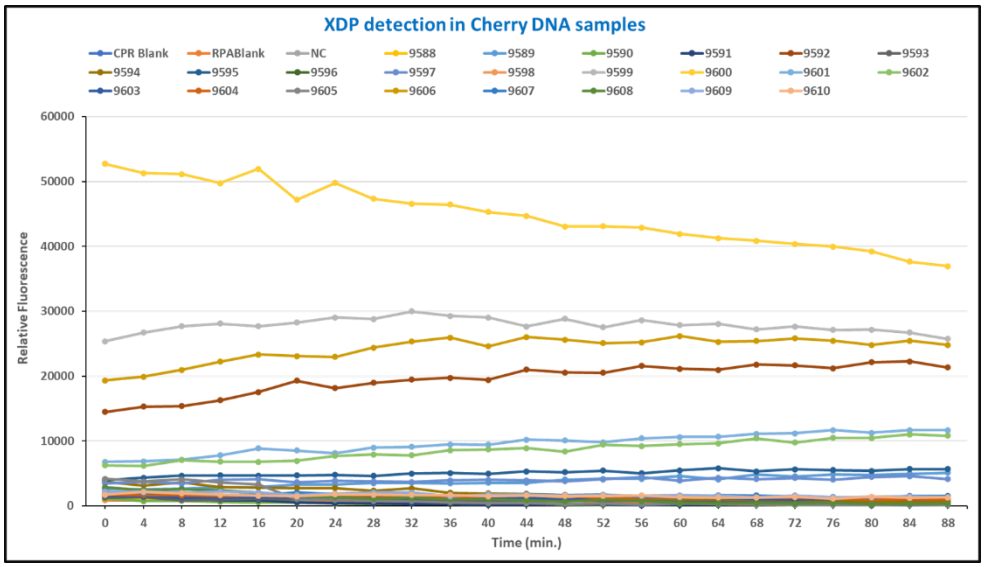


Fig. 10. Double blind tests of 23 cherry samples. Only 9 samples tested positive with CT values ranging from 31 to 39.

In addition, we adapted and optimized a 30-second rapid genomic DNA extraction method based on cellulose dipsticks (**Fig. 11**). This method comprises the grinding of 1-cm cherry tissues, including leaf, stem, bark, fruit, and flower in DNA extraction buffer. The cellulose dipstick dipped 5 times each in DNA extraction buffer and washing buffer. The purified DNA on the cellulose dipstick was then eluted in the dH_2O or RPA reaction solution for amplification. This DNA extraction method is simple and can rapidly extract DNAs from various plant tissues.

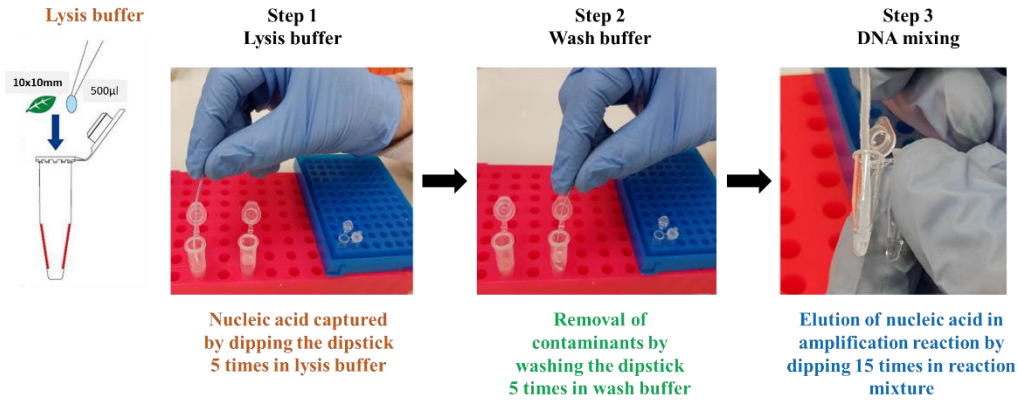


Fig. 11. Rapid extraction of genomic DNAs from the cherry tree samples.

Significance to the industry, and potential economic benefits. The cherry industry has been in an uncharted territory due to the cherry X-disease, which causes significant economic losses to growers and nurseries in the past several years. One major challenge in effectively managing the X-disease phytoplasma (XDP) is to find effective methods of early disease detection for non-fruit-bearing trees and for non-symptomatic trees/rootstocks in both orchards and nurseries. However, early detection of XDP is particularly challenging due to unculturable nature, low titer, and uneven distribution of the pathogens within infected plants as well as the ever changing genotypes of the pathogen. The RPA/Cas12a assay for XDP detection developed in the current study specifically addressed the industry's need by providing an early detection platform with the following advantages: a) highly sensitive, detecting as low as one to two copies of DNA within one microliter sample; b) highly specific, detecting the XDP target DNA with no cross reactivity; c) very rapid and have results within an hour; and d) can detect all current known genotypes of the pathogen. On the other hand, current laboratory-based testing by real-time PCR is the industry's standard for diagnosing an XDP infection not expressing symptoms, which could cost $60 per sample. The method we currently developed costs much less than that, which will provide the industry with an inexpensive alternative method for XDP diagnosis. Therefore, the significant and potential economic benefits to the cherry industry of the current study are multifaced. It not only provides the industry with much-needed tool for fast, supersensitive, and accurate diagnosis, but also saves time and money for both growers and nurseries.

Executive Summary

Project Title: Cas12a-based rapid method for early detection of X-disease phytoplasma

Key words: X-disease Phytoplasma, Diagnosis, Detection, CRSPR-Cas12a, Cherry

Abstract:

The cherry X-disease is at epidemic levels in the Pacific Northwest and caused significant economic losses in the past several years. To effectively manage the X-disease phytoplasma (XDP), it is critical that infected trees be identified as rapidly as possible, especially before symptoms appear. Recently, the clustered regularly interspersed short palindromic repeats/CRISPA-associated (CRISPR-Cas) system has been used as an extremely sensitive and rapid diagnostic tool for pathogen detection besides its common applications in genome editing. In this study, we have developed the recombinase polymerase amplification (RPA)/Cas12a diagnostic assay and a quick DNA extraction method to facilitate the rapid and super-sensitive detection of XDP. Based on the DNA sequence alignment and analysis of the *SecY* genes from the phytoplasma 16SrIII subgroups, XDP-specific RPA primers and crRNAs have been identified and validated for its high efficiency of RPA and for the XDP Cas12a detection assay. Thus, a two-pot RPA/Cas12a assay has been successfully developed for highly sensitive and specific detection of different XDP strains and demonstrated to be capable of specific and rapid detection of XDP-infected samples from the field. Double blind assay results from Cas12a-based tests for more than 100 samples of sweet cherries, weeds and insects correlated well with the current industry standard qPCR assay results. In addition, we also developed a 5-min genomic DNA extraction method for the RPA/Cas12a assay and established one-pot RPA/Cas12a assay based on the cap/spin approach, which will facilitate the field-deployable detection of XDP in the near future. In summary, the highly sensitive, specific, and rapid RPA/Cas12a assay for XDP detection not only provides the industry a much-needed platform for fast, supersensitive, and accurate diagnosis, but also saves time and money for both growers and nurseries.

Towards the identification of Little cherry disease linked volatile biomarkers

Report Type: Final Report

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Equipment cost match: $60,000 (FAIMS-Lonestar VOC Analyzer, Owlstone Medical, UK)
WTFRC Budget: none

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

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

Item	2022	2023	2024/2025
Salaries	46,200	48,048	
Benefits			
Wages			
Benefits	16,014	15,120	
Equipment			
Supplies	15,120	16,655	
Travel	2,697	2,697	
Miscellaneous			
Plot Fees			
Total	80,031	82,520	0

Footnotes: Year-1: 11-month salary support ($46,200 plus $16,014 benefits) for a postdoctoral researcher is requested. Postdoc will work closely with the PIs in planning and conducting experiments, data analytics and reporting. $1,500 requested to procure FAIMS sampling experiment lab consumables such as PTFE tubing, sampling glass jars, gloves, chem-wipes, headspace trapping cling films and N2 carrier gas. $7,120 requested to procure Tedlar bags ($178/ pack of 10 bags x 40 pack) for plant volatile trapping, and $5,000 towards PI-Ruddell's GC/MS consumables and maintenance. $2,697 is requested for field sampling related travel (150 miles /trip x 20 trips) as well as GC/MS analysis travel from Prosser to USDA-ARS Wenatchee, WA (5 nights per diem + 270 miles/trip). PI Serban requests $1,500 towards extension outreach activities. **Year-2**: request is similar to Year 1 expect salary rate is adjusted by 4% per WSU policies. **Year-3.** Approved No Cost Extension until 12/31/2025.

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

Significant Findings. This project evaluated FAIMS technique (objective 1) for early detection of LCD/X-disease in 'Bing' and 'Skeena' cherry cultivars by analyzing volatile profiles across seasons and different growth stages. The pit hardening stage consistently showed significant differences in volatile emissions between symptomatic (S) and asymptomatic (AS) samples, with AS samples having higher ion current values in both cultivars. Temporal variations were also observed at other stages, though less consistently, indicating the importance of the pit hardening stage for early detection of X-disease. Cultivar-specific differences and environmental conditions across seasons also influenced volatile emissions. Project also identified key volatile biomarkers, using GC-MS static and dynamic sampling technique (objective 2), associated with the X-disease infestation. Potential biomarkers include, Ethanol, (E)-2-hexenal, propanoic acid, and acetone, ethyl acetate, (Z)-2-penten-1-ol, (Z)-3-hexen-1-ol*, 2-hexenal, acetaldehyde, acetoin. *Practical Implications*: 1) *Early Detection*: Identification of the pit hardening stage as critical for detecting X-disease, 2) *Targeted Monitoring*: Growers can focus on non-destructive (e.g., Canine based) scouting efforts during the pit hardening stage, 3) *Biomarker Utilization*: The volatile biomarkers identified in this project can be used to develop more precise diagnostic tools, enhancing the accuracy of X-disease detection, 4) *Cultivar-Specific Strategies*: Understanding cultivar-specific differences in volatile emissions can help tailor disease management practices to specific cherry varieties, improving overall crop health, and 5) *Seasonal Adaptation*: Recognizing the influence of environmental conditions on volatile emissions enables growers to adapt their monitoring and intervention strategies according to seasonal variations.

Methods

Field sampling and preparation. Table 1 summarizes details about field sites and objective/analysis technique specific sampling during the three-year (2021-2023) project period. In each case, samples from the lower canopy zone of sweet cherry trees were collected from orchard sites located in Central Washington. The samples were typically collected at the key growth stages: flowering, shuck fall, pit hardening, first straw, harvest, and post-harvest for the commercially grown cultivars (Table 1). Trees in the experiment were selected based on the preliminary molecular analysis done by PI-Harper's lab (detailed below) and symptomology. Trees and branches were labeled with colored tape and labels for consistent data collection throughout the experiment duration. Sub-sections below detail methods specific to each of the volatile analysis technique.

Molecular analysis (qPCR). Stem samples were collected for conducting qPCR-based molecular analysis as a validation of the volatile sensing approaches. For all sites, samples were collected within a similar time frame (see table 1). Post-sample collection, a small section of stem from each branch was used for phloem tissue extraction. These extracted tissues were chopped using a razor blade and stored in a bead-beating tube. For further processing, 0.1 g tissue sample was scaled in labeled tubes. Similarly, the plant tissue stored at -80 °C was also weighed in labeled tubes (GC/MS samples). The Nucleic acids from these samples were extracted using the cetyltrimethylammonium bromide (CTAB) extraction method. Resulting samples were stored at -20 °C. The amplified samples were used as the template for the qPCR reaction. All the samples were diagnosed with X-disease phytoplasma (XDP), Little Cherry Virus-2 (LChV-2), Prunus Necrotic Ringspot Virus (PNRSV) and Prunus Dwarf Virus (PDV). The bacterial canker symptoms were

observed based on visual scouting of the trees at the time of sampling. The results of the molecular analysis were used for comparative analysis.

Table 1. Field sites and volatile headspace analysis sampling details.

Season	Site	Cultivar	Growth Stage	Analysis Method*	Trees Sampled** S	Trees Sampled** AS
2021	Site 1	Bing	Flowering	1,4	6	3
			Shuck fall	1		
			Pit hardening	1		
			First straw	1		
			Harvest	1,4		
			Post-harvest	-		
	Site 2	Benton	Post-harvest	1,4	12	12
	Site 3	Cristalina	Post-harvest	1,4	3	3
		Tieton	Post-harvest	1,4	3	3
2022-23	Site 4	Bing	Flowering	1,3,4	5	4
			Shuck fall	1,4		
			Pit hardening	1,4		
			First straw	1,2,3,4		
			Harvest	1,2,3,4		
			Post-harvest	1,4		
	Site 5	Skeena	Flowering	1,3,4	4	4
			Shuck fall	1,4		
			Pit hardening	1,4		
			First straw	1,3,4		
			Harvest	1,2,3,4		
			Post-harvest	1,4		

*1: FAIMS; 2: GC-MS-static; 3: GC-MS-dynamic; 4: qPCR technique; S: Symptomatic; AS: Asymptomatic

**1 replicate/tree for FAIMS and GC-MS dynamic sampling technique, 5 replicate/tree for GC-MS static sampling technique.

Field Asymmetric Ion Mobile Spectrometry (FAIMS). Field collected samples were stored in 1-gallon glass (sterilized) jars. Each sample jar was covered with a food-grade cling film for aerobic storage conditions (Fig. 1). Samples were then analyzed using a portable FAIMS system using a custom-developed unit (Arasaradnam et al., 2016; Kothawade et al., 2021). The glass jars were covered by a Teflon lid having two stoppers with two holes, one as an inlet for the carrier gas source (nitrogen air) and another to flush out the volatiles to the FAIMS ionization chamber. A total of six scans were collected for each sample jar at the optimized operation parameters (flow rate: 1.5 L min^{-1} and pressure: 60 kPa). The FAIMS unit takes ~4 minutes to collect these six scans (i.e., 60 s/scan). Blank jar was scanned as a reference in data analysis for each sampling day.

Figure 1. *Portable FAIMS system analyzing cherry leaves volatile profile.*

The FAIMS scans output is an ion current spectrum that is proportional to the mass of distinct volatile organic compounds (VOCs) under a range of dispersion fields (DF: 0 to 100%) and compensation voltages (CV: -6 to 6V). The ion current spectrums from all scans generate three-dimensional data consisting of 51 DFs, 512 CVs, and resultant ion currents (I_c, arbitrary units, AU). The system needed to be purged for about 40 minutes using nitrogen air before scanning the next sample jar to remove the residues from the previous sample. The purge time can be shortened in future, commercial use, by reducing sample size, i.e., introducing a lesser amount of VOC headspace to the unit.

Data analysis. Raw FAIMS scans from the volatile's headspace analysis were extracted to *.csv file format. Noise removal and feature engineering was then performed to identify the important features. A threshold value (I_c> 0.02) was, determined using histogram-based thresholding, used to remove background noise. Subsequently, a total of four generalized regions of interest (ROIs) were defined based on the I_c clusters and were used to extract the sum of I_c (I_{sum}) at all CV-DF combinations. I_{sum_all} as sum of entire spectra was also computed as a feature. Additional features such as, maximum ion current (I_{max}) and area under the curve (I_{AUC}) were extracted at each DF within a given range of CV for the DF specific analysis. A scanline at a given DF value and CV range is a 2D representation of the spectra. The I_{AUC} was calculated for each DF value and compressed along with the sample names as rows and DF values in columns and respective I_{AUC} value. The I_{AUC_sum} feature was then used for temporal analysis of the VOC profiles over the growth stages. The number of phytoplasma copies in the sample were calculated using a calibration curve and quantification cycles (Cq) values (Wright et al., 2022) from qPCR results. Correlation analysis was performed between important features and the number of phytoplasma copies in a sample. All the analyses and visualizations were conducted in Python software (version 3.6, Python Software Foundation, Wilmington, DE, USA) packages using libraries, 'NumPy', 'pandas', 'seaborn', 'sklearn' and 'matplotlib'.

Gas Chromatography-Mass Spectrometry (GC-MS). Experiments were conducted during 2022 and 2023 growing seasons. For the 'Bing' cultivar, samples were collected from a commercial orchard located near Sunnyside, WA. For the 'Skeena' cultivar samples were collected from the orchard at the WSU Roza experiments block in Prosser, WA. All samples were collected from the lower canopy zone. From each tree, five leaves were collected per replicate, with five replicates per tree for both symptomatic and asymptomatic trees.

Static headspace volatile analysis was conducted at the first straw and harvest stages. Figure 2 depicts the typical sampling and analysis protocols. For the sample collection, 50 ml centrifuge tubes were used to store the samples (5 leaf/replicate). Liquid nitrogen was used to flash freeze the samples. These samples were then stored in dry ice until moving them to the -80 °C facility. Prior to volatile analysis, scaling of ground plant tissue (0.5g) in 20 ml glass vials was performed in a box filled with liquid nitrogen to avoid sample thawing at room temperature.

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

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

In case of *dynamic volatile headspace analysis*, samples including leaf tissue were collected from the same sites as reported above and stored in the 1-gallon glass jars. The volatile headspace from the sample jars was trapped on the adsorbent Tenax TA packed in glass collectors using a vacuum pump. The sorbent tubes were then analyzed using the GC-MS system as reported above.

Data analysis. Deconvolution and peak identification of volatile biomarkers was performed using quantitative analysis software (Quant. 10.1, Agilent Technologies, Palo Alto, CA, USA). Compounds were identified using custom libraries containing retention indices and mass spectra. The data was compared with standard spectral catalogs (NIST05) and authentic standards. Compounds without definitive matches were labeled as 'tentative' or 'unknown' along with their spectral information. Raw data of VOCs relative abundances were preprocessed, including normalization and missing value imputation. Samples were then classified as asymptomatic (AS) or symptomatic (S) based on qPCR results. Partial Least Squares-Discriminant Analysis (PLS-DA) was used for classification, evaluating model performance with cross-validation and R2/Q2 metrics. To identify influential VOCs, used was Variable Importance in Projection (VIP) scores, considering compounds with VIP scores >1 as significant. Further validation was performed using these findings using ANOVA and Tukey's Honest Significant Difference test ($p < 0.05$). The relationship between significant VOC abundances and pathogen infection levels were analyzed using Pearson's correlation analysis, with significance levels set at $p < 0.05$ and $p < 0.1$. All the analysis were performed in MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/), R (version 3.6.1, R Core Team 2022, Vienna, Austria, and R Studio Inc., Boston, MA, USA).

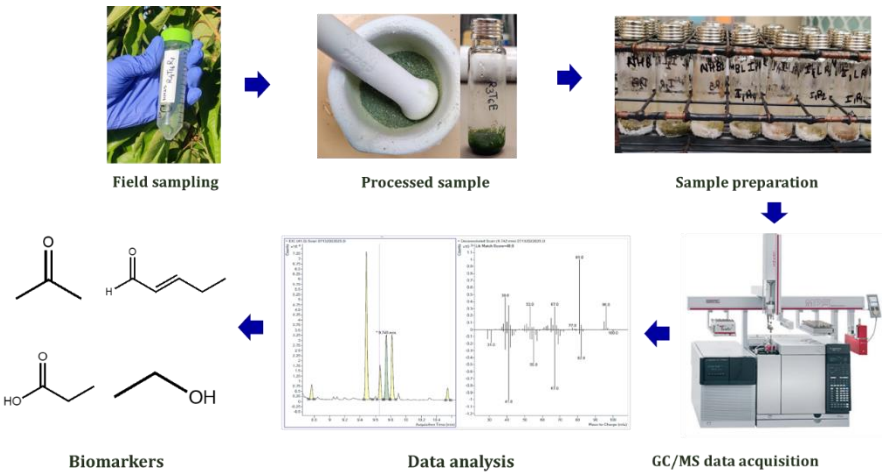

Figure 2. *Workflow of destructive headspace sampling combined with GC/MS analysis for volatile biomarkers identification.*

Results and Discussion

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

Feasibility of the volatile-based detection of LCD/X-disease was evaluated on Benton cultivar samples at post-harvest growth stage of 2021 season. Typical spectra (positive polarity) for a representative LCD/X-disease symptomatic (S) and asymptomatic (AS) samples is shown in Figure 3. Three I_c peaks (clusters) were observed in AS samples while an additional peak curving to the left and then top (labelled as signature peak; CV: -0.11 to 0.51 V and DF: 64–84 %) was present in S samples (Fig. 3a). FAIMS spectral dataset, combined for all seasons for given cultivar and given growth stage, was processed to extract ion current sum (I_{sum}), maximum ion current (I_{max}) and area under the curve (I_{AUC}). Higher ion currents were observed at selective regions in the FAIMS spectra for S samples compared to AS samples ($p < 0.05$). Such

discrimination was prominent at ion current features at different CV- DF ranges of -3 to 3 V and 20–98 %. The ion current plots at the identified DF ranges also confirmed a clear distinction between the symptomatic and asymptomatic samples. Cultivar-specific variation in the ion current spectra was observed in all the samples.

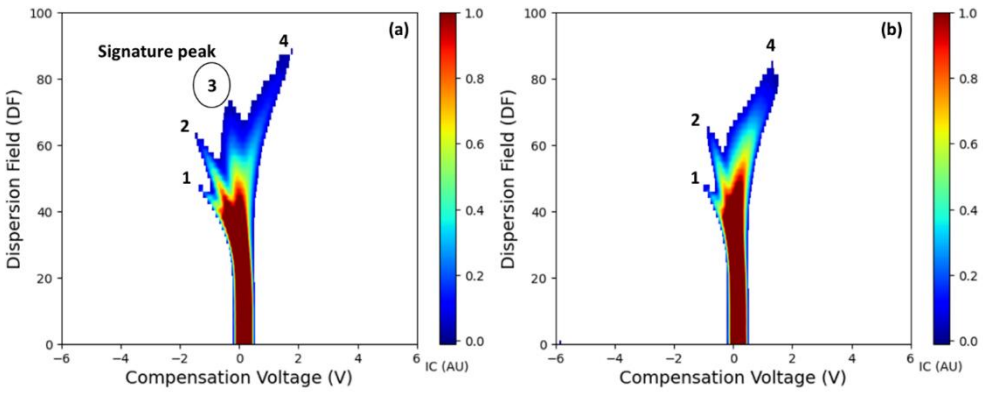

Figure 3. *FAIMS spectra with volatile headspace signature specific to (a) LCD/X-disease symptomatic, and (b) asymptomatic samples for 'Benton' cultivar. The potential signature peak observed in infected samples is highlighted with a circle.*

Validation with other host species of LCD/X-disease. The FAIMS spectra in the LCD/X-disease infected samples were observed distinct from the AS samples for the other host species, such as peach and nectarines. While LCD/X-disease infection in peaches and nectarines exhibits visual symptoms on both leaves and fruits, and trees may exhibit decline with time and eventually die. The FAIMS spectra obtained from these hosts showed distinct characteristics compared to the spectra of sweet cherry samples. The maximum I_c values in both peaches and nectarines were lower than those observed in sweet cherry samples. Specific to peach samples, a distinct peak was observed in S samples and could be a signature peak for LCD/X-disease. This confirms the diagnostic potential of FAIMS-based detection in other host species of these diseases. While similar observations could be inferred from the nectarine samples, the limited number of samples prevented further conclusions from being drawn.

Early detection feasibility

Bing Cultivar. The FAIMS spectra for the S and AS samples varied between the growth stages and across the seasons. FAIMS derived features I_{AUC_sum} for the respective ROIs were used for the temporal analysis of the volatile profile of the cherry stem samples. The Box–whisker plot (Fig. 4) shows the comparison between the S and AS samples. It illustrates the temporal variations in FAIMS-derived I_{AUC_sum} features for ROIs 3 and 4 across the three growing seasons (2021, 2022, and 2023). The data indicates that the volatile profile varies throughout the different growth stages. Significant differences between the S and AS samples were more prominent during the pit hardening stage across multiple years, where AS samples tend to have higher I_{AUC_sum} values. At the flowering and shuck fall stage, variations were observed but with less consistency across the years. This variability could potentially be linked with the titer level, symptom expression and dynamic nature of the disease. Overall, both the 2022 and 2023 seasons were not environmentally favorable for XDP symptom development in *Prunus* species. Specifically, 2022 was characterized by cold/freeze in early spring and 2023 season had high temperatures in late June. This might have impacted on the season-specific variation of volatiles release.

Skeena Cultivar. The box-whisker plots (Fig. 5) depict the temporal variation in the FAIMS-derived I_{AUC_sum} features for the ROIs 3 and 4 in Skeena samples across the 2022 and 2023 seasons. The volatile profiles of S and AS samples vary across different growth stages. The pit hardening stage consistently showed a significant difference between the S and AS classes, particularly in 2023, where AS samples

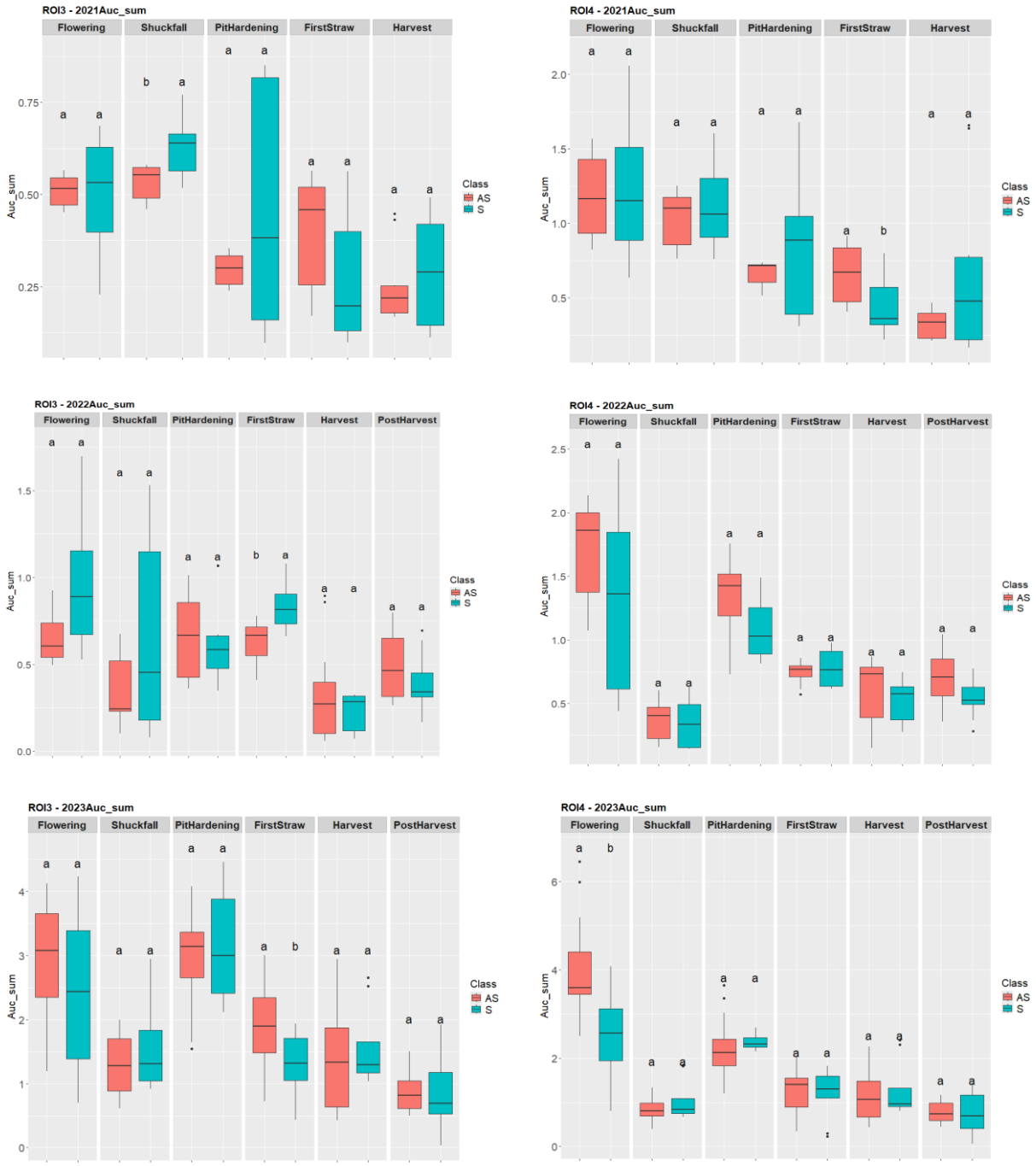

Figure 4. *Temporal variation in the FAIMS data derived* I_{AUC_sum} *feature for ROI's 3 and 4 (cultivar: Bing, combined dataset for 2021, 2022 and 2023 seasons).*

exhibit higher I_{AUC_sum} values. A similar pattern was observed for the Bing cultivar samples. This pattern suggests that pit hardening is a critical stage for detecting changes in volatile emissions linked to disease or stress responses. Similar to the previous analysis, the first straw and post-harvest stages show minimal differences between S and AS samples, indicating comparable metabolic activity during these periods. The flowering stage exhibits significant variability in both years but less consistency in terms of differentiation

between the S and AS classes. Overall, the results emphasize the importance of the pit hardening stage in detecting differences between S and AS Skeena samples, suggesting its potential for use in early disease detection or monitoring. *Correlation of FAIMS spectral features with titer level.* The correlation analysis between the I_{AUC_sum} for ROIs 3 and 4 with the Cq values was performed. For Bing cultivar and ROI 3 data, the correlation was weak and not statistically significant (r = 0.27). This indicates that there was no strong relationship between the titer level and I_{AUC_sum}. ROI 4 showed a moderate and statically significant correlation (r = 0.53) suggesting higher titer levels are associated with lower I_{AUC_sum} values. For the Skeena cultivar, a weak correlation (r = 0.28) between ROI 3 I_{AUC_sum} and Cq values was observed. ROI 4 I_{AUC_sum} showed moderate correlation (r = 0.38), indicating that as the titer increases, the AUC_sum values increase for the ROI4.

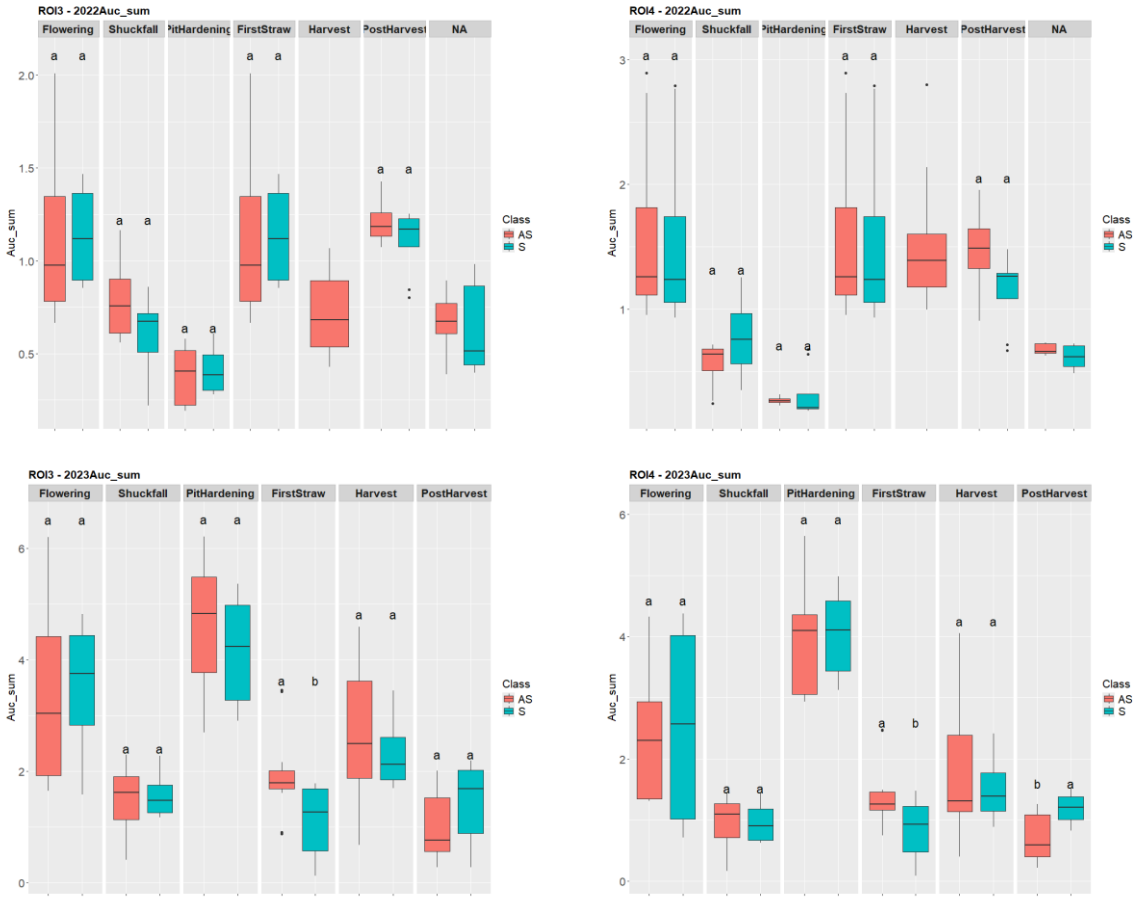

Figure 5. *Temporal variation in the FAIMS data derived I_{AUC_sum} feature for ROI's 3 and 4 (cultivar: Skeena, combined dataset for 2022 and 2023 seasons).*

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

The GC-MS analysis suggested that sweet cherry leaves release a broad range of primary and secondary metabolites. These metabolites were analyzed towards identification of XDP-linked volatile biomarkers (Fig. 6). The volatile compounds that significantly add importance to the class separation, for both first straw and harvest growth stages, were identified using the PLS-DA VIP scores. Compounds with high VIP scores in both years and growth stages (listed in Table 2) might be closely associated with the presence or absence of XDP infection in the leaf samples.

Static headspace sampling technique.

Bing cultivar. A few compounds were consistently found across both seasons at specific growth stages. For example, (Z)-2-pentanal was present in harvest stage samples for both years. At the first straw stage, compounds such as hexanol, acetic acid, butanoic acid, propanoic acid, and n-hexane had a VIP score greater than 1 in both years. At harvest, significant compounds included (E)-2-hexenal, acetic acid, acetone, octane, and propanoic acid. Only acetic acid and propanoic acid were found in both stages over both years. At the first straw stage, five compounds, including hexanal (a stress-response aldehyde), were significantly different in abundance between AS and S samples. AS samples had higher levels of hexanal and Z-3-hexenal, while S samples showed a higher abundance of ethanol. Unknown compounds (1334, 71) and (1167, 57) also showed variations. Overall, volatile biomarkers associated with XDP infection were detected in Bing cultivar (leaves) at both the first straw and harvest stages. Ethanol levels were higher in symptomatic samples at the first straw stage, while propanoic acid, octane, n-hexane, and acetone were elevated in symptomatic samples at harvest. Some compounds, like vinyl aldehydes, GLVs (Z-3-Hexen-1-ol, (E)-2-hexenal), and unknowns (1967, 57) and (1418, 109), showed strong correlations with disease severity and titer levels.

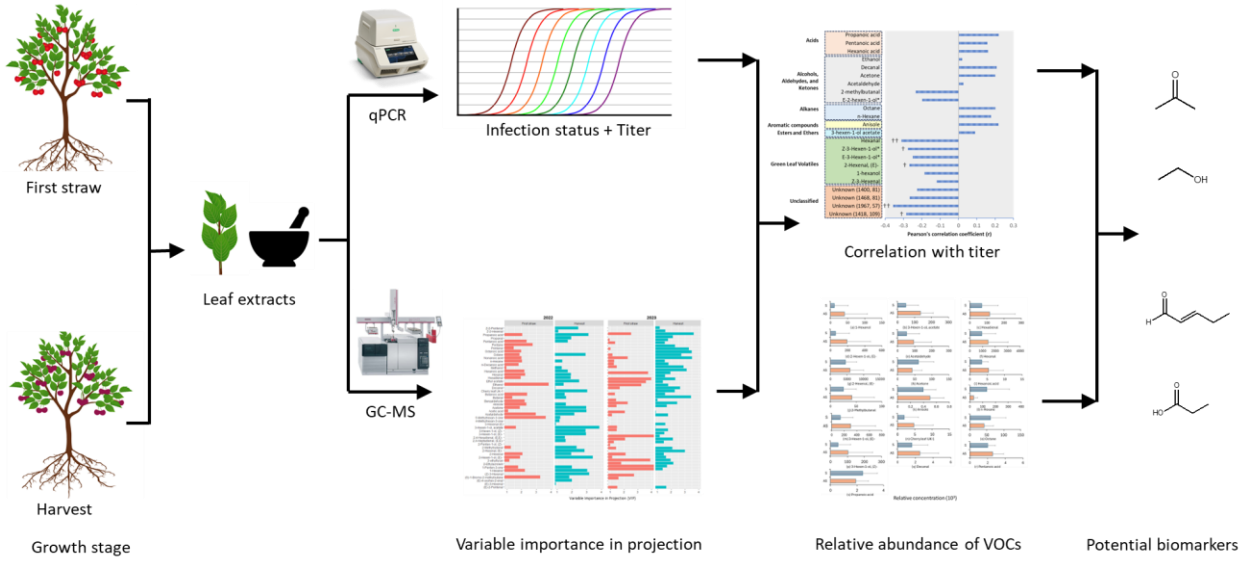

Figure 6*. XDP associated volatile biomarker identification workflow using GC-MS technique.*

Skeena cultivar. Volatile biomarkers associated with X-disease in the sweet cherry cultivar 'Skeena' were identified at the harvest growth stage. Several compounds were consistently significant across two seasons, including methanol, ethyl acetate, (Z)-2-penten-1-ol, and three unidentified compounds: unknown (1169, 83), unknown (1412, 81) and unknown (1468, 81). In 2022 season, E-2-Hexen-1-ol, E-3-Hexen-1-ol, acetaldehyde, and unknown (1412, 81) compound showed higher abundance in S trees compared to AS trees. The 2023 season data identified a broader range of compounds including several green leaf volatiles with higher abundance in S trees. Several GLVs exhibited strong positive correlations with infection levels. E-2-Hexenal, Z-3-Hexen-1-ol*, 2-Hexenal, acetaldehyde, acetoin, methanol and unidentified compounds, unknown (1334, 71) and unknown (1412, 81) showed positive correlations with the XDP titer levels (Table 2).

Dynamic headspace sampling technique.

A dynamic volatile headspace sampling method was evaluated to identify X-disease-linked biomarkers in stem samples of sweet cherries (cv. 'Bing' and 'Skeena'). Key compounds were identified using PLSDA based on variable importance in projection scores, with statistical significance was confirmed with the Mann Whitney U-test at $p < 0.05$. In case of Bing cultivar, key compounds identified were n-Hexane, methanol, unknown (1141, 41), E-3-Hexen-1-ol, 2-methylbutanal, Z-2-Hexenal with varying levels of relative abundance at different growth stages (Table 2). Whereas for Skeena, the significant compounds were, E-3-Hexenal, 1-Penten-3-one, unknown (1141, 41), pentane and butanal. A weak correlation was observed between the compound's relative abundance and pathogen level for both the cultivars and could be attributed to the type of sample and sampling technique. Overall, this project developed a dynamic volatile headspace analysis technique and assessed its applicability towards XDP detection. Identified biomarkers and variation between cultivar and growth stages, suggesting complex interaction between plant metabolism, stress responses and pathogen attack by insects.

Table 2. Potential biomarkers associated with XDP symptoms in sweet cherry cultivar 'Bing' and 'Skeena' for leaf tissue and stem samples.

Leaf tissue samples (GC-MS static)		**Stem samples (GC-MS dynamic)**	
Bing	**Skeena**	**Bing**	**Skeena**
Methanol	Methanol	n-Hexane	E-3-Hexenal
(E)-2-hexenal	Ethyl acetate	Methanol	1-Penten-3-one
Propanoic acid	(Z)-2-pentenol	Unknown (1141, 41)	Unknown (1141, 41)
Acetone	(Z)-3-hexenol*	E-3-Hexen-1-ol	Pentane
n-hexane	Unknown (1412, 81)	2-methybutanol	Butanal
(Z)-3-hexenol	Unknown (1468, 81)	Z-2-Hexenal	
Unknown (1967, 57)	E-2-Hexenal		
Z-3-Hexen-1-ol	Z-3-Hexen-1-ol*		
(E)-2-hexenal	2-Hexenal		
Unknown (1418, 109)	Acetaldehyde		
E-3-Hexen-1-ol	Acetoin		
1-hexanol	Unknown (1334, 71)		
Z-3-hexenal	Unknown (1412, 81)		
Unknown (1400, 81)			
Unknown (1468, 81)			
2-methylbutanal			

Objective 3. Conduct pertinent extension education and technology demonstrations.

Throughout the project period, we have actively disseminated pertinent technology and findings to stakeholders via various platforms. These include field days such as Little Cherry Disease Field Day (2022, 2023) and Little Cherry Disease Day (2022), research news flash talks at the WSTFA Annual Meeting & NW Hort Expo (2022, 2023), and a research update presentation at the Columbia Basin Tree Fruit Club (2023). Additionally, our work was highlighted at the 2024 Annual X-disease Research Summit and the X-disease Research Summit for Nurseries and Growers, both organized by USDA-ARS in Wapato, WA. Our

research story was featured in the February 2022 issue of Good Fruit Grower magazine (https://www.goodfruit.com/testing-the-testing-tools/), as well as in other electronic media such as the Yakima Herald-Republic News (2022) and a podcast (2023). The project outcomes were also presented to the research community during a session talk at ASABE-AIM 2022 in Houston, TX. The culmination of this work is a durable product: a PhD Dissertation by Gajanan Kothawade (August 2024), titled "Identifying Biomarkers of Little Cherry/X-Disease in Prunus avium Using Volatile Sensing Technologies" at Washington State University. Furthermore, four peer-reviewed research articles are currently being prepared for submission based on these efforts.

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Executive Summary

Project title: Towards the identification of Little cherry disease linked volatile biomarkers

Key words: Little Cherry Disease, Presymptomatic detection, Volatile Biomarkers, FAIMS, GC-MS

Abstract: This project evaluated FAIMS technique for early detection of LCD/X-disease in 'Bing' and 'Skeena' cherry cultivars by analyzing volatile profiles across seasons and different growth stages. The pit hardening stage consistently showed significant differences in volatile emissions between symptomatic (S) and asymptomatic (AS) samples, with AS samples having higher ion current values in both cultivars. Temporal variations were also observed at other stages, though less consistently, indicating the importance of the pit hardening stage for early detection of X-disease. Cultivar-specific differences and environmental conditions across seasons also influenced volatile emissions. Project also identified key volatile biomarkers, using GC-MS static and dynamic sampling technique, associated with the X-disease infestation. Potential biomarkers include, Ethanol, (E)-2-hexenal, propanoic acid, and acetone, ethyl acetate, (Z)-2-penten-1-ol, (Z)-3-hexen-1-ol*, 2-hexenal, acetaldehyde, acetoin. *Practical implications* of this research include: 1) Early Detection: Identification of the pit hardening stage as critical for detecting X-disease, 2) Targeted Monitoring: Growers can focus on non-destructive (e.g., Canine based) scouting efforts during the pit hardening stage, 3) Biomarker Utilization: The volatile biomarkers identified in this project can be used to develop more precise diagnostic tools, enhancing the accuracy of X-disease detection, 4) Cultivar-Specific Strategies: Understanding cultivar-specific differences in volatile emissions can help tailor disease management practices to specific cherry varieties, improving overall crop health, and 5) Seasonal Adaptation: Recognizing the influence of environmental conditions on volatile emissions enables growers to adapt their monitoring and intervention strategies according to seasonal variations.

Project Title: Real-time Detection of Little Cherry Disease using Detector Canines

Report Type: Continuing Project Report

Primary PI: Jessica Kohntopp
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Cooperators: Daisy Arias (Stemilt Growers LLC), Teah Smith (Zirkle Fruit Company), Craig Harris (Harris Farms), Bill Howell (NNII), Todd Cameron (Cameron Nursery), Garrett Bishop (G.S. Long Co), Rodney Cooper (USDA-ARS)

Project Duration: 2-Year

Total Project Request for Year 1 Funding: $74,267
Total Project Request for Year 2 Funding: $116,045

Other related/associated funding sources: None

Budget 1
Primary PI: Jessica Kohntopp
Organization Name: Ruff Country K9
Contract Administrator: Jessica Kohntopp
Telephone: (208) 602-1293
Contract administrator email address: jesskohntopp@gmail.com

Item	2023	2024
Salaries	$40,000.00	$40,000.00
Benefits	$13,600.00	$13,600.00
Wages		
Benefits		
RCA Room Rental		
Shipping		
Supplies	$2,640.00	$500.00
Travel	$8,338.00	$12,276.00
Plot Fees		
Miscellaneous		
Total	$64,578	$66,376

Footnotes: Year-1: PI Jessica Kohntopp requests salary based on below median detection dog trainer salary survey conducted by Highland Canine Training LLC School for Dog Trainers. Supplies for boxes/tins, harnesses, booties, canine training supplies, camera for videos, tripod, mini freezer etc. Travel to cover 5 trips of 5 days each to bring the canines to WA for blind studies, mock orchards, demonstration and outreach days. **Year-2** travel costs were doubled because canines will be in WA more times for in depth studies.

Budget 2
Co-PI 3: Scott Harper
Organization Name: Washington State University
Contract Administrator: Anastasia (Stacy) Mondy
Telephone: (509)-335-2885
Contract administrator email address: arcgrants@wsu.edu

Item	2023	2024
Salaries		
Benefits		
Wages	$6,233.00	$43,631.00
Benefits		
RCA Room Rental		
Shipping		
Supplies	$955.80	$3,538.20
Travel		
Plot Fees		
Miscellaneous		
Total	$7,189	$47,169

Footnotes: Year-2: Co-PI Harper requests allocation of $30,996 in salary (0.5 FTE) and 11,159 in benefits, with 12,203 in supplies and consumables for the generation of peptides for detector dog training.

Budget 3
Co-PI: Corina F. Serban
Organization Name: Washington State University
Contract Administrator: Anastasia (Stacy) Mondy
Telephone: (509)-335-2885
Contract administrator email address: arcgrants@wsu.edu

Item	2023	2024
Salaries		
Benefits		
Wages		
Benefits		
RCA Room Rental		
Shipping		
Supplies	$500.00	$500.00
Travel	$1,809.00	$1,809.00
Plot Fees		
Miscellaneous	$191.00	$191.00
Total	$2,500	$2,500

Footnotes: Year-1: Co-PI Serban requests $500 towards extension and outreach activities; $1,809 for travel and accommodation; and $191 for miscellaneous items such shipping costs. **Year-2:** request is similar to Year-1.

Objectives:

Year one (May 10, 2023 - May 10, 2024):

(1) Train canines to detect the three pathogens that cause Little Cherry Disease: Little Cherry Virus-1 (LChV-1), Little Cherry Virus-2 (LChV-2), and X-disease phytoplasma (XDP) by using infected plant samples. (Target odors 1-3)

(2) Validate the accuracy, sensitivity, and specificity of the canines to detect Little Cherry Disease (LCD) pathogens in young cherry trees in a Blind Controlled Study.

(3.0) When canines are proficient (95% or higher) in Objective 2, canines will be brought to a mock field setting. (Due to the season, titer levels will not be at their highest. This first step will be to introduce canines to a field setting and for trainer to observe their behaviors)

(4) Extension and Outreach – monthly meetings, demonstration at field day and technical factsheet (Year one-two)

Year two (May 10, 2024 - May 10, 2025):

(3.5) Due to timing and season a majority of mock field training will be done

in the beginning of year two when titers are high.

(5) Orchards: When canines are proficient in both parts of Objective 3, Blind studies will be performed in cherry orchards where the observer knows where positive trees are but the handler does not.

(6) Dormant study: Determine if canines can detect LCD in dormant trees in both young nurseries and older orchard blocks.

(7) Temporal Study: Determine how early of infection canines are able to detect LCD in comparison to molecular PCR methods of artificially infected trees.

(8) Nursery study: Determine if canines can find LCD in newly grafted rootstocks, young trees in storage bins, etc.

Objective Deviations: The day the Blind Study was supposed to happen, both canines were sick from minor heat exhaustion the day before. On the trip up, the handlers truck broke down and both canines were stuck in a very hot vehicle with only the use of battery powered fans for three hours. The handler did the best she could to keep the canines cool while waiting for the tow truck. In a real life scenario, the handler would have called off the trip and rescheduled but due to timing and everyone else being ready, they continued with the blind study. Aika was affected the most and was trying to eat grass the entire time, because of this, there were a lot of missed positive plants. The handler asked to redo the blind study at a later time as she did not believe it was a fair representation of Aika's abilities. Everyone agreed and a mini blind study was conducted a couple weeks later. Deviations to the Temporal study were made as well. Due to lack of greenhouse space and resources we did a three month study with 20 leafhopper infected plants that were being used in a different project. From the short study, the handler/trainer expressed the need to be able to confidently 'stair-step' the canines down in odor recognition. Dr. Harper has been tasked with expressing the proteins from LChV-2 and XDP to be used to train the canines at lower levels.

Significant Findings:

- Distractor training showed canines have odor discrimination of all three target odors.
- Canines found 7 positive potted trees blindly arranged in a greenhouse scenario as well as one additional tree that was tested and came back qPCR positive
- Blind study showed canines are running 99.72% Accuracy, 96% Sensitivity, and 100% Specificity in a controlled setting using potted plants
- Canines were successfully transferred from searching potted trees to trees in the field using two qPCR tested mapped out grids
- Canines have alerted on 21 unknown younger trees in a commercial orchard - 4 have come back qPCR positive
- Canines have alerted on 4 unknown older trees in a commercial orchard - 3 have come back qPCR positive

Methods:

Distractor Plants:
'Distractor Plants' are plants that are infected with other diseases that are commonly found in cherry orchards. This could also include environmentally stressed plants i.e. water deprived, nutrient deficient etc. This step ensures the canines are only alerting on the Target Odor and not other pathogens or a generally sick plant. The first set of distractor plants were acquired December 14th, 2023. This included 4 cherry trees infected with prune dwarf virus (PDV), prunus necrotic ringspot virus (PNRSV), and cherry virus A (CVA). Aika showed no interest in the distractor plants and ran them like they were negative trees, meaning she initially imprinted on the correct target odor. On the other hand, Humma hesitated on every distractor plant and alerted on one of the four. This means Humma did not solely imprint on the target odors. 'Proofing off' training commenced and by the end of the week Humma stopped alerting on the distractor plants. A second set of 5 distractor plants were picked up on February 5th, 2024. These consisted of additional plants with PNRSV, PDV, CVA, and cherry necrotic rusty mottle virus. This time around neither dog showed interest or hesitated at the new distractor plants. On March 4th, 2024 all distractor plants were returned and replaced by seven new plants, these consisted of different species of trees ie hazelnut, plum, apple, pear, etc. Again, neither canine showed interest meaning the proofing off training worked for Humma and both canines have odor discrimination of the three target odors.

Blind Greenhouse Study:
The first time canines were brought to Washington, a scenario was set up at the WSU-IAREC in Prosser in one of the greenhouse bays. Dr. Harper and his technician placed seven known LCD positive plants randomly throughout the greenhouse amongst 187 other plants (both healthy and infected with various diseases). Neither of the canines had ever seen these positive plants nor the negative/distractor plants before and the handler was unaware of the location and number of positive plants within the greenhouse. Both canines ran the scenario, finding all seven positive plants plus one additional plant. This additional plant was sampled, tested and did come back qPCR positive for XDP with a Ct value of 34, equating to approximately 50 phytoplasma cells.

Blind Study:
A blind study was conducted on July 17th, 2024 to test the canines' accuracy, sensitivity, and specificity in a controlled setting using potted plants. The blind study consisted of 101 LCD positive plants and 1,279 negative plants for a total of 1,380 plants per canine. Since there were not enough potted plants for one run, it was divided into a total of 23 runs per canine on a 60 plant grid. Each run was completely randomized via a computerized generated program randomizing both the position of the hot plant(s) as well as the incident rate (ranging between 0% to 10%). Ms. Serban and various interns set up the grid with the corresponding hot plants for each run. The handler did not know the position of the hot plant and when the canine alerted, the handler raised their hand to call the alert. Ms. Serban then verbally confirmed or denied the alert so the canine could be rewarded or pulled off and the run continued. This ensured the handler was unaware of the hot plant positions so she could not cue the dog or force them to sit on a hot plant. The canines alternated between runs and hot plants were moved after each run. The

canines responses were evaluated as a true positive rate (TPR; sensitivity), a true negative rate (TNR; specificity), and overall accuracy (TP+TN/n) as defined below and described by Gottwald et al., 2020. The definitions and formulas for the evaluation of canines performance have been utilized for previous successful efforts to train canines to detect pathogens causing plant diseases and are listed below.

Where n = total population assessed.
True Positive (TP) correct canine alert on positive sample
True Negative (TN) correct rejection, no alert on negative sample
False Positive (FP) false alert on negative sample, Type I error
False Negative (FN) missed positive target, Type II error

Sensitivity (SEN) or true positive rate, = TP/(TP + FN)
Specificity (SPE) or True Negative Rate = TN/(FP + TN)
Precision or Positive Predictive Value (PPV) = TP/(TP + FP)
Negative Predictive Value (NPV) = TN/(TN + FN)
False Positive Rate (FPR) = FP/(FP + TN)
False Negative Rate (FNR) = FN/(FN + TP)
False Discovery Rate (FDR) = FP/(TP + FP)
Accuracy (ACC) = (TP + TN)/n

The goal is to get the canines to **>95% accuracy, >90% sensitivity, and >90% specificity.**

Mini Blind Study:
A mini blind study was conducted on August 6th, 2024 using the same methods listed above but this time each canine only ran the 60 plant grid 12 times instead of 23. This study consisted of 50 LCD positive plants and 670 negative plants for a total of 720 plants per canine. Each run was newly computer generated and none of the scenarios from the initial study were rerun. Results were evaluated based on the same formulas listed above. The purpose of this study was to allow the canines to run the scenario in a healthy condition. Due to unfortunate circumstances both canines were sick from minor heat exhaustion during the initial blind study. It did not affect Humma as much as Aika but to give the canines a fair shot and a fair representation of their abilities this mini study was performed.

Moxee USDA-ARS Research Farm:
This was the canine's first introduction to cherry trees in the ground rather than in pots. This plot had eight rows of ten younger trees. Each tree has been tested making a fully mapped out training grid. Seven of the 80 trees came back positive and their titer levels ranged from 34.29 (~40 phytoplasma cells) to 39.46 (<5 phytoplasma cells) for XDP (*Figure 1*). Initially both canines struggled switching from potted plants to trees but did overcome that relatively quickly. We did have an incident with the bird cannon that set Aika back a couple steps however she has since overcome that as well. Neither dog has alerted on any new trees in that block.

Tree	USDA Moxee Fully Mapped Training Block							
10	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
9	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
8	N/A	N/A	N/A	N/A	N/A	38.08 (#5)	N/A	N/A
7	N/A	N/A	37.44 (#2)	N/A	N/A	39.2 (#4)	N/A	N/A
6	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
5	N/A	38.28 (#1)	N/A	N/A	N/A	N/A	N/A	N/A
4	N/A	N/A	N/A	N/A	N/A	N/A	39.46 (36)	34.29 (#7)
3	N/A	N/A	N/A	37.55 (#3)	N/A	N/A	N/A	N/A
2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Row	1	2	3	4	5	6	7	8

Figure 1: Moxee Field Map

Older Cherry Block A:

This was the canine's first introduction to older cherry trees. Mrs. Serban and Dr. Harper sampled and tested two complete rows of 45 plants. The first row came back all negative whereas the second row came back with nine LCD positive plants. Eight were XDP positive ranging from 35.67 to 38.53 (20 and <5 cells respectively) and one LChV-2 at 36.19 (~15 virions). Several of these positive plants were back to back as seen in the map in *Figure 2.* Canines initially struggled with the positive plants being so close together. More training was done back in Idaho and the following trip the canines were much better at alerting on positive trees side by side.

Younger UFO Cherry Block B:

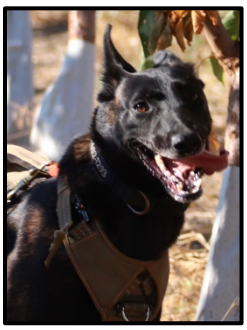
This block consisted of 1,442 plants within 35 rows. Each row had two plants randomly selected and tested in the Fall of 2023. Of those samples, one came back qPCR XDP positive with a CT value of 38.1 (<5 cells) and the rest were non-detect. Canines were brought to this block on August 20th, 2024 and ran the first nine plants in each row. Canines alerted on the plant that had tested positive in 2023 along with five unknown plants. Samples were taken and tested from all plants canines alerted on, two qPCR negative plants from 2023, and two randomly selected non-alert plants. The canines were also brought back on September 17th and 19th to run 672 more trees. Of those trees, both canines alerted on 16 more. Again, samples were taken and tested from all trees the canines alerted on plus 4 randomly selected non-alert trees.

Older Cherry Block C:

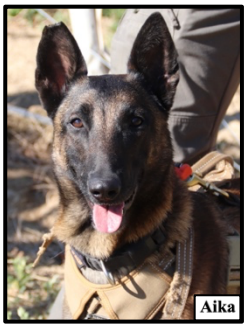
This block had symptomatic trees that were flagged at harvest but not qPCR tested. Both canines ran down one and a half rows before the handler decided not to run anymore and await the qPCR results. Running too many unknown trees in these initial stages can be detrimental to the canine's training and progression. Each row chosen had one symptomatic tree and neither canine alerted on those trees. Both canines alerted on four other trees. Samples were taken and tested from all plants canines alerted on, both symptomatic trees, one they both hesitated on, and one randomly selected non-alert plant.

Older Cherry Block A		
Tree #	Row 1	Row 2
1	N/A	Removed
2	N/A	N/A
3	Removed	N/A
4	N/A	N/A
5	N/A	XDP 38.25 (#9)
6	N/A	XDP 38.53 (#8)
7	N/A	LChV-2 36.19 (#7)
8	N/A	N/A
9	N/A	N/A
10	N/A	XDP 36.78 (#6)
11	Removed	N/A
12	N/A	XDP 38.31 (#5)
13	N/A	XDP 35.67 (#4)
14	N/A	XDP 37.9 (#3)
15	N/A	N/A
16	N/A	N/A
17	N/A	N/A
18	N/A	N/A
19	N/A	N/A
20	N/A	N/A
21-25	Removed	Removed
26	N/A	XDP 37.66 (#2)
27	N/A	N/A
28	N/A	N/A
29	N/A	N/A
30	N/A	N/A
31	N/A	N/A
32	N/A	N/A
33	Removed	N/A
34	N/A	N/A
35	N/A	XDP 37.9 (#1)
36	N/A	N/A
37	N/A	N/A
38-41	Removed	Removed
42	N/A	N/A
43	N/A	N/A
44-45	Removed	Removed

Figure 2: Field Map



Humma



Aika

Results and Discussion:

Blind Studies

The three main things that are being looked at in the blind study is the accuracy, sensitivity, and specificity of the canines. Accuracy: The total amount of correct alerts on positive trees and non-alerts on negative trees. Sensitivity: The total amount of correct alerts on positive trees. Specificity: The total amount of correct non-alerts on negative trees. Results from the blind study can be seen on the tables below. In the initial blind study, both canines were sick but it affected Aika the most. Humma was able to stay on task and had very similar numbers to the mini study. Aika on the other hand was constantly eating grass resulting in 12 missed positive plants. The second time around she was feeling better and was much more focused. Both canines missed two plants during the mini blind study. It should be noted that in both blind studies, the dogs never missed the same plant. It was always in a different run or a different hot plant location. It should also be noted that neither dog has ever false alerted. They would miss some hot plants but never alerted at a negative plant as shown in the highlighted red below. The number of positive plants they missed (excluding Aika's initial run) were relatively low. The mini blind study showed both canines are running at a 99.72% Accuracy, 96% Sensitivity, and 100% Specificity in a controlled setting.

Table 1: Blind Study

	Initial Blind Study		Mini Study
Canine	**Humma**	**Aika**	**Both Canines**
Sensitivity	**95.05%** (96/101)	**88.12%** (89/101)	**96%** (48/50)
Total Positives Missed	**5**	**12**	**2**
Specificity	**100%** (1279/1279)	**100%** (1279/1279)	**100%** (670/670)
Total False Alerts	**0**	**0**	**0**
Positive Predictive Value	**100%**	**100%**	**100%**
Negative Predictive Value	**99.61%**	**99.07%**	**99.70%**
False Positive Rate	**0.00%**	**0.00%**	**0.00%**
False Negative Rate	**4.95%**	**11.88%**	**4.00%**
False Discovery Rate	**0.00%**	**0.00%**	**0.00%**
Accuracy	**99.64%** (1375/1380)	**99.13%** (1368/1380)	**99.72%** (718/720)

Footnotes: ***Accuracy****: The total amount of correct alerts on positive trees and non-alerts on negative trees.* ***Sensitivity****: The total amount of correct alerts on positive trees.* ***Specificity****: The total amount of correct non-alerts on negative trees.*

Older Cherry Block C: qPCR Results

Samples were taken on Sept 3, 2024 from the nine trees in the table below with their corresponding dog responses and qPCR test results. Both canines alerted hard on Tree D

running from both directions and did not alert on nor showed interest in the symptomatic Tree F that came back positive with a CT Value of 20.13 (~480k phytoplasma cells) for XDP. This could be due to the canines' odor threshold at the time. The canines have seen qPCR positive potted plants in those high ranges before but the trees were much younger and smaller than in this block. It is believed the canines were within their 'normal reward threshold' when they were smelling the adjacent tree shown in the *Figure 3* on the right. The handler was concerned the same sort of scenario could have happened with Tree G. Samples were retaken from that tree on Sept 24, 2024 and both trees on either side of it. When results came back, both the adjacent trees came back non-detect and this time Tree G did come back qPCR positive (XDP 33.9, ~54 cells). Tree J could have been missed by both canines as there are times they struggle finding back to back trees. Another explanation could be due to the canines alerting on a 33.58 (~66 cells) right before the 39.66 (<5 cells). Looking back to the diagram above, odor is continuously dispersing from the tree and the canines are constantly having to work through the residual rings. With such a weak infection beside a higher infected tree, the canines could have marked it as residual odor and kept searching.

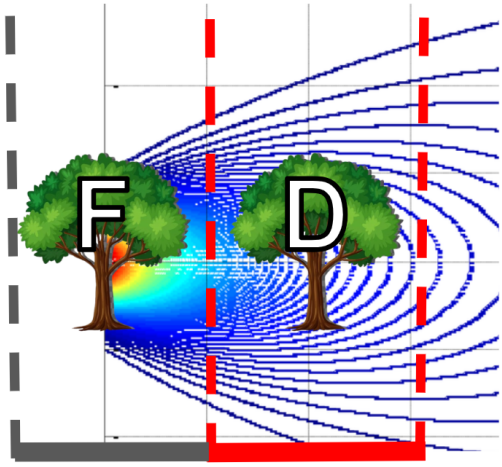


Figure 3: Odor Threshold

Table 2: Block C qPCR Results

Tree Sample	Humma Alert	Aika Alert	Humma Hesitate	Aika Hesitate	qPCR Result Sept. 3	Retested Sept. 24
Unknown Tree A	X	X			LChV-2 (37.89)	
Unknown Tree B	X	X			XDP (33.58)	
Unknown Tree J (Adjacent to Tree B)					XDP (39.66)	
Unknown Tree C		X	X		-	
Unknown Tree D (Adjacent to Tree F)	2x	2x			-	
Symptomatic Tree F					XDP (20.13)	
Symptomatic Tree E					-	
Unknown Tree G	X	X			-	XDP (33.9)
Unknown Tree H			X	X	-	

Younger UFO Cherry Block B: qPCR Results

Samples were taken on Sept 3rd and Sept 24th, 2024 on 41 total trees. So far, four unknown samples that both canines alerted on, have come back qPCR positive for XDP and LChV-2. We will monitor the rest of the non-detect plants and test later on when

titers should be higher. It should be noted that samples were taken and retested from the tree that showed positive in fall of 2023 came back non-detect in 2024.

Table 3: Block B qPCR Results

Tree Sample	Humma Alert	Aika Alert	Humma Hesitate	Aika Hesitate	PCR Results 2024	Fall 2023 PCR Result
Unknown Tree 1	X	X			XDP (37.63)	
Unknown Tree 2	X	X			XDP (39.41)	
Unknown Tree 3	X	X			LChV-2 (38.83)	
Unknown Tree 4	X	X			XDP (37.63)	
qPCR Positive Tree 5	X	X			-	XDP (38.1)
Unknown Trees 6-29	X	X			-	
Unknown Trees 30-31		X	X		-	
Unknown Trees 32-34			X	X	-	
Non-Alert Controls (8 trees total)					-	

With the large economic impact of Little Cherry Disease paired with the inability to feasibly test mass amounts of trees and the minimal timeframe of symptomatic trees, it is imperative to find a year-round, real-time, non-destructive diagnostic tool. The use of canines for detection of Little Cherry Disease could be the solution that is needed for this industry to help mitigate the spread of LCD.

Future Steps: Within the upcoming months we will run the canines on dormant trees in locations we have been to before as well as a newly tested area. If canines are able to find LCD infections in dormant trees, it might help canines be more accurate in the older blocks. When the trees go dormant the disease within the tree starts moving towards the root system. Hopefully with the disease more condensed within the tree, the canines with have an easier time pinpointing trees with higher infection rates.

Project Title: Evaluation of simple, cheap tests for X-disease in bare root trees

Report Type: Final Project Report

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Cooperators: Tobin Northfield, WSU

Project Duration: 1-Year

Total Project Request for Year 1 Funding: $ 10,000

Other related/associated funding sources: Awarded - USDA-ARS/Washington State University Little Cherry Disease Research Partnership
Funding Duration: 2022 - 2027
Amount: $319,000
Agency Name: USDA-ARS

WTFRC Collaborative Costs: None

Budget 1
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Item	2024
Salaries	$0.00
Benefits	$0.00
Wages	$0.00
Benefits	$0.00
RCA Room Rental	$0.00
Shipping	$0.00
Supplies	$10,000.00
Travel	$0.00
Plot Fees	$0.00
Miscellaneous	$0.00
Total	$10,000.00

Footnotes: N/A

Objectives

1. Identify a simple staining system that can be used to discriminate between woody tissues from phytoplasma-infected and uninfected plants and compare the accuracy of stain methods to qPCR.

2. *If successful*, develop a training guide to teach the staining protocol to end users.

This study's goal is to support the WTFRC research priorities of 1) optimizing or new testing methods for early disease detection, and 2) using research-informed solutions to reduce costs, turn-around time, and for non-symptomatic trees, by examining whether cheap histochemical stains can be used to determine X-disease phytoplasma presence in sections taken from, for example, dormant young trees prior to planting.

Significant Findings:

- Localization of X-disease phytoplasma cells is not readily achievable with light microscopy and histological stains.
- Identifying cytopathological effects of specific strains of the phytoplasma is feasible. However, this is not suitable as a diagnostic for PNW growers – better options exist.

Methods:

Objective 1:
To determine whether simple histochemical stains could be used to detect the presence of the X-disease phytoplasma in infected plants we collected root, stem, and leaf samples from a series of one- to three-year-old potted own-root and grafted cherry plants maintained as part of our positive control collection and/or from other ongoing X-disease experiments.

As hand sectioning with a razorblade or similar results in thick sections in which is difficult to determine individual cells and/or contents, we first tested the staining methodology on fixed, embedded ultrathin (10 µm) sections. Briefly, tissues were collected from the plants, cut them into 2-3 mm blocks and placed these in 2 ml of 1.25% (v/v) glutaraldehyde, 2% paraformaldehyde (v/v), 0.025% tween-20 (v/v) in 1x PBS (pH 7.4) for 24 hours at 4°C, then dehydrated in a series of increasing ethanol concentrations ranging from 30% to 100% for 30 minutes each. These were then transitioned to from ethanol to tert-butanol through 3:1, 1:1, and 1:3 steps for 24 hours each, then from tert-butanol to paraffin also through

3:1, 1:1, and 1:3 steps for 24 hours each. The infiltration of paraffin was completed with three 100% paraffin incubations for 24 hours each, then the sections were cast in 7 mm blocks and left to set overnight. Ultrathin sections were prepared using a rotary microtome, to a thickness/depth of 10 µm, heat-fixed onto glass microscope slides for 2 hours at 60°C, then deparaffinized using histoclear II. Deparaffinized sections were stained for visualization. Stains used, and expected results are listed in table 1.

Table 1. Stains used for histochemical examination of X-disease phytoplasma infected tissues.

Stain	Concentration	Expected effect under Light Microscopy
Toluidine blue	0.01%	Blue or violet accumulations in the sieve elements
Dienes Stain	1:20	Blue accumulations in sieve elements
Thionin/Acridine Orange	0.5%	Purple accumulations along the walls of sleeve elements.
Iodine	0.01M	Black staining of starch containing tissues/vesicles.

Finally, and based on the results of the staining and the cytopathology observed, leaf and woody stem samples from select plants were hand sectioned with a razor blade, with an average thickness of 0.1 mm being achieved for leaves and 0.2 mm for woody stem tissues. These were stained with Toluidine Blue as above, mounted in water examined under a compound microscope.

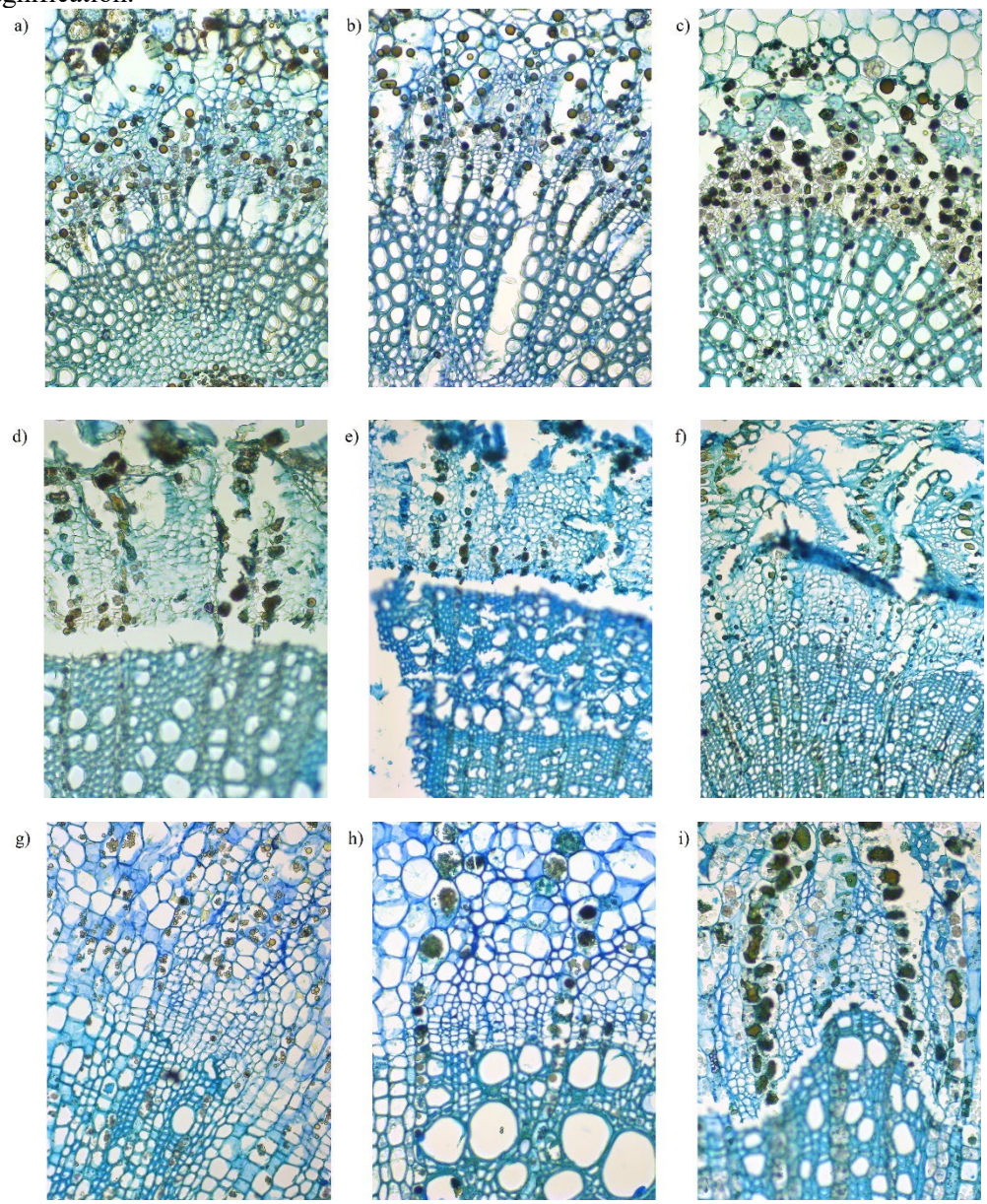
Results and Discussion

Objective 1:

Staining sectioned plant tissues for phloem-infecting pathogens is an older and less used method as better, more sensitive and specific methods exist. However, these newer methods require more specialized equipment and training and are more expensive to perform. Therefore, in this pilot study, we aimed to determine whether histochemical stains that have been reported in the literature for the detection of phytoplasmas could be used to show the presence of the X-disease phytoplasma (‘*Ca*. P. pruni’). Here we first collected root, stem, and leaf midrib samples, depending on timing and availability, of phytoplasma-negative, weakly infected, and heavily infected *Prunus* cultivars. These were fixed, embedded in paraffin and ultrathin (~10 µm) sections were produced using a rotary microtome.

Staining with 0.1% Toluidine Blue (Figure 1) will stain most plant tissue various shades of light blue/turquoise to purple, while phytoplasmas or bacterial in the phloem would appear as a blue accumulations or aggregates around the interior of the phloem sieve elements. This was unsuccessful and no differences between infected and uninfected plants could be found. Interestingly, heavily infected plants with specific strains not prevalent in Washington or Oregon did show some cytopathology with distortion of phloem rays and disorganization of the cambial tissue between the vascular cylinder and epidermis in leaf, stem and root sections. This reaction appeared to have been strain-specific as it was not observed in plants infected with the common northwestern strains of X-disease.

Figure 1. Toluidine Blue stained leaf (a-c), stem (d-f), and root (g-i) tissues from plants that were negative (a, d, & g), weakly infected (b ,e, & h), or heavily infected (c, f, & i) with '*Ca*. P. pruni' at 400x magnification.



Next, we applied Diene's stain (Figure 2), which like Toluidine Blue, stains cells various cell types blue-purple, and cellular aggregates such as phytoplasmas blue, and Thionine-Acridine Orange (Figure 3), which stains different cell types blue/purple through to orange, with cellular aggregates. Neither of these stains indicated the presence of any phytoplasmas, bacteria or cellar aggregates in the phloem in any of the tissue types. As with Toluidine Blue, the cellular distortion associated with non-WA/OR strains were apparent in all tissue types.

Figure 2. Diene's-stained leaf (a-c), stem (d-f), and root (g-i) tissues from plants that were negative (a, d, & g), weakly infected (b ,e, & h), or heavily infected (c, f, & i) with '*Ca*. P. pruni' at 400x magnification.

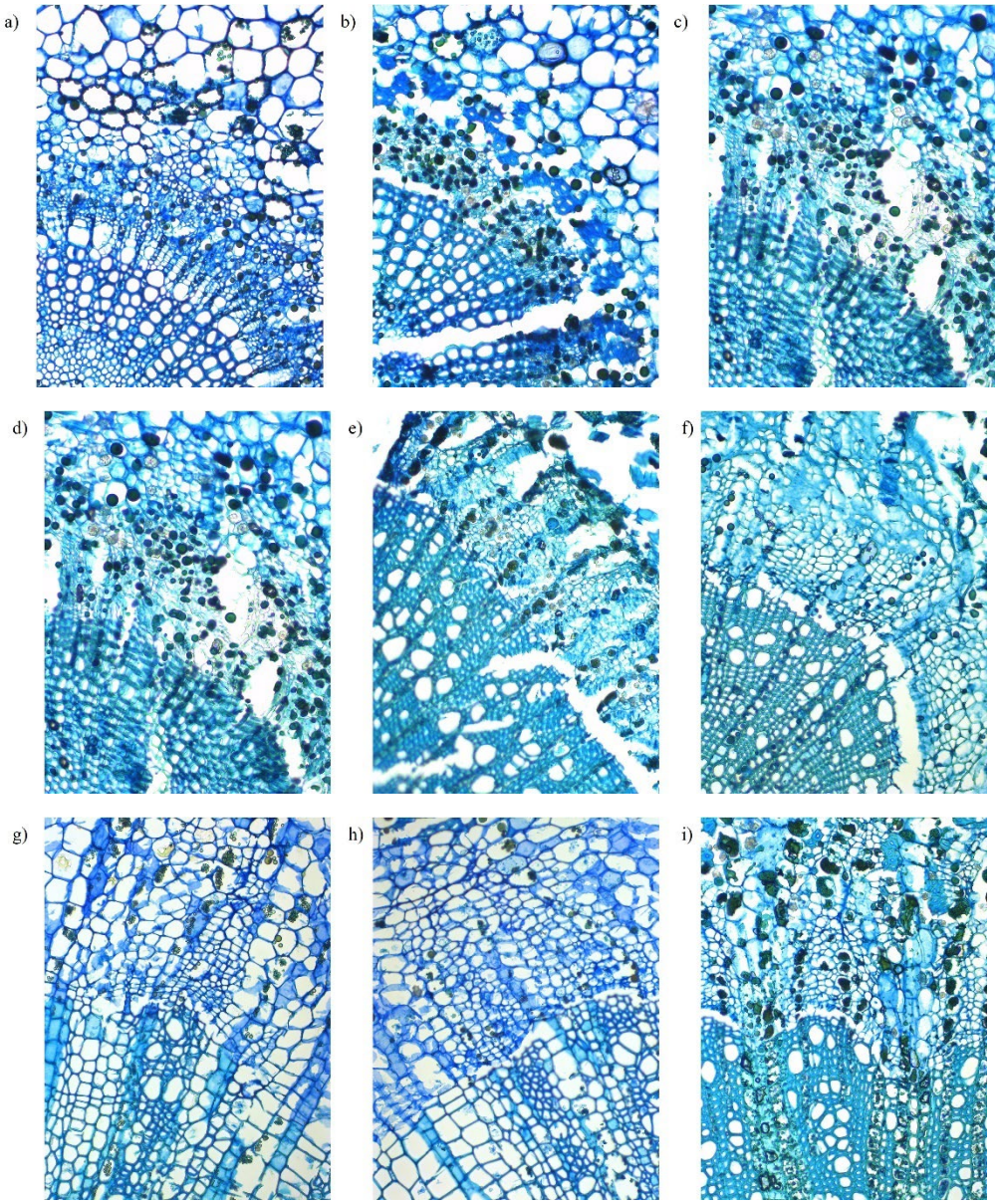
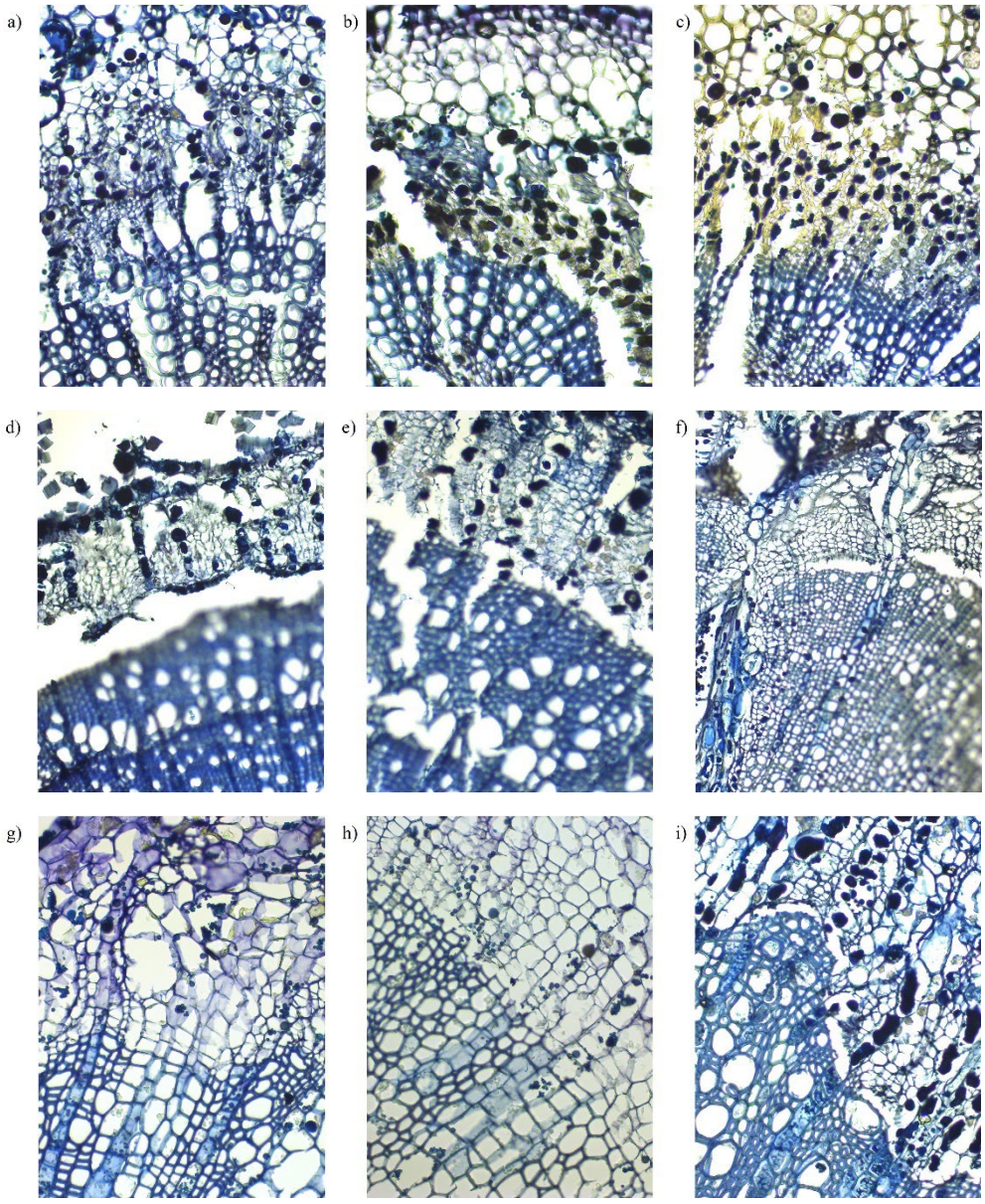
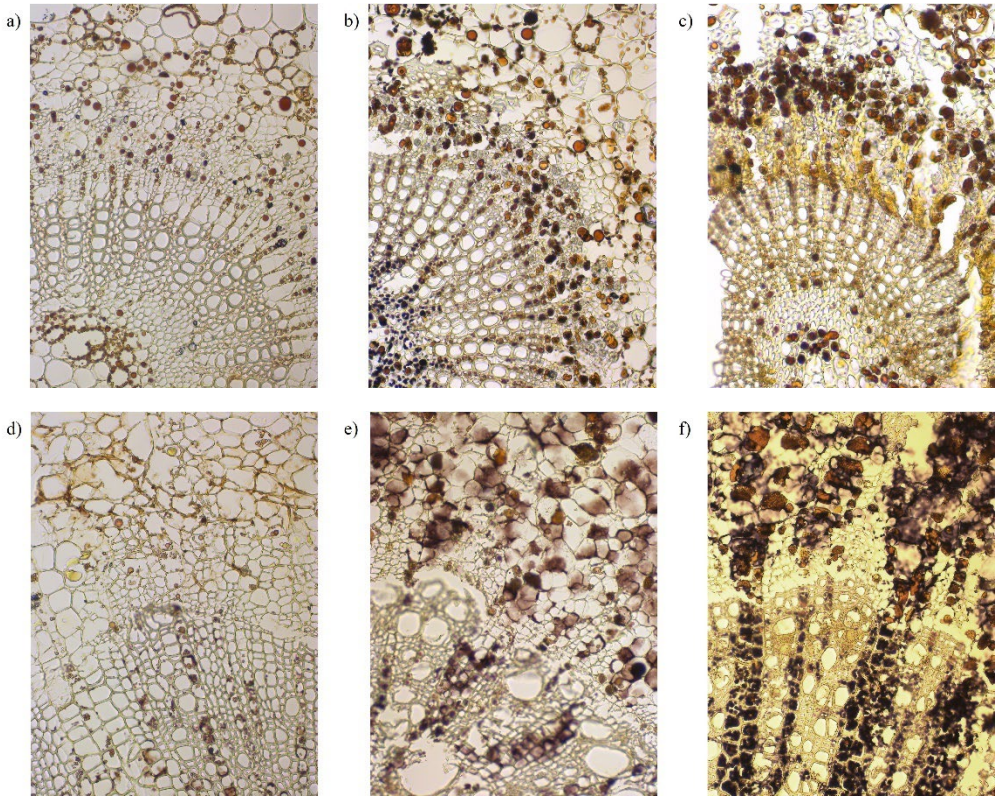


Figure 3. Thionin-Acridine Orange stained leaf (a-c), stem (d-f), and root (g-i) tissues from plants that were negative (a, d, & g), weakly infected (b ,e, & h), or heavily infected (c, f, & i) with '*Ca*. P. pruni' at 400x magnification.



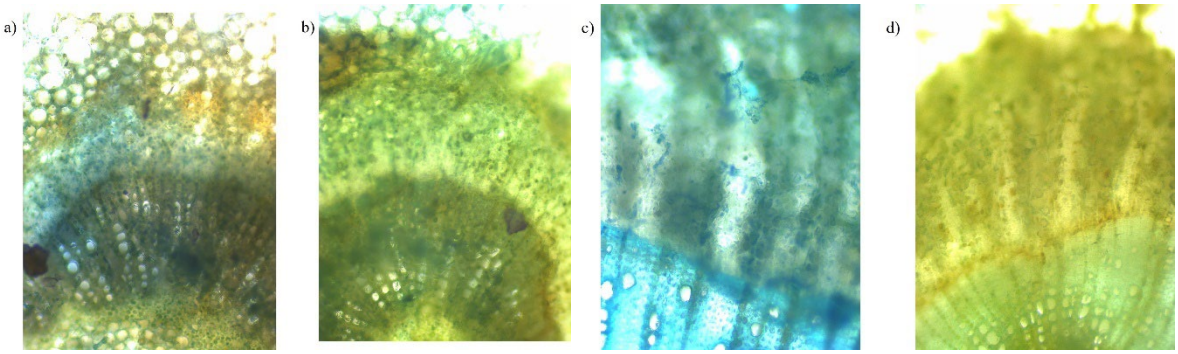
Finally, staining with iodine (Figure 4) was also not effective as an indicator of phytoplasma presence. While uninfected plants had less staining than infected plants, we observed that this was influenced by plant age and the time when samples were collected. Data from other research projects (i.e. the WTFRC-funded project "*Understanding Little Cherry Disease Pathogenicity*") indicates that the phytoplasma infection does affect photosynthesis, sugar metabolism and transport, but at a local/cellular level the change may be below the limit of what can be visually detected.

Figure 4. Iodine stained leaf (a-c) and root (d-f) tissues from plants that were negative (a & d), weakly infected (b & e), or heavily infected (c & f) with '*Ca*. P. pruni' at 400x magnification.



Given the cellular distortion, we investigated whether hand sectioning could be used as method to screen or identify plants infected with the more severe strains that are not present in Washington or Oregon in any significant frequency, i.e. as a method to check plants that have been brought in from out of state. To do this, leaf and stem sections of 0.1 and 0.2 mm respectively were prepared using a razor blade and cutting tile, then stained using Toluidine Blue as this is the most cost-effective and rapid stain assessed in this study, and therefore the most accessible for an end user.

Figure 5. Hand-cut cross-sections of a) uninfected and b) infected leaf, and c) uninfected and d) infected woody stem tissues, stained with Toluidine Blue and imaged at 400x magnification.



Unfortunately, hand sectioning (Figure 5) produces (for the purposes of microscopy) thick sections in which anything beyond the general location of basic cell types cannot be readily distinguished. Furthermore, there was some variation in the depth and angle of the sections made so it cannot be recommended as a method.

Objective 2: As objective 1 failed to produce a viable method, objective 2 was not pursued.

Conclusions

In summary, this project aimed to see whether simple histochemical stains could be used to indicate the presence of the X-disease phytoplasma, '*Candidatus* Phytoplasma pruni', in infected plants. The hope was that his could be a simple diagnostic tool. While the stains, particularly Diene's stain, have been shown to indicate the presence of phytoplasma in phloem sieve elements in other plant species, primarily herbaceous species, in *Prunus* it did not work. The likely reasons for this are twofold. First, herbaceous species are both easier to preserve and section and contain cells that are less lignified or have thinner cell walls than woody perennials, therefore herbaceous species have much less 'background' when stained, making it easier to distinguish cellular aggregates in the phloem under light microscopy. Second, the titer or concentration of the phytoplasma may be lower and/or more scattered in its distribution in a tree than in a much smaller, or potentially more susceptible, herbaceous plant.

We did, however, observe cytopathological symptoms in association with specific strains, i.e. what has been classified as 'Eastern-X' in recent work based on the sequences of this phytoplasma from different parts of the country. In leaf, stem, and root tissue cross-sections there was a distortion of the rays extending into the cortical tissue beyond the vasculature. Cell layers in the cortical tissues were disorganized, which may explain the stunting and other growth abnormalities observed with this strain as opposed to the 'Northwestern-X' strains in which the cells and growth appear comparable the uninfected controls. How this is occurring requires further research.

Executive Summary:
This project aimed to determine whether histological staining could be used as diagnostic method to test trees for the presence of the X-disease phytoplasma, in response to the need for rapid screening of planting stock. Three stains, Toluidine Blue, Diene's, and Thionin-Acridine Orange were tested based on reported phytoplasma studies in herbaceous plants, on uninfected, weakly infected, and heavily infected leaf, woody stem, and root tissues. Unfortunately, in contrast to previous studies on other phytoplasma species and host systems, here the presence of the X-disease phytoplasma in phloem sieve elements could not be observed using light microscopy. This may be due to host-specific background staining or phytoplasma titer being too low or unevenly distributed.

We did, however, observe cytopathological effects associated the infection of 'Eastern-X' strain of this phytoplasma, with distortion and/or disruption of the rays and cortical cells in all tissues. This was not observed in plants infected with the 'Northwestern-X' strains and correlates with the greater virulence of the 'Eastern-X' strains observed in other studies. While it may be of potential use as a diagnostic for this particular stain, the histological process is time consuming, and molecular methods provide greater utility.

In summary, histopathological staining is not recommended as a rapid or reliable option for the detection of the X-disease phytoplasma.

Report Type: Continuing Project Report

Project Title: Precision Agriculture: Innovating Cherry Disease Management with DPI

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Cooperators: Michelle Heck USDA-ARS, Ithaca, NY; Scott Harper; Tobin Northfield

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 35,000
Total Project Request for Year 2 Funding: $ 35,000
Total Project Request for Year 3 Funding: $ 35,000

Other related/associated funding sources: None

Budget 1
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Item	2024	2025	2026
Salaries			
Benefits			
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$35,000.00	$35,000.00	$35,000.00
Travel			
Plot Fees			
Miscellaneous			
Total	$35,000.00	$35,000.00	$35,000.00

Anticipated benefits to industry

The successful adaptation and implementation of DPI in cherry orchards promises to revolutionize disease and pest management practices. The ability to deliver therapeutic molecules directly to the plant's vascular system offers several key benefits to the industry:

- **Reduced Pesticide Use**: DPI's targeted delivery system enables the use of significantly lower insecticide dosages compared to traditional foliar sprays, reducing environmental impact and potential residues on fruit.
- **Improved Efficacy**: By directly targeting the pathogen and its insect vector within the plant, DPI can potentially achieve higher efficacy in disease and pest control, leading to healthier trees and increased crop yields.
- **Enhanced Sustainability**: DPI's reduced reliance on chemical pesticides and its potential for delivering bio-based solutions contribute to a more sustainable and environmentally friendly approach to crop protection.
- **Cost Savings**: The reduced insecticide dosages and potential for increased yields can translate into significant cost savings for cherry growers.

Note on Project Funding:
Despite the delayed receipt of the grant funding, we have made significant progress towards the project objectives by leveraging alternative funding sources. We remain optimistic that the grant funding will be released soon, as the lack of funds is currently hindering further advancement of this research. We appreciate your understanding and continued support.

Original Objectives

1. **Investigate the mobility of therapeutic molecules and marker signals delivered through direct plant infusion.**

2. **Investigate the effectiveness of therapeutic molecules delivered through direct plant infusion in decreasing phytoplasma titer and improving plant fitness.**

3. **Investigate the effectiveness of therapeutic molecules delivered through direct plant infusion in decreasing *Colladonus reductus* fitness.**

4. **Initiate field evaluation with commercially available injection systems.**

Significant Findings

- Successful adaptation of DPI to cherry trees: The direct plant infusion (DPI) method, previously developed for citrus, has been successfully adapted for use in cherry trees.
- Effective delivery and distribution of marker molecules: Marker molecules (dye) were successfully delivered to cherry trees using DPI, with rapid uptake and distribution observed.
- Dye distribution throughout the plant: The dye was observed in various plant tissues, including vascular tissue, flowers, leaves, roots, and even in flowers and leaves that developed after dye introduction. This demonstrates the potential for DPI to target phytoplasma bacteria residing in the phloem.
- Dye uptake by leafhoppers: *Colladonus reductus* feeding on dye-inoculated trees exhibited red coloration in their bodies, eggs, and gut within 24-48 hours, indicating the potential of DPI to deliver molecules targeting insect vectors.
- Successful dye uptake in orchard trees: Large orchard trees inoculated with red dye showed good uptake, suggesting the feasibility of DPI for larger-scale applications.

Materials and Methods

Procurement and Planting:

- Approximately 2400 cherry trees, representing various cultivars and rootstocks commonly used by growers, were obtained. These trees will be used for both DPI and Symbiont for the other grant WTFRC funded.
- These trees were planted in different pots, including apple bins, to accommodate their sizes and experimental requirements.
- Smaller potted trees were moved into a highly infected orchard to acquire natural phytoplasma infections for future studies.

Direct Plant Infusion (DPI) Adaptation and Implementation

- The DPI method, initially developed for citrus trees, was successfully modified and optimized for use in cherry trees.
- Marker Molecule Selection and Delivery: Non-toxic, easily visible blue food dye and floral red dye (2.5 mL per DPI) were chosen as marker molecules for tracking their distribution within the plant.

Field Trunk Injection

- Initiation of Field Trunk Injection: To evaluate DPI effectiveness in mature orchard trees, trunk injections were conducted using passive inoculation**:** A 250 ml red dye solution was introduced using passive inoculation, harnessing the tree's natural transpiration to facilitate dye uptake and distribution.

Insect Assay with Bagged Leafhoppers

- Initiating Insect Assay: *C. reductus* were confined in mesh bags on tree leaves, allowing for air circulation while preventing escape.
- DPI Application: DPI was used to deliver 2.5 ml of either red or blue dye to the trees for a 24-hour duration.
- Leafhopper Dissection: After dye exposure, *C. reductus* were dissected to examine dye uptake and distribution within their bodies.

Insecticide Selection and Dosage Calculations

- Insecticide Classes Evaluation: A range of insecticides representing different chemical classes were considered for potential use in the DPI system. These classes included botanical essences, diamides, limonoids, neonicotinoids, pyrethrins, pyrethroids, spinosyns, and sulfoximines.
- Dosage Calculation: The recommended foliar application rates for each insecticide were used as a starting point for calculating potential injection dosages, ranging from 100 to 10000 dilution per tree compared to the current usage.
- Significant Reduction in Dosage: Due to the targeted delivery nature of DPI, it is anticipated that significantly lower amounts of insecticide will be required compared to traditional foliar applications. Preliminary calculations suggest a potential reduction in the range of 50-75% for injection dosages.

Results and Discussion

Trees from Nursery

- Procurement and Acclimatization: Over 2400 cherry trees were successfully acquired and are currently growing in pots and bins, based on their size (Fig. 1A, B, C).
- Orchard Trees and qPCR Analysis: Over 200 trees were exposed to X-disease leafhopper vectors from a highly infected orchard during the second-generation peak abundance of *C. reductus* to ensure optimal phytoplasma inoculation. Yellow sticky traps were strategically deployed to monitor the presence of infected *C. reductus*, and subsequent qPCR analysis revealed 68% of the captured *C. reductus* were positive with X-disease phytoplasma. qPCR analysis will be performed to confirm infection levels of the trees and prepare them for future DPI experiments targeting phytoplasma.
- Mesh-Protected Space: A mesh-enclosed space is under construction to protect the cherry trees from harsh conditions while maintaining a near-natural environment (Fig. 1D).

Figure 1: Cherry trees, (A) planted, (B) larger trees in apple bin, (C) smaller trees in pots and (D) new structure for trees

Successful Adaptation of DPI to Cherry Trees

- DPI Adaptation and Limitation: DPI was successfully adapted for cherry trees (Fig. 2A). However, due to delayed grant funding, a 3D printer for creating custom adapters is unavailable, limiting testing to trunk sizes of 8- and 10-mm diameter.
- Successful Implementation: Despite this limitation, DPI was effectively executed using existing limited equipment. This achievement opens doors for targeted therapeutic molecule delivery in cherry orchards, offering a potential breakthrough in disease management and crop protection.

Figure 2: DPI. (A) dpi on cherry trees, (B) blue dye color spreading into leaves, (C) flowers, (D) Suckers, and (E) section of the tree showing blue color dye

Rapid and Widespread Distribution of Marker Molecules

- Rapid Uptake and Systemic Movement: DPI application resulted in rapid uptake and distribution of both blue and red dyes throughout the cherry trees (Fig. 2 b-c-d-e).
- Dye Presence in Various Tissues: The dyes were observed in vascular tissue, flowers, leaves (both mature and new), roots, and secondary growth suckers, indicating efficient transport and bi-directional movement.
- Rapid Coloration: Coloration was visible within 30 minutes of a 2.5 ml dye dose, highlighting the system's rapid uptake and distribution capabilities.
- Potential for Phytoplasma Targeting: This widespread dye distribution suggests DPI's potential to effectively reach and target phytoplasma within the phloem.

Dye Uptake by Leafhoppers

- Insect Assay System: A mesh bag system was developed to contain leafhoppers on tree leaves for dye exposure experiments (Fig. 3a).
- Clear Evidence of Dye Uptake: *C. reductus* feeding on dye-inoculated trees exhibited distinct red coloration in their bodies, eggs, and gut contents within 24-48 hours (Fig. 3 b-c-d).
- Potential for Vector Control: This result demonstrates DPI's capability to deliver molecules targeting insect vectors, critical for disrupting phytoplasma transmission.

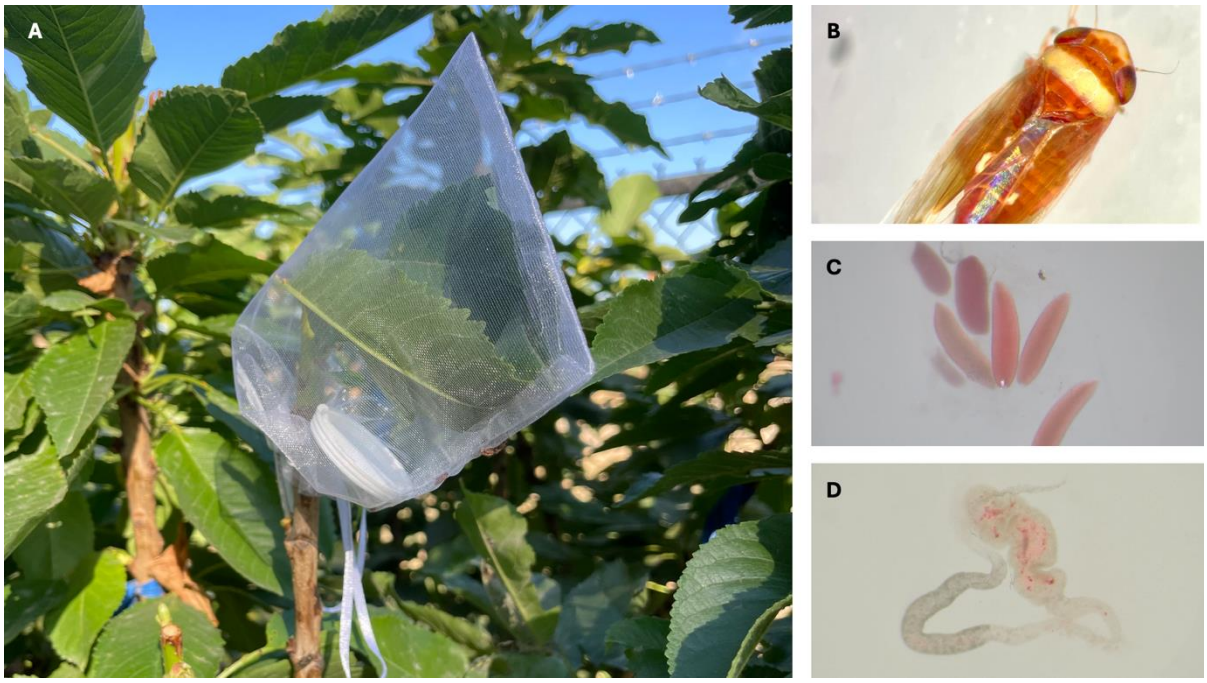

Figure 3: *C. reductus* dye uptake using (A) mesh bag cage. (B*) C. reductus* 24 h after treatment (C) eggs and (D) gut showing red coloration after injection from leaves.

Successful Dye Uptake in Orchard Trees

- Trunk Injection and Dye Uptake: Mature orchard trees inoculated with 250 ml of red dye via trunk injection showed good dye uptake within 24 hours (Fig. 4)
- Feasibility and Scalability: This successful uptake in large trees supports the feasibility and scalability of DPI for commercial orchards, paving the way for potential large-scale implementation of DPI-based therapies for disease management and crop protection.

Figure 4: Cherry tree in orchard showing red coloration on branches

List of insecticide

- Insecticide Selection: Insecticides were chosen based on their efficacy against *C. reductus*.
- Dosage Calculation and Preparation: Injection dosages were calculated based on recommended foliar application rates and adjusted downwards considering the targeted delivery of DPI. Insecticide solutions were prepared according to manufacturer instructions and adjusted for injection volume.

Class	Active Ingredient(s)	Formulation	Trade Name	Manufacturer	Rate/Acre	Rate/Hectare
Botanical essences	Cinnamon oil 60% Oils: geraniol 30%, peppermint 1% cottonseed 0.1%, rosemary 0.01%	Botanical concentrate Concentrate	‡Cinnerate Wrath	Seipasa S.A., Huesca, Spain GoPro, Burnsville, MN	60 fl oz 48 fl oz[b]	4,384 ml 3508 ml[b]
Diamide	Cyantraniliprole 10.2%	Suspoemulsion	Exirel	FMC Corp. Philadelphia PA	20.5 fl oz	1,498 ml
Limonoid	Azadirachtin 4.5%	Botanical concentrate	‡Neemix 4.5	Certis USA LLC Columbia MD	16 fl oz	1,169 ml
Neonicotinoids	Thiamethoxam 75%	Soluble granule	Platinum 75 SG	Syngenta CropProt LLC Greensboro NC	3.76 oz	263 g
Pyrethrin	Pyrethrins 1.4%	Emulsifiable concentrate	‡PyGanic EC 1.4 II	MGK Minneapolis MN	64 fl oz	4,677 ml
Pyrethroid	Esfenvalerate 8.4%	Emulsifiable concentrate	Asana XL	Valent USA Corp. Walnut Creek CA	14.5 fl oz	1,060 ml
Pyridinecarboxamide	Flonicamid 50%	Soluble granule	Beleaf 50SG	FMC Corp. Philadelphia PA	2.8 oz	196 g
Spinosyn	Spinosad 22.5%	Suspension concentrate	‡Entrust SC	Corteva Ag. LLC Indianapolis IN	8 oz	560 g
Sulfoxamine	Sulfoxaflor 50%	Water-dispersible granule	Transform WG	Corteva Ag. LLC Indianapolis IN	2.75 oz	193 g

Chemical Class	Amount per tree (grams, foliar)	Potential Reduction Range (grams)	Possible Injection Amount (grams)	Amount per tree (grams) - Small Tree	Amount per tree (ml) - Small Tree
Botanical essences	4.26	2.13 - 3.20	1.06 - 2.13	1.595	1.66
Diamide	1.46	0.73 - 1.10	0.36 - 0.73	0.545	0.57
Limonoid	1.14	0.57 - 0.86	0.28 - 0.57	0.425	0.44
Pyrethrin	4.54	2.27 - 3.41	1.13 - 2.27	1.7	1.77
Pyrethroid	1.03	0.52 - 0.78	0.25 - 0.52	0.385	0.4
Spinosyn	0.57	0.29 - 0.44	0.14 - 0.29	0.215	0.22

Figure 5: List of chemicals for *C. reductus* control and concentration for DPI

Conclusion

This research has successfully demonstrated the adaptation of Direct Plant Infusion (DPI) for targeted delivery in cherry trees, opening new avenues for managing diseases and pests. The rapid distribution of marker dyes throughout the plant, including uptake by *C. reductus*, emphasizes the potential of DPI to target both the phytoplasma pathogen and its insect vector. Successful dye uptake in mature orchard trees underscores the practicality and scalability of trunk injection for commercial applications. The preliminary findings on insecticide dosage reduction provide encouraging evidence for the development of more sustainable and cost-effective pest control strategies. Furthermore, this research lays the groundwork for future exploration into utilizing DPI as a tool for screening molecules against phytoplasma pruni (X-disease).

Future Work

Building on these promising results, future research will focus on expanding the applications of DPI in cherry and refining its implementation. This includes:

- Optimizing Insecticide Delivery: We will conduct comprehensive trials to assess the effectiveness of various insecticides delivered through DPI in managing X-disease leafhopper vector populations and mitigating phytoplasma transmission. Optimization will involve refining injection dosages, timings, and formulations for different insecticides and environmental conditions.

- Evaluating Therapeutic Molecules: We will leverage DPI to deliver and evaluate therapeutic molecules aimed at directly targeting phytoplasma and disrupting its lifecycle within the plant. This will involve assessing the efficacy of these molecules in reducing phytoplasma titer.
- Expanding DPI's Applications: We will continue to explore the potential of DPI as a versatile tool for delivering various molecules within the plant.
- Tree Injection: We will initiate tree injection evaluation with commercially available injectors

Project Title: Sustainable Cherry Protection with SYMBIONT

Report Type: Continuing Project Report

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Cooperators: Michelle Heck USDA-ARS, Ithaca, NY; Scott Harper; Tobin Northfield

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 48,000
Total Project Request for Year 2 Funding: $ 48,000
Total Project Request for Year 3 Funding: $ 48,000

Other related/associated funding sources: None

Budget 1
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Item	2024	2025	2026
Salaries			
Benefits			
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	48,000	48,000	48,000
Travel			
Plot Fees			
Miscellaneous			
Total	$48,000.00	$48,000.00	$48,000.00

Anticipated benefits to industry

The successful development and implementation of engineered symbionts as a delivery system for anti-phytoplasma AMPs constructs hold immense promise for the cherry industry. This innovative approach has the potential to revolutionize disease and pest management practices by providing a targeted, sustainable, and environmentally friendly solution. By effectively combating phytoplasma infections and their insect vectors, this technology could significantly reduce crop losses, improve fruit quality, and enhance the overall productivity and profitability of cherry orchards. The reduced reliance on chemical pesticides would also contribute to a healthier and more sustainable agricultural ecosystem.

Note on Project Funding:
Despite the delayed receipt of the grant funding, we have made significant progress towards the project objectives by leveraging alternative funding sources. We remain optimistic that the grant funding will be released soon, as the lack of funds is currently hindering further advancement of this research. We appreciate your understanding and continued support.

Original Objectives

1. **Symbiont Development in Cherry: Assess the development of engineered symbionts in cherry, comparing with citrus findings.**

2. **AMPs Screening: Inoculate infected cherry trees with various symbiont plasmids, screen for disease reduction, and develop improved plasmids.**

3. **Combined AMPs Efficacy: Evaluate new plasmids in greenhouse trials, focusing on titer reduction and plant health improvements.**

4. **Field Evaluation: Plan and initiate field trials for top-performing symbionts, leveraging citrus trial experience.**

Significant Findings

- Successful Symbiont Development in Cherry: A major milestone was achieved with the successful development of engineered symbionts within cherry plants. These symbionts not only exhibited robust growth but also demonstrated the expression of marker genes like GFP, confirming their functionality and potential for further applications.
- Plant Material Acquisition and Preparation: A diverse collection of over 2400 cherry trees, with various cultivars and rootstocks, was bought from nurseries. A portion of these trees were utilized for optimizing symbiont inoculation and growth protocols. The remaining trees were strategically relocated to a highly infected cherry orchard to facilitate natural infection with phytoplasma, serving as valuable material for future experiments involving the delivery of anti-phytoplasma AMPs.

Materials and Methods

Procurement and Planting:

- Approximately 2400 cherry trees, representing various cultivars and rootstocks commonly used by growers, were obtained. These trees will be used for both Symbiont and for the other grant WTFRC funded on DPI.
- These trees were planted in various pots, including apple bins, to accommodate their sizes and experimental requirements.
- Smaller potted trees were moved to a highly infected orchard to acquire X-disease phytoplasma through natural infection for future studies.

Symbiont Generation

Bacteria for inoculum

A small amount of frozen *Agrobacterium tumefaciens* containing the desired plasmid is scraped from the glycerol stock using a sterile micropipette tip. This bacterial sample is then introduced into 5 mL of LB liquid media enriched with 100 mg/L kanamycin within a 50 mL centrifuge tube. The inoculated culture is incubated in a shaker at 200 rpm and 28°C for 16-18 hours, allowing the bacteria to multiply. Following incubation, the culture is centrifuged at 5000 rpm for 10 minutes to pellet the bacterial cells. The supernatant is discarded, and the pellet is resuspended in 30 mL of Agrobacterium Infiltration Solution (AIS). This centrifugation and resuspension process is repeated twice to remove any residual media components. Finally, the pellet is resuspended in 5 mL of AIS, and the optical density (OD600) is measured. If needed, the cell concentration is adjusted to an OD600 of 1.0 using AIS to ensure optimal concentration for subsequent inoculations. The Agrobacterium Infiltration Solution (AIS) used in this study consists of 10 mM MgCl2, 10 mM MES buffer at pH 5.6, and 400 μM Acetosyringone. This solution facilitates the infection process and the transfer of the desired plasmid from *Agrobacterium* to the plant cells.

Agrobacterium Inoculation of Cherry Species

Two sections on the cherry stem, located above the rootstock but below any branching points, are selected for biopsy punch locations are chosen either symmetrically on opposite sides of the stem or asymmetrically, maintaining at least a 2 cm distance and a 90-degree rotation between them. The appropriate biopsy punch size (4 mm for stems <10 mm in diameter, 6 mm for stems ≥10 mm) is selected based on the stem's diameter.

Inoculation

Using the selected biopsy punch, two wounds are created in the stem, removing the bark layer. The prepared *A. tumefaciens* solution is then applied around the interior circumference of each biopsy site using a micropipette. The volume of the applied solution depends on the biopsy punch size: 10 µl for 4 mm punches and 20 µl for 6 mm punches.

Sealing and Light Protection

The inoculation sites are tightly sealed with parafilm to prevent moisture loss and contamination. Optionally, K-flex tape can be wrapped around the stem to cover the inoculation points, shielding them from light and preventing chlorophyll synthesis in any potentially transformed cells.

GFP Detection

One-month post-inoculation, the parafilm is carefully removed, and the inoculation sites are examined for GFP production using two techniques:

- Fluorescence microscopy: Thin sections of plant tissue from the inoculation site and surrounding areas are prepared and observed under a stereo and fluorescence microscope to visually detect GFP expression.
- Western blot analysis: Total protein is extracted from both the symbiont tissue and adjacent plant tissues. This extracted protein is then subjected to western blotting using an anti-GFP antibody to confirm the presence and movement of GFP.

Protein Extraction

The collected symbiont and plant tissue samples are flash-frozen in liquid nitrogen and ground into a fine powder. The powdered tissue is homogenized in lysis buffer containing protease inhibitors to disrupt the cells and release proteins. The homogenate is centrifuged to separate cellular debris from soluble proteins in the supernatant. The extracted proteins are then separated by SDS-PAGE, transferred onto a membrane, and probed with an anti-GFP antibody to detect GFP expression and movement. The presence of GFP in tissues above or below the symbiont inoculation site is investigated to assess the effectiveness of signal peptides in facilitating GFP movement within the plant.

AMPs Construct Preparation and Inoculation

All plasmids engineered to express antimicrobial peptides (AMPs) in cherry trees are now ready for screening. These plasmids will be delivered into over 200 cherry trees previously exposed to phytoplasma infection. These trees will first be screened to confirm the presence of phytoplasma, and then those testing positive will be used for the symbiont inoculation process. Following the delivery, we will utilize DNA extraction and qPCR techniques on this tree to assess the titer levels of the expressed AMPs.
The engineered plasmids incorporate a stacked arrangement of AMPs, targeting both bacteria and insects. These AMPs are strategically separated by P2A sequences, a self-cleaving peptide sequence that allows the expression of multiple, distinct proteins from a single open-reading frame. Additionally, different versions of the AMPs have been fused to various Signal Peptides, with the aim of potentially enhancing their export into the vascular system of the tree, ensuring wider distribution and efficacy.

Some of the peptides included in these constructs are: Oncocin, a proline-rich AMP known to disrupt bacterial protein synthesis by targeting the ribosome; Jaburetox, a natural insecticide derived from Jack Bean Urease that modulates insect behavior through the activation of voltage-gated sodium channels; and Nodule-specific cysteine-rich (NCR) peptides, which have demonstrated antimicrobial activity against a spectrum of bacteria and fungi; Trypsin Modulating Oostatic Factor (TMOF) is a decapeptide which inhibits the biosynthesis of trypsin-like enzymes in the midgut of several insect species. For the delivery of these AMP-expressing plasmids into the cherry tree, we will also evaluate an L-methionine auxotrophic strain of *Agrobacterium tumefaciens*. This strain has a limited survival capacity within the plant, which is expected to minimize any potential environmental impact associated with the delivery process.
Once the inoculation of the cherry tree is complete, we will closely monitor the expression levels of the AMPs and carefully assess their efficacy in combating the existing phytoplasma infection and insect vector mortality.

RNAi Constructs for Cherry

The phytoene desaturase (PDS) gene has been selected as the target for evaluating RNA interference (RNAi) in cherry trees. The engineered symbiont will act as a delivery vehicle for double-stranded RNA (dsRNA), the key trigger for RNAi-mediated gene silencing. Silencing the PDS gene is anticipated to induce a visible whitening of plant tissue, serving as a clear marker for RNAi effectiveness.
To enhance the production of dsRNA within the cherry trees, the RNAi constructs designed for this study incorporate a strategic approach. The target gene sequence (PDS) is divided and separated by an intron sequence within the plasmid construct. This intron facilitates the formation of a hairpin RNA structure upon transcription, which is subsequently processed into dsRNA by the plant's cellular machinery. This ensures efficient generation of dsRNA, thus increasing the likelihood of observing the desired phenotypic change (whitening) and validating the efficacy of the RNAi system in cherry trees.
This established RNAi system holds the potential to be further utilized for targeted gene silencing in the context of phytoplasma disease management. By adapting the system to target phytoplasma susceptibility genes or essential genes in insect vectors, we can explore novel strategies for disease control and pest management in cherry trees.

Results and Discussion

Symbiont Development in Cherry

Plant Material Acquisition and Preparation:

Over 2400 cherry trees, representing a diverse range of cultivars and rootstocks, were procured and are currently being maintained in pots, bins, and an orchard setting, plants will be used for both Symbiont and DPI grant WTFRC funded grants (fig. 1). A subset of these trees was intentionally exposed to natural phytoplasma infection in the orchard, providing valuable material for future experiments evaluating the efficacy of AMP delivery against the pathogen. A dedicated mesh-protected space will be constructed to provide a controlled environment for the cherry trees from USDA internal funds, safeguarding them from harsh weather conditions while allowing for natural light and airflow more similar to an orchard setting compared to greenhouse environment.

Figure 1. Cherry tree preparation for symbiont inoculation. (A) Large cherry tree potted in apple bins. (B) Smaller cherry trees in pots. (C) Cherry tree inoculated with the symbiont. (D) Dedicated mesh-protected space under construction.

Successful Establishment of symbiont:

The engineered symbionts were successfully introduced and established within cherry plants. Robust growth of the symbionts was observed using different wrapping system (fig. 2 a-c).

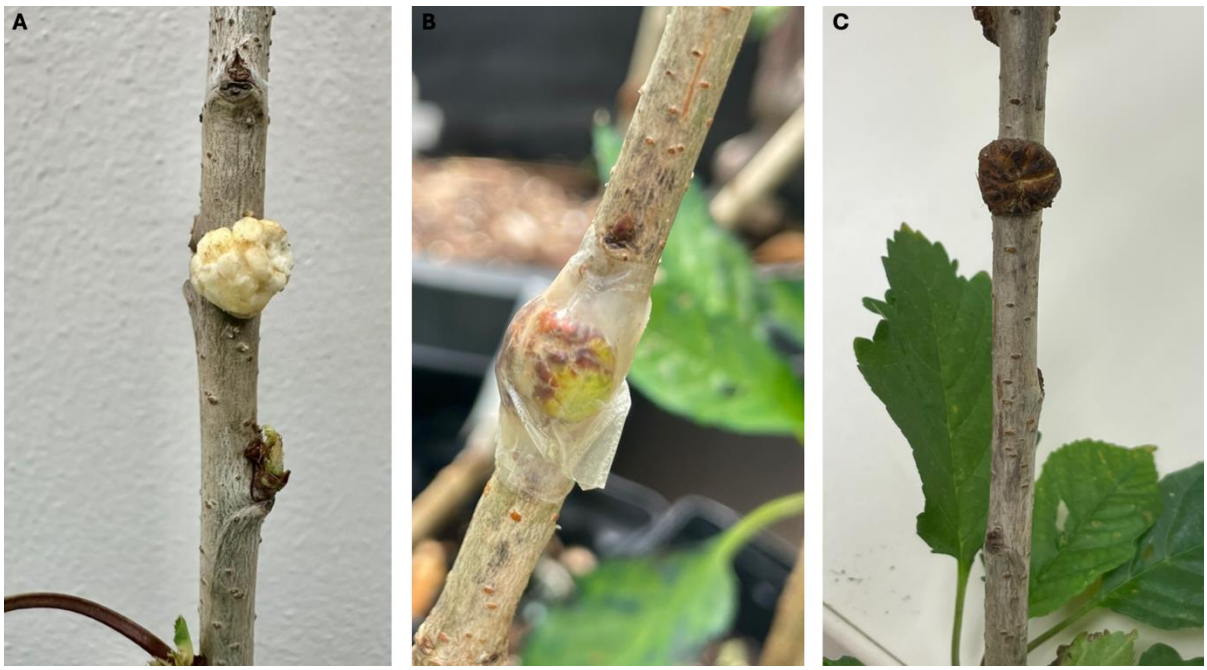

Figure 2. Different inoculation systems used to evaluate symbiont growth on cherry. (A) Dark cover to prevent light and inhibit chloroplast generation. (B) Hydrogel tape. (C) No cover

GFP Detection

One-month post-inoculation, the presence of GFP was successfully confirmed in symbiont tissue using fluorescence microscopy (fig. 3).

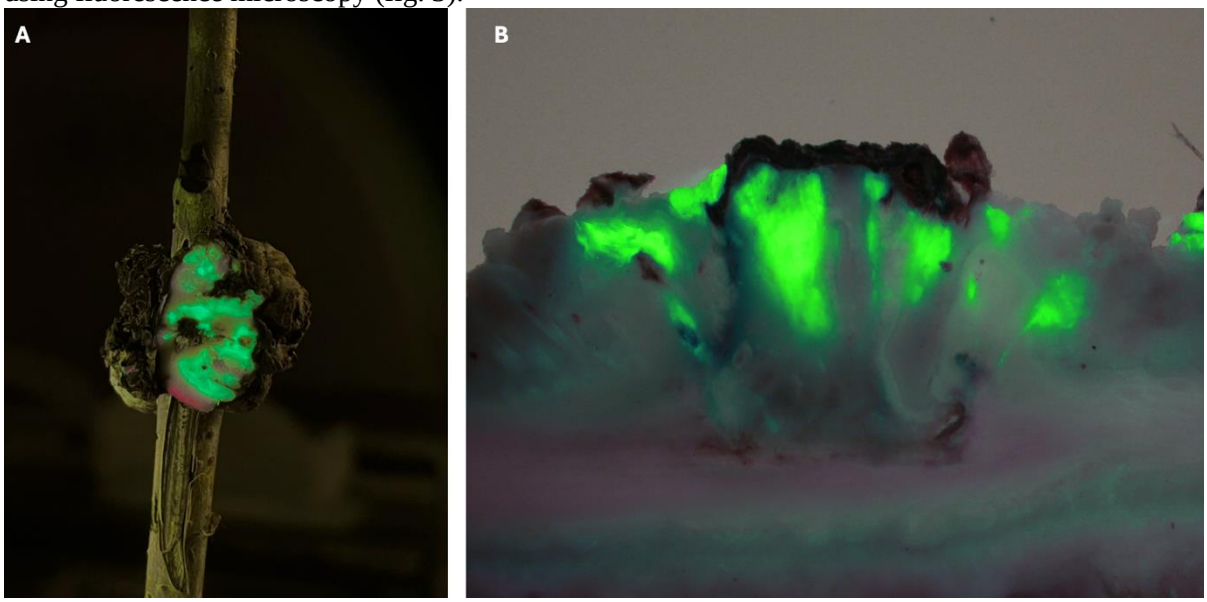

Figure 3. Symbiont expressing GFP. (A) Cross-section of the symbiont showing GFP expression under UV light. (B) Longitudinal section of the symbiont under a stereomicroscope and UV light, showing GFP expressed in the symbiont.

Infected plants and AMP Delivery Preparation:

Over 200 trees were exposed to X-disease leafhopper vectors from a highly infected orchard during the second generational peak abundance of *Colladonus reductus* to ensure optimal phytoplasma inoculation. Yellow sticky traps were strategically deployed to monitor the presence of infected *C. reductus*, and subsequent qPCR analysis revealed a 68% of the captured *C. reductus* tested positive for X-disease phytoplasma.

Leaf tissue samples from these potentially infected trees will be collected at the end of the growing season, just before they enter dormancy. Inoculation with the engineered symbiont will commence in early spring as the trees awaken from their winter dormancy. Throughout the spring and summer, we will evaluate the symbiont's effectiveness in preventing the spread of phytoplasma within these trees.

RNAi Constructs for Gene Silencing:

Two constructs targeting the phytoene desaturase (PDS) gene have been designed to evaluate the potential of RNAi-mediated gene silencing in cherry trees (fig. 4). The successful delivery and expression of these constructs are expected to result in a visible whitening phenotype, serving as a clear indicator of effective gene silencing. This RNAi system holds promise for future applications targeting phytoplasma susceptibility genes or crucial genes in insect vectors, offering innovative approaches for disease and pest control.

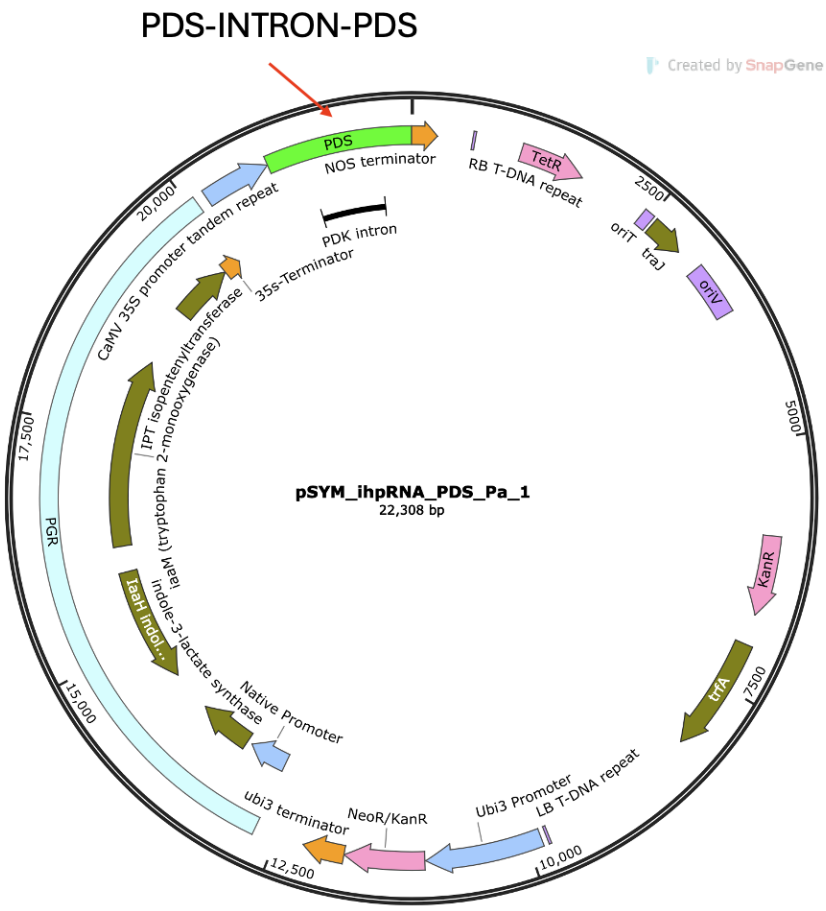

Figure 4. Plasmid pSym map featuring the sequence with intron to generate dsRNA for phytoene desaturase (PDS).

Conclusion

Despite the challenges posed by delayed grant funding, this project has achieved remarkable progress towards its objectives. We have successfully developed engineered symbionts in cherry, established a diverse collection of cherry trees for future experiments, and prepared AMP-expressing plasmids for phytoplasma control. Additionally, RNAi constructs have been designed for potential gene silencing applications. The successful establishment of symbionts in cherry, coupled with the confirmation of GFP expression, underscores the feasibility of utilizing Symbiont for further research and applications. The acquisition and preparation of plant material, including exposure to natural phytoplasma infection, provides a valuable resource for evaluating the efficacy of AMP delivery and RNAi-mediated gene silencing.

Future Work

In the coming months, we anticipate significant advancements in several key areas. The screening of AMP-expressing plasmids in phytoplasma-infected trees will enable us to pinpoint the most effective candidates for further development. Simultaneously, the evaluation of RNAi constructs targeting the PDS gene will provide crucial insights into the potential of RNAi-mediated gene silencing as a tool for disease and pest control in cherry. We will also screen the bioinsecticide produced by the symbiont against leafhoppers to assess its efficacy in controlling the insect vector. Following these screenings, we will develop new plasmids that combine the most promising AMPs and bioinsecticide

genes. These combined plasmids will then be evaluated for their ability to simultaneously target both the phytoplasma pathogen and its insect vector, offering a comprehensive approach to disease management.

We appreciate your continued support and understanding as we navigate these challenges and strive to make significant contributions to the field.

Project Title: Coordinating SWD and X Disease Management

Report Type: Final Project Report

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Project Duration: 3 Years

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 Specialty Crop Research Initiative
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); total amount $5,355,186.

WTFRC Collaborative Costs: none

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

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

Objectives:
(Objectives 1-5 are the objectives in the leveraged SCRI grant 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 final 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 vector management on SWD populations in Eastern Washington cherry orchards.
    b. Given the potential horticultural benefits of Extenday and Surround WP kaolin clay foliar application 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 (Obj. 6):
There are multiple lines of evidence that soil barriers such as Extenday and weed mats will suppress SWD populations used either pre-harvest or post-harvest. The mechanism appears to be physical rather than behavioral. These methods are may also be used for leafhopper vectors of X-disease, and thus are complementary in sweet cherry IPM.
2024:
- Growth chamber experiments indicate that soil barriers (Extenday, weed mat) do not affect SWD pupation or adult emergence; however, the arena may have allowed cryptic places for pupation.
- A field experiment demonstrated that the majority of SWD fall from the fruit as larvae to seek a pupation site, and do so during the daylight hours; thus, they should be negatively impacted by soil barriers, supporting the results of the 2021-2022 field experiments.

2023:
- Behavioral disruption: Extenday and weed mat do not significantly reduce SWD oviposition in comparison to the control.
- Physiological effects: Over a 24-hour exposure period, Extenday and weed mat did not significantly reduce egg viability (lab-emerged adults post experiment) compared to the control.

2022:
- At Cashmere 1, Extenday reduced SWD adult trap counts by 58.2% in comparison to the control.
- At Cashmere 2, Extenday applied postharvest reduced SWD adult trap counts by 67.6% in comparison to the control.

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

Methods (Objective 6)

Growth Chamber Soil Barriers 2024: Based on the negative results for behavioral influences of soil barriers in 2023, we investigated their ability to cause physical disruption in 2024. The first was a growth chamber experiment using small cages to determine the effect of covering the soil, preventing SWD from reaching their natural pupation site.

This experiment was performed in February of 2024 in controlled atmosphere room as a preliminary test of the concept of a physical barrier as the mechanism of reducing SWD. Three treatments were tested: Extenday, a black plastic weed barrier, and a soil control in a 10 x 10 square container in a small cage. The test used organic red sweet cherries purchased at Safeway. Oviposition arenas were set up with 5 cherries. Each arena contained a 94 mm filter paper and a cotton ball in a plastic cup moistened with 1 ml of water to provide humidity. The cherries were exposed to 10 adult female SWD for ≈5 hours, then the females removed. The number of ovipositions in each cherry was counted, and the treatments were randomly assigned in a RCB design. Cherries were moved to the barrier arenas to incubate (larval maturation, pupation, adult emergence). The cherries with eggs were suspended over square containers with one of the three treatments in small mesh insect cages. The number of emerged adults (male and female) were evaluated after 24 days later using a sticky card and aspirator. Three variables were analyzed: ovipositions/cage (this occurred prior to treatment, and was a check on bias in randomization); adults/cage, and fertility (adults/ovipositions). Data were analyzed with SAS (SAS ver. 9.4) using a mixed model analysis of variance (PROC MIXED) for an RCB and the Tukey adjustment for multiple comparisons.

Larval Drop Field Experiment, 2024: This experiment was performed in a block of mixed tree fruit species on the TFREC home farm. The test had 10 replicates, spaced around the periphery of a 'Montmorency' tart cherry tree. SWD females were taken from a laboratory colony in the Overley lab growth room, and used in the trial when they were ca. 7 d old. The replicate branches had ≈5 undamaged cherries, and surrounding vegetation was trimmed away. A fine mesh paint strainer bag was tied to the branch, enclosing the cherries (Plate 1). Five females from the colony were introduced into the bag on 27 June, and allowed to oviposit until they were removed on 30 June.

Plate 1. Mesh bag enclosing SWD-infested cherries.

Plate 2. SWD pupa in cherry.

Twice-daily checks of the bags (roughly 12 h apart, or ca. 8 am and 8 pm) began on 3 July (6 days after the introduction of the female SWD), and concluded on 14 July. Counts on a given replicate ceased when all fruit had dropped. The bag was replaced at each collection time, and the bag and its contents were labelled and stored in a cold room to arrest development until the sample was counted. The contents were categorized by stage (larva or pupa), and whether the SWD dropped to the bottom of the bag, or were contained in a cherry (Plate 2). The number of SWD were summed over all collection dates for each replicate, and comparisons made between SWD stage (larva v. pupa), type of drop (free or in a cherry), and time (daylight vs dark). Data were analyzed with a mixed model analysis of variance (PROC MIXED, SAS 2024) using a model for a completely randomized design, and means of the key variables (drop type, collection time, and stage) were separated with the

least significant difference test. The interaction between two key factors, drop type and stage, was also tested.

Behavioral Mesocosm Test, 2023: There were two primary mechanisms that would explain the trap suppression seen in 2021-2022: behavioral or physical. In 2023, we tested the behavioral hypothesis that the observed reduction in SWD trap capture in Extenday blocks was due to reflected light in those blocks disrupting host orientation behavior. This mechanism has been observed for other orchard pests such as pear psylla (Nottingham and Beers 2020, Nottingham et al. 2022). We speculated that the reflective nature of Extenday would disrupt female host-finding and oviposition, whereas a non-reflective black weed mat (providing the same soil coverage) would not. This trial was conducted in late September, providing environmental conditions conducive for improved fly survival in our experimental cage setup.

Three treatments (Extenday, weed mat, and uncovered sod control) were assessed in mesocosm cages (56 in x 23 in x 23 in screen cages, Raising Butterflies LLC, Salt Lake City, UT). Five replicate cages for each treatment were placed in three adjacent rows in a randomized complete block design at the TFREC pear orchard (15 × 20 ft spacing). The pear orchard provided an environment with the shade normally associated with mature trees, along with the temperature and RH of an orchard floor. Cages were spaced 10 ft apart within rows (Plate 3). Each cage contained two trays (20 in x 10 in plastic growing trays) of bluegrass sod (Harmony Outdoor Brands, Lakewood Ranch, FL) (Plate 2). The sod in the Extenday and weed mat cages were covered with a 56 in x 23 in rectangle of Extenday reflective groundcover or black polypropylene landscape fabric (Greenscapes Inc, Calhoun, GA) respectively. In each cage, a 1 gal plastic container holding four cherry branches was placed between the two sod trays in the center of the cage (Plate 4). Cherry branches (~1-1.5 ft in length) were sourced from an unsprayed cherry orchard at WSU Sunrise Research Orchard. Each cherry branch had five 'Sweetheart' cherries attached with a binder clip resulting in 20 cherries per cage. No other food sources or oviposition substrates were present. Adult female SWD (100/cage + 20 males) were released into each cage at 12 pm on Sept 21. They were allowed to oviposit for 24 hours before adults were removed and ovipositions were counted (Plate 5). The fruit was kept for 3 weeks in the lab to assess SWD emergence.

Plate 3. Mesocosm cages in an orchard row.

Plate 4. Cherry branches and sod trays in mesocosm cages.

One cage of each treatment hosted light sensors and environmental dataloggers. The datalogger (HOBO Pro v2 dataloggers, Onset Computer Corporation, Bourne, MA) was hung from the south facing cherry branch, and continuously measured temperature and relative humidity. Reflected light (measured as Photosynthetic Photon Flux Density; SQ-520 Full Spectrum quantum sensors, Apogee Instruments Inc, Logan, UT) measured reflected light. The quantum sensor was affixed to cage's ceiling, suspended over the

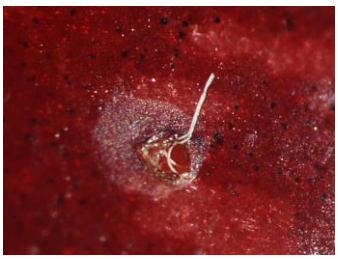

Plate 5. SWD oviposition in sweet cherry.

various surfaces. Point measurements of the intensity of different wavelengths of light (Lighting Passport Essence Pro spectrometer; Asensetek Inc, New Taipei City, Taiwan) reflected by the surfaces were taken at 2 pm (21 Sept.), 9 am, and noon (22 Sept).

Large Plot Field Trials, 2021-2022: In 2021, the impacts of Extenday ground cover (Plate 6) and Surround (kaolin clay) (Plate 7) canopy sprays on SWD trap capture were compared to an untreated control. Both treatments were candidate control measures for suppression of the leafhopper vectors of X-disease. The treatments were deployed in two sweet cherry orchards near Wapato and Wenatchee, respectively. The Wapato cherry orchard consisted of 28 acres of 'Sweetheart' cherries (12 × 18 ft spacing) and was conventionally managed. The Wenatchee cherry orchard consisted of ~25.9 acres of 'Coral Champagne' cherries (10 × 15 ft spacing) and was in the first year of transitioning from conventional to organic management. The three treatments were 1) Extenday ground cover (Extenday USA Inc, Union Gap, WA), 2) Surround kaolin foliar application (NovaSource, Phoenix, AZ) and 3) an untreated control. Each treatment had 4 replicate blocks 200 ft x 12 rows arranged in a RCB design. Treatments were maintained from mid-July until early November.

Plate 6. Extenday ground cover

Plate 7. Kaolin clay spray application.

Adult SWD were monitored throughout the treatment period using a modified 32 oz plastic jar containing a lure (Scentry Biologicals Inc., Billings, MT) suspended over a water-based drowning solution (Plate 7). Each replicate had two traps, one at 50 ft and one at 150 ft from the block's edge in the middle (seventh) row. 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 were counted. The lures were replaced every 6 weeks.

The Wapato site was similar to the Wenatchee site, except there were only 2 replicates, but with 4 traps each (at 50 feet in row 5, 100 feet in rows 6 and 8, 150 feet in row 7). The treatment deployment period was mid-July through mid-October; the block was removed in November due to the high incidence of X-disease.

In 2022, we assessed two candidate control practices (Extenday and herbicide) compared to an untreated control at two conventionally managed orchard sites near Cashmere, WA. The first orchard (Cashmere 1) consisted of 5.37 acres of 'Rainier' sweet cherries (9 × 15 ft spacing), and the second orchard (Cashmere 2) (Plate 8) consisted of 3.13 acres of 'Rainier' sweet cherries (10 × 18 ft spacing).

At each orchard, the three treatments (Extenday, herbicide, and untreated control) were replicated twice. Each replicate block was 130 ft long and 6 rows wide. Treatment blocks were set up in late May and maintained until the end of October. The herbicide treatments consisted of one preharvest groundcover application of SPUR (Clopyralid, Albaugh LLC, Ankenny, IA) on 20 May and one postharvest groundcover application of Venue (Pyraflufen ethyl, Nichino America Inc, Wilmington, DE) on 25 July. These herbicides were applied to row middles to control broadleaf weeds. At Cashmere 1, the Extenday blocks were maintained for the duration of the experiment. At Cashmere 2, Extenday was deployed by the grower across all 3 treatments from 30 May 30-27 June to improve color during fruit ripening. The Extenday was removed from all Cashmere 2 blocks by June 2 for harvest and was then re-applied solely to the designated Extenday Blocks on 15 July. As such, our Cashmere 2 analyses consist only of post-harvest comparisons.

Plate 8. Cashmere 2 site showing treatment arrangement (Yellow: Extenday; Pink: Herbicide; Blue: Control).

Monitoring for SWD adults was conducted as described for 2021, except the traps were checked weekly. Each replicate had two traps, one in the second row at 30 ft from the block's edge and another in the third row at 65 ft from the block edge.

Results and Discussion:

Growth Chamber Soil Barriers 2024: There were no differences in oviposition numbers at the beginning of the trial. After larval development and adult emergence, there were no significant differences total adults emerged (Fig. 1) or in adults/initial oviposition (data not shown).

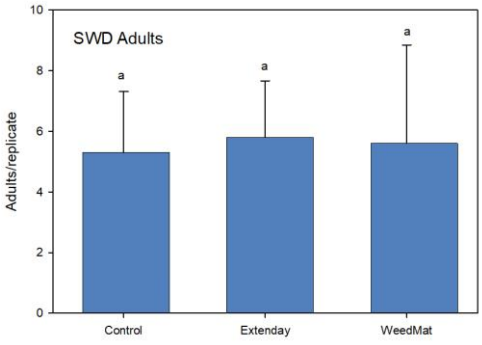

Fig. 1. SWD adults produced in three soil barrier treatments in a growth chamber.

There are several possible explanations why neither of the physical barriers interfered with adult emergence. First is that pupation occurred within the fruit, and thus the larvae never contacted the barrier. Second is the small size of the arena, and incomplete nature of the barrier (larvae seeking a pupation site could crawl under the barrier into the soil beneath, and pupate there). These results bring up two additional questions to determine if the physical nature of the barrier is the mechanism for reductions in trap catch. First is the location of pupation sites. Pupation has been observed in the fruit (Plate 1); if this is true for the majority of the population in cherry, then soil barriers should have little or no impact. However, research in berry crops indicates that the majority of pupation occurs in the soil (Woltz & Lee 2017), thus barriers should be a major impediment. Secondly is the potential for survival if the larva does drop onto one of the barriers vs the soil. This could depend on the temperature of the barrier's surface, which could vary depending on the amount of solar radiation it is exposed to (day/night, sun/shade). The first of these questions was explored in the following field experiment (larval drop experiment).

Larval Drop Field Experiment, 2024: A total of 17 SWD (larvae+ pupae) were found in all replicates at end of the experiment. There was no significant difference between the numbers of SWD that dropped directly to the bottom of the bag vs those that dropped inside a cherry (Fig. 2), although the number dropping into the bag was ca. 3.25× higher. This group represents larvae or pupae that would have dropped to the ground, and thus potentially affected by a soil barrier. Those SWD which were still in a fruit when it dropped may potentially have been protected to some extent. It should be noted that a third possible outcome (fruit retained on the tree, with pupation occurring inside it) was not found in this trial; this could be due to the cultivar, or the amount of manipulation the fruit were subject to (removal and replacement of the bags twice daily). Observations in sweet cherry orchards indicate that while the majority of unpicked fruit drop to the ground, some are retained until fall; in this case, any SWD contained in these fruits would not be subject to the influence of soil barriers.

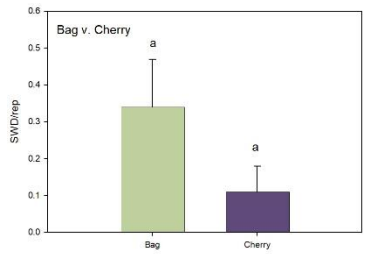

Fig. 2. Numbers of SWD dropping into the bottom of the cage vs in a cherry.

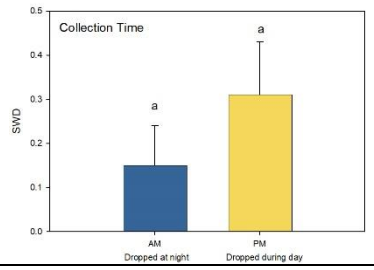

Fig. 3. Numbers of SWD dropping during the night vs during the day.

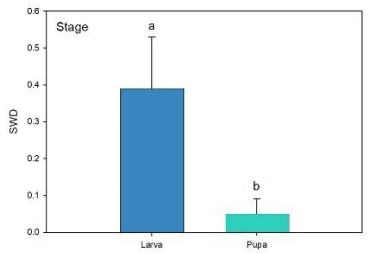

Fig. 4. Numbers of SWD dropping as larvae vs pupae.

Similarly, there was no significant difference found in the numbers of SWD in the morning vs evening collection, although about twice as many dropped during the day (pm collection) (Fig. 3). Presumably, larvae or pupae that dropped during daylight hours would be more subject to higher surface temperatures on soil barriers. We hypothesize that larvae that drop onto a heated surface of a barrier have a very poor chance of survival and will die before reaching an appropriate pupation site (soil or litter).

Significantly more SWD were found in the larval than in the pupal stage (Fig. 4). In theory, larvae would be more mobile, and better able to seek a suitable pupation site after reaching the ground. When the interaction between the drop type and stage was examined, no significant interaction was detected. However, the interaction-level means indicate that larvae dropping into the bag [ground] was the most common outcome which supports the idea that SWD will be vulnerable to soil barriers.

Although statistical differences were lacking, this work supports the findings of Woltz & Lee (2017) which found that the majority of SWD pupated in the soil vs in the fruit (blueberry and raspberry). The same behavior appears to hold in cherry, although these fruits are typically larger than berries, and theoretically better able to support pupae.

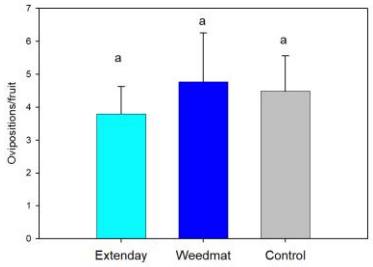

Fig. 5. SWD ovipositions/fruit in soil barrier field cage treatments compared to a control (sod).

Behavioral Mesocosm Test, 2023. There was no significant effect of Extenday or weed mat on SWD ovipositions/cage, with ovipositions in being comparable to control cages (Fig. 5).

As expected, Extenday had the highest levels of reflected light, followed by the grass control, with black weed mat having the lowest levels (Fig. 6). The relative intensity of red, orange, yellow, green, blue, violet, and ultraviolet light reflected by Extenday was greater than those same wavelengths reflected by the weed mat or control (Fig. 7). Although recorded temperatures were similar among the three treatments (Fig. 8A), the relative humidity in the Extenday and weed mat cages was lower than in the control (Fig. 8B).

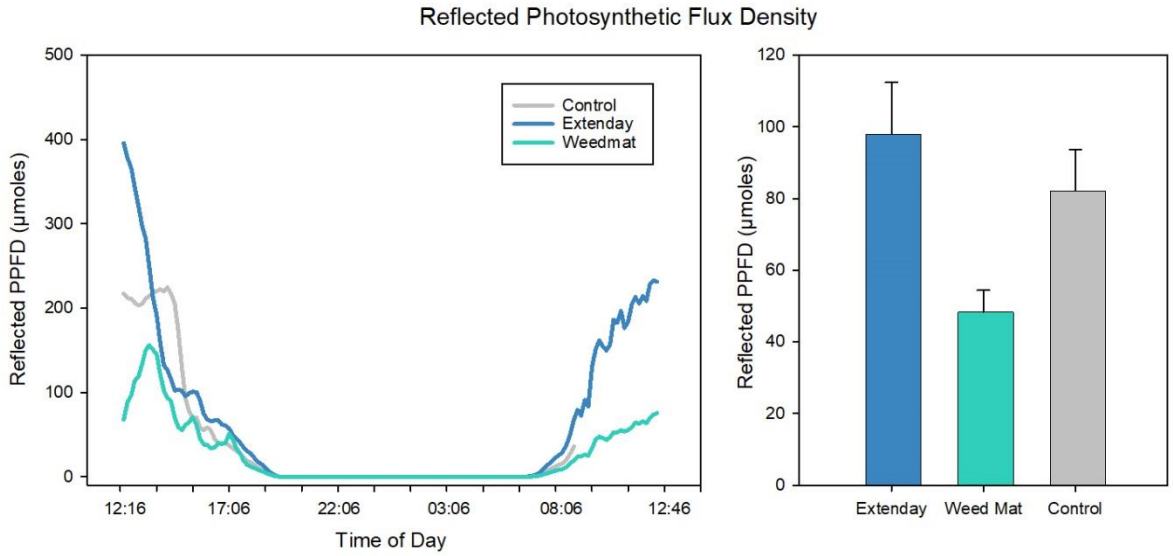

Fig. 6. Reflected photosynthetic flux density in three soil barrier treatments

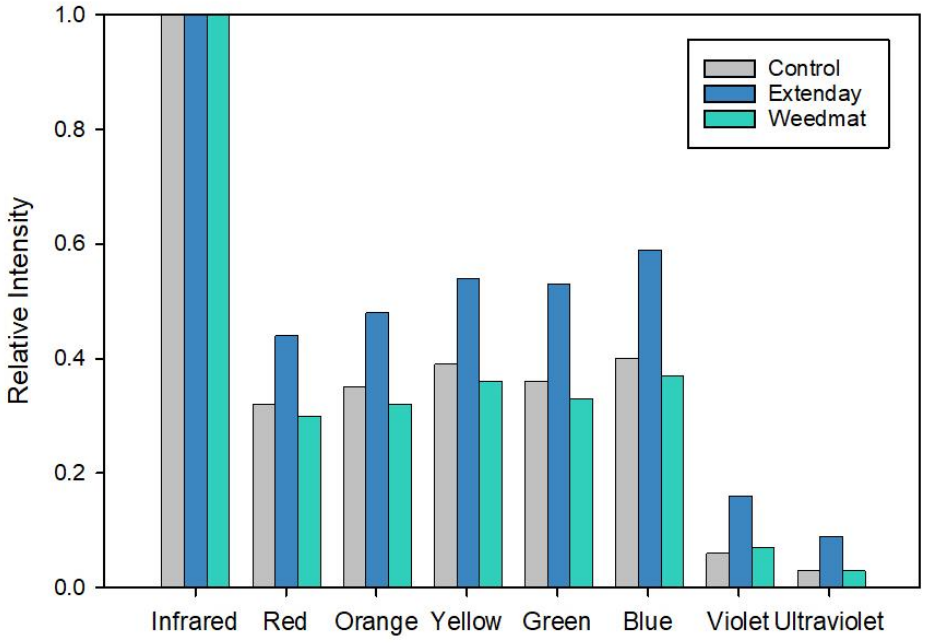

Fig. 7. Relative intensity of different wavelengths of light in three soil barrier treatments.

Although the amount and intensity of light was greater in Extenday than in the other blocks, the reflected light did not interfere with SWD egg laying behavior; this suggests that mechanisms other than reduced oviposition are responsible for the suppression of SWD seen in Extenday blocks. Prior

studies with plastic mulches in raspberry noted that plastic mulches (metallic, black, and white) significantly reduced SWD larval counts in fruit as well as adult trap capture compared to the control (McIntosh et al. 2021, McIntosh et al. 2023). Those studies noted that reduced larval development in fruit might be due to plastic mulch induced changes in canopy microclimate. It is also possible that the Extenday serves as a physical barrier that prevents SWD larvae and pupae from completing their development in the soil. In raspberry, dropped larvae and pupae suffered increased mortality after 4 h on plastic mulches compared to a grass control (C. Guédot, personal communication). With leafhopper vectors, Extenday is thought to reduce trap capture by preventing access to broadleaf weeds and other ground cover hosts (Marshall et al. 2023), thus the mechanisms may be analogous for the two pests.

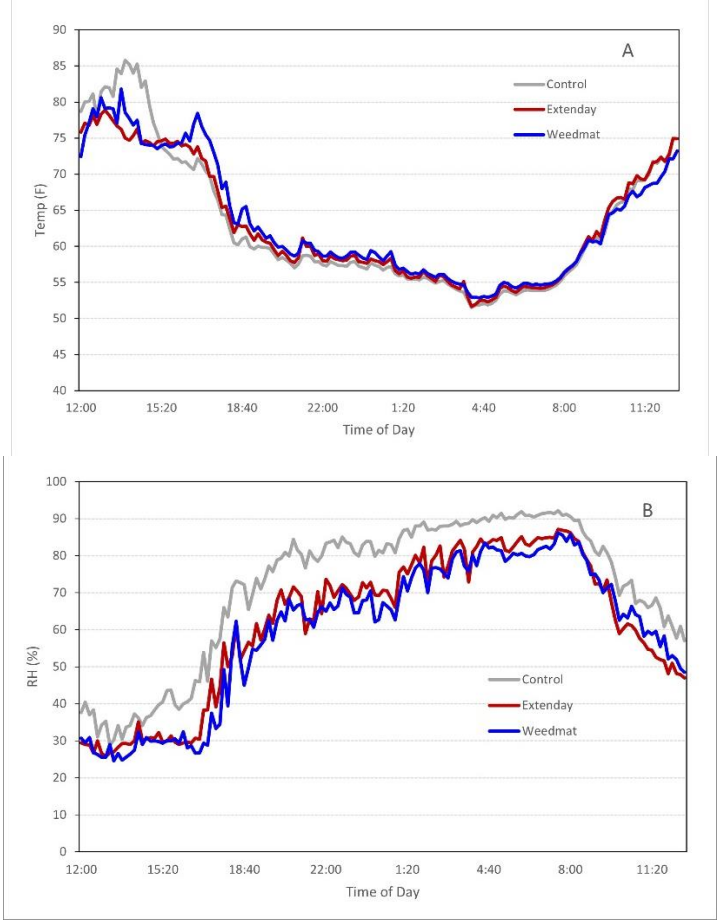

Fig. 8. Temperatures (A) and relative humidity (B) in mesocosm cages (three treatments) during the 24 h experiment.

Large Plot Field Trials, 2021-2022: In 2021, SWD counts across all treatments were initially low, likely due to the extreme heatwave events that the Pacific Northwest experienced during the summer of 2021. At Wapato, SWD counts in traps started to increase in late August while at Wenatchee, SWD counts remained low until late September (Fig. 9A, B). At the Wenatchee site, there was a significant effect of treatment on SWD collected per trap. Extenday applied postharvest reduced SWD adult counts by 72% while Surround reduced SWD adult counts by 71% in comparison to the control (Fig. 9C,). At the Wapato site, there was a significant effect of treatment on SWD collected per trap. Extenday applied postharvest reduced SWD adult counts by 47.9% while Surround reduced SWD adult counts by 41.3% (Fig. 9D).

In 2022, SWD counts in traps at both Cashmere sites remained low until mid-September (Fig. 10A, B). At Cashmere 1, there was a significant effect of treatment on SWD collected per trap. Extenday reduced SWD adult counts by 58.2% while herbicide reduced SWD adult counts by 17.8% in comparison to the control (Fig. 10C). At Cashmere 2, there was a significant effect of treatment on SWD collected per trap. Extenday applied postharvest reduced SWD adult counts by 67.6% while herbicide treated blocks increased SWD adult counts by 37% (Fig. 10D).

The results from 2021 suggest that postharvest canopy and groundcover management practices may provide a dual benefit in suppression of SWD and leafhopper vectors in cherry orchards. The 2022 trials support the use of Extenday, but not herbicides, to suppress SWD; however, herbicide use that reduces weed hosts may suppress leafhopper densities. These preliminary results suggest that these integrative management options may be viable under a wide scale of potential pest pressure.

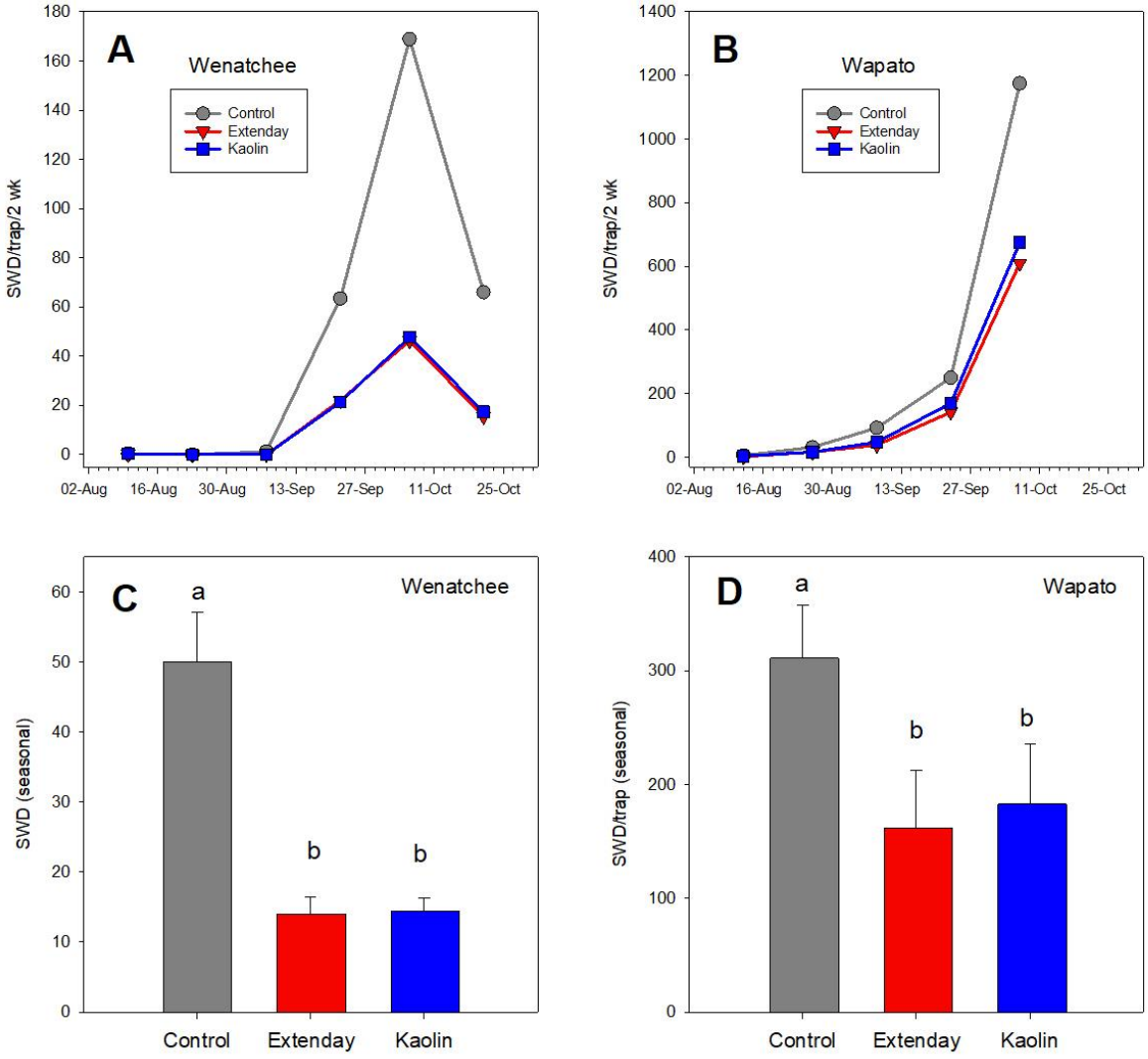

Fig. 9. SWD trap capture over time (A, B) and seasonal means (C, D) in large plot field trials, 2021.

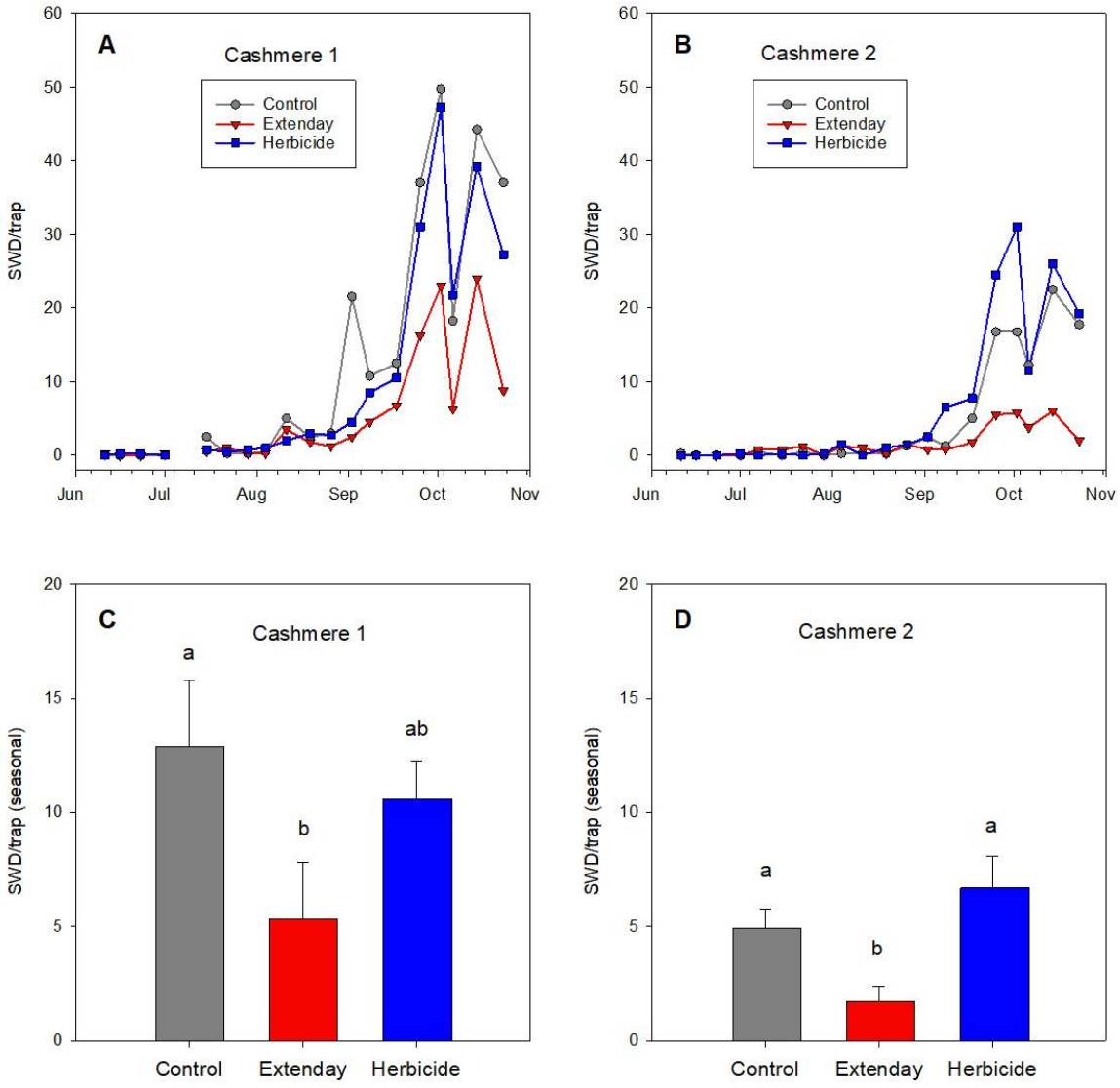

Fig. 10. SWD trap capture over time (A, B) and seasonal means (C, D) in large plot field trials, 2022.

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Executive Summary

Project Title: Coordinating SWD and X Disease Management
Key words: spotted-wing drosophila, *Drosophila suzukii*, X-disease, Kaolin, Extenday

Abstract: The detection of spotted-wing drosophila (SWD) in eastern Washington in 2010 started a new era in insect control in sweet cherry. Up until that point in time, western cherry fruit fly had been the only direct pest of consequence, because of the permanent quarantine restrictions placed on it. Counterbalancing insect pests were lethal diseases caused by viruses or phytoplasmas; the rise of X-disease in the late 2010s shifted the focus to vectors of a disease that, if left unchecked, could kill an orchard (as opposed to destroying the current year's crop). As always, growers were left facing the dilemma of managing their orchard both for the present and the future. This project sought to integrate two of the major concerns of a pest management program, specifically with the use of non-pesticide tactics.

This project examined the use of tactics were more sustainable that prophylactic sprays for SWD and leafhopper vectors which are currently the mainstay of control for these two pests. The work on SWD was done in tandem with the work on leafhoppers, with the idea of finding tactics that were effective for both. This project focused on SWD suppression; work on leafhopper vectors may be found in other projects. The field tests examined the use of a geotextile, Extenday, which was used for horticultural purposes (fruit coloration and maturation); and a postharvest spray whose original use was to reduce doubling of fruit in the subsequent year's crop (Surround, a particle film of kaolin clay). Both tactics were found to suppress trap capture of SWD in the post-harvest period (the period critical for leafhopper vector control). Subsequent tests examined Extenday and herbicides for control of broadleaf weeds in the row middles; the latter was directly more specifically at leafhopper vectors and nymphal development. The continuing theme is that Extenday worked for both pests, while herbicides (effective for leafhoppers) were not helpful in SWD suppression. Regardless, the mechanisms for the various tactics became important to understanding what they would work for, and why.

For the leafhopper vectors, both Extenday and herbicides limited access to the nymphal hosts, and disrupted the life cycle of leafhoppers in the orchard. For SWD, the mechanism was less clear. The reflective properties of Extenday might exert a behavioral influence that prevented correct orientation to the host plant for oviposition; examples of this effect in other crops had been demonstrated. In addition to possible behavioral influences, Extenday functioned as a soil barrier that could limit access to pupation sites. This physical function, however, could also be served by other soil barriers (e.g., black plastic mulch). To this end, we tested the physical effect of soil barriers, but found no effect on the smaller scale of research trials. Drilling down into the underlying biology, it would appear that larvae are most likely to drop directly to the ground (thus potentially influenced by a soil barrier), and are more likely to do it during the daylight hours, when surface temperatures are more likely to be lethal. The studies of mechanism underscore the importance of scale in research experiments. The smaller scale gives more repeatability and precision, but may miss effects that operate at a larger scale.

The effect of kaolin clay sprays remains open to question; its mechanism was not pursued for SWD suppression, in part because preharvest use (the critical period for SWD) is impractical because of the difficulty in removing it from the fruit during packing. Post-harvest, it appear to be a useful, cost-effective method of suppression and for both leafhoppers and SWD. The higher cost of Extenday may be offset by the horticultural benefits, providing an additional incentive for its use.

Project Title: *Ganaspis brasiliensis* for Biological Control of SWD
Report Type: Continuing Project Report

Primary PI: Christopher Adams
Organization: OSU
Telephone: 248-850-0648
Email: chris.adams@oregonstate.edu
Address: 3005 Experiment station drive
Address 2:
City/State/Zip: Hood River, OR 97031

Cooperators:
Mike Omeg, Stacey Cooper, Brian Nix

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $10,120
Total Project Request for Year 2 Funding: $10,422
Total Project Request for Year 3 Funding: $10,922
Total Request: $31,464

Other related/associated funding sources: N/A

WTFRC Collaborative Costs:

Budget 1
Primary PI: Christopher Adams
Organization Name: OSU
Contract Administrator: Charlene Wilkinson
Telephone: 541-737-3228
Contract administrator email address: Charlene.wilkinson@oregonstate.edu
Station Manager/Supervisor: Brian Pearson
Station manager/supervisor email address: brian.pearson@oregonstate.edu

Item	2023	2024	2025
Salaries[1]	$5,794.00	$5,967.00	$6,147.00
Benefits	$4,326.00	$4,455.00	$4,775.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies[2]			
Travel[3]			
Plot Fees			
Miscellaneous			
		*total	
Total	$10,120.00	$10,422.00	$10,922.00

Footnotes:
[1]FRA salary: $4,166 x 12 mo x 10% FTE = $5,794 for year 1 x 1.03 inflation rate /yr. Benefits at 73%

***Total - I have three copies of this document in my files. I have copies where this middle year is wrong at $4,000. Not sure which version you have on file at WTFRC, but if there is a discrepancy that is why.**

Objectives

1. Establish and increase a colony of *Ganaspis brasiliensis* wasps.

Deviations: We struggled to establish a thriving colony of our target wasp *Ganaspis brasiliensis,* and our small colony collapsed in the winter of 2023.

2. Conduct releases at selected orchards (years 2 & 3)

Deviations: we did not occomplish and releases of *Ganaspis brasiliensis* in 2024.

3. Measure establishment of wasps (year 3)

Deviations: This objective was planned for 2025.

Significant findings

- We had a colony of *Ganaspis brasiliensis* wasps in fall of 2023 but it struggled and failed over the winter.
- In our surveys for SWD parasitoids we have found and brought into our colony, both *Leptopilina japonica* an introduced SWD parasitoid, and *Pachycrepoideus vindemiae* a native generalist egg parasitoid of Drosophila and Tephritidae. We are currently rearing **all three** parasitoids for release against SWD.
- We have released 185 *Leptopilina japonica* at 7 locations in both Hod River and Wasco counties.

Methods

1. **Establish a colony of *Ganaspis brasiliensis* wasps**. Rearing protocols have been published by several authors (Rossi-Stacconi et al. 2022) and I have visited with ODA to see their colony rearing procedures (Figure 1). We spent year one trying to get sufficient numbers of wasps to conduct effective releases. Rearing these wasps is very labor intensive and requires daily maintenance including providing a supply of SWD larvae of specific age to adult wasps to parasitize. Mass rearing has not been perfected and lots of wasps die even with daily attention. Development time is slow and female wasps only produce a few dozen eggs in their lifetime. Wasps require host volatile cues of infested blueberries. Fresh blueberries kept in humid conditions tend to turn moldy quickly. A slight decrease in humidity causes blueberries to desiccate and dry up before SWD larvae and wasp parasitoids can develop.

We have been in contact with regional collaborators for tips on rearing protocols and help with reestablishing our collapsed colony. Both ODA and USDA have experienced similar struggles and loss of their colonies. No one in the PNW was able to help us reestablish our G. *brasiliensis* colony during the 2024 season. We continue to communicate with regional researcher partners and hope to re-establish this colony this winter.

We continue to build our colonies of the other two wasps (*Leptopilina japonica* and *Pachycrepoideus vindemiae*) as part of this same effort.

2. **Conduct releases of *G. brasiliensis* at selected orchards**. Several orchard locations have been selected and we are scouting for others. We have been collecting year zero data on the parasitoid wasp populations during the first year as we build the colony. Release sites will be selected based on suitable habitat outside cherry orchards where SWD populations are likely escaping pesticide sprays.

3. **Measure establishment of wasps**. In year three we will measure establishment of introduced wasps by collecting fruit from release locations. Fruit will be brought back to the lab and held to observe the emergence of flies and wasps. Emerged wasps will be sent for positive identification. We will describe the habitats where wasp establishment is most successful and report findings in extension and peer reviewed publications. The effectiveness of outside-orchard habitat supporting beneficial insects will be communicated to stakeholders at grower meetings.

Figure 1. *Ganaspis brasiliensis* rearing container (left). Blueberries are placed in SWD colony to allow flies to lay eggs. Blueberries with eggs and young SWD larvae are placed in containers with newly emerged wasps, *Ganaspis brasiliensis* (right) on a penny for scale.

Figure 2. Blueberries experiencing both desiccation (left) and mold (right). Because these wasps must be reared in fresh fruit, we struggle with both too much and too little humidity. Mold is the biggest challenge to rearing these wasps.

Results and Discussion

We had successfully started a colony of *Ganaspis brasiliensis*, however we lost our colony from excessive mold. Using fresh blueberries is a major bottleneck for this project and mold is the biggest hurdle to scaling this up. Despite our best efforts to keep the insectary clean mold spores are everywhere and it takes only a tiny amount of mold to mushroom into a huge problem. A regional working group is working on ways to improve rearing techniques and to develop an artificial diet to replace the need for fresh fruit.

We will continue to work with regional partners to help improve rearing methods and to help us rebuild our colony.

We will continue to rear additional parasitoid wasp to increase the probability of successful biological control in outside orchard habitats.

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

Report Type: Continuing Project Report

Note* - This is a winter project. We have just started the 3rd year of data collection.

Primary PI: Christopher Adams
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Address 2:
City/State/Zip: Hood River, OR 97031

Cooperators:
M3 Agriculture Technologies

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $43,952
Total Project Request for Year 2 Funding: $41,137
Total Project Request for Year 3 Funding: $42,254
Total Request: $127,343

Other related/associated funding sources: not funded
Agency name: Helium Foundation, IoT grant
Amount: $100,000
Funding duration: 2021 - 2023
Status: Preproposal approved and highly ranked. Leadership at the foundation changed while in review for the full proposal, and project was not funded.

WTFRC Collaborative Costs:

Budget 1
Primary PI: Christopher Adams
Organization Name: OSU
Contract Administrator: Charlene Wilkinson
Telephone: 541-737-3228
Contract administrator email address: Charlene.wilkinson@oregonstate.edu
Station Manager/Supervisor: Brian Pearson
Station manager/supervisor email address: brian.pearson@oregonstate.edu

Item	2021	2022	2023
Salaries[1]	$21,266.00	$21,904.00	$22,561.00
Benefits	$14,886.00	$15,333.00	$15,793.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies[2]	$6,800.00	$2,900.00	$2,900.00
Travel[3]	$1,000.00	$1,000.00	$1,000.00
Plot Fees			
Miscellaneous			
Total	$43,952.00	$41,137.00	$42,254.00

Footnotes:
[1]Adams lab Faculty Research Assistant at 0.40 FTE (2 days /wk), with 3% increase in years 2 and 3; OPE 70%
[2]Dragino LHT65 temp & humid sensor ($50 each) x 80, Long Range Transceiver ($450 each) x2
& Research consumables, SWD bait and traps (40 traps per rep x 3reps = 120 traps) bait replaced weekly for 24 weeks (Jul-Dec) aprox. $100 x 24 wk = 2400,
[3]Travel to field plots

Objectives

1) Collect micro-climate temperature data and trap for overwintering SWD. (Year 1 beginning fall/winter 2022)

   Progress: We established 4 orchard blocks in The Dalles and installed 20 remote temperature and humidity sensors in each, connected to a wireless network, paired with 20 insect traps. Blocks were between 5 and 10 acres. Because of early snow in 2022 we did not catch many flies in the first year. 2023 data showed flies in overwinter habitats just outside orchards. Habitats included conifers and oak fragments.

   Deviations: Because of an early snow in 2022 we collected very little catch data in the first year.

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

   Progress: Although we only have one year of trapping data, our data showed that in late season, catch data was highest on the borders of the orchard. Spotted wing drosophilas are either not surviving or are leaving the interior of managed orchards in the winter. The specific habitat (oak or conifer) did not seem to be determinative in predicting higher trap catch. We did not have enough oak fragment habitat to make a comparison. Microclimate temperatures and elevation were not correlated with higher overwinter trap catch.

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

   Progress: Erythritol (non-sugar sweetener) was applied as a non-pesticide winter control tactic in a separate un-funded on-farm field trial in The Dalles. Results were inconclusive. We plan to look at Combi-ProTec in combination with a pyrethroid or OP in subsequent experiments.

   Deviations: We had planned to target overwintering locations with bait sprays in the 3rd year of the project. However, because the early snow fall cut off trapping data in the first year, we would like to first collect two good seasons of trapping data before we attempt to target these populations with a spray. We will plan to target overwintering populations outside the managed orchard, in year four. This assumes that we see a consistent pattern in winter catch data this winter.

Significant findings

- We established 4 orchard blocks in The Dalles and installed 20 remote temperature and humidity sensors in each, with wireless a network, paired with 20 insect traps. (Figure 2). Winter micro-climate temperatures are not correlated with trap catch data.
- Snow cover (not cold weather) significantly affects SWD winter catch data (SWD activity).
- In year two we saw a pattern in all four orchards. Large numbers of SWD are present throughout managed orchards in late fall (October). By early November, the majority of catch data is recorded in traps just outside the border of the managed orchard, in oak or pine habitats. In some cases, “habitat” might just be 2 or 3 pine trees.
- It is not clear if SWD are *surviving* better in off site habitats or if they are *moving* to these habitats for shelter or food.
- There is a larger SWD population in the off season than during the growing season. This means that sprays applied at this time would produce a much greater return on investment (kills per dollar) than sprays applied during the growing season.

Brief Overview

The inspiration for this research came from visiting an orchard in The Dalles (Figure 1) and observing that there were no wild resources outside of the managed cherry orchard. The conventional wisdom is that SWD leave cherry orchards after harvest to find suitable overwintering sites. In addition, this area experiences high winds most of the year, so flies might struggle with directed migration, and might need to seek shelter in place, alternatively we might see catch increase in downwind populations. At the center of the orchard is a valley with some wild blackberry habitat. Our hypothesis was that overwintering SWD would move to the valley at the center of the orchard to seek shelter for the winter, or find microclimate locations with favorable conditions. To capture the microclimate across the orchard we partnered with M3 Agriculture Technologies to equip the orchard with 20 temperature and humidity readers and connected them to a central hub modem with cell phone connectivity for remote data access (Figure 1). Each temperature reader was paired with a baited trap. While catch data from the traps in the valley was the highest (>4000), other traps still caught SWD at very high levels (1000s). This would suggest that flies are not moving out of the orchard, but are seeking shelter within the orchard. How and where SWD overwinter, and what environmental conditions SWD need to survive is still unclear. Many questions remain to be answered about winter movement. This project was designed to expand this initial research project to three other orchards and try to replicate these results, to understand winter survival of this important invasive species.

Proposed Timeline (amended for winter control sprays in 2025)

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

Methods

1. Collect micro-climate temperature data and trap for overwintering SWD.

Site selection. In fall of 2022 four orchards were equipped with an array of temperature and humidity sensors and a central solar powered modem to collect and transmit data. Each temperature sensor was paired with a baited (apple cider vinegar and wine) cup trap. Traps were deployed in a grided pattern across each block, with the last row of traps on a border (where possible) outside the orchard.

SWD data collection. Traps were checked weekly or bi-weekly (every other week) from October through February in 2022/2023, and 2023/2024. Because of the volume of traps (80 traps spread across The Dalles) SWD numbers were estimated* when catch in the traps was high, e.g. above 100 (*note even numbers in catch data on maps of Fig.2). SWD parasitoids were also collected when they got caught in monitoring traps.

Temperature monitoring. Remote Sensors collect temperature and humidity data hourly and send data to the cloud for storage (Figure 1)

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

Data analysis. We tried to find correlation of temperature, humidity, elevation, and habitat with SWD trap catch data. Only habitat was correlated with high late season trap catch data.

3. Determine if overwintering SWD can be targeted with off season sprays or attractive baits. (*amended* to yr 4)

Insecticidal trails. Bait sprays will be applied in year four after (we hope) we see two consecutive years of catch data to document overwintering locations in all four blocks.

Results and Discussion

SWD catch data showed a similar pattern in most orchards (Figure 2). Traps hung at the edge of the blocks in pine, cedar, or along oak fragment habitat caught more flies in late winter. Often the catch in these border traps was in the thousands in November and December. In some cases, the "outside orchard" habitat was just a couple of pine trees within the larger orchard. A severe cold snap in January of 2024 did not completely shut down catch of SWD; catch followed a downward trend, but we still saw catch data in February (Figure 4). Snowfall did completely shut down all catch in traps early in the fall 2022.

There was some difference in catch data between orchards, with some blocks still catching hundreds of SWD in the interior of the block, while others dropped to zero within the cherry trees. Elevation and micro-climate humidity and temperature were not correlated with high catch data. High catch within the trapping grid did not correlate with prevailing winds at any of the locations.

Orchards 2, 3, and 4* had no other wild berries in the orchard or in the surrounding outside orchard habitat (*orchard 4 has a personal raspberry planting. A trap placed at this location was not the highest catching trap of that block). Orchard 1 had extensive wild Himalayan blackberry throughout the orchard that was *all* removed in the winter/summer of 2022, and currently has no more fruiting (non-cherry) food sources for SWD in or around the orchard.

We do not have any data to understand if flies are moving from the interior of the orchard to the pine of oak habitat where we get thousands of flies in the monitoring traps in November and December. There are no resources within the orchards post-harvest, other than a handful of fallen cherries. SWD has a *winter morph,* a slightly different form that is more robust looking, that is designed to over-winter. This winter-morph is thought to suppress reproductive activity, slow its metabolism and simply survive the winter. We do not know how the population goes from near zero (most monitoring traps in Wasco Co. catch zero during June, July, and August, see Fig. 5) to the thousands we find in November. It is likely that flies are reproducing across the landscape in Himalayan blackberries.

Additional work in 2025 will look at the final objective of applying an attractive bait spray that will last and be effective through the end of the calendar year to reduce overwintering populations. We will also request pesticide records to see if we can find any correlation with catch data and spray programs.

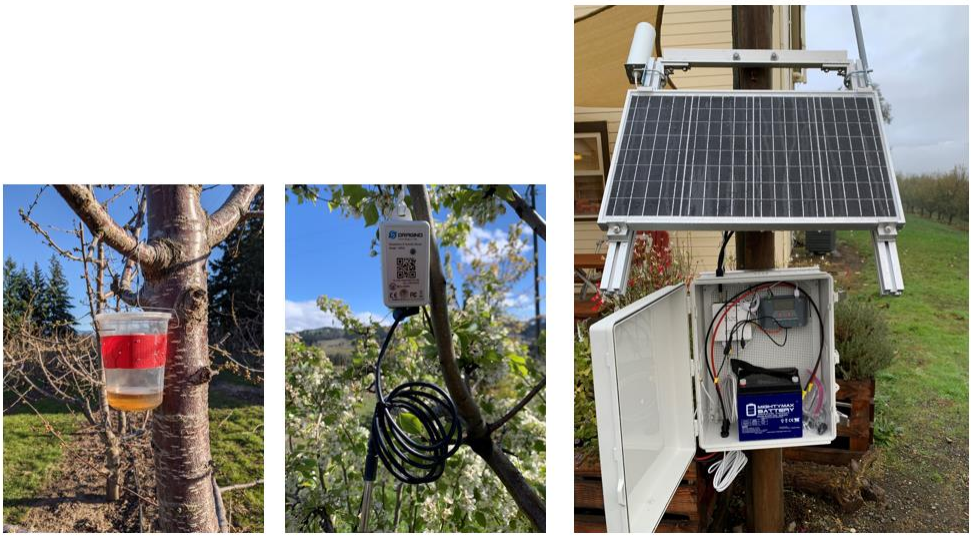
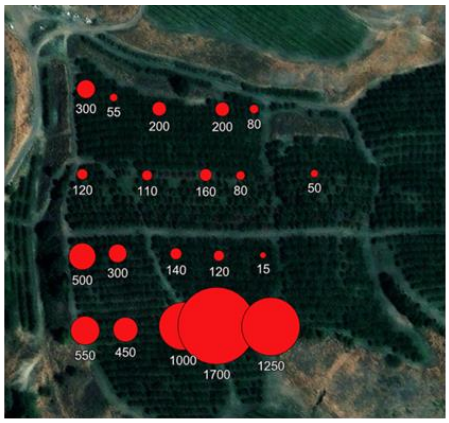
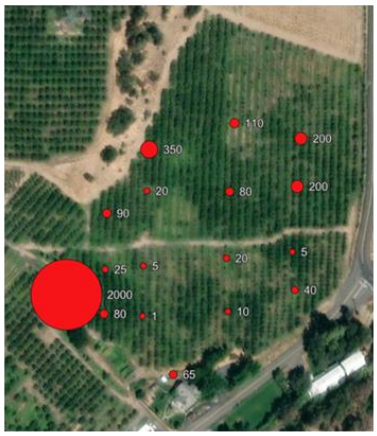


Figure 1. SWD trap, temperature sensor, and solar panel powered modem for data collection.

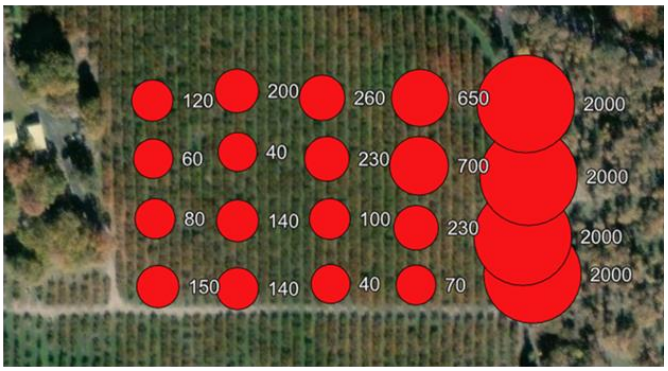


Orchard #1 – 11/21/2023

Orchard #2 - 12/9/2023



Orchard #4 – 11/27/2023



Orchard #4 – 11/13/2023

Figure 2. Catch data in the four orchard blocks in late November and early December of 2023. Red dots indicate locations of temperature sensors and SWD traps. Size of the red dots is relative to catch data. Numbers indicate estimated catch for the week. Blocks are between 5 and 10 acres with elevation change of 5 – 20 meters.

Figure 3. Visualization of hourly temperature data across block #4, showing variable temperatures across the orchard. Micro climate temperature and humidity did not correlate with high catch.

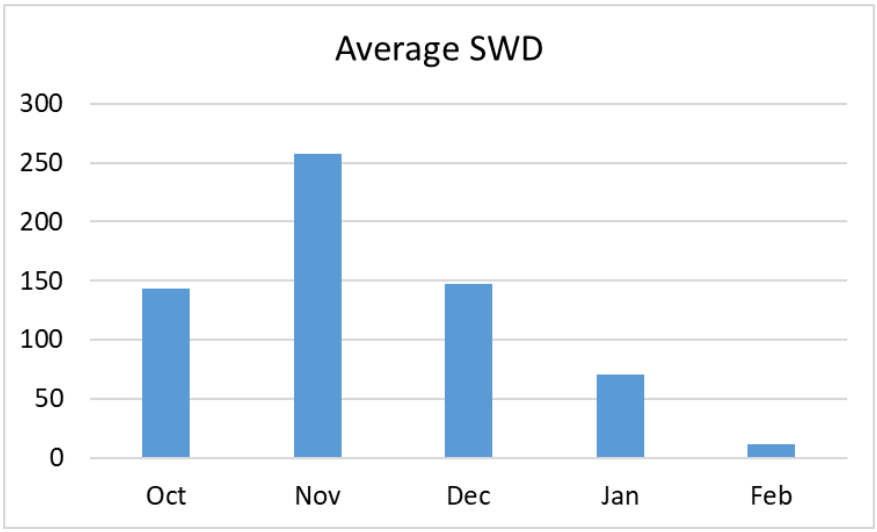


Figure 4. Winter 2023/2024, average catch per trap across all four sites (n = 80 traps) by month. The graph shows peek catch of SWD is in November. This indicates when a pesticide spray or an attract and kill bait would be most effective. This indicates where the control tool would need to be effective, not necessarily when it would need to be applied. For example, an attractive bait applied in September would need to still be viable and effective in November and December to target peek winter populations.

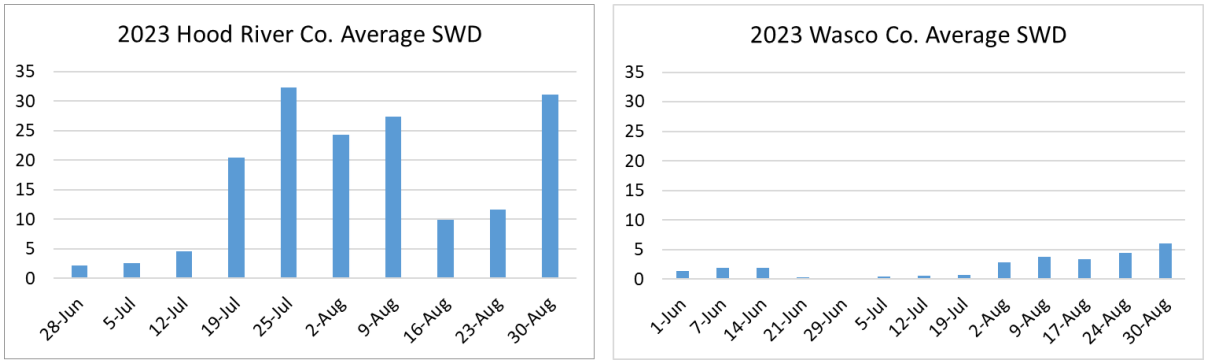


Figure 5. Summer 2023 average catch per trap data across our trapping network in both Hood River and Wasco counties. Data shown for comparison to winter catch (Figure 4). Prophylactic protective sprays are necessary when fruit is present on the trees and *must* be applied regardless of catch in monitoring traps. However, control tactics targeting off-season SWD could be much more impactful and possibly reduce the pressure/population in the following season much more that in-season sprays.

Project Title: Etiology of Cherry Cankers and Dieback in the Pacific Northwest

Report Type: FINAL Project Report (NCE 2024)

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Cooperators: Garrett Bishop, GS Long

Project Duration: 1-Year

Total Project Request for Year 1 Funding: $ 9960 (**NCE**)

Other related/associated funding sources: None

WTFRC Collaborative Costs: None

Budget 1

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Wages[1]	$591.00
Benefits	$59.00
RCA Room Rental	
Shipping	
Supplies[2]	$3,720.00
Travel[3]	$3,590.00
Plot Fees	
Miscellaneous[4]	$1,000.00
Total	$8,960.00

Footnotes: [1] = timeslip labor for media preparation; [2] = petri plates, growth media (2 types), antibiotics, and orchard tools; [3] overnight (Grove) and local travel (Grove and DuPont); [4] = autumn canker/ dieback workshop

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Salaries	
Benefits	
Wages	
Benefits	
RCA Room Rental	
Shipping	
Supplies[1]	$500.00
Travel[2]	$500.00
Plot Fees	
Miscellaneous	
Total	$1,000.00

Footnotes: [1] = lab, field, outreach costs; [2] = local travel

Objectives
A. Sample symptomatic cherry trees in multiple orchards in Eastern WA and OR, where dieback has previously documented and include additional orchards in other regions in the PNW (no more than 5 per orchards per county).
B. Remove bark on and adjacent to diseased tissue and (using magnification) search for fungal fruiting bodies that could enable field identification.
C. Isolate, purify, morphologically characterize and store fungal and bacterial isolates from symptomatic tissue using standard sterile microbiological techniques. Photographically document canker morphology and isolate growth habit/color on growth medium.
D. Summarize known information about cherry dieback in the PNW and distribute the information to industry.

Methods
Over 70 orchards were sampled in 2023 while 16 additional orchard were sampled in 2024. Samples were collected in Adams, Benton, Grant, Hood River, Okanogan, Chelan, Yakima, Franklin, Walla Walla, Klickitat, Douglas, and Wasco Counties (Table 1). Cankers and dieback were found in most locations. Bark on and adjacent to diseased tissue was removed and (using magnification) observed for fungal fruiting bodies that in some cases facilitated identification (Figures 6 and 7). Six to eight fragments (~8 mm; 0.31") from the margin between healthy and necrotic tissue (Figures 2 and 3) were be taken from each sample. Fungal isolation was performed by surface sterilization using 95% ethanol or 0.055 NaOCL followed by immediate flaming and subsequent placement onto Potato Dextrose Agar (PDA) and Malt agar (MA) amended with 100 ppm (0.1g/L) tetracycline and 2.5 ml / l lactic acid. Pure cultures of each fungal isolate were obtained using single hyphal tip sub-culturing methods on PDA and MA (Figure 4). Cultures were incubated at ambient temperature conditions in darkness and then photographed to document colony color and morphology.

Hyphal transfers from pure cultures were made to glass microscope slides and observed for conidia and conidiophore morphology at 40 - 250 X. If fruiting bodies were observed in pure cultures, representative samples were transferred to glass slides observed at 100 X to determine fruiting body and ascospore or conidia morphology.

Significant findings:
- Canker / dieback issues are widespread and quite severe
- Bacterial, Leucostoma (=Cytospora), Eutypa, Calosphaeria, and Botryosphaeria cankers were documented in the region
- Bacterial canker and Leucostoma cankers were the most frequently encountered
- Calosphaeria canker was documented in 12 orchards (10 of those 12 were in Oregon).
- Multiple instances of mixed infections were documented
- > 30 fungi distinct from those listed above were collected from diseased tissue and stored in long-term culture. These await positive identification and proof of pathogenicity.
- Fungal fruiting bodies were easier to detect during late summer /fall *in situ*

Results and Discussion

Leucostoma (=Cytospora) and bacterial cankers were documented in the late 20th century as the primary canker issues facing cherry growers in the Pacific Northwest (PNW). However, cankers of dieback of

cherry have become more prevalent over the last decade and in some cases appear to different from known diseases. A canker survey was conducted during the 2023 and 2024 growing seasons when 86 orchards were sampled (Table 1). Many of these sites had disease symptoms and signs different from those of a typical bacterial canker infection. Plant material was observed for disease signs (fungal reproductive structures; Figures 6 and 7) and then tissue was removed from the edges of cankers (Figures 1 and 2) and cultured (Figure 2) on potato dextrose agar (PDA) and malt agar (MA). Mycelium from the edges of colonies were transferred to PDA and MA and incubated 14-21 days in darkness at 22 C (71.6 F) to purify cultures (Figure 4).

Canker and dieback was widespread and in some sites *quite* severe, far more than what was apparent in similar surveys conducted in late 20th century. Both bacterial and Leucostoma cankers were quite common but cankers caused by Eutypa (Figures 1 and 2), Calosphaeria (Figures 6 and 7), and Botryosphaeria were also documented in the region. Mixed infections (bacterial canker + Leucostoma, Leucostoma+ Eutypa, and Leucostoma + Calospshaeria) were also discovered (Figures 4-5) in multiple regions. However, over 30 fungi distinct from *L. cinctum*, *E. lata*, *C. pulchella,* and *Botrysphaeria* were isolated from diseased tissues. The pathogenicity of these isolates is unclear as are their respective roles in the cherry canker complex. Aside from the notable prevalence of *C. pulchella* in Oregon, no discernable geographic patterns were observed for the distribution of the other fungal pathogens. Canker/dieback causation varied significantly within production regions (Figure 4).

Table 1. Number (out of 86 orchards sampled) and percentage of orchards affected by Leucostoma, Eutypa, Calosphaeria, Botryosphaeria, Pseudomonas, and mixed infections.

	Leucostoma (Cytospora)	**Eutypa**	**Calosphaeria**	**Botryosphaeria**	**Pseudomonas (bacterial canker)**	**Mixed Infections[3]**
Number of orchards affected[1]	41	8	10[2]	2	12	14
Percent of orchards sampled	47.7	9.3	11.6	2.4	14	16.3

Footnotes: [1] of 86 orchards sampled; [2]80% of these orchards were in Oregon; [3]Leucostoma: Eutypa, Leucostoma:Calofsphaeria, Leucostoma: Pseudomonas

Figure 1. Tissue was taken from the margins of diseased tissue and plate on potato dextrose (PDA) and malt (agar) amended with oxytetracycline and lactic acid.

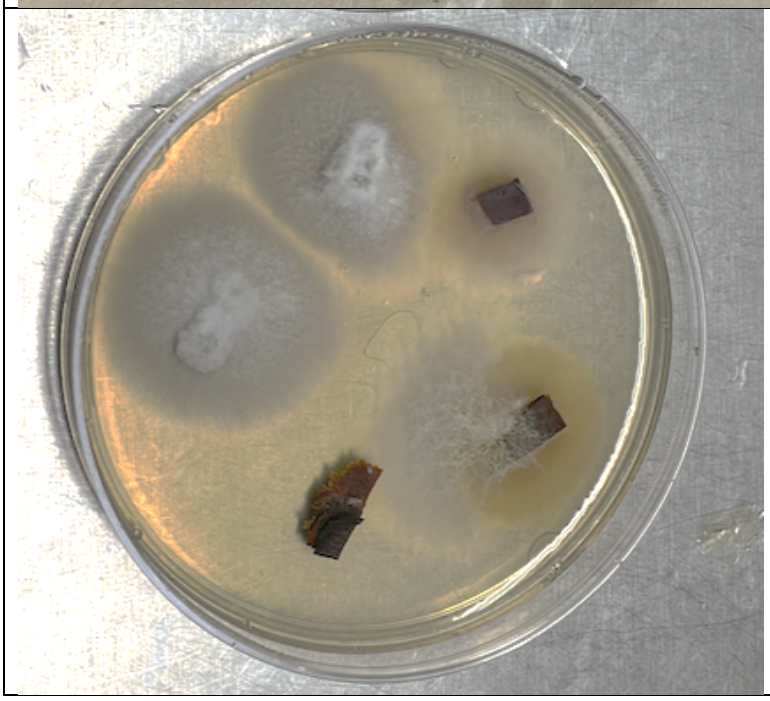

Figure 2. *Eutypa lata* growing from diseased cherry wood. Tissue was taken from the margins of diseased tissue and plate on potato dextrose (PDA) and malt (agar) amended with oxytetracycline and lactic acid.

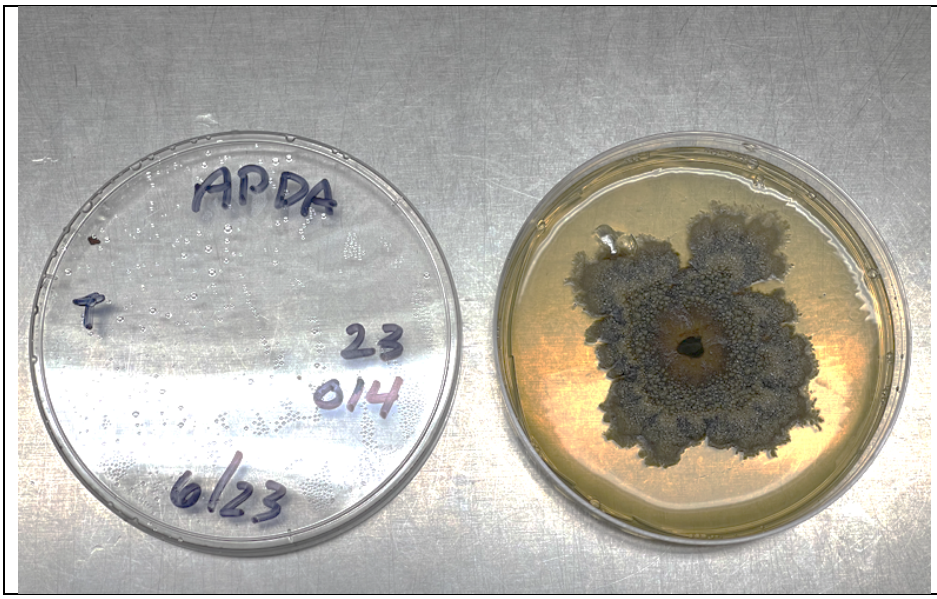

Figure 3. Leucostoma (=Cytospora) spp. Isolated from symptomatic "Chelan" trees near Wallula, WA. Isolate was obtained from a tree infected by both *Calosphaeria pulchella* and this isolate. See Figs. 6 and 7 below.

Figure 4. Results from various orchard sites in Franklin and parts of Adams and Walla Walla counties. Note that variation in causal agents and mixed infections.

	**Figure 5 (mixed infection).** Diseased Chelan tree near Wallula, WA. Tree is infected by both *Calosphaeria pulchella, Leucostoma* (=Cytospora) *cinctum,* and a second undetermined Leucostoma species (see Figure 4).
	**Figure 6**. Signs of *Calosphaeria pulchella*. Black areas are clusters of the sexual state (perithecia) of this pathogen.

Figure 7. Signs of *Calosphaeria pulchella.* Black-gray area is a clustes of the sexual state (perithecia) of this pathogen at higher magnification than in Figure 7. Each club-like structure is a perithecium (source of ascospores).

Extension. Current information on cankers of cherry was distributed via oral presentations at The Columbia Basin Tree Fruit Club (April 26, 2023), OSU Wasco County Preharvest Cherry Tour (June 6, 2023), Cherry Day in The Dalles (February 27, 2024), NCW Stone Fruit Day (January 17, 2027) Okanogan Horticultural Association Summer Field Day (August 3, 2023), Legacy Fruit Annual Meeting (March 14, 2024), G.S. Long Hood River Grower Meeting (January 5, 2024), and publications on the Tree Fruit Web Site / Fruit Matters:

Dupont, T., and Grove, G., and Thompson, A. 2023. Fungal Canker and Dieback Pathogens of Stone Fruit. *Fruit Matters*, August 2023. https://treefruit.wsu.edu/fungal-canker-and-dieback-pathogens-of-stone-fruit/.

Dupont, T., and Grove, G., and Thompson, A. 2024. Fungal Canker and Dieback Pathogens of Stone Fruit. *Fruit Matters*, August 2023. https://treefruit.wsu.edu/fungal-canker-and-dieback-pathogens-of-stone-fruit/.

Grove, G.G., and Sallato, B. 2023. The Fungi Among Us. *Fruit Matters*, August 2023. https://treefruit.wsu.edu/article/the-fungi-among-us/.

Executive Summary

A total of 86 orchards were sampled (70 in 2023, 16 in 2024) for wood dieback/cankers of *unknown (bacterial canker was not identified as the primary pathogen prior to the orchard visit)* etiology. Tissue samples were taken from the edges of cankers using a sterile razor blade, surface-disinfested using either sodium hypochlorite or ethanol / brief exposure to flame and placed on potato dextrose agar amened with tetracycline and lactic acid. Pure cultures of putative isolates were obtained by taking hyphae from the edges of fungal cultures and transferred to fresh PDA or malt agar plates. Colony morphology and the presence/morphology of fungal fruiting bodies and spore size/morphology were used to identify isolates. Leucostoma (=Cytospora) was isolated from samples taken from 47.7% of orchards sampled. Eutypa, Calorphaeria, and Botryosphaeria were isolated from samples taken from 9.3%, 11.6%, and 2.4% of orchards, respectively. Calosphaeria was the primary fungal pathogen isolated from Oregon orchards (9.3% of the aforementioned 11.6% of total orchards). Mixed infections were detected in 16.3% of the orchards sampled. Leucostoma and bacterial cankers were the most common. Numerous other fungi were isolated during the course of the study but their pathogenicity to cherry remains unclear; they are most likely saprophytic organisms common in diseased wood tissue. The epidemiology of Eutypa and Calosphaeria in the context of our regional climate and cultural practices needs further study.

Proposal Title: Investigating bacterial canker disease of cherry in young orchards

Report Type: Continuing Project Report

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Cooperators: Garrett Bishop (G. S. Long); Todd Cameron (Cameron Nursery); Bennett Mayo (Mike and Brian Nursery)

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $51,014

Total Project Request for Year 2 Funding: $52,575

Total Project Request for Year 3 Funding: $54,198

Other related/associated funding sources: None

WTFRC Collaborative Costs: None

Budget 1
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Station Manager/Supervisor: Naidu Rayapati
Station manager/supervisor email address: naidu.rayapati@wsu.edu

Item	1/3/2024	1/3/2025	1/3/2026
Salaries[1]	$28,800.00	$29,952.00	$31,150.00
Benefits[1]	$10,131.00	$10,536.00	$10,957.00
Wages			
Benefits			
RCA Room Rental			
Shipping[2]	$83.00	$87.00	$91.00
Supplies	$9,000.00	$9,000.00	$9,000.00
Travel	$2,000.00	$2,000.00	$2,000.00
Plot Fees	$1,000.00	$1,000.00	$1,000.00
Miscellaneous			
Total	$51,014.00	$52,575.00	$54,198.00

Footnotes: [1]Postdoc salary for 6 months at $4,800/month and postdoc benefit rate at 35.2%. 4% inflation for years 2 and 3.
[2]Shipping materials.

Objectives:

1. To determine the etiology of BCD and their copper and kasugamycin resistance status;

2. To survey nurseries and young orchards and investigate factors contributing to the outbreak of BCD;

3. To develop and recommend BMP for BCD in nurseries and young orchards.

Significant Findings:

- A total of 470 fluorescent pseudomonads isolates were collected from 73 orchards, representing 12 varieties of cherries;
- *Pseudomonas syringae* pv. syringae (Pss) is the main pathogen causing BCD of sweet cherries in WA; representing 75% of all isolates;
- *Pseudomonas amygdali* pv. morsprunorum (Pam) was found for the first time in WA to be the causal agent of BCD of sweet cherries; representing 5% of all isolates;
- The identify of about 94 isolates still needs to be determined, representing 20% of all isolates;
- New symptoms of BCD were found on cherries for the first time in summer 2024;
- Copper resistance among Pss and Pam is widespread. Resistance isolates were recovered from 72 orchards and 12 varieties of sweet cherries;
- Among all pathogenic isolates, 98%, 96.5% and 63% exhibited resistance to 1, 1.8 and 3 mM copper, respectively;
- Among 352 isolates of Pss, 100%, 99%, and 66% showed resistance to 1, 1.8 and 3 mM copper, respectively;
- Among 24 isolates of Pam, 70.8%, 58% and 20.8% exhibited resistance to 1, 1.8 and 3 mM copper, respectively;

- We found that 12 isolates of Pss and Pam were able to grow on plates containing100 ppm kasugamycin. However, after storage for a few months at -80ºC (-112ºF), the isolates no longer can grow at plates containing 100 ppm kasugamycin.
- We determined the minimal length of shoot below canker needed to be removed from infected trees;
- We conducted chemical efficacy tests on 200 cherry trees in field condition for protection of heading cut wound infection by BCD pathogen in early and mid-May. The experiments are still on-going and the results are still being analyzed.

Methods:

Procedures and Methodology:

Samples of diseased shoots (canker/ooze) from 12 different cherry varieties were collected from 73 sweet cherry orchards in WA/OR/CA, including newly planted and up to 10-year-old orchards. Samples were immediately put on ice and transferred to lab or stored in a cold room until further processing.

Bacteria were isolated on King's B (KB) medium at 82°F (28ºC) and slow-growing fluorescent *Pseudomonas*-like colonies were selected. Bacterial isolates were subsequently cultured routinely on KB medium and single colony was purified for each isolate and stored in 15% glycerol at -112°F (-80°C). Bacterial isolates were first identified using two sets of specific primers (*syrB, Psm1*) as *Pseudomonas* pathovars and positive PCR amplification from one or the other primer sets indicates the isolate is either *P. syringae pv.* syringae or *P. amygdali* pv. morsprunorum. All isolates were confirmed by conducting pathogenicity tests on immature green cherry fruits.

To determine the status of copper and kasugamycin resistance of the isolates, we first streaked each isolate in triplicate on mannitol glutamate (MG) medium and MG amended with copper sulfate (MGcu) at 250 ppm (1 mM copper concentration). Copper-resistant isolates were then streaked in duplicate on copper-amended MG plates with copper concentrations ranging from 1, 1.8 and 3 mM. Similarly, kasugamycin resistance status was determined by streaking isolated on KB medium amended with 100 ppm kasugamycin.

200 cherry young trees, cv. Coral Champagne, were purchased from nurseries and planted in greenhouse and field plots in Roza in April 2024. Head cutting experiments were conducted during early May, characterized by sunny, dry, and hot weather, and late May, marked by rainy, humid, and wet conditions (**Fig. 1**). Nine treatments included lime sulfur (T1), actigard (T2), tetracycline (T3), latex paint (T4), latex paint + badge X2 (T5), badge X2 (T6), kasugamycin (T7), control with head-cut and bacterial inoculation (T8), and control with head cut without bacterial inoculation (T9) (**Fig. 2**). In both early and late May, treatments were applied either pre-inoculation or post-inoculation (**Fig. 1**). Cut sites were either inoculated with a rifampicin-resistant isolate of *Pss* (S2 Rif^R) or left uninoculated as a negative control. Canker length and ooze formation on each tree was recorded at week 3, 5, and 8 after inoculation and treatment. Cankers were removed again after 8 weeks in July and dissected for final measurement of canker length. Then, trees without cut wound treatment were monitored for canker development once a month until May 2025. The experiment is still on going and the results are currently being analyzed.

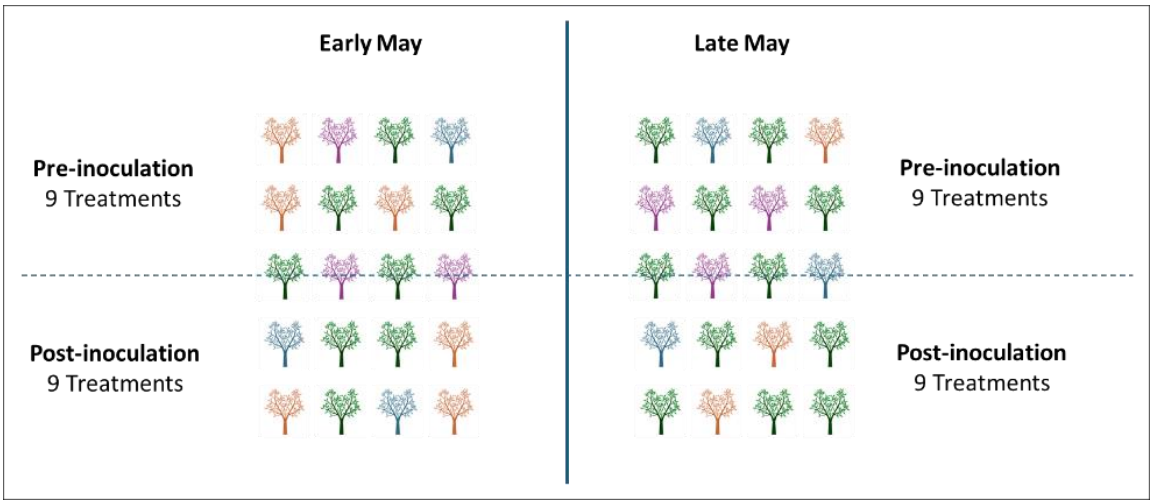

Fig. 1. Experiment design of field assay in early and mid-May.

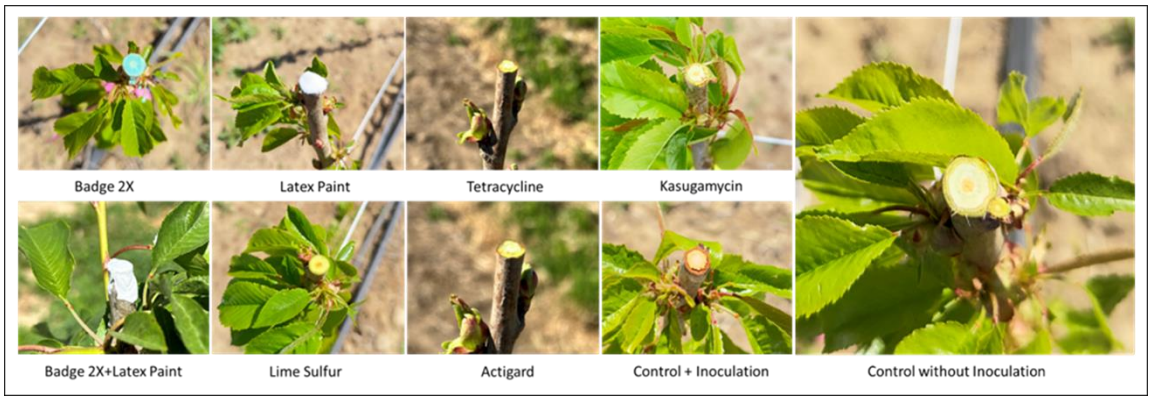

Fig. 2. A total of nine treatments were conducted as above**.**

To answer one of the questions growers asked: how far to cut below cankers. Samples with obvious canker symptoms collected from our cut wound experiments above as well as greenhouse inoculated trees after heading cut and canker formation were used to isolate bacteria from 0.5, 1.5, 3 and 5 inches below the obvious canker symptoms. Isolated bacteria were confirmed by PCR.

Based on our initial observation and results, we worked with nurseries and consultants to promote our recommendations, including limited copper usage, drop irrigation, clean cultivation of row middle, no paper tree guards (protectors), and gave presentations at the 81st cherry institute, 2023 and 2024 WSTFA annual meeting, three GS Long field Staff and growers meetings as well as numerous field trips. We also published our initial findings in scientific journals, i.e. Plant Disease and Good Fruit Growers.

Types and timing of anticipated results: We determined the major pathovars and species of pseudomonads present in WA, and their status of copper and kasugamycin resistance. We found that copper resistance is widespread at very high level (3 mM). We also determined that more than 3-inch cut below canker is required to remove the pathogen from cankers.

Potential problems or limitations: We did not encounter any problems and limitations so far as the method is technically straightforward.

Results and Discussion

Findings during the prior year(s) of the project:

In the PNW, we had experienced a worst outbreak of bacterial canker disease (BCD) in 2023 during the past 40 plus years, which led to tree death up to 40% to 70% in severe cases in WA and OR, especially during the first 2 years after planting. In WA and OR, the main symptom of BCD included canker with ooze (gummosis) and dead bud, leading to tree death (**Fig. 3, left**); Another major issue was canker development after heading cut in the spring. In worst cases, canker length reached 10 to 15 inches within one month after cutting (**Fig. 3, right**). In 2024, BCD was not as bad as in 2023, but we observed never-reported symptoms in first-year orchards in the summer (**Fig. 4**). Young succulent shoots developed fire-blight like shoot blight symptoms with ooze (**Fig. 4 left**) or shoot tips were turning brown and dead (**Fig. 4, middle**). However, these symptoms were not observed in less vigor shoots (**Fig. 4, right**). These symptoms were observed in several orchards in Pasco and Matawa and nitrogen fertilizer was applied almost every week before symptoms appeared. Later, fluorescent pseudomonads were isolated from these samples and confirmed to be Pss.

Fig. 3. Representative of typical canker, dead bud, and gummosis symptoms of sweet cherries in young orchards (left) and newly planted orchards (right) in WA**.**

Fig. 4. New disease symptoms of bacterial canker disease of sweet cherries in newly planted orchards (left & middle) and control healthy plants (right) in summer 2024**.**

We collected diseased samples of 12 cherry varieties (mostly Coral Champagne) from 73 orchards in WA and OR and isolated a total of 470 fluorescent pseudomonads isolates. These isolated were characterized by PCR to be either *Pseudomonas syringae* pv. syringae (Pss) or *Pseudomonas*

amygdali pv. morsprunorum (Pam) by PCR (**Fig. 5, left top**). Among the 470 isolates, 75% (352) were Pss, whereas 5% (24) were Pam (**Table 1**). However, we were not able to determine the identity of those 20% (94) isolates. Pathogenicity tests on immature cherry fruits confirmed all Pss and Pam isolates are pathogenic, causing either necrotic lesions or whole fruit decays (**Fig. 5, left bottom**). These results suggest that Pss is the main pathogen causing BCD of sweet cherries in WA, while Pam was found to be the causal agent of BCD of sweet cherries in WA for the first time.

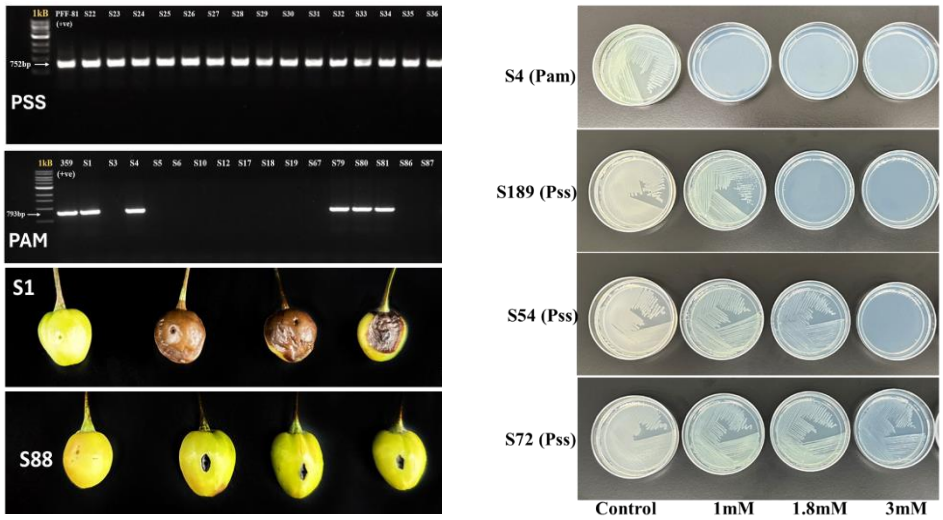

Figure 5. PCR confirmation of BCD pathogens and pathogenicity tests on immature cherry fruits (left); representative of BCD pathogen isolates that are resistant to different levels of copper (right).

We then screened these 376 isolates for their resistance to copper and kasugamycin (**Fig. 5, right**). We found that 98%, 96.5% and 63% of these isolates exhibited resistance to 1, 1.8 and 3 mM copper, respectively (**Fig. 6, left; Table 1**); and those resistant isolates were recovered from 72 orchards on 12 varieties of sweet cherries. Among 352 isolates of Pss, 100%, 99%, and 66% showed resistance to 1, 1.8 and 3 mM copper, respectively; whereas, 70.8%, 58% and 20.8% of the 24 Pam isolates exhibited resistance to 1, 1.8 and 3 mM copper, respectively (**Fig. 6, right; Table 1**); These results suggest that copper resistance among Pss and Pam is widespread in WA and precautions should be taken whether to apply copper for control BCD or not. However, we initially found that 12 isolates of Pss and Pam were able to grow on plates containing100 ppm kasugamycin. After storage for a few months at -80ºC (-112ºF), these isolates no longer can grow at plates containing 100ppm kasugamycin.

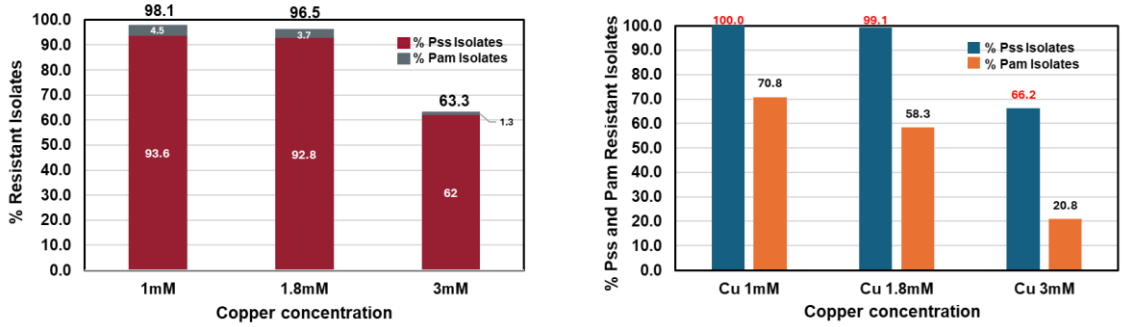

Figure 6. Percentage of BCD pathogens that are resistant to different levels of copper

Table 1. **Numbers of BCD pathogenic isolates that are resistant to different levels of copper**

1mM (MGY)	1.8mM (MGY)	3mM (MGY)	Pss	Pam	Total	Isolates (%)
-	-	-	0	7	7	1.9
+	-	-	3	3	6	1.6
+	+	-	116	9	125	33.2
+	+	+	233	5	238	63.3
			352	24	376	100.0

Chemical efficacy tests were performed on 190 cherry trees which were planted in an orchard in April 2024, for protection of head cut wound in early and mid-May. We selected to conduct filed experiments in early May 2024 (sunny, dry, and hot weather) and late May 2024(rainy, humid, and wet conditions) and to compare the infection rate and disease severity. A total of nine treatments, including lime sulfur (T1), actigard (T2), tetracycline (T3), latex paint (T4), latex paint + badge X2 (T5), badge X2 (T6), kasugamycin (T7), control with head-cut and bacterial inoculation (T8), and control with head cut without bacterial inoculation (T9), were compared both pre-inoculation and post inoculation. Since the data are still being analyzed, we will present these data next year. After rating the disease severity after 8 weeks, we then determined the minimal length of shoot below canker needed to be removed from infected trees (**Fig. 7, right**). Among 38 trees analyzed, we isolated Pss back 100%, 97% and 18% at 1, 1.5 and 3 inches, respectively, but none at 5 inches (**Fig. 7, left**). These results suggest that more than 3 inches of shoot needed to be cut in order to remove all the pathogens in the canker.

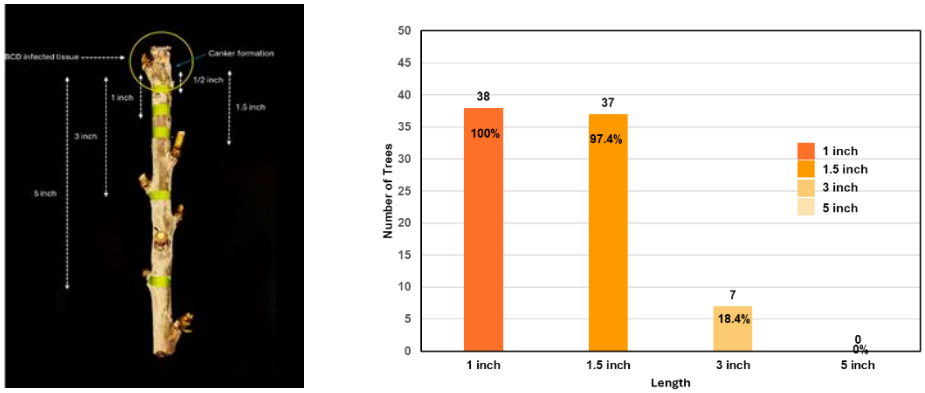

Figure 7. Minimum inches of shoot below canker need to be removed to be free of BCD pathogen

Based on our initial observation and results, we worked with nurseries and consultants to promote our recommendations, including limited copper usage, drop irrigation, clean cultivation of row middle, no paper tree guards (protectors), and gave presentations at the 81st cherry institute, 2023 WSTFA annual meeting, three GS Long field Staff and growers meetings as well as numerous field trips. We also published our initial findings in scientific journal Plant Disease.

Significance to the industry and potential economic benefits:

The outbreak of BCD in 2023 has caused significant economic losses to growers in WA, including a total removal of 24-acres of five-old orchard and a 100% infection rate of 13-acres newly planted orchard, which probably will be replanted. One major challenge in effectively managing BCD

is that the pathogen can survive on leave surface of host plants and weeds as epiphyte and its ice nucleation activity, which led to frost damage as a predisposing factor of infection. In addition, the leading commercial cultivar Coral Champagne since 2012 is the most susceptible one to BCD infection. Furthermore, growers mostly rely on copper for BCD management. However, we do not know whether copper resistance exists in WA orchards or not as copper spray may result in more BCD due to pathogen resistance, leading to control failure. In the current year, we determined the major pathovars and species of pseudomonads causing BCD in WA. We also found that copper resistance is widespread at very high level in WA. In addition, we determined that more than 3-inch cut below canker is required to remove the pathogen from cankers. Therefore, our findings are significant and could provide direct economic benefits to growers and the cherry industry by limiting copper use and reducing cost. Our result not only provides the industry with a standard for removal of cankers, but it will also save money and time for both growers and nurseries by avoiding copper as a main chemical for control. We recommend that due to copper resistance, growers should rely more on cultural control measures for management of BCD, including but not limited to timely prune cankered limbs under warm and dry conditions, remove wild Prunus species, keep middle row free of weeds for the first three years, paint trunks to prevent winter damage, avoid all types of injury, esp. frost damage, use dripping instead of sprinkler system for irrigation, and promote early defoliation in the fall etc. In terms of new symptom observation in 2024, we think proper fertilization is critical to avoid new shoot infection in summer.

Project Title: Evaluation of UV-C for management of cherry diseases and pests

Report Type: Continuing Project Report

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Cooperators: None

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 12,292
Total Project Request for Year 2 Funding: $ 12,757
Total Project Request for Year 3 Funding: $ 13,245

Other related/associated funding sources: USDA-ARS In-House Funding
8080-21000-032-000-D Integrated production and automation systems for temperate fruit crops.
Funding Duration: Jun 16, 2020 – Jul 15, 2025
Amount: 422,774
Agency Name: USDA ARS
Notes: In-house USDA ARS funding will be used to cover cost of permanent technician time and growth chamber costs.
WTFRC Collaborative Costs: None

Budget 1
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Item	2024	2025	2026
Salaries	$6,544.00	$6,871.00	$7,215.00
Benefits	$2,748.00	$2,886.00	$3,030.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$3,000.00	$3,000.00	$3,000.00
Travel			
Plot Fees			
Miscellaneous			
Total	$12,292.00	$12,757.00	$13,245.00

Footnotes:

OBJECTIVES

1) Evaluate cherry fungal pathogens and spotted wing drosophila (SWD) sensitivity to UV-C (254nm) or far UV (222nm) light in direct laboratory assays.
In this objective, cherry fungal pathogens and SWD will be exposed to UV-C (254nm) or far UV (222nm) in a laboratory setting to determine the minimum duration and dose required to kill the pathogen or pest.

2) Evaluate control of fungal rots and SWD on sweet cherry fruit after of UV-C (254nm) or far UV (222nm) light treatment.
In Objective 2, sweet cherry fruit will be harvested at commercial maturity and inoculated with fungal pathogens or fruit will be held with mature female SWD or infested with SWD eggs prior to treatment with UV-C or far UV. We will measure incidence of disease or SWD oviposition, and egg and larval survivorship after treatment to determine the optimal dosage and exposure time to eliminate disease and pests without damaging the fruit.

3) Evaluate UV-C or far UV-C treatments in combination with biological control products on sweet cherry fruit.
Antagonists or biocontrol products such as Bio-Save (*Pseudomonas syringae*) will be applied after UV exposure to fill in the microbial void left after irradiation. We will measure incidence of disease after treatment to determine if UV treatments are more effective when used in combination with biological control products.

Proposed Timeline

Objective	2024	2025	2026
1. UV-C and far UV direct laboratory assays	X	X	
2. Evaluate control of fungal rots and SWD on fruit	X	X	X
3. Evaluate UV in combination with biological control on fruit		X	X

Significant Findings

- UV-C (254nm) treatment of *Monilinia fructicola* for 60 seconds in plate assays reduced the growth of the fungus by 27% and treatment for 120 seconds completely abolished growth when a high concentration (1 x 10^4 conidia/mL) of the pathogen was used.
- There were no significant differences observed in fruit weight, firmness, or color for cherry cultivars ‘Regina’ and ‘Rainier’ after treatment with UV-C (254nm) for 60 seconds.
- UV-C (254nm) treatments of 120 seconds or less did not reduce the survivorship of adult male or female SWD.

METHODS

Objective 1: Evaluate cherry fungal pathogens and spotted wing drosophila (SWD) sensitivity to UV-C (254nm) or far UV (222nm) light in direct laboratory assays.

Cherry Fungal Pathogens
M. fructicola, the causative agent of brown rot, was grown on potato dextrose agar (PDA) media and evaluated with direct UV-C exposure to assess pathogen sensitivity to UV treatment. UV-C treatment was conducted using a conventional germicidal UV-C lamp with maximum emission at 254 nm (55-W Phillips Model TUV PL-L, Phillips North America Corp., Andover, MA USA). The UV-C lamp was mounted 30 cm from the targeted irradiation surface. Conidia of *M. fructicola* was harvested

from 5 to 10-day old cultures grown on PDA with 0.05% Tween 20 by gently scraping culture surfaces with a glass rod and filtering through two layers of cheese cloth. The pathogen suspension was adjusted to a concentration of 1 x 10^3 or 1 x 10^4 conidia/ml using a hemocytometer. 10 ul of the conidia suspension were spotted in the center of a 100 mm diameter Petri dish containing PDA. Plates were randomized then placed on trays by treatment group, with 3 plates per treatment group. Plates were exposed to UV-C for 0, 30, 60 or 120 seconds then incubated at 25°C for seven days. The growth of the pathogen on each plate was measured at 5- and 7-days post treatment. The entire experiment was repeated twice.

In year 2, we plan to perform direct plate assays with additional isolates of *M. fructicola* collected in 2024 from sweet cherry fruit in a commercial orchard as well as isolates of *Alternaria alternata*, the causative agent of Alternaria rot. We will also add additional treatment time points between 60 and 120 seconds to determine the minimum duration of UV-C exposure needed to prevent growth of the pathogens. Far UV treatment will also be tested using a 500-W lamp (Sterilray.com, Somersworth, NH USA) with a maximum emission at 222 nm.

Spotted Wing Drosophila (SWD)
SWD were exposed to UV-C to assess the lethal dose requirement for adults. In this case, 5 mature adult females or males were held in each container. There was a total of 10 replicates (each with 5 SWD) per treatment group. SWD were exposed to UV-C for 0, 15, 30, 45, 60, or 120 seconds. Survivorship was assessed 4, 24, and 48 hours after treatment. Adults not exposed to UV-C treatments served as a control.

In year 2, we plan to expose newly deposited SWD eggs to UV-C (254nm) and SWD adults and eggs to far UV. Survivorship will be assessed at 4, 24, and 48 hours after treatment. Newly deposited eggs recovered from standard and drosophila diet cups will be transferred to filter paper. Five eggs will serve as a replicate. Following the same exposure intervals described for adults, eggs will be transferred back to diet cups with hatching tracked for up to five days. Adults and eggs not exposed to UV-C and far UV treatments will serve as controls.

Objective 2: Evaluate control of fungal rots and SWD on sweet cherry fruit after of UV-C (254nm) or far UV (222nm) light treatment.

‘Rainier’ and ‘Reginia’ sweet cherry fruit was harvested from a local commercial orchard. Fruit that was free from damage was randomized and sorted into experimental treatment groups. Ten fruits were used per treatment group. To test the effect of UV-C on fruit quality, fruit was treated with UV-C light for 60 seconds, and fruit not exposed to UV-C was used as a control. Fruit was weighed before and 4 or 24 hours after treatments. Fruit firmness was also measured 4 or 24 hours after treatment for UV-C and control non-treated groups using a fruit penetrometer.

For pathogen inoculations, fruit was rinsed with tap water, surface sanitized with 70% ethanol for 1 min and rinsed three times with sterile water. Fruit was arranged on sterilized test tube racks with 10 fruit per treatment group and allowed to air dry in a laminar flow hood overnight. Fruit was wounded with a sterile sewing pin to a depth of 3 mm and inoculated with 10 µL of *M. fructicola* Mf-7 conidia suspension (1 x 10^4 conidia/mL) or sterile water for non-inoculated controls. One hour post inoculation, fruit was irradiated with UV-C for 0, 15, 30, 45, or 60 seconds. After irradiation, fruit was placed in plastic fruit bin and kept in the dark for 4 hours. After the dark period, lids were removed, and fruit bins were covered with saran wrap and incubated in a humid environment at room temperature for 7 days under continuous light. Disease incidence was determined after 7 days as percentage of infected fruit.

In year 2, we plan to begin experiments with SWD on fruit. Each replicate will be comprised of five sweet cherry fruit held in individual small screened containers with two mature female and male SWD. Fruit will be irradiated using the same treatments and intervals as described above. Following the treatment, adult survivorship will be assessed at 0, 4 and 24h later, and fruit will be examined for evidence of oviposition. Fruit will be held for up to two weeks to assess infestation using standard salt water techniques. Unexposed fruit in absence of SWD will serve as a control.

Objective 3: Evaluate UV-C or far UV-C treatments in combination with biological control products on sweet cherry fruit.

Work on objective 3 will begin in year 2. We plan to acquire sweet cherry fruit from a local wholesale market and/or commercial orchard at commercial maturity and wound inoculate with fungal pathogens ad described in objective 2. Infected fruit will be irradiated after infection for 0-120 sec based on results from objectives 1 and 2. After irradiation, solutions containing biocontrol agents such as Bio-Save (*Pseudomonas syringae*), *Aureobasidium pullulans*, or *Metschnikowia fructicola* will be applied to fill in the microbial void left after irradiation. At least three replicates of 10 cherries per treatment group will be used. After treatment, fruit will be covered and incubated in a humid environment for 7 days. Disease incidence will be measured after 5 and 7 days. Disease severity will be rated based on the percentage of cherry surface covered by fungal mycelia (0, uninfected cherry; 1, surface mycelia just visible to 25% of the cherry surface; 2, 26% to 50% of the cherry surface covered with mycelia; 3, 51% to 70% of the cherry surface covered with mycelia; and 4, >75% of the cherry surface covered with mycelia). Experiments will be conducted over two years to account for annual differences in weather, natural disease pressure, and cherry fruit quality.

Problems/Limitations Encountered

- We did not get consistent infection in untreated cherry fruit when we wound inoculated with *M. fructicola* Mf-7 isolate in year 1, so we could not compare disease incidence and severity in UV-treated and untreated fruit. In year 2, we plan to use a different wounding tool to get more consistent infections in fruit. We have also acquired additional *M. fructicola* isolates collected from infected cherry fruits in local commercial orchard in 2024. Inoculation methods and *M. fructicola* isolates will be tested on immature cherry fruit sourced from trees in research blocks at our station to refine the infection protocol before commercial cherry harvest next year. Since we have planned to do fruit inoculations over all 3 years of the proposal, we anticipate still being able meet the proposed goal of 2 years of fruit inoculation data in year 2 and year 3.

RESULTS & DISCUSSION

Objective 1: Evaluate cherry fungal pathogens and spotted wing drosophila (SWD) sensitivity to UV-C (254nm) or far UV (222nm) light in direct laboratory assays.

Cherry Fungal Pathogens

In the direct plate assays, we saw a reduction of *M. fructicola* growth on PDA plates after UV-C treatment of 30, 60 or 120 seconds compared to untreated (0 second) controls at both low (1×10^3) and high (1×10^4) concentrations of the pathogen. In plate assays with a high concentration of the pathogen (1×10^4) treatment with UV-C for 60 seconds reduced growth by 27%, and no growth was observed on plates exposed to UV-C for 120 seconds (Figure 1). The UV-C treatment time to prevent growth of *M. fructicola* (120 seconds) was longer than was previously observed for *Botrytis cinerea* (45 seconds), *Penicillium expansum* (30 seconds), and *Colletotrichum* spp. (30-60 seconds) using the same light source (Janisiewicz et al 2021). In year 2, we will repeat the direct plate assays with additional timepoints between 60 and 120 seconds to determine the minimum exposure time needed to prevent growth of *M. fructicola*.

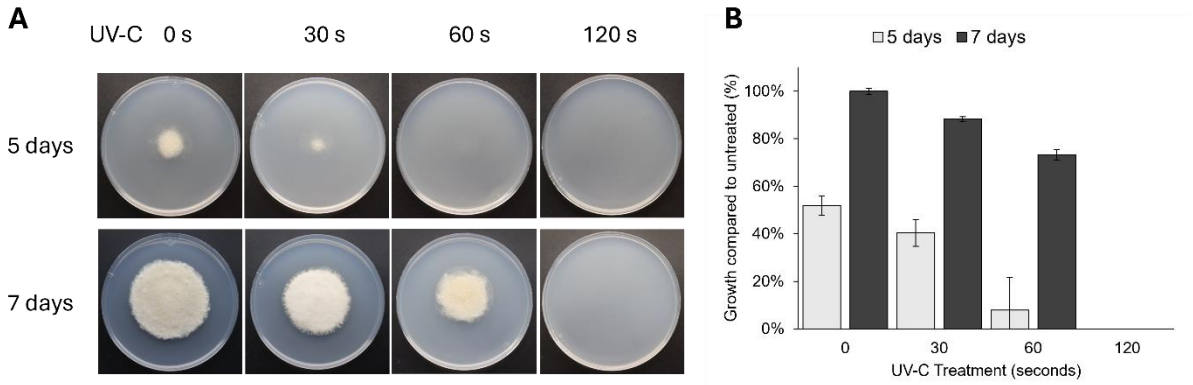

Figure 1: A) Representative images of *Monilinia fructicola* at a concentration of 1 x 10^4 conidia/mL on potato dextrose agar media after 0, 30, 60 or 120 seconds of UV-C treatment. Images were taken at 5 or 7 days after UV-C treatments. **B)** Bars represent the mean of three replicates ± standard error. Growth of *M. fructicola* is relative to the control treatment of 0 seconds at 7 days post treatment, which was set at 100%.

Spotted Wing Drosophila (SWD)

Adult male or female SWD were exposed to UV-C for 0, 15, 30, 45, 60, or 120 seconds. Survivorship was assessed 4, 24, and 48 hours after treatment. Adults not exposed to UV-C treatments served as a control. In all treatment group >88% of males and >96% of females survived 48 hours post treatment. A statistically significant difference between male and female survivorship was only observed at one timepoint (30 seconds), but no significant differences between males and females were observed at longer durations of UV-C treatment (Figure 2). UV-C treatments of up to 120 seconds did not decrease SWD survivorship. Efforts in year 2 will focus on evaluating the effect of UV-C on SWD eggs and the effect of far UV (222nm) light on SWD adults and eggs.

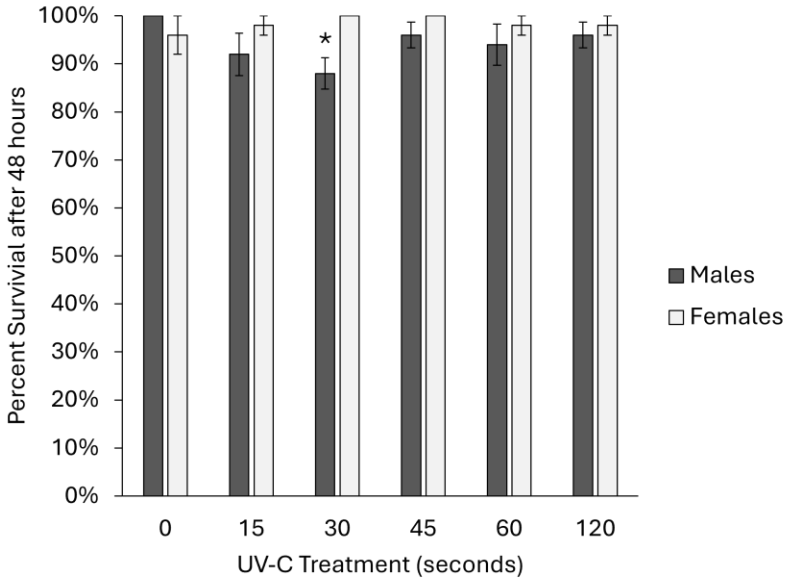

Figure 2: Bars represent the mean of 10 replicates, each containing 5 male (black bars) or female (gray bars) SWD ± standard error. Survivorship was determined 48 hours after exposure to UV-C light (254nm) for 0, 15, 30, 45, 60, or 120 seconds.

Objective 2: Evaluate control of fungal rots and SWD on sweet cherry fruit after of UV-C (254nm) or far UV (222nm) light treatment.

'Rainier' and 'Regina' fruit was harvested in June 2024 from a local commercial orchard. Fruit was treated with UV-C for 60 seconds and compared to control fruits that were not exposed to UV-C. Fruit was weighed before and after treatments. Fruit firmness was also measured 4 or 24 hours after treatment for UV-C and control non-treated groups using a fruit penetrometer. No significant differences were observed for UV-C treated fruits compared to non-treated controls for 'Rainier' or 'Regina' cultivars (Figure 3). Efforts in year 2 will include testing longer durations of UV-C exposure effect on fruit to determine if the doses needed to prevent growth of fungal pathogens has any adverse effects on fruit quality traits in different cherry cultivars.

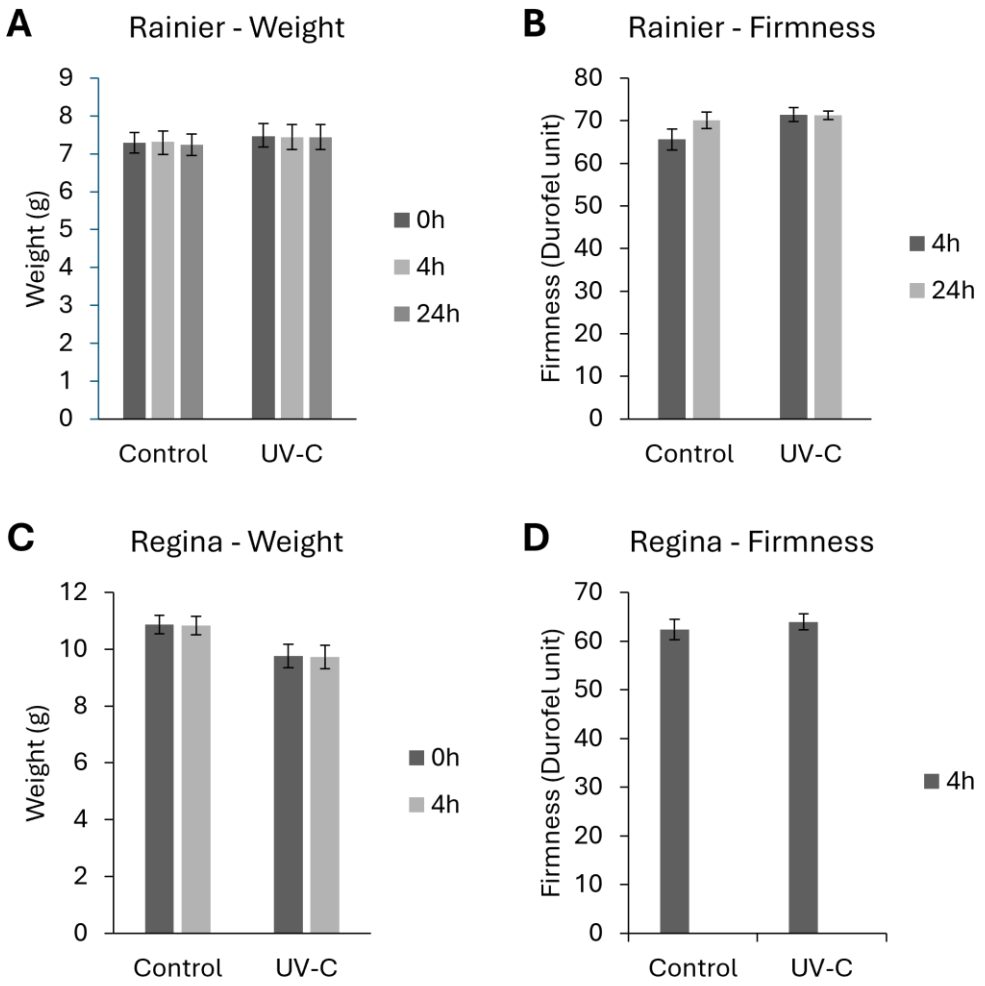

Figure 3: Fruit weight and firmness was measured in control (non-treated) and 60 second UV-C treated fruit for 'Rainier' (A, B) and 'Regina' (C, D) cultivars. Bars represent the mean of 10 replicates ± standard error.

In year 1, ‘Rainier’ and ‘Regina’ fruit were wound inoculated with a *M. fructicola* Mf-7 conidia suspension (1 x 10^4 conidia/mL) or sterile water for non-inoculated controls. One hour post inoculation, fruit was irradiated with UV-C for 0, 15, 30, 45, or 60 seconds. Fruit was incubated at room temperature in a humid environment. However, after 7 days we saw no fungal infection in any of the treatments including the control fruit which were not exposed to UV-C light. To troubleshoot our infection protocol, additional *M. fructicola* isolates were collected from sweet cherry fruit in 2024. Since these isolates were collected from the same cherry cultivars we are using for experiments, they may be more aggressive on cherry than our lab strain *M. fructicola* Mf-7. In year 2, these new *M. fructicola* isolates will be evaluated for consistent disease progression in cherry fruit that is not treated with UV light. We also plan to try different wounding tools, and an increased concentration of the pathogen (1 x 10^5 conidia/mL) to optimize our infection protocol. Additionally, we will use the knowledge gained this year in objective 1 and in experiments planned to be conducted in the winter of 2024-2025 to optimize the duration of the UV-C or far UV treatment used in fruit experiments for year 2 and 3.

Project Title: Understanding Food Safety Risks During Post Harvest Cherry Production

Report Type: Continuing Project Report

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Cooperators: Four cherry packinghouses in Washington, the identity of the operations serving in cooperator roles will be kept confidential.

Project Duration: 3 Years

Total Project Request for Year 1 Funding: $74,255
Total Project Request for Year 2 Funding: $76,231
Total Project Request for Year 3 Funding: $69,785

WTFRC Collaborative Costs:

Item	2024	2025	2026
Salaries	$3,900.00	$4,056.00	$4,218.00
Benefits	$1,218.00	$1,267.00	$1,317.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies			
Travel			
Plot Fees			
Miscellaneous			
Total	**$5,118.00**	**$5,323.00**	**$5,535.00**

Footnotes:
Salary/benefits: an estimate of the percent of time spent for Mendoza (4%) at a 31.2% benefit rate and 2% annual increases.

Budget 1

Primary PI: Claire Murphy
Organization: Washington State University Irrigated Agriculture Research and Extension Center
Contract Administrator: Hollie Tuttle
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Contract administrator email address: prosser.grants@wsu.edu
Station Manager/Supervisor: Naidu Rayapati
Station manager/supervisor email address: 509-786-9215

Item	2024	2025	2026
Salaries	$32,981.00	$34,300.00	$35,672.00
Benefits	$2,848.00	$2,848.00	$2,848.00
Wages	$9,955.00	$10,353.00	$10,767.00
Benefits	$1,353.00	$1,407.00	$1,463.00
RCA Room Rental			
Shipping			
Supplies	$20,500.00	$20,500.00	$13,000.00
Travel	$1,500.00	$1,500.00	$500.00
Plot Fees			
Miscellaneous			
Total	**$69,137.00**	**$70,908.00**	**$64,250.00**

Footnotes:
Salaries: $32,981, $34,300, and $36,672 are requested in years 1, 2, and 3, respectively, for a graduate research assistantship for a Ph.D. student to work on all objectives
Benefits: $2,848 is requested in years 1, 2, and 3 for benefits tied to the graduate research assistantship
Wages: $9,955, $10,353, and $10,767 are requested in years 1, 2, and 3, respectively, for summer student hourly employees to assist with sample collection and conducting experiments related to all three objectives
Benefits: $1,353, $1,407, and $1,463 are requested in years 1, 2, and 3, respectively, for benefits tied to the hourly employees
Supplies: $20,500, $20,500, and $13,000 are requested in years 1, 2, and 3, respectively, to purchase disposal supplies such as glassware, inoculation loops, pipette tips, sponge swabs, microbiological media, Petri dishes, PCR reagents, etc.
Travel: $1,500, $1500, and $500 are requested in years 1, 2, and 3, respectively, for milage and travel associated cost adhering to all university policies

Objectives:

1. Evaluate the prevalence of *Listeria* spp., as an indicator for *L. monocytogenes*, on food contact surfaces on the packing line (e.g., conveyor belt surfaces, stainless steel, etc.) within commercial cherry packinghouses at three time points (beginning, middle, and end of the season) over two packing seasons (2024 and 2025).
2. Evaluate the microbiological status of postharvest water and cherries to understand the change in microbial status from receiving to packing over a packing day.
3. Evaluate the risks associated with hydrocooling during laboratory-simulated hydrocooling with water contaminated with generic *E. coli* or *L. monocytogenes* at varying levels (i.e., low and high contamination) under a variety of relevant industry conditions, including cherry physiology state (i.e., cracked/split cherries and whole/intact cherries), contact time, and water temperatures.

Significant Findings:

Objective 1:

- Among 960 food contact surfaces samples tested in year one, 1.9% (n=18) were positive for *Listeria* spp.
- Bristle flaps (6.9%) followed by solid conveyor belts (4.2%) demonstrated the highest prevalence of *Listeria* spp. among food contact surfaces.
- The prevalence of *Listeria* spp. on FCS increased throughout the packing season
- The prevalence of *Listeria* spp. was generally higher during processing compared to pre-processing, suggesting that cleaning and sanitation efforts are effective, and that *Listeria* spp. is likely introduced with the incoming product.
- Only 1 sampling site, out of 160, tested positive more than once, suggesting that when present, *Listeria* spp. is transient within the packinghouse, unable to establish a persistent presence.

Methods:

Objective 1 (Years 1 and 2)

Study Design: Four commercial apple packinghouses were recruited into this study and swabs of food contact surfaces were collected from each of the packinghouses during three separate visits (beginning, middle, and end of the season). The food contact surfaces for sampling were selected at each operation prior to the first sampling event. Sampling sites for each packinghouse were photographed and described in detail to ensure consistency during all sampling events. Samples were collected prior to the start of packing as well as 5 hours in packing. Sampling was conducted during the 2024 season and will be repeated during the 2025 season.

Sample Collection: Swab samples were collected from each site using sponge sticks that are pre-moistened with 10mL of Dey Engley neutralizing broth. Each sponge stick was aseptically removed from the bag with sterile gloves, used according to the manufacturer's specifications: returned to the bag, sealed, and placed in a cooler (on ice). All samples were processed within 24h at the Murphy Bio-Safety Level 2 Laboratory at Washington State University's Irrigated Agriculture Research and Extension Center.

Sample Processing: The isolation, detection, and confirmation of *Listeria* spp. was conducted following a modified FDA Bacteriological Analytical Manual method (FDA, 2022). Briefly, 90 mL

of buffered *Listeria* enrichment broth was added to each sample bag, stomached, and incubated at 30ºC. Following a 4h incubation period, a 360µL aliquot of *Listeria* selective enrichment supplement was added to each bag and returned to the 30ºC incubator for 20h. At both 24h and 48h, the enriched sample was sub-streaked onto modified oxford agar and incubated for 48h at 30ºC. Presumptive positive colonies were re-streaked onto modified oxford agar and incubated for 48h at 30ºC before being PCR-confirmed.

Objective 2 (Year 2):

Protocol development: A protocol for validating, verifying, and monitoring postharvest water systems and produce through the postharvest water system was developed at WSU. The protocol is set up so that once validated by the team at WSU, it can be created into an extension document that producers can follow to use within their system, including what samples to collect, what to ask a third-party lab for, and how to analyze data. To generate feedback on the protocol, it has been sent to extension and industry professionals to provide feedback on the layout, feasibility, etc.

Sample collection and processing: Triplicate water samples (100 mL) and cherries (whole) will be collected from each distinct water system in the postharvest process until recirculated water is changed. Water and produce samples will be tested for *Enterobacteriaceae*, total coliforms, and generic *E. coli*. Water physicochemical properties and antimicrobial levels in water will be monitored throughout the sampling day as well. Trends in microbial populations in water and on produce throughout the production run will be evaluated to determine the microbial target(s) and physiochemical parameters that best correlate with water quality. For validation, populations of target microorganisms must remain the same or decrease compared to Time 0.

Objective 3 (Years 2 and 3)

Study Design: Simulated cherry hydrocooling trials will be performed in duplicate, at minimum, for each combination of the following experimental variables treatments: (i) bacteria (generic *E. coli*, *L. monocytogenes*) (ii) inoculum [low (3 log CFU/mL) and high (6 log CFU/mL)], (iii) water temperature [2°C (35.6°F) and 6°C (42.8°F)], (iv) contact time (5min and 15 min), and (v) physiological state (cracked and whole). To simulate the maximum field temperatures of cherries exposed to direct sunlight prior to harvest, fruits will be equilibrated to 40°C (104°F) before use. Temperatures and times are subject to change, based on input from stakeholders, to ensure industry-relevant conditions. For each treatment combination option, cherries will be immersed in simulated hydrocooler water. After immersion, cherries will be removed from the inoculum tank.

Sample processing: For evaluating cross-contamination on the surface of cherries during hydrocooling, cherries will be placed into a stomacher bag with 1:10 mL (weight/volume) of peptone water and hand-rubbed for 90 min to detach cells for cherry surfaces. The rub solutions will be 10-fold serially diluted and surface on duplicate appropriate agar plates.

Results and Discussion

The prevalence of *Listeria* spp. on food contact surfaces was accessed in four Washington State cherry packinghouses during the 2024 packing season. *Listeria* spp. was isolated from all four packinghouses during the 2024 packing seasons. Among all tested samples during the 2024 packing season, 1.9% (18/960) were confirmed positive for *Listeria* spp. (Table 1). Of the 14 food contact

surface types sampled, *Listeria* spp. was most frequently isolated from bristle flaps (6.9%; 5/72), followed by transfer points (2.3%; 3/132) and stainless-steel flumes (1.9%; 3/156; Table 1).

Only a handful of previous studies have examined the prevalence of *Listeria* spp. on food contact surfaces (zone 1) of whole fresh market produce packinghouses. On tomato packing lines in Tennessee, 10.9% (62/565) of food contact surfaces sampled from three packinghouses were positive for *Listeria* spp. (Hamilton, 2018). A previous study assessing the prevalence of *Listeria* spp. in apple packing houses in Washington state over two seasons found the prevalence on food contact surfaces to be 4.6% (136/2,988; Ruiz-Llacsahuanga et al., 2021), with the greatest prevalence of *Listeria* spp. on polishing brushes (19.6%), dividers under fans/blowers (17.4%), dryer rollers (10.5%), and brushes under fans/blowers (9.7%). This previous study in Washington noted that *Listeria* spp. was most frequently isolated from the wax coating unit operation (Ruiz-Llacsahuanga et al., 2021). Unlike apples, cherries are not waxed and do not utilize brushes throughout the packing line. However, the bristle materials that make up many of the brushes within the apple packing line are used in cherry packing lines as a way to help direct the flow of fruit as the cherries move on the line. Food contact surfaces that utilize this bristle material can retain debris and moisture, thus maintaining an environment that can support *Listeria*. In a two-year study of *L. monocytogenes* prevalence in three tree fruit packinghouses on non-food contact surfaces (i.e., zones 2, 3, and 4), it was found that equipment under brush beds (e.g., drip pans, drip pan drainage funnels, brush scraper bars, structural supports, and floors) had the highest prevalence of *L. monocytogenes* (Simonetti et al., 2021). This research by Simonetti et al. underscores the difficulties posed by bristle materials on the packing line, showing that the accumulation of moisture and debris may lead to contamination on adjacent surfaces within the facility. Given that bristles are frequently recognized as common harborage sites for *Listeria* spp. in tree fruit operations due to their complex hygienic design, the cherry industry should prioritize mitigation strategies for these materials. This includes replacing them with alternative materials when feasible, regularly cleaning and sanitizing them, and replacing them when they show signs of wear.

TABLE 1: Frequency of isolation of *Listeria* spp. by food contact surface during the 2024 packing season

Food Contact Surface	No. of Total Samples	No. of Positive Samples	Frequency
Bristle Flap	72	5	6.9%
Cherry Elevator	84	1	1.2%
Cluster Cutter	42	0	0.0%
Interlocking Conveyor Belt	114	0	0.0%
Packing Guide	30	1	3.3%
Plastic Flap	12	0	0.0%
Plastic Guide Rails	66	0	0.0%
Sizing Rollers	42	1	2.4%
Solid Conveyor Belt	72	3	4.2%
Sorter Cups	48	0	0.0%
Sorter Flap	42	1	2.4%
Stainless Steel Flume	156	3	1.9%

Textured Conveyor Belt	48	0	0.0%
Transfer Point	132	3	2.3%
Total	960	18	1.9%

In addition to food contact surface types, *Listeria* spp. prevalence was evaluated based on three operation units and it was found that *Listeria* spp. was most frequently isolated from the packing unit operation (3.75%; 9/240; Table 2). Thus, when time is limited between operational runs, sanitization should focus on the packing step, which accumulates organic matter and moisture without the benefit of antimicrobial agents that are used in flumes.

TABLE 2: Frequency of isolation of *Listeria* spp. by processing step

Processing Step	Percentage (%) of Samples Positive		
	Pre-processing	**In-processing**	**Total**
Washing	2.2% (1/45)	0.0% (0/45)	1.11% (1/90)
Sorting	1.3% (4/315)	1.3% (4/315)	1.3% (8/630)
Packing	0.8% (1/120)	6.7% (8/120)	3.75% (9/240)

In addition to the food contact surface and unit of operation, the timing of sample collection had an impact on *Listeria* spp. prevalence. In pre-processing samples, 1. 3% (6/480) were positive for *Listeria* spp. compared to twice as many positive samples five hours into processing (2.5%; 12/480). *Listeria* spp. isolation was also evaluated based on whether the sampling site (i) tested positive pre-processing and negative in-process, (ii) tested negative pre-processing and positive in-process, or (iii) positive both pre-processing and in-process (Table 3). The majority of positive samples tested negative pre-processing and positive in-process (64.7%), while 29.4% tested positive pre-processing and negative in-process (Table 3). However, 1 sampling site, a transfer point, did test positive both pre-processing and in-process during the end-of-the-season sampling event of the 2024 cherry season. Pre-processing sampling occurred after any cleaning and/or sanitation events but before the product was run for the day, which provides an indication of the effectiveness of the cleaning and sanitation. The higher prevalence of *Listeria* spp. during processing compared to pre-processing and only 1 sampling site testing positive both pre-processing and in-process most likely indicates that cleaning and sanitation is relatively effective and that incoming crop is cross-contaminating the FCS. Previous research in apple packinghouses in Washington (Ruiz-Llacsahuanga et al., 2021), a stone fruit packinghouse in Virginia (Bardsley et al., 2024), and an avocado processing facility in South Africa (Strydom et al., 2016) also found that *Listeria* spp. prevalence was greater during the in-process sampling.

TABLE 3: Frequency of *Listeria* spp. isolation for a specific sampling site based on sampling time

Scenario	Operational Timepoint		No. of Sites	Frequency (%) (n = 17)[a]
	Pre-processing	**In-processing**		
1[b]	Positive	Negative	5	29.4%

2[c]	Negative	Positive	11	64.7%
3[d]	Positive	Positive	1	5.9%

[a] Total number of sampling sites with at least one positive detection of *Listeria* spp. From the total of positive samples (n = 18), 1 sampling site belonged to scenario 3, thus n = 17
[b] Sampling sites where *Listeria* spp. was detected only in pre-processing samples
[c] Sampling sites where *Listeria* spp. was detected only in in-processing samples
[d] Sampling sites where *Listeria* spp. was detected in both pre-processing and in-processing samples

Lastly, the prevalence of *Listeria* spp. on food contact surfaces was observed during the end-of-the-season sampling event, compared to the beginning and middle (Figure 1). Of the 18 positive samples, only 1 was collected at the beginning of the season, 1 during the middle of the season, and 16 during the end of season sampling (Figure 1). Over the course of the short cherry packing season, food contact surfaces and equipment may accumulate organic materials, biofilms, or residues that create an environment where *Listeria* spp. can thrive. By the end of the season, if the cumulative effects of organic residue, biofilm formation, and insufficient cleaning have occurred, the risk of contamination on food contact surfaces is greater.

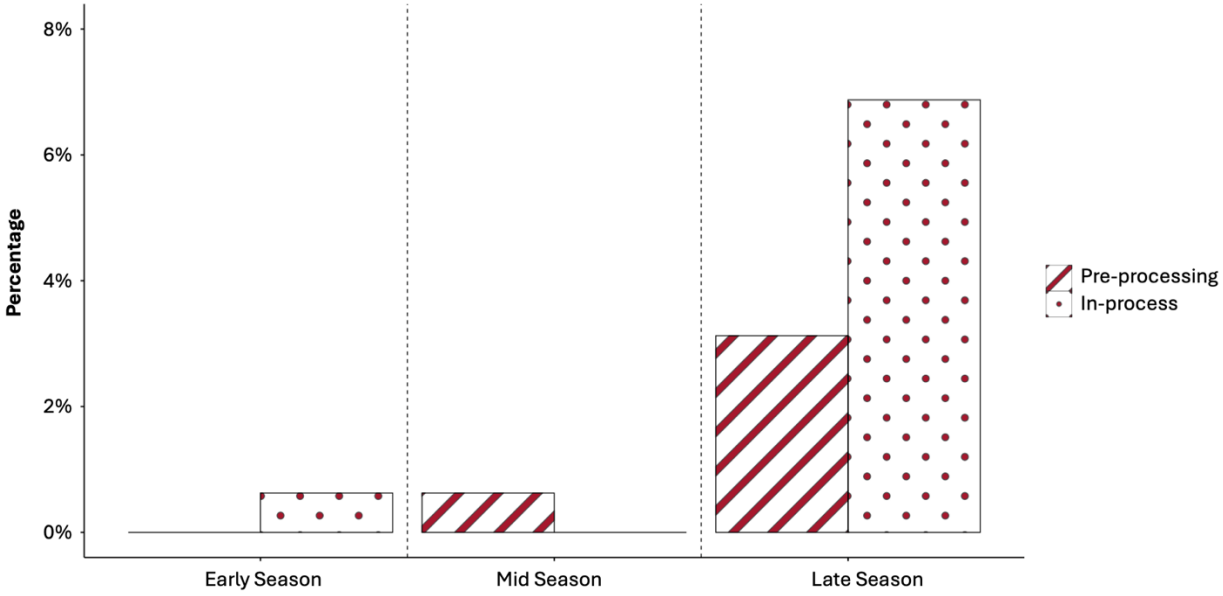

FIGURE 1: Prevalence of *Listeria* spp. by operational timepoint and packing season timepoint.

References:

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Simonetti, T., Peter, K., Chen, Y., Jin, Q., Zhang, G., LaBorde, L.F. and Macarisin, D., 2021. Prevalence and distribution of *Listeria monocytogenes* in three commercial tree fruit packinghouses. *Frontiers in Microbiology*, *12*.

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Project Title: Pesticide residues of PNW cherries
Report Type: Continuing report

Primary PI: Tory Schmidt
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Cooperators: Gerardo Garcia (WTFRC), Northwest Horticultural Council, Pacific Agricultural Labs (Sherwood, OR), Gale Fource Orchards

Project Duration: 3 Years

Total Project Request for Year 1 Funding: $ 6000
Total Project Request for Year 2 Funding: $ 6250
Total Project Request for Year 3 Funding: $ 6500

Other related/associated funding sources: Most chemical products donated by registrants

Primary PI: Tory Schmidt
Organization Name: WTFRC
Contract Administrator: Paige Beuhler
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Contract administrator email address: paigeb@treefruitresearch.com

Item	2023	2024	2025
Salaries			
Benefits			
Wages1	$1,500.00	$1,600.00	$1,700.00
Benefits1	$800.00	$850.00	$900.00
RCA Room Rental			
Shipping2	$400.00	$425.00	$450.00
Supplies	$300.00	$300.00	$300.00
Travel3	$1,000.00	$1,025.00	$1,050.00
Plot Fees			
Miscellaneous			
Analytical lab fees	$2,000.00	$2,050.00	$2,100.00
Total	$6,000.00	$6,250.00	$6,500.00

Footnotes:
Schmidt estimates 8% of his time is dedicated to this project on an annual basis
Most pesticides tested are donated by their registrants or an ag chemical supply company
1 Wages & benefits primarily for Garcia (spray applications), crew help for Garcia, and Stone (data entry & review)
2 Est. costs to ship cherries overnight to Sherwood, OR
3 Travel costs include hauling equipment to & from plots

2024 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 https://nwhort.org/export-manual/.

Spraying new trial block

TRIAL DETAILS

- New trial block established in mature 'Skeena'/K.6 central leader trees on 10' x 16' spacing near East Wenatchee, WA
- 11 insecticides/acaricides & 3 fungicides applied at or near maximum rates and minimum pre-harvest and re-treatment intervals; products were applied twice if allowed by labels
- Applications made by Rears PakBlast PTO-driven airblast sprayer with 8 oz non-ionic surfactant (Regulaid)/100 gal water at 200 gal water/acre
- A total of roughly 0.06" of rain fell on the trial block on June 2 & 3; this precipitation was unlikely to affect residues on the fruit
- Fruit samples shipped overnight to Pacific Agricultural Labs (Sherwood, OR) for chemical analysis

RESULTS & DISCUSSION

Through the years, the primary objective of these studies has been to simulate a *worst case scenario* for residues of legally applied pesticides by using aggressive rates, timings, and spray intervals. As in the past, 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 2024 residues complied with domestic tolerances but some exceeded foreign MRLs for important export markets**:

Insectides/acaricides: Bexar, Esteem, Asana XL, Carbaryl 4L

Fungicides: Torino, Miravis

Dried residues on cherries at harvest

It should be noted that some key export markets in Asia have adopted MRLs for several popular pesticides set at the Limit of Quantitation (LOQ), or the lowest amount of residue that can be reliably measured; these low tolerances make it virtually impossible to meet these standards if the product applied leaves any residue at all. On a positive note, compliance with MRLs on some products continues to get easier as those tolerances relax or are posted for the first time rather than relying on default values. These positive developments are due in part to the efforts of the Northwest Horticultural Council to encourage regulators around the world to adopt and publish reasonable pesticide residue standards for imported Northwest cherries. MRLs are known to change frequently and cherry producers should routinely monitor the most current information (https://nwhort.org/export-manual) to facilitate compliance with constantly evolving foreign standards.

Measured residue levels vs. MRLs for pesticides applied to cherry fruit at 200 gal water/acre. 'Skeena'/K.6, East Wenatchee, WA. WTFRC 2024.

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*
tolfenpyrad	Bexar	27 oz	28, 14	**0.19**	2	0.01 (many)
pyriproxyfen	Esteem	16 oz	28, 14	**0.31**	1	0.01 (THA)
thiamethoxam*	Actara	5.5 oz	21, 14	0.106	0.5	0.5 (many)
esfenvalerate	Asana XL	14.5 oz	21, 14	**0.044**	3	0.01 (THA)
chlorantraniliprole	Altacor	4.5 oz	21, 10	0.15	2.5	0.5 (KOR)
cyclaniliprole	Verdepryn 100SL	11 oz	14, 7	0.059	1	0.6 (TWN)
cyflufenamid	Torino	8 oz	14, 7	**0.073**	0.6	0.01 (THA)
flonicamid	Beleaf 50SG	2.8 oz	14, 7	0.24	0.6	0.6 (many)
emamectin benzoate	Proclaim	4.8 oz	14, 7	<0.01	0.09	0.005 (AUS,THA)
carbaryl	Carbaryl 4L	96 oz	10, 3	**2.6**	10	0.01 (THA)
pydiflumetofen	Miravis	5.1 oz	10, 1	**0.12**	2	0.01 (JPN)
mefentrifluconazole	Cevya	5 oz	10, 1	0.25	4	4 (many)
hexythiazox	Onager	24 oz	7	0.18	1	0.2 (KOR)
pyrethrins	Pyganic 5.0EC	15.6 oz	3, 1	<0.05	1	0.01 (THA)

[1] All materials were applied by Rears PakBlast sprayer with 8 oz Regulaid/100 gal water

[2] 14 August 2024. http://mrldb.nwhort.org/#top_markets

[3] Major export markets for Pacific Northwest cherries; 14 August 2024. http://mrldb.nwhort.org/#top_markets

* Reported thiomethoxam values reflect sum total of thiomethoxam and clothiandin residue levels

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

WASHINGTON TREE FRUIT RESEARCH COMMISSION

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

Report Type: Final Project Report

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

Project Duration: 3 Year

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

Other related/associated funding sources:

Awarded
Amount: $458,022
Funding Duration: 2020-2024 (No-cost extensions in 2022, 2023)
Agency Name: WTFRC/OSCC
Notes: "Understanding little cherry disease pathogenicity". PI. Scott Harper. Co-PIs: Alice Wright, Per McCord

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

WTFRC Collaborative Costs: None

Budget 1
Primary PI: Per McCord
Organization Name: Washington State University
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Contract administrator email address: arcgrants@wsu.edu
Station Manager/Supervisor: Naidu Rayapati
Station manager/supervisor email address: naidu.rayapati@wsu.edu

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

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

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

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

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

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

Original Objectives

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

Significant Findings

- An average of 285 Phase 1 seedlings evaluated in the fruit lab each year (2022-2024). **Three Phase 1 seedlings were advanced** to Phase 2 trials.
- Phase 2 trials (planted 2021) harvested for two seasons (will be one more in 2025)
    - Most promising are selections R47, CR01T078, and R35B
    - A new Phase 2 location added near Naches (Valley View) to focus on late-season selections
- Phase 3 selections **R19 and R3** harvested for two seasons (will be one more in 2025)
    - **R19** ('Chelan' timing) performed well in 2024, **expected to be released** as new cultivar as early as 2025
    - **R3** ('Black Pearl' timing) performed well in 2024, **expected to be released** as new cultivar (pending sufficient performance in 2025) as early as 2025
- Optical sorter (single-lane Tomra InVision2) installed in 2023, used for grading fruit from Phases 2 and 3 for 2023 and 2024 seasons
    - Sorter maps developed for both harvest and postharvest grading
    - **Labor savings → 2 fewer summer employees in future years**
- ~**3100** seedlings planted in Phase 1 field2022-2024. DNA tests for self-fertility, powdery mildew resistance, low cracking, and high firmness were used to eliminate **>1200** seedlings prior to planting, with expected **saving of ~$47,000** during Phase 1 compared to planting all seedlings
- Seed production (2022-2024) totaled 27,325, with 99% from targeted bi-parental crosses
- PCR testing for Little Cherry Disease (183 samples in 2024) identified 6 weakly-infected trees, which will be removed during fall/winter
- PCR pathogen testing of breeding parents in 2024 identified higher incidence of Prune dwarf virus and Prunus necrotic ringspot virus than in prior years

Results and Discussion

Breeding Pipeline (Objective 1)

In 2021 (the year prior to this project), 3 selections had been advanced and planted to Phase 3 (larger scale pre-commercial trials), and 6 selections had been advanced and planted to Phase 2 (small replicated trials) (Table 1). These selections began to fruit in 2023 and have now been evaluated for two of the three seasons slated for Phase 2 and 3 trials.

Table 1. List of cherry selections planted in 2021 Phase 3 and Phase 2 trials.

Phase	Selection	Market Class	Status
3	R19	Early Mahogany	Under evaluation
"	R3	Early Mahogany	Under evaluation

“	R29	Midseason Mahogany	No data yet (delays in tree establishment); first harvest 2025
2	R46	Early Mahogany	Dropped in 2024 (soft fruit)
“	R47	Early Mahogany	Under evaluation
“	CR01T078	Midseason Mahogany	Under evaluation
“	R50	Midseason Mahogany	Dropped prior to 2023 (small fruit)
“	R45	Mid-late Mahogany	Under evaluation
“	R35B	Mid-late Blush	Under evaluation

R19 is early ripening (‘Chelan’ timing), but self-fertile with larger fruit, greater firmness, and higher SSC (°Brix) than ‘Chelan’. Although color and sizing was poor in 2023 (likely due to the shortened development time caused by a late bloom followed by warm temperatures), it performed much better in 2024 (Table 2). Small amounts of certified budwood of R19 were sent to local nurseries in 2022 and again in 2024.

R3 ripens 4-7 days later than ‘Chelan’ but is considerably larger and sweeter, with comparable firmness. Budwood of R3 was sent to interested nurseries in Fall 2024. While R3 mother trees tested positive for cherry virus A (which has no known negative impacts), they tested negative for X-disease, Little Cherry virus 2, Prune dwarf virus, and Prunus necrotic ringspot virus. The breeding program will continue its efforts to obtain certified wood of R3.

Pending a favorable harvest in 2025, Dr. McCord will recommend to the WSU Variety Release Committee that R3and R19 be released as new cultivars. Upon release, Plant Variety Protection or a Plant Patent will be sought. R19 could be available for commercial pre-orders as early as spring 2026. R3 will likely be 1-2 years behind this due to budwood availability.

Table 2. Characteristics of R19 and R3 (Phase 3), with ‘Chelan’ as a standard. Performance results are from 2024 season. Estimated yield and packout data are from the entire trial, which was harvested and run over a commercial packing line; all other results are based on fruit pooled from 5 trees and evaluated in the breeding program fruit lab.

Selection	**S-alleles**	**Harvest Date**	**Weight (g)/ Row size**	**Firmness (g/mm)**	**°Brix/TA**	**Estimated Yield/Packout**	**Location**

R19	S4'[1]S9	6/6	8.3/10.0	335	23.1/0.28	7.3 tons/89%	Cherry Barn
R3	S1--	6/10	11.4/9.0	285	25.3/--	-- (small trees)	"
'Chelan'	S3S9	6/6	5.8/Under	319	17.3/0.44	--/93%	"
R3		6/10	11.4/9.5	224	--/--	3.76 tons/78%	Area 51
'Chelan'		6/3	7.0/11.5	212	17.8/0.31		"

Footnotes:. [1]Self-fertile allele.

Of the Phase 2 selections (Table 3), R47 is the earliest, ripening more than a week before 'Bing'. It is the firmest cherry in our current Phase 2 trials, with excellent size (especially for the timing) and strong flavor. CR01T078 ripens generally a few days after 'Bing' and has very large fruit with good flavor. R45 is the latest of the group, ripening with or slightly later than 'Skeena'. It is self-fertile, with very large fruit. R35B is the lone blush selection in this group. It ripens 3-4 days after 'Rainier'. Crops are lighter, but fruit size is very large with excellent firmness, and the tree is self-fertile. The coloring of R35B was light this year; we will observe this carefully in 2025 and postpone harvest as needed to allow for color development. Pending a favorable harvest in 2025, we expect to advance several of these selections (resources permitting) to Phase 3 trials.

Table 3. Characteristics of R47, CR01T078, R45, and R35B (Phase 2), with 'Benton', 'Bing', 'Rainier', and 'Skeena' as standards. performance results are from the 2024 season. Results are averaged (where possible) across 3 locations [IAREC (Prosser), Sagemoor (Pasco), and MCAREC (Hood River)].

Selection	**S-alleles**	**Timing (vs. 'Bing')**	**Weight (g)/ Row size**	**Firmness (g/mm)[1]**	**°Brix/TA**	**Notes**
R47	S1S9	-8	12.5/8.4	303	22.6/0.73	Firm, strong flavor
'Benton'	S4'[2]S9	-5	9.3/9.8	248	25.4/0.92	Standard
'Bing'	S3S4	0	9.2/9.9	206	22.0/0.59	Standard
CR01T078	--	+3	12.6/9	252	22.2/0.60	Good size, flavor
'Skeena'	S1S4'	+9	11.2/9.6	243	22.7/0.45	Standard
R45	S4'S9	+8	13.4/8.7	258	23.6/0.49	Consistent yield across sites

'Rainier'	S1S4	-1	9.7/10	208	21.4/0.41	Standard
R35B	S4'--	+2	13.9/8.6	292	22.7/0.42	Lighter color (need to pick later), lighter crops vs. 'Rainier'

Footnotes: [1]Firmness measured on fruit at room temperature. [2]Self-fertile allele.

In addition to the Phase 2 selections described above, we have planted or are awaiting nursery trees for an additional 9 selections (Table 4).

Table 4. List of additional Phase 2 selections in the breeding program pipeline.

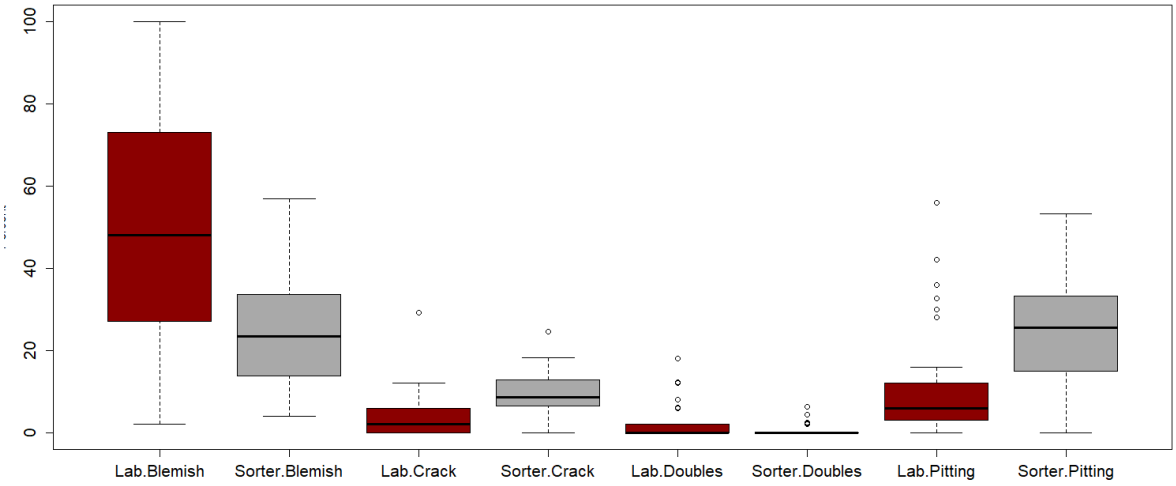
Selection	**Market Class**	**Planting Year**
FR09T084	Early Mahogany	2022
CR11T019	Late Mahogany	2022
FR31T011	Mid-season Mahogany	2023 (2024 at MCAREC)
R37B	Late Blush	2023
CR21T043	Late Mahogany	2023
R25	Early Mahogany	2024
CR20T046	Late Mahogany	2025
PSC2019003-WPM	Early Mahogany	2026
PSC2020019-120	Early Mahogany	2026

Optical Sorter (Objective 1a)

The purchase and installation of the optical sorter was made possible by generous financial support from AgWest Farm Credit and the WSU Tree Fruit Endowment Advisory Committee, and industry members also participated in reviewing bids. A two-lane (one active) Tomra InVision2 sorter was selected. The former pilot winery in the West Building on the Prosser campus (IAREC) was remodeled, and the sorter was installed in time for the 2023 harvest season. A cross-feed conveyor belt was donated by Monson Fruit and installed for the 2024 season. Breeding program personnel have received extensive training from Tomra and local Van Doren Sales trainers. We have developed harvest maps (sorting algorithms) for dark red and blush varieties, and a postharvest map for dark red

cherries. In 2023 and 2024 we gained valuable experience in operating the sorter, and compared the results of the sorter with our current fruit lab protocols in which breeding personnel record systematic observations. An example of the comparison for the 2024 season is shown in **Figure 1.** The sorter detected fewer blemishes, but this is likely due to the fact that the fruit went over the sorter first and were damaged before going to the fruit lab. It is also likely that some samples scored by lab personnel as having blemishes were scored by the sorter as having pitting. The sorter is more consistent, and can routinely be run with only two people (one to operate the sorter, one to load and move fruit). In addition, entire plots of hundreds to potentially thousands of fruit can quickly be graded, compared to only 50 fruit per plot currently tested in the fruit lab. We will continue to use the fruit lab for firmness testing, juice quality, and some postharvest traits (stem color and skin luster), but moving forward we expect that using the sorter will remove the need for 2 temporary worker positions devoted to these evaluations, saving the breeding program > $9000 per year or redirecting such resources to other critical breeding operations.

Figure 1. Comparison of defect detection (percentage of total fruit) between fruit lab personnel and the new Tomra Invision2 optical sorter.



Effects of PGRs on Phase 3 Selections (Objective 2)

We utilized the older Phase 2 planting at MCAREC to test the effects of PGRs on R19 (Retain®, Parka®), R3 (ProGibb®) and R29 (ProGibb®). These experiments suffered in 2022 and 2023 from combinations of poor weather, animal predation, and high *Pseudomonas* pressure in the old Phase 2 block. In 2022, applications of ProGibb® (GA_3) did not have significant effects on color, diameter, individual fruit weight, firmness, stem retention force, soluble solids concentration, or titratable acidity for R29 or R3. Parka® did not have significant effects on cracking, color, diameter, individual fruit weight, or firmness for R19. Retain® did not have significant effects on fruit set, color, diameter, individual fruit weight, firmness, stem retention force, soluble solids concentration, or titratable acidity for R19. We suspect that the small amounts of fruit that survived to be harvested and quality tested limited the robustness of the data and as a result, we elected not to continue the PGR studies in 2024. The old Phase 2 block at MCAREC will be removed in the near future.

Crossing, Seedlings, and DNA Testing (Objective 3)

In 2024, the breeding program made 74 crosses that produced an estimated 7188 seed. Favorable pollination weather enabled a greater number of crosses compared to 2022 and 2023. We continued the successful use of bumblebees in the crossing greenhouse, and increased the number of hives from one to two. We also purchased custom-built insect-proof cages for use in the crossing greenhouse. The bumblebees were then moved to the field (which blooms later) to supplement the mason bees we traditionally use for controlled crossing in cages. No open-pollinated seed was collected in 2024, because we focused all crosses on those where both parents were carefully chosen. Priority target traits for crossing included early/late ripening, powdery mildew resistance (especially combined with late ripening), and fruit size, firmness, and cracking resistance. Additional traits targeted included self-fertility, resistance to X-disease, late blooming, and genetic diversity. We embryo-rescued approximately 2170 seeds in 2024, including 1500 from crosses targeting early ripening. Across the project period of 2022-2024, the breeding program made a total of 191 crosses, producing an estimated 27,325 seed. Also during this period, approximately 3100 seedlings enriched for target attributes were transplanted to the field. Prior to transplanting, we utilized DNA tests for self-fertility, powdery mildew resistance, cracking, and firmness to eliminate more than 1200 seedlings. These less-desirable seedlings would otherwise have taken up more than 1.3 acres of land and cost approximately $47,000 to maintain over the life of the seedling orchard (~ 5-6 years).

Orchard Management (Objective 4)

As in prior years, the orchards at Prosser (Roza and IAREC blocks) were sprayed regularly for insect and disease control. A delayed dormant spray was applied on 30 March. Aside from the delayed dormant and postharvest sprays for leafhopper control (which ended on 21 September), spraying occurred at 2-week intervals. In addition to leafhoppers, target insects included aphids, cherry fruit fly, cherry maggot, spider mites, and spotted wing *Drosophila* (SWD). Fungicide sprays were applied to control powdery mildew, except in the Phase 1 plantings where we encourage disease pressure to enable selection for resistance. Natural powdery mildew incidence was relatively mild in 2024.

Trees in the Roza orchard were winter-pruned. The younger trees at IAREC were managed by a combination of winter and summer pruning, including summer hedging of the most vigorous seedling blocks. Fertilizer recommendations were provided by Jeff Sample based on soil test results, and feeding occurred via fertigation (IAREC) or broadcast (Roza).

We routinely test parental trees for the presence of Prune dwarf virus (PDV) and Prunus necrotic ringspot virus (PNRSV). In prior years, we have detected one or both viruses at a relatively low rate (24% in 2022, 10% in 2023). However, in 2024, the rate of detection was much higher (93% for PDV, 42% for PNRSV). The most likely reason for the higher rate of detection is cleaner extractions in 2024, resulting in higher sensitivity. For samples with mild infections (high Cp values in the PCR test), it is also possible that these trees only recently became infected (perhaps by the use of infected pollen). The majority of trees do not display obvious symptoms. We are removing the most highly infected trees, and are exploring the possibility of using cryotherapy (a new tissue culture technique) to clean up our parental collection. Preliminary results of cryotherapy from the Clean Plant Center Northwest (CPCNW) are promising, but the technique needs to be confirmed as both effective and cost-conscious before we proceed.

For Little Cherry Disease (LCD), the breeding program tested 101 samples. These samples included parental trees in the B53 and C53 blocks at the Roza, symptomatic trees at IAREC, mother trees of

budwood sent for propagation (IAREC), newly planted nursery-grown trees (IAREC), and a sample of R3 and R19 trees in the Phase 3 plantings at Cherry Barn. All samples tested negative for X-disease and Little cherry virus 2. An additional 82 trees in the B53 block were tested by Scott Harper's lab, and six were found to have low-level X-disease infection. These trees will be removed this fall/winter.

Executive Summary

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

Key words: breeding, DNA testing, optical sorting

Abstract
The PNW cherry breeding program continues its progress since the 2018 relaunch. Two promising Phase 3 selections, the early-ripening R19 and R3, are nearing the end of their 2023-2025 trial period. Pending favorable results in 2025, these two are expected to be released as new cultivars later that year. Newer selections in Phase 2 are also performing well, and one or more of these are expected to advance to Phase 3 after the 2025 season. The deployment of a new optical sorter, tested in 2023 and 2024, will enable cost-saving labor reductions as well as producing more consistent results. More than 3000 new Phase 1 seedlings have been planted in the last three years, derived almost exclusively from bi-parental crosses targeting industry priority traits. The breeding program has also successfully deployed newly developed DNA tests for fruit firmness and cracking along with existing tests for self-fertility and powdery mildew, eliminating > 1200 inferior seedlings at the greenhouse stage, with projected savings of thousands of dollars in planting and management costs.

Project Title: Nutrient management for high quality sweet cherries

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Cooperators: Denny Hayden, Craig Harris, Luke Anderson (Allan Brothers), Rob Blakey (Stemilt)

Report Type: Final Report

Project Duration: 3 Year

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

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

Budget 1

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

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

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

OBJECTIVES

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

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

2) Determine nutrient demand of 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.

SIGNIFICANT FINDINGS

- Differences in year explained 12% and 15% of fruit firmness and size variability, respectively. In 2022, fruit firmness was 16% higher.
- Variety differences explained only 5% of firmness and size variability, when compared across years, while the interaction of year and cultivar, explained 20% of firmness variability, and 23% of size variability.
- Firmness and size were highly variable within samples. (e.g., ranging between 89 and 480 $g \cdot mm^{-1}$ in Skeena). This level of variability was also observed with fruit size. Addressing fruit quality variability within orchards should be a key goal for Washington growers.
- Very soft fruit (firmness < 200 $g \cdot mm^{-1}$) had consistently lower N and S concentration. However, above this level there was no strong relationship between fruit quality and nutrients ($r < 0.60$) despite the large number of samples and wide range of quality conditions.
- Macronutrients were always higher in the small fruit, suggesting a dilution factor due to other components associated with bigger fruit (sugars, acids, water).
- There is a lack of relationship between fruit quality parameters and nutrient concentrations that we attribute to the high levels of nutrients found in all samples, being within or above the critical values reported for sweet cherry in the literature.
- Nutrient extraction (lbs. per ton of fruit) was determined for Skeena, Coral Champagne, and Chelan. Given the consistency of the results across sites, years and cultivars, these values are likely representative of most sweet cherry cultivars grown in Washington.
- Postharvest differences were found associated to the cultivar, year and site. For example, stem retention was twice as high in Chelan compared to Coral Champagne and Skeena, but also there was a strong influence of the year.
- Some postharvest defects correlated strongly with nutrient levels; however, these correlations varied among cultivars, with Chelan and Coral Champagne having more correlations compared to Skeena, which had none.

METHODS

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

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

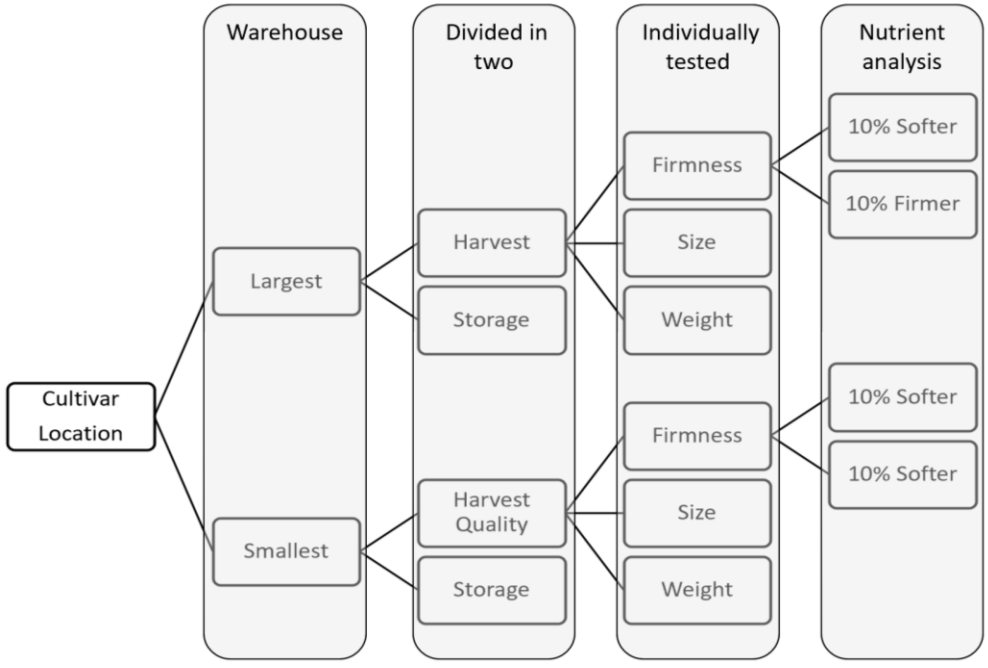

Figure 1.

Fruit sampling scheme for nutrient and storability analyses.

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

RESULTS AND DISCUSSION

Fruit quality summary by year, cultivar, and site

Fruit quality varied widely across years, cultivars, and sites (Table 1). When evaluating all the fruit received from the packing houses, differences from years explained 12% and 15% of fruit firmness and size variability, respectively ($p<0.001$). Fruit firmness was 16% higher in 2022, ranging between 269 and 388 $g \cdot mm^{-1}$ across cultivar and sites. Fruit size was also 3% and 14% higher in 2022, compared to 2021 and 2023, respectively (Table 1). The variety, on the other hand, explained only 5% of firmness and size variability, when comparing across year ($p<0.001$), while the interaction of year and cultivar, explained 20% of firmness variability and 23% of size variability.

Skeena fruit were consistently larger than Coral Champagne and Chelan (4 to14% larger), and Chelan was larger than Coral Champagne in 2021 and 2023, but smaller in 2023. In relation to fruit firmness, Coral Champagne was always softer (238 – 292 $g \cdot mm^{-1}$) than the other two varieties, while Chelan was firmer than Skeena in 2021 and 2023, but not in 2022 (Table 1). The impact of site on fruit firmness and size was also significant ($p<0.05$), however among the explanatory variables, year and cultivars were the most influential. Across all years, the variability in firmness among sites, cultivars and years is very high. Figure 1 represents firmness and size variability among orchards for Skeena in 2022, underscoring the importance of managing variability in orchards to maximize the proportion of higher quality fruit.

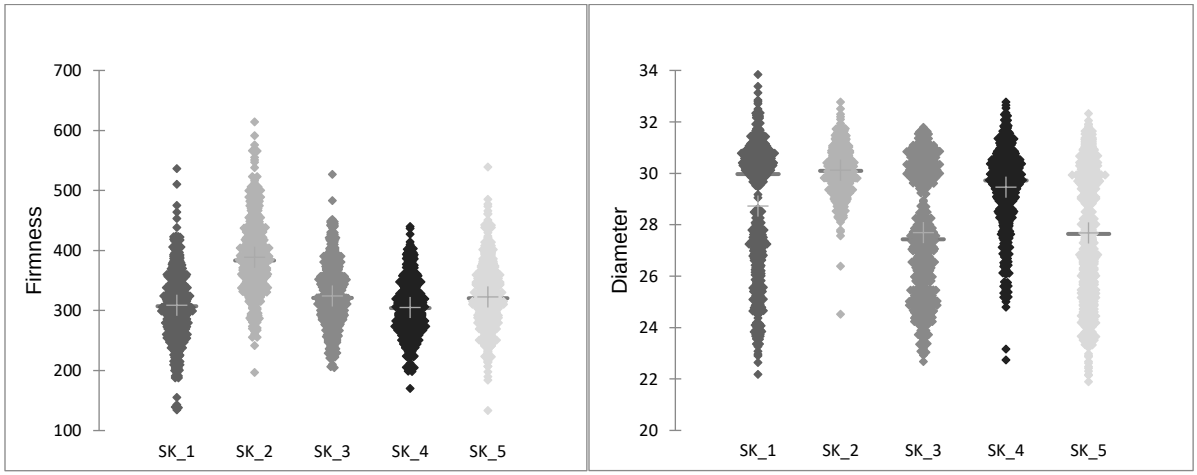

Figure 1. Firmness (left) and fruit size (right) variability across Skeena orchards in 2022. Middle cross indicates mean value.

Table 1. Fruit firmness and diameter differences by year, cultivar, and site. Different letters indicate statistical differences between years (bold capital), cultivars within years (bold) and among sites within year and cultivar (small letters) (Tukey test and $p < 0.05$)

			Firmness ($g \cdot mm^{-1}$)				Diameter (mm)			
Year	Variety	Site	Mean	Min	Max	StdDev	Mean	Min	Max	StdDev
2021	Chelan	1	295b	139	427	51.5	25b	20	33	3.4
		2	301a	159	444	49.4	26b	20	30	3.3
		3	255c	140	367	43.8	29a	25	35	2.9
	Chelan		**285.1a**	**139**	**444**	**52.4**	**26.6b**	**20**	**35**	**3.7**
	Coral	1	233b	123	377	44.8	25b	19	32	4.3
		2	236b	131	382	43.6	27a	22	32	2.8
		3	247a	140	360	36.2	25b	20	32	4.1
	Coral		**238.6c**	**123**	**382**	**42.1**	**25.6c**	**19**	**32**	**3.9**
	Skeena	1	304a	176	422	43.6	27b	24	32	2.1
		2	278c	161	394	36.7	28a	22	32	1.9
		3	289b	172	419	42.9	26c	22	30	2.0
		4	260d	146	394	40.4	26c	22	31	2.3
		5	260d	164	378	33.4	28a	25	31	1.2
	Skeena		**277.3b**	**146**	**422**	**42.5**	**26.8a**	**22**	**32**	**2.1**
2021			**269.3B**	**123**	**444**	**49.5**	**26.4B**	**19**	**35**	**3.2**
2022	Chelan	1	269c	134	434	48.3	25c	20	32	3.5
		2	350a	150	613	60.8	27b	21	34	3.6
		3	313b	154	544	58.7	29a	22	35	3.3
	Chelan		**310.9b**	**134**	**613**	**65.6**	**26.9b**	**20**	**35**	**3.8**
	Coral	1	275b	130	457	62.3	26b	21	31	3.4
		2	302a	199	636	45.2	27a	22	34	4.0
		3	298a	140	448	47.3	26b	20	34	4.5
	Coral		**292.0c**	**130**	**636**	**53.3**	**26.0c**	**20**	**34**	**4.0**
	Skeena	1	309c	134	537	54.9	29c	22	34	2.5
		2	388a	197	614	62.9	30a	25	33	1.0
		3	324b	205	527	49.1	28d	23	32	2.4
		4	305c	170	440	43.4	29b	23	33	1.5
		5	322b	133	539	49.9	28d	22	32	2.5
	Skeena		**326.5a**	**133**	**614**	**58.2**	**28.6a**	**22**	**34**	**2.3**
2022			**311.4A**	**130**	**636**	**60.9**	**27.3A**	**20**	**35**	**3.6**
2023	Chelan	2	267b	139	478	47.0	22a	18	30	2.4
		3	303a	169	628	57.0	22a	18	26	2.1
	Chelan		**285.0a**	**139**	**628**	**55.1**	**22.2c**	**18**	**30**	**2.3**
	Coral	1	245b	156	401	37.2	23a	17	31	4.1
		3	265a	137	408	37.1	24a	17	30	2.3
	Coral		**255.1c**	**137**	**408**	**38.6**	**23.5b**	**17**	**31**	**3.3**
	Skeena	1	268a	101	455	55.4	25b	21	32	2.3
		3	265ab	97	480	54.7	26a	20	31	2.1
		4	261b	89	468	47.0	25b	21	31	2.2
	Skeena		**264.8b**	**89**	**480**	**52.6**	**25.4a**	**20**	**32**	**2.2**
2023 Total			**268.2B**	**89**	**628**	**51.2**	**23.9C**	**17**	**32**	**2.9**

Nutrient distribution by cultivar

The distribution of nutrient concentration by cultivars also was highly variable (Figure 2). Fruit nutrient concentration distribution in these Washington orchards were either within or above the critical levels reported in the literature (Figure 2).

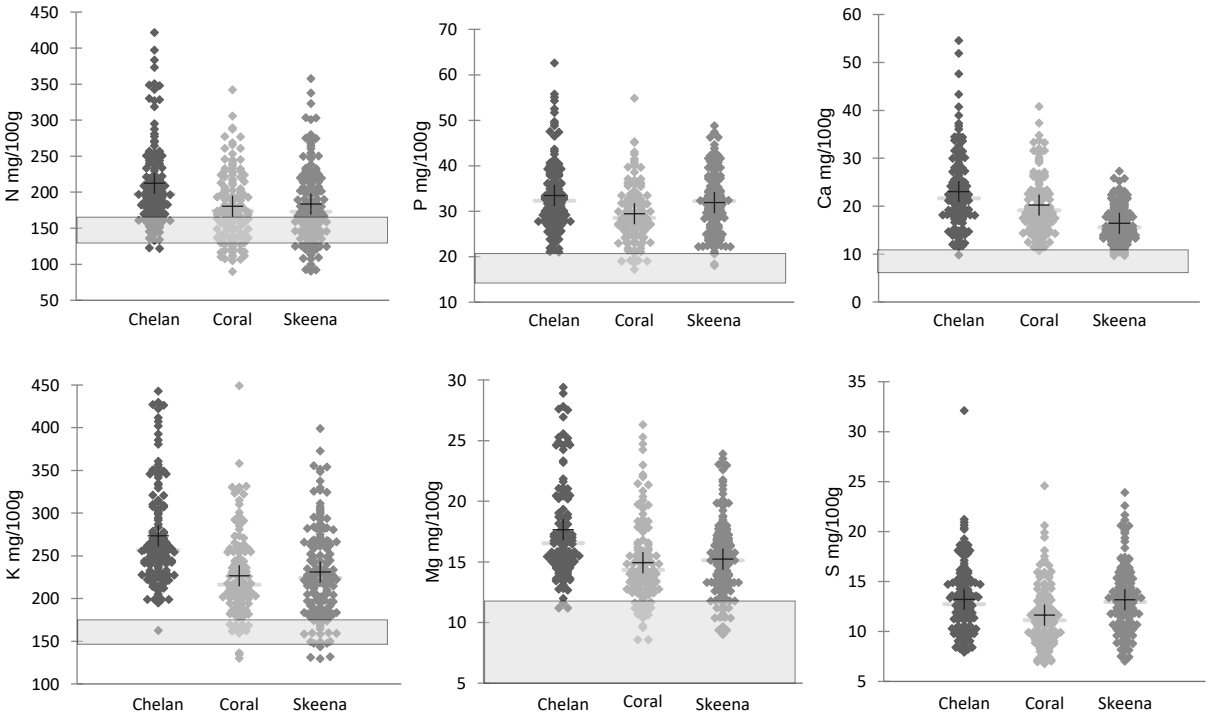

Figure 2. Fruit macronutrient distribution by cultivar. The gray boxes corresponds to the critical range reported in the literature for sweet cherries.

Nutrient differences of segregated fruit by year, cultivar and site

Within each size category, fruit from the 10th and 90th percentile ranking of firmness were selected for individual fruit quality analyses and nutrient analyses. When combining all categories and sites, fruit concentration of N, K, Mg and S was different among years, and all macronutrients were different among cultivars (Table 2).

Nitrogen was 6 and 15% higher in 2022 when compared with 2021 and 2023 respectively. However, K and Mg were higher in 2021, and S lowest in 2023, with no relation to fruit firmness or size. Among cultivars, Chelan had more than 15% higher N concentration, with no differences between Coral Champagne and Skeena, and no relationship with fruit quality (i.e., Chelan and Skeena were the firmest and Skeena were the largest). Coral Champagne had the lowest P and S, while highest K, Ca, Mg, again, with no relation with fruit firmness and size (Table 2). Overall, the cultivar and cultivar*year interaction had a greater influence in fruit nutrient variability.

Nutrients also varied by site and year (Table 3), however with no clear relation with fruit firmness and size differences. For example, Chelan site 2 had softer fruit in 2021, when compared with the other two sites, while there were no associated differences in nutrient levels. In 2022 and 2023, the same site 2 had firmer fruit, and again with no differences in nutrient levels, except higher B in 2023. For Coral

Champagne, site 1 had firmer and larger fruit, and higher N and P levels. However, in 2022, even though site 1 also had the largest fruit, nutrient concentration was not different from site 2 that had the smallest fruit. Similarly, for Skeena, site 1 having the firmest fruit in 2021 and 2022, only in 2021 had the highest N and B levels, while there were no differences in 2022. Regardless of the firmness and size differences between sites, note that Ca concentration only showed differences among sites in 2021 for Chelan and Skeena, and those differences did not align with firmer fruit, as it is sometimes perceived.

Table 2. Fruit firmness, size, weight, dry matter (DM) and macronutrient differences among years and variety. Different letters in the same column indicate significant differences within year and variety based on Tukey test ($p<0.05$). R^2 indicates the percentage of the variability in nutrient concentration (%) explained by the interaction of year and variety, shown only for factors with significant p value.

Factor		Firmness ($g.mm^{-1}$)	Size (mm)	Weight (g)	DM	N	P	K	Ca	Mg	S
						mg $\cdot 100g^{-1}$ (fresh)					
Year	2021	273.0 b	26.7 b	9.0 b	19 a	189.0 b	32.1	261.0 a	19.5	16.8 a	12.9 a
	2022	316.0 a	29.9 a	10.8 a	19 a	201.4 a	30.8	234.3 b	19.6	15.3 b	13.2 a
	2023	275.0 b	22.8 c	8.2 b	18 b	174.6 b	32.1	216.7 c	20.3	15.0 b	11.0 b
p value		<0.001	<0.001	<0.001	0.01	0.003	0.18	<0.001	0.702	<0.001	0.001
Variety	Ch*	295.5 a	26.5 b	8.3 c	19	212.6 a	33.5 a	273.4 a	23.0 a	17.7 a	13.2 a
	CC	268.2 b	26.7 b	9.1 b	19	180.5 b	29.4 b	226.8 b	20.2 b	14.9 b	11.6 b
	Sk	306.5 a	28.7 a	11.1 a	19	184.0 b	31.9 a	231.1 b	16.4 c	15.2 b	13.2 a
p value		0.000	<0.001	<0.001	0.51	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Year*Variety		0.89	0.22	0.007	0.99	0.000	0.270	0.001	0.642	0.188	0.000
	R^2	-	-	0.24	-	0.15	-	0.27	-	-	0.12

*Ch; Chelan, CC; Coral Champagne, Sk; Skeena.

Nutrient relationships with fruit quality

Correlation analysis is a useful tool to identify relationship between variables, especially when there is a large and wide range of values within each variable. When combining all cultivars, sites and years, firmness correlated significantly ($p<0.001$) with N and S concentrations in dry and fresh weight, however the relations were weak (R2 below 0.23) (Table 4). Fruit diameter and weight were negatively related with P, K, Ca, Mg concentrations, but again the correlations were weak (R2 below 0.5) (Table 4).

Given the influence of years and cultivar in fruit quality (described above), we evaluated the correlation after grouping by year or cultivar. For firmness, the correlations with nutrients were either not significant ($p>0.05$) or weak ($R2 < 0.37$) across all years and cultivars (data not shown). When grouping by year, fruit size (diameter or weight) was strongly and negatively correlated Ca concentration ($R2 > -0.70$) and Ca content ($R2 > -0.69$), but only in 2021 (data not shown). While there were no strong correlations between fruit quality and nutrients when grouping by cultivar. The negative relation between nutrient concentration and fruit size appears to be a consequence of dilution, rather than a cause effect relationship. For example, when mean fruit nutrient levels were compared by size category, nutrients were always higher in the small fruit, while no differences were found between firm and soft fruit (Table 5). The interaction of fruit quality categories firmness x size, was a secondary factor for nutrient levels, being significant ($p<0.05$) for P, K, Ca, Mg and S (Table 5), however the percentage explained by the interaction was generally low.

Table 3. Firmness, size and nutrient concentration by cultivar, site and year. Different letters in the same column indicate significant differences based on Tukey test ($p<0.05$). Lines in grey highlight sites described in the paragraph.

Cultivar	Year /Site		Firmness (g.mm-1)	Diameter (mm)	Nutrient concentration fresh (mg.100g-1)					
					N	P	K	Ca	Mg	S
Chelan	2021	1	333 a	28.1 b	188 a	30.5	249	12.4 b	14.6	12.0 a
		2	283 b	32.2 a	178 a	27.3	246	14.4 ab	16.3	13.9 a
		3	339 a	28.5 b	145 b	27.9	251	16.3 a	14.3	9.2 b
	p value		<0.0001	<0.0001	0.005	0.340	0.946	0.008	0.082	0.001
	2022	1	471 a	33.3 a	254 a	41.1 a	354 a	33.6	20.0 a	15.4 a
		2	400 b	32.5 b	203 b	27.6 b	249 b	20.7	16.2 b	16.2 a
		3	329 c	31.6 c	178 b	31.4 b	250 b	20.3	14.7 b	10.1 b
	p value		<0.0001	<0.0001	0.005	0.001	0.002	0.058	0.000	<0.0001
	2023	2	402 a	24.4	231	36.1	254	19.3	16.2	14.6 a
		3	345 b	24.0	221	42.8	250	31.8	18.0	12.1 b
	p value		0.000	0.111	0.632	0.397	0.827	0.172	0.164	0.002
Coral Champagne	2021	1	315 a	30.0 a	167 a	29.2 a	208	14.4	13.2	11.5
		2	270 c	29.6 b	155 ab	22.8 b	183	14.2	11.6	9.7
		3	291 b	29.7 b	133 b	20.6 b	164	12.9	10.9	9.4
	p value		<0.0001	0.015	0.021	0.010	0.107	0.341	0.158	0.188
	2022	1	357	34.5 a	247 a	36.6 a	282 a	16.3	14.8	19.4 a
		2	396	32.3 c	263 a	32.2 ab	262 ab	22.0	16.4	15.8 ab
		3	375	33.2 b	187 b	28.9 b	236 b	21.8	15.8	12.6 b
	p value		0.078	<0.0001	0.008	0.046	0.052	0.124	0.423	0.026
	2023	1	314	25.8 b	160 a	29.1	191	19.1	14.3	8.7
		3	304	27.7 a	123 b	33.1	221	18.3	12.7	10.2
	p value		0.177	<0.0001	0.023	0.221	0.129	0.704	0.133	0.153
Skeena	2021	1	377 a	29.8 a	246 a	29.3	247 ab	13.7 ab	15.6	17.4 a
		2	365 a	28.1 b	165 bc	36.9	298 a	20.1 a	17.8	12.3 b
		3	343 b	29.1 a	203 ab	32.6	211 b	18.3 ab	14.7	11.6 b
		4	304 c	28.1 b	169 bc	28.1	221 ab	12.9 b	13.8	12.2 b
		5	313 c	28.1 b	109 c	26.9	219 b	14.9 ab	13.1	9.1 b
	p value		<0.0001	<0.0001	0.000	0.083	0.022	0.023	0.101	0.002
	2022	1	510 a	32.1 bc	281	41.8 a	234	19.4	20.0 a	18.7
		2	411 b	30.2 d	214	31.2 b	206	12.7	14.9 ab	15.4
		3	395 b	33.1 a	212	34.2 ab	235	18.0	14.6 b	13.3
		4	385 bc	32.7 ab	199	34.0 ab	189	17.2	16.1 ab	14.0
		5	361 c	31.6 c	168	30.0 b	190	13.7	13.8 b	13.1
	p value		<0.0001	<0.0001	0.083	0.018	0.269	0.048	0.023	0.059
	2023	1	353	27.6 a	197 a	47.8	335	18.3	19.5	13.2 a
		3	353	22.7 b	147 b	42.0	280	20.6	15.5	10.2 ab
		4	349	22.3 b	114 b	39.6	266	15.9	15.2	7.9 b
	p value		0.754	<0.0001	0.001	0.392	0.212	0.649	0.153	0.023

Table 4. Pearson correlation between fruit quality indicators and dry nutrient concentration (%) and fresh nutrient concentration (mg/100g). Bold values indicate significance level of $p < 0.05$.

Variables	Firmness ($g \cdot mm^{-1}$)	Diameter (mm)	Weight (g)
N %	**0.197**	0.091	**-0.132**
P %	0.012	**-0.329**	**-0.225**
K %	-0.049	**-0.254**	**-0.382**
Ca %	-0.052	**-0.363**	**-0.501**
Mg %	-0.038	**-0.276**	**-0.423**
S %	**0.198**	**0.209**	-0.021
Dry Matter %	**0.108**	-0.006	**0.105**
N mg/100g	**0.247**	**0.107**	-0.071
P mg/100g	0.078	**-0.252**	**-0.093**
K mg/100g	0.024	**-0.201**	**-0.220**
Ca mg/100g	0.005	**-0.326**	**-0.407**
Mg mg/100g	0.035	**-0.224**	**-0.277**
S mg/100g	**0.250**	**0.211**	0.024

Table 5. Fruit nutrient concentration differences between size, firmness, and the interaction of size x firmness categories across all three years and cultivars. Different letters in the same column indicate significant differences within size and firmness category based on ANOVA test ($p<0.05$). R^2 indicates the percentage of the variability in nutrient concentration (%) explained by the interaction of fruit size and firmness.

Fruit Quality Category			Nutrient concentration dry (%)[1]						
		Unit[2]	Dry Matter	N	P	K	Ca	Mg	S
SIZE	Small	24.6 b	18%b	1.05 a	0.17 a	1.36 a	0.12 a	0.09 a	0.07 a
	Big	30.0 a	19%a	0.98 b	0.16 b	1.22 b	0.09 b	0.08 b	0.07 b
	p value	<0.0001	0.004	0.002	<0.0001	<0.0001	<0.0001	<0.0001	0.006
FIRMNESS	Firm	361 a	0.19	1.02	0.17	1.28	0.10	0.08	0.07
	Soft	221 a	0.19	1.00	0.17	1.29	0.10	0.08	0.07
	p value	<0.0001	0.683	0.482	0.474	0.462	0.373	0.361	0.299
SIZE x FIRMNESS	p value		0.595	0.098	0.002	0.040	0.067	0.034	0.013
	R^2		0.02	0.03	0.08	0.11	0.24	0.15	0.04

[1]Means of 208 fruits/category. [2]Unit of category, being diameter (mm) for size, and force ($g.mm^{-1}$) for firmness.

Nutrient extraction to determine demand

Fruit nutrient extraction varied slightly among years, cultivar and site ($p < 0.001$). However, these differences are of agronomic irrelevance (data not shown). For example, Ca extraction was lowest in 2022 with mean value of 0.34 lbs per ton of fruit, and highest in 2023 with 0.38 lbs per ton of fruit. When translating this to a per acre rate for an orchard producing 10 tons of fruit, the difference between 2022 and 2023 is only 0.4 lbs. Thus, we opted to provide a range of nutrient extraction values to account for the variability across years and sites. Note that regardless of the differences in fruit quality and yields between years, the extraction remained stable and within a small range. Thus, nutrient extraction values

determined in our study provide a confident estimation of nutrient demand in mature orchards, and should guide nutrient management rates to prevent excessive use of fertilizers.

Table X. Nutrient extraction ranges for Washington Sweet cherry cultivars.

	N	P	K	Ca	Mg	S	Zn	Mn	Cu	B
Nutrient / Cultivar	Lb/USTon						g/USTon			
Chelan	3.2 - 4.6	0.5 - 0.7	3.9 - 6.4	0.4 - 0.6	0.3 - 0.4	0.2 - 0.3	0.2 - 1.5	0.7 - 1.8	0.6 - 1.2	3.4 - 11
Coral Champagne	2.4 - 4.9	0.5 - 0.7	3.2 - 5.4	0.3 - 0.4	0.2 - 0.3	0.2 - 0.4	0.3 - 1.8	0.1 - 2.3	0.6 - 1.4	1.3 - 7.7
Skeena	1.9 - 3.4	0.6 - 0.9	2.9 - 6.3	0.3 - 0.4	0.2 - 0.4	0.2 - 0.4	0.2 - 0.9	0.1 - 1.5	0.5 - 1.2	3.7 - 21
Range	**1.9 - 5.0**	**0.5 - 0.9**	**2.9 - 6.3**	**0.3 - 0.4**	**0.2 - 0.4**	**0.2 - 0.4**	**0.2 - 1.8**	**0.1 - 2.3**	**0.5 - 1.4**	**3.7 - 21**
Literature	2.7 - 11.7	1.50	7.60	0.40	-	-	-	-	-	-

Postharvest differences

Postharvest condition and disorders were influenced by the site, cultivar, and year. In this report, we focus on key finding and their relationship with fruit nutrient levels.

Firmness after storage varied between 234 and 497 ($g.mm^{-1}$), with Chelan showing higher firmness compared to Coral Champagne and Skeena (Table 6). In 2021, site 1 and 2 had firmer fruit, whereas in all other cultivars and years, firmness was higher in 2023. Soluble solids (SS) were largely influenced by the cultivar, with Skeena showing the highest levels (mean: 21 Brix), and Chelan and Coral Champagne being similar (17 – 18 Brix). Year had a lesser influence, with the lowest SS observed in 2022. Stem retention was strongly influenced by both year and cultivar, with these variables explaining 78% of the variability (data not shown). Overall, Chelan required twice the force compared to Coral Champagne and Skeena, and stem retention was consistently higher in 2022 across all cultivars and sites (Table 6).

Stem decay was more influenced by the cultivar than by the year, with Skeena showing the highest level (3.5 N) and Coral champagne the lowest (2.1 N). Differences between years and sites were inconsistent. The incidence of pitting varied between 10% to 93% across all sites, years, and cultivars, being highly influenced by year and cultivar (accounting for 61% of the variability). Pitting was two to three times higher in 2021 compared to 2022 and 2023, respectively. Chelan and Skeena experienced twice as much pitting as Coral Champagne. Interestingly, differences between sites were inconsistent in Chelan, nonexistent for Coral Champagne, and higher in Skeena site 4 when comparing across years (data not shown).

Fruit splits were higher in 2022, ranging from 13% in Skeena to 16% in Chelan, but no differences were observed between cultivars (data not shown). In 2021 and 2023, split percentage were below 3%. However, differences appeared when comparing split incidence by site and year (Table 6). For example, Chelan Site 1 had 16% splits in 2021 but 0% in 2022, while Chelan Site 3 had 0% in 2021 and 18% in 2022. This variability suggests that environmental factors, rather than management, play a key role in fruit splitting.

Mechanical damage varied widely, from 0 to 54%, with Chelan showing the highest percentage compared to Skeena and Coral Champagne. Similar to fruit splits, the incidence of mechanical damage varied across sites and years, without a consistent relation to any variable (Table 6).

Other fruit defects were observed at lower incidence or not every year (data not shown). Bruising was only observed in 2021, but at a low percentage (<5%). Fruit decay was also generally low (< 1%) across years and cultivars. However, in 2023, Skeena site 1 and 2 had 28% decay, much higher than the other sites. Sunburn damage was observed only in 2021, ranging from 2.8% to 30%, and its incidence was closely related to site and cultivar. Russet and browning were observed only in 2022, but at low levels (mean: 2.4%). Soft shoulders ranged from 1% to 28%, being highest in 2021 (mean: 18%) and in Coral Champagne (mean: 28%), while not detected in 2023. Similarly, shrivel ranged from 1% and 31%, with

the highest level in 2021 and 2022 (mean: 8%), and more prominent in Chelan (mean: 13%) and Coral Champagne (mean: 8%) compared to Skeena (< 1%). Lizard skin was highly influenced by the year, being highest in 2023 (mean: 32%) and being more severe in Chelan and Coral Champagne (averaging 17%), with four orchards showing more than 35% incidence.

Postharvest relation with nutrients

Given the strong influence of the cultivar in most postharvest attributes, correlation with nutrient levels were conducted by cultivar. Here we report only on strong correlations ($r < -0.55$, or > 0.55). In Chelan, a positive relationship was found between K:Ca and SS ($r = 0.64$), soft shoulder ($r = 0.63$) and pitting ($r = 0.71$) (Figure 3), while negative relation with lizard skin ($r = -0.62$). Pitting incidence also correlated positively with N:Ca ($r = 0.58$) and negatively with N ($r = -0.57$), P ($r = -0.55$), Ca ($r = -0.67$) and Mg ($r = -0.61$). Fruit P also correlated positively with lizard skin ($r = 0.60$). Fruit browning showed strong positive correlation with K ($r = 0.84$), dry matter ($r = 0.62$), Mg ($r = 0.67$) and N ($r = 0.57$). Fruit stem retention force was positively correlated with dry matter ($r = 0.72$).

In Coral Champagne, fruit K:Ca correlated with SS ($r = 0.55$). Stem decay correlated negatively with Ca ($r = -0.58$) and Mg ($r = -0.55$), and stem retention force correlated strongly and positively with dry matter ($r = 0.69$), N ($r = 0.88$), K ($r = 0.69$), Mg ($r = 0.62$) and S ($r = 0.71$). Fruit browning correlated positively with dry matter ($r = 0.59$), N ($r = 0.77$), K ($r = 0.78$), Mg ($r = 0.56$) and S ($r = 0.78$), while pitting was negatively correlated with dry matter ($r = -0.79$), K ($r = -0.58$), Ca ($r = -0.63$) and Mg ($r = -0.56$).

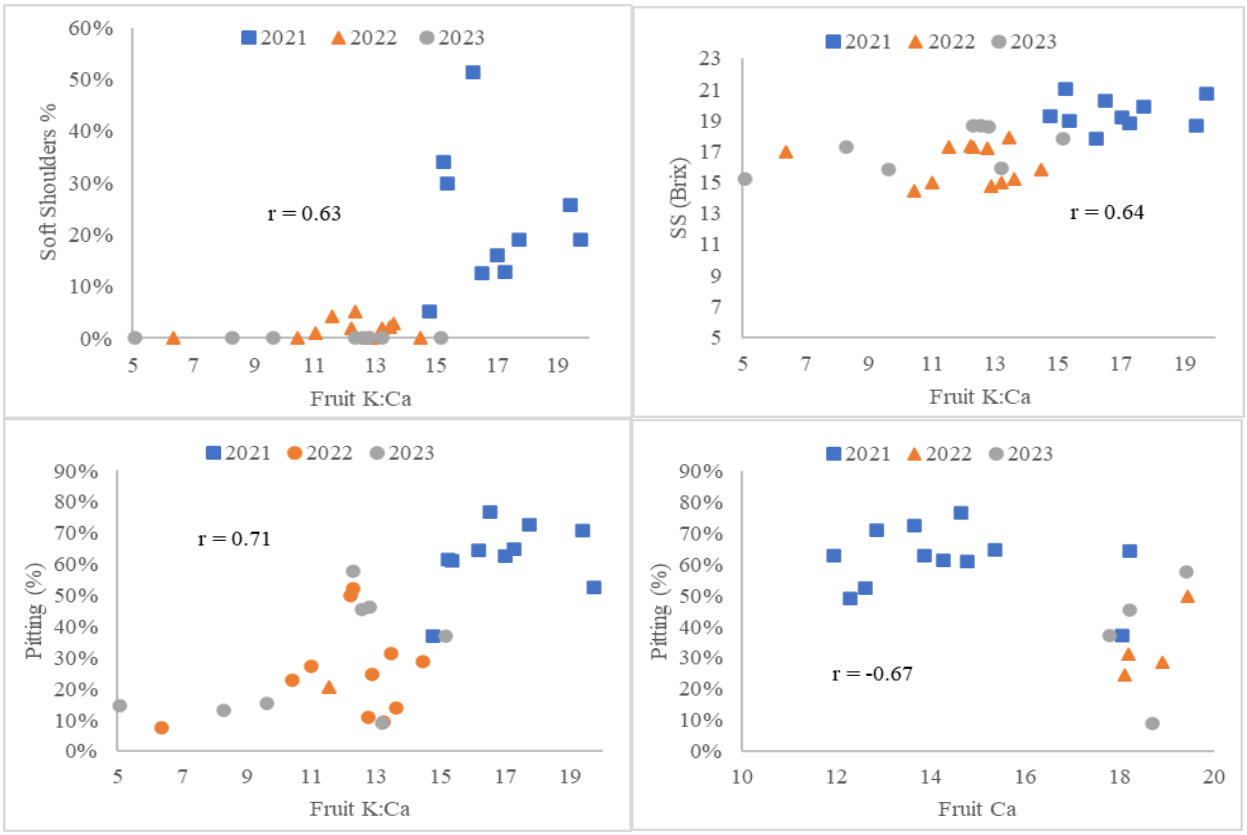

Figure 3. Top: correlation between fruit K:Ca with soft shoulder (left) and soluble solid SS (right), and bottom: correlation between pitting and fruit K:Ca (left) and Ca (right), in 2021 (●), 2022 (▲), and 2023 (■). Correlation across all years represented by r ($p < 0.001$).

Table 6. Fruit quality and condition across cultivars, sites and years, after four weeks of storage at 39 F and regular atmosphere. Different letters in the same column indicate significant differences within cultivar and site (Tukey test $p<0.05$).

Cultivar	Site	Year	Firmness AVG (gr/mm2)	SS (Brix)	Stem retention (N)	Stem Decay (1-5)[1]	Pitting %		Splits %		Mechanical Damage %	
Chelan	S1	2021	484 a	19.3 a	1.6 b	2.9	56%		2%	b	38%	b
		2022	300 b	17.5 b	6.6 a	3.0	38%		13%	a	79%	a
	S2	2021	441 a	16.1 b	7.3 a	3.3 a	10%	b	16%	a	9%	b
		2022	390 b	19.5 a	3.8 b	3.3 a	59%	a	0%	b	30%	b
		2023	398 b	16.1 b	2.3 c	2.1 b	13%	b	5%	b	74%	a
	S3	2021	379 b	19.6 a	3.1 b	3.3 a	69%	a	0%	b	17%	b
		2022	352 b	15.0 b	7.0 a	2.6 b	26%	c	18%	a	62%	a
		2023	472 a	18.5 a	4.2 b	2.9 ab	47%	b	1%	b	33%	b
Coral	S1	2021	350 b	19.3 a	1.4 b	2.5 a	47%	a	2%	ab	10%	b
		2022	371 ab	15.1 b	7.2 a	1.7 b	12%	b	5%	a	3%	b
		2023	392 a	19.0 a	0.9 b	2.3 a	16%	b	0%	b	35%	a
	S2	2021	273 b	18.2	1.4 b	2.3	36%	a	1%	b	66%	a
		2022	316 a	18.8	5.2 a	2.1	12%	b	6%	a	8%	b
	S3	2021	336 b	18.1 a	1.1 b	2.4 a	43%	a	1%	b	28%	a
		2022	313 c	15.4 b	4.0 a	1.8 b	10%	b	29%	a	1%	b
		2023	383 a	15.5 b	1.6 b	1.8 b	23%	b	2%	b	35%	a
Skeena	S1	2021	358	20.5 a	1.2 b	2.5 b	60%	a	0%	b	17%	c
		2022	327	18.8 b	3.6 a	2.8 b	24%	b	16%	a	51%	a
		2023	337	21.1 a	1.2 b	4.0 a	9%	c	2%	b	32%	b
	S2	2021	301 b	20.0 b	4.2	3.5 b	56%		8.9%		23%	
		2022	356 a	24.5 a	4.3	4.7 a	73%		15.4%		14%	
	S3	2021	317	21.0	2.3 b	2.9 b	56%	a	1%	b	7%	b
		2022	331	20.7	3.9 a	2.3 c	28%	d	16%	a	30%	a
		2023	354	19.5	1.9 b	4.0 a	0%	c	9%	ab	30%	a
	S4	2021	257 b	24.7 a	3.4 a	4.0 b	69%	a	3.1%		24%	
		2022	272 b	21.7 b	3.0 ab	2.7 c	68%	a	4.9%		25%	
		2023	372 a	24.3 a	2.1 b	4.6 a	27%	b	6.4%		32%	
	S5	2021	255 b	21.1 a	2.4	2.9	57%	a	2%	b	34%	b
		2022	344 a	18.2 b	3.2	4.1	13%	b	13%	a	56%	a

[1]Stem decay scale 1 to 5, with 1 being green stem with no decay and 5 being brown stems with severe decay.

In contrast, there were no strong correlations between nutrient levels and Skeena postharvest quality and condition (data not shown).

Executive Summary
Project Title: Nutrient management for high quality sweet cherries

Key words: sweet cherry nutrients, firmness, size, postharvest, calcium

The project aimed to enhance nutrient management strategies for sweet cherries by analyzing the relationship between fruit nutrient levels, and fruit quality parameters such as size, firmness, and storability. The key objectives were to identify optimal nutrient conditions for sweet cherry quality, determine the nutrient demand for Chelan, Coral Champagne and Skeena and improved fruit quality and storability. We found yearly differences explained 12% of fruit firmness and 15% of size variability. Cultivar differences had a minimal effect on firmness and size across years, explaining only 5% of the variance, but the interaction between year and variety increased to 20% and 23%, respectively.

Very soft fruit (<200 $g \bullet mm^{-1}$ firmness) showed consistently lower nitrogen (N) and sulfur (S) concentrations, but there was no strong correlation between fruit nutrient levels and fruit quality beyond this firmness level. Nutrient extractions were consistent across varieties and sites, values provided by this study can be utilized to estimate the rate of nutrients required per ton of fruit produced with greater confidence.

Postharvest attributes such as stem retention and firmness, varied significantly across cultivars and years. For instance, Chelan fruit had higher firmness and stem retention than Coral Champagne and Skeena. Also, retention was two to three times higher in 2022. Fruit disorders such as pitting, mechanical damage, fruit splits, and other defects were influenced by year, variety, and site. Pitting, for example, was higher in Chelan and Skeena and was most severe in 2021. Postharvest defects such as browning, soft shoulders, and pitting were linked to nutrient levels, especially in Chelan and Coral Champagne. But no strong nutrient correlations were found for Skeena.

Managing variability in fruit quality within orchards is crucial for growers. In Washington, nutrient levels were either within or above the reported adequate ranges for sweet cherry, which might explain the lack of relationship. Nutrient levels, especially K:Ca ratio were related to postharvest disorders. Note that relations do not represent causation, however they could be utilized as indicators to predict storability.

Project Title: Precision Nutrient Management for sweet cherry orchards

Report Type: Continuing Project Report Year 2

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Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 70,004
Total Project Request for Year 2 Funding: $ 20,321
Total Project Request for Year 3 Funding: $ 20,650

Budget 1
Primary PI: Bernardita Sallato
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Item	2023	2024	2025
Salaries			
Benefits			
Wages[1]	7,190	7,478	7,777
Benefits	734	763	793
RCA Room Rental			
Shipping			
Services	56,680	6,680	6,680
Supplies	4,400	4,400	4,400
Travel	1,000	1,000	1,000
Total	70,004	20,321	20,650

Footnotes: [1] Salary for 50% FTE for 4 month per year for Juan Munguia, Research Assistant at Sallato's laboratory for data collecting processing fruit in the laboratory. [2] Services include root monitoring equipment and PNNL services. Supplies include laboratory analysis and processing of samples for nutrient test.

OBJECTIVES

Our objectives are to investigate new technology for determining biomass partitioning and root growth, through an integration of existing resources, expertise, and technology. This study will provide information to validate (or not) the current premise for calculating nutrient demand based on fruit extraction only, determine the differences between growing conditions and rootstocks, and provide an estimation of nutrient demand for new sweet cherry plantings

1. Demand: Investigate biomass and nutrient partitioning differences in three distinct rootstocks and systems.

In 2024, a replicated set of trees were excavated, and components of tree biomass and nutrient partitioning are under analysis for 1) A 6th leaf Benton MxM14, Gisela 12 and Gisela 5. Rootstocks, 2) 12th leaf Skeena and Selah on G.12, and 3) A first-leaf Black Pearl on G.6, G.12 and Mazzard.

Sites 1 and 2 are the only ones bearing fruit, and fruit yield, biomass partitioning of the fruit and nutrient levels were determined after harvest. Site 3 is a new planting, where 3 trees per rootstock were selected for complete destructive analysis.

2. Timing: Identify strategies to monitor root growth in sweet cherry.

2.1 Root growth was evaluated in sites 1 and 2. Root windows were installed in 2023, on one of each rootstock (Example of the root windows can be found in Good Fruit Grower, link https://www.goodfruit.com/a-window-to-the-roots/). Monitoring started prior to bloom and continued a biweekly basis until leaf fall. Images were captured on a 1 ft grid for the root window.

In 2024, we incorporated the minirhizotron (CI-600 In-Situ Root Imager, CID-Bioscience) root growth monitoring tool and the Electrical resistivity tomography (*ERT*). The ERT was implemented in April 2024 by Dory Linneman and Dana Sirota (PNNL collaborators) and monitored at frequent intervals.

2.2. Soil Nutrient Availability and Leaching

To determine the availability and potential leaching of nutrients throughout the season, nutrient levels were evaluated at three depths throughout the season: bloom, active shoot growth, end of shoot growth, leaf yellowing, after leaf drop.

3. Deliver outreach and extension of new findings.

A good fruit grower reporting on our project in https://www.goodfruit.com/research-to-help-growers-schedule-the-sustenance/

SIGNIFICANT FINDINGS

- Fruit nutrient extraction of K, Mg and S were highest in MxM14. However, the highest difference was 1.3 lbs of K per ton of fruit, less than 0.3 lbs for Mg and S, and less than 7 g per ton of fruit for micronutrients. Thus, nutrient extraction differences among rootstocks were irrelevant for practical applications.

- Fruit extraction difference, calculated based on each fruit component versus flesh alone, were less than 1 lb per ton of fruit. Thus, fruit nutrient extraction can be estimated by measuring only the flesh, at a third of the cost associated for fruit nutrient testing.

- Fruit nutrient extraction by 'Benton' sweet cherry were within the same ranges reported for Chelan, Coral Champagne and Skeena (Sallato et al, 2024). Confirming that there are minimal differences in nutrient extraction among cultivars.

- After the second year of growth, Gi6 and Gi12 allocated a higher proportion of biomass to aerial parts of the tree (leader and laterals) compared to Mazzard. While weight of trees was higher in Gi12 and Mazzard, compared to Gi 6.

- Total nutrient content (grams of nutrient in the whole tree) was equivalent among rootstocks.

- Preliminary data of the Electrical resistivity tomography (*ERT*) for monitoring root growth suggest high correlation to soil temperature. However, temperature does not account for all the variability observed.

- Nitrate levels in sites 1 and 2 were within normal ranges, with consistently higher levels in the first 8 inches, with lower and equivalent values at 12 and 24 inches deep. However, nitrate fluctuation across the season were observed across all the depth and soil types, suggesting fast movement of nitrate through the profile.

METHODS

1. Demand: Investigate biomass and nutrient partitioning differences in three distinct rootstocks and systems.

Biomass partitioning is being evaluated in the following conditions:

- 5^{th} leaf Benton on MxM14, Gisela 12 and Gisela 5. Steep leader training
- 12^{th} leaf Skeena and Selah on G.12. UFO
- 1^{st} leaf Black Pearl on G.6, G.12 and Mazzard

At harvest, all fruit will be harvested to determine yield and nutrient extraction of fruit, from four replicates per rootstock / cultivar – this will determine fruit nutrient demand. After harvest, a subsample of leaves was collected to determine nutrient levels in recently mature leaves to monitor nutrient status following standard methods.

Before leaf fall a net was placed around the trees to collect all the leaves dropped and determine leaf allocation, partitioning and nutrient levels at drop. Between winter and spring, three trees per site and rootstock were excavated for whole tree partitioning and biomass allocation: total weight of each tree component, dry matter of each component, and total macronutrients N, P, K, Ca, Mg and micronutrient B (Figure 1).

Figure 1. Whole tree excavation (left) one year old shoot weighed and measured (center) subsample of spurs and wood before drying (right).

2. Timing: Identify strategies to monitor root growth

Root growth was evaluated in sites 1 and 2, with different rootstocks and systems. During year one, root windows were installed on each rootstock (Example of the root windows can be found in Good Fruit Grower, link https://www.goodfruit.com/a-window-to-the-roots/). Root growth was monitored starting at bloom, on a biweekly basis until leaf fall. Images were captured on a 1 ft grid for the root window.

In year 2, we will contrast the information with two newer technologies: a minirhizotron (CI-600 In-Situ Root Imager, CID-Bioscience) and an Electrical resistivity tomography (*ERT*). Both are non-destructive root monitoring systems that can help monitor root growth in established orchards. The minirhizotron is a 360 scanner that can be inserted in clear plastic tubes buried around the trees to scan roots throughout the season, while the ERT uses electrical sensors to monitor growth, a technology yet to be validated in orchard settings. The latter will be evaluated by our collaborators at the Pacific Northwest National Laboratory located in Richland, WA.

All images will be analyzed using the WinRHIZOTRON image analysis software (Regent Instruments Canada, Quebec, Canada).

3. Soil Nutrient Availability and Leaching

To determine the availability and potential leaching of nutrients throughout the season, nutrient levels were evaluated in the root zone and below the root zone, six times through the season; bloom, active shoot growth, end of shoot growth, leaf yellowing, after leaf drop. This will inform fertilization timing to increase efficiency in nutrient uptake.

RESULTS AND DISCUSSION

1. Demand: Investigate biomass and nutrient partitioning differences in three distinct rootstocks and systems.

Bearing sites

Biomass partitioning was determined for site 1) a 6- year-old sweet cherry 'Benton' block, grown on MxM14, Gisela 12 and Gisela 5. The average fruit size ranged from 9.8 to 10.7 with no differences between rootstocks. There were no differences in flesh and pit partitioning, and their dry matter concentration (Table 1), while stem partitioning and dry matter were lowest on Gi12 (Table 1).

Table 1. Fruit fresh partitions into flesh, pit and stems, and tissue dry matter by rootstock. Different letters indicate statistical differences within columns.

Rootstock	Fruit Weight (g)	Proportion (%)			Dry Matter (%)		
		Flesh	Pit	Stems	Flesh	Pit	Stems
Gi5	9.8	94%	5%	1.0% a	23%	58%a	52%
Gi12	10.7	94%	6%	0.7% b	22%	53%b	48%
MxM14	10.6	94%	5%	1.0% a	23%	59%a	47%
Pr > F(Model)	0.418	0.463	0.290	**0.005**	0.566	**0.003**	0.321

Fruit nutrient extraction was calculated considering all fruit components (flesh, pit and stems), as each of these are removed from the orchard at harvest. Fruit extraction of macronutrients N, P, Ca were not different between rootstocks ($p<0.05$), while K, Mg and S extraction were highest in MxM14 (Table 2). MxM 14 demanded 1.3 lbs. and 0.7 lbs. more K, compared to Gi12 and Gi5, respectively (Table 2). When translating this difference to a 10 tons/acre block, the maximum difference was only 13 lbs. for K, and less than 3 lbs. for Mg and S. For micronutrients, the differences were much smaller (< 70 g). Thus, differences across rootstocks are irrelevant for practical estimation of nutrient demand.

Table 2. Fruit nutrient extraction considering all fruit components; flesh, pit and stems, for 'Benton' sweet cherry on three rootstocks.

Nutrient / Cultivar	N	P	K	Ca	Mg	S	B	Zn	Mn	Cu
	Lb/USTon						g/USTon			
Gi5	3.2	0.9	5.1 b	0.51	0.31 ab	0.20 b	4.3 b	0.82 a	1.2 a	1.0 ab
Gi12	3.2	0.8	4.5 b	0.41	0.27 b	0.17 b	4.2 b	0.54 b	1.0 b	0.9 b
MxM14	4.0	1.0	5.8 a	0.45	0.33 a	0.26 a	6.8 a	0.57 b	0.8 b	1.2 a
p value	ns	ns	0.002	ns	0.032	0.009	0.038	0.00	0.001	0.024

Note: to estimate rate of nutrient required per acre, multiply the value in the table by the production in tons (US tons).

Fruit extraction differences, calculated based on each fruit component versus flesh alone, were less than 1 lb per ton of fruit (Table 3), which relates to the high relative contribution of fruit flesh to the total weight (94%) and total dry matter (86%). These findings suggest that the estimation of nutrient extraction can be obtained by analyzing only the flesh, saving two thirds of the resources needed for nutrient testing.

Table. 3. Difference in the amount of nutrient extracted by fruit when calculated based on each fruit component versus only flesh.

	N	P	K	Ca	Mg	S	B	Zn	Mn	Cu
Cultivar	Lb difference /USTon						g difference /USTon			
Benton	0.4	0.1	0.1	0.13	0.04	0.02	0.4	0.2	0.2	0.2

Fruit nutrient extraction by 'Benton' sweet cherry were within the same ranges reported for Chelan, Coral Champagne and Skeena reported by Sallato et al (2024). This confirms that there are minimal differences in nutrient extraction among cultivars (Table 3).

Table 3. Ranges of fruit nutrient extractions for WA cultivars.

Nutrient /	N	P	K	Ca	Mg	S	B	Zn	Mn	Cu
Cultivar	Lb/USTon						g/USTon			
Chelan	3.2 - 4.6	0.5 - 0.7	3.9 - 6.4	0.4 - 0.6	0.3 - 0.4	0.2 - 0.3	3.4 - 11	0.2 - 1.5	0.7 - 1.8	0.6 - 1.2
Coral C.	2.4 - 4.9	0.5 - 0.7	3.2 - 5.4	0.3 - 0.4	0.2 - 0.3	0.2 - 0.4	1.3 - 7.7	0.3 - 1.8	0.1 - 2.3	0.6 - 1.4
Skeena	1.9 - 3.4	0.6 - 0.9	2.9 - 6.3	0.3 - 0.4	0.2 - 0.4	0.2 - 0.4	3.7 - 21	0.2 - 0.9	0.1 - 1.5	0.5 - 1.2
Benton*	2.8 - 3.5	0.8	4.5 - 5.6	0.3	0.2 - 0.3	0.2	3.8 - 6.4	0.4 - 0.5	0.7 - 1.0	0.4 - 1.1
Range	**1.9 - 5.0**	**0.5 - 0.9**	**2.9 - 6.3**	**0.3 - 0.4**	**0.2 - 0.4**	**0.2 - 0.4**	**3.7 - 21**	**0.2 - 1.8**	**0.1 - 2.3**	**0.5 - 1.4**

* Information for Benton was obtained in this project. Note: to estimate rate of nutrient required per acre, multiply the value in the table by the production in tons (US tons).

Non-bearing site

After the second year of growth, Gi6 and Gi12 allocated higher proportion of biomass to aerial parts of the tree (leader and laterals) compared to Mazzar, which has higher proportion of big and fine roots, while there were no differences in the proportion of biomass allocated to the stump (aerial portion of the rootstock) (Table 4). Dry matter of each component did not vary among rootstocks (Table 4)

Table 4. Fresh weight partitioning and dry matter of different tree components in a 2nd leaf Black Pearl grown on G6, G12 and Mazzard.

Rootstock	Fresh Partitioning (%)					Dry Matter (%)				
	Leader	Laterals + Buds	Stump	Big Roots	Fine Roots	Leader	Laterals + Buds	Stump	Big Roots	Fine Root
Gi6	48 a	15 a	16	16 ab	5 b	54%	49%	49%	54%	51%
Gi12	55 a	15 a	17	10 b	2 c	52%	47%	61%	43%	48%
Mazzard	33 b	8 b	16	33 a	10 a	53%	39%	60%	35%	47%
Pr > F(Model)	0.006	0.050	0.858	0.018	0.000	0.676	0.055	0.195	0.145	0.77

Whole tree weight was higher in Gi12 and Mazzard ($p < 0.10$) compared to Gi 6. However, total nutrient content (grams of nutrient in the whole tree) was equivalent for all nutrients, except K, where Mazzard had higher amount compared to Gi6 (data not shown).

Table 5. Tree weight and nutrient content (total grams per tree) on a leaf Black Pearl grown on G6, G12 and Mazzard.

Rootstock	Tree Weight (g)	Woody Tissue Nutrient Content (g)				
		N	P	K	Ca	Mg
Gi6	464.78	4.13	0.55	0.8 b	1.43	0.46
Gi12	773.22	5.55	0.74	1.3 ab	2.27	0.73
Mazzard	793.48	5.25	0.63	1.5 a	1.84	0.72
Pr > F(Model)	0.079	0.348	0.273	0.036	0.329	0.080

In 2024, whole tree destruction of 6-year old Benton trees showed no difference in tree partitioning among rootstocks (Table 6).

Table 6. Fresh weight partitioning for different tree components in a 6th leaf Benton grown on G5, G12 and MxM14.

Rootstock	Fresh Partitionin (%)							
	FineRoots	BigRoots	Rootstock	Trunk	Leader	2nd.Yr.	1st.Yr	Spurs
G5	10%	15%	8%	19%	39%	5%	3%	1%
G12	6%	9%	9%	26%	37%	8%	4%	1%
MxM14	6%	11%	15%	21%	36%	7%	4%	0%
p value	0.609	0.237	0.111	0.267	0.613	0.162	0.813	0.026

Note: There were no differences among rootstocks

Nutrient extraction by the woody tissue (all tissue except fruit and leaves), on a per ton of tree basis were not different among rootstocks.

Rootstock	Tree Weight (Kg)	Nutrient Extraction (Kg/Ton of Trees)				
		N	P	K	Ca	Mg
G5	67.9	5.0	0.9	1.9	6.4	0.8
G12	66.2	4.9	1.1	1.8	6.2	0.8
MxM14	87.4	4.5	0.8	1.8	5.1	0.8
p value	0.179	0.767	0.330	0.910	0.493	0.864

2. Timing: Identify strategies to monitor root growth

Reported by Dory Linneman and Dana Sirota (PNNL)

Electrical resistivity tomography (ERT) is a direct-current imaging method that can be used to estimate the distribution of electrical resistivity. Bulk resistivity is related to several properties of the subsurface including rock/soil type, porosity, ionic strength of the pore fluids, and surface conductivity of geologic materials. Each study tree was instrumented with 30 electrodes in 5 rows of 6 electrodes centered around the trunk of the tree (Figure 2). The electrodes were spaced 18 inches apart and contained within protective boxes.

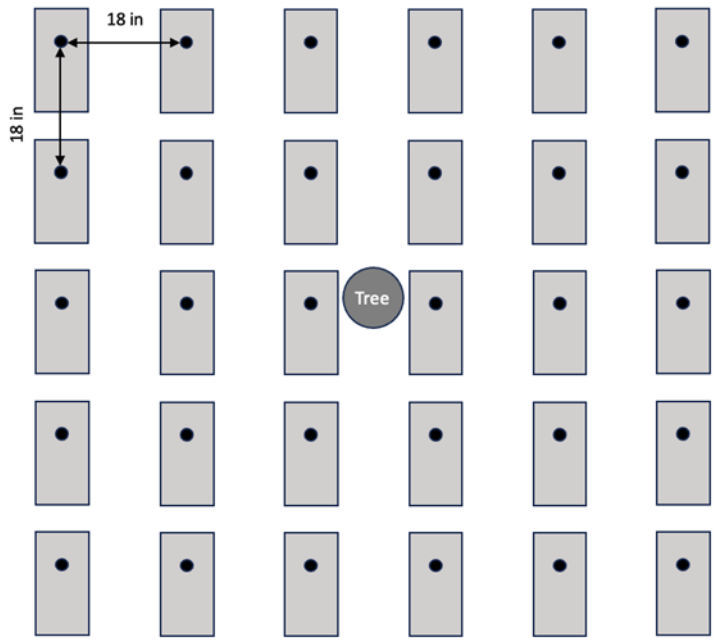

Figure 2. Schematic diagram of the electrode layout around each study tree. The electrodes (shown as black circles) were 10-inch steel nails inserted approximately 6 inches into the ground. The electrodes are spaced 18 inches apart and are contained within protective enclosures (shown as gray boxes).

In this study, this procedure is repeated for 230 combinations of source and receiver electrodes per survey. This survey design was conducted on the six study trees and was repeated 13 times between April and September 2024. Figure 3 shows a field photo of data being collected.

Figure 3. Photograph from the field site showing the data collection set up at one target tree.

The resistance measured by the field instrument is converted to an "apparent conductivity" between electrode pairs with a geometric factor determined by the source and receiver electrode positioning.

To provide a preliminary look at changes over time, the average apparent conductivity for each survey was calculated. In addition to subsurface structures, electrical properties are somewhat temperature dependent. To show this relationship, in Figure 4 soil temperature data from a meteorological station on the Roza site is plotted along with the average apparent conductivities of each survey over time. Qualitatively, we observe that while changes in conductivity seem somewhat correlated to temperature, it does not account for all the variability seen in apparent conductivity.

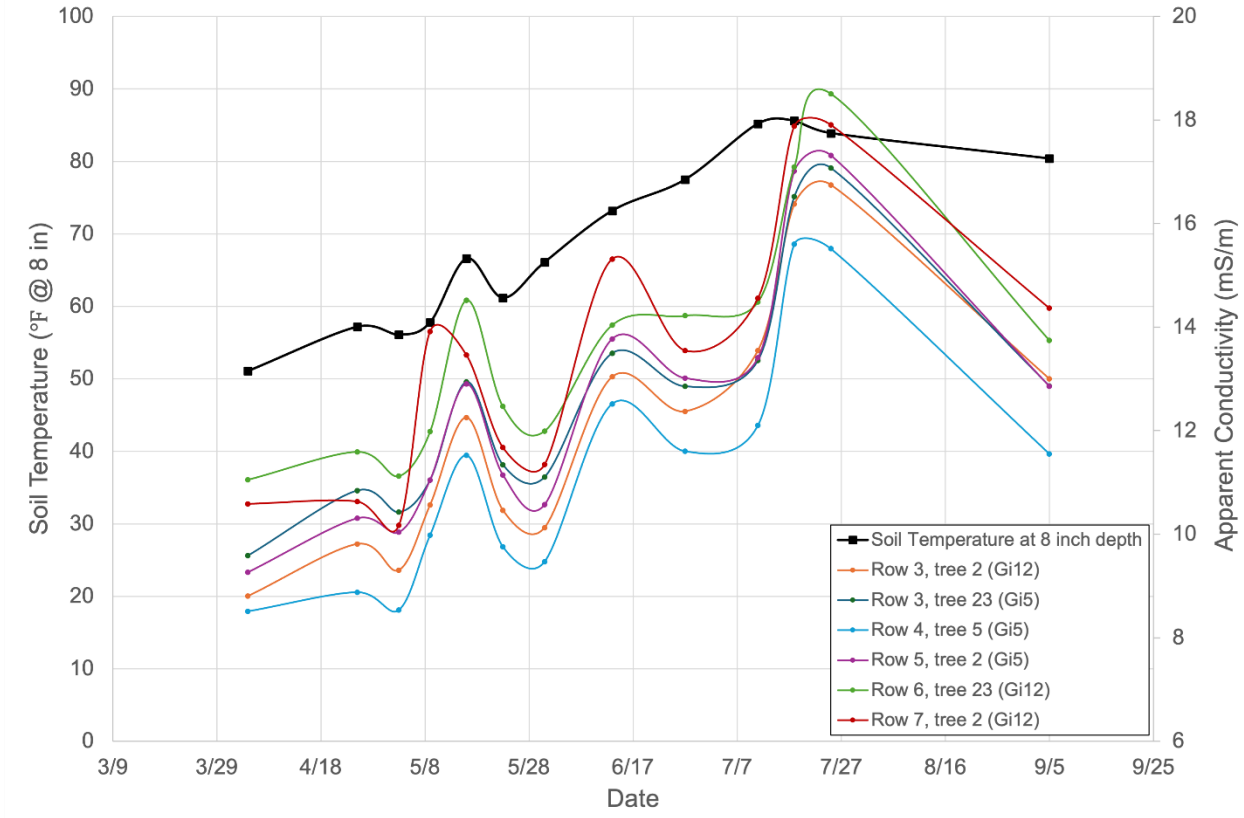

Figure 4. Average apparent conductivity of each survey at each target tree over time. 8-inch soil temperature at noon on each survey day is also plotted.

In this study, we discarded data with standard deviations exceeding 5 percent, current below 2A, and contact resistance over 15 kΩ. Of the nearly 18,000 data points collected, approximately 3% were culled using these criteria. The tree with the highest number of 'bad' data points culled less than 5% of the data. This is a strong indication of high data quality throughout the dataset.

3. Soil Nutrient Availability and Leaching

Soil nutrient levels were measured monthly from May to October at three depths: 8, 12 and 24 inches. Soil nitrate (NO_3) levels were low at the beginning of the season, ranging from 2.9 to 5.5 mg/kg. As the soils start to warm up, NO3 increased slightly in all soils. Nitrate levels were higher in the top layer of soil (first 8 inches) (Figure X top), decreasing to about half the amount in the 12 and 24 inches. In all sites, nitrate levels across the season followed the same pattern, (e.g. increasing in all

the depths at the same time), suggesting there is fast movement from the upper strata to the 24 inches depth.

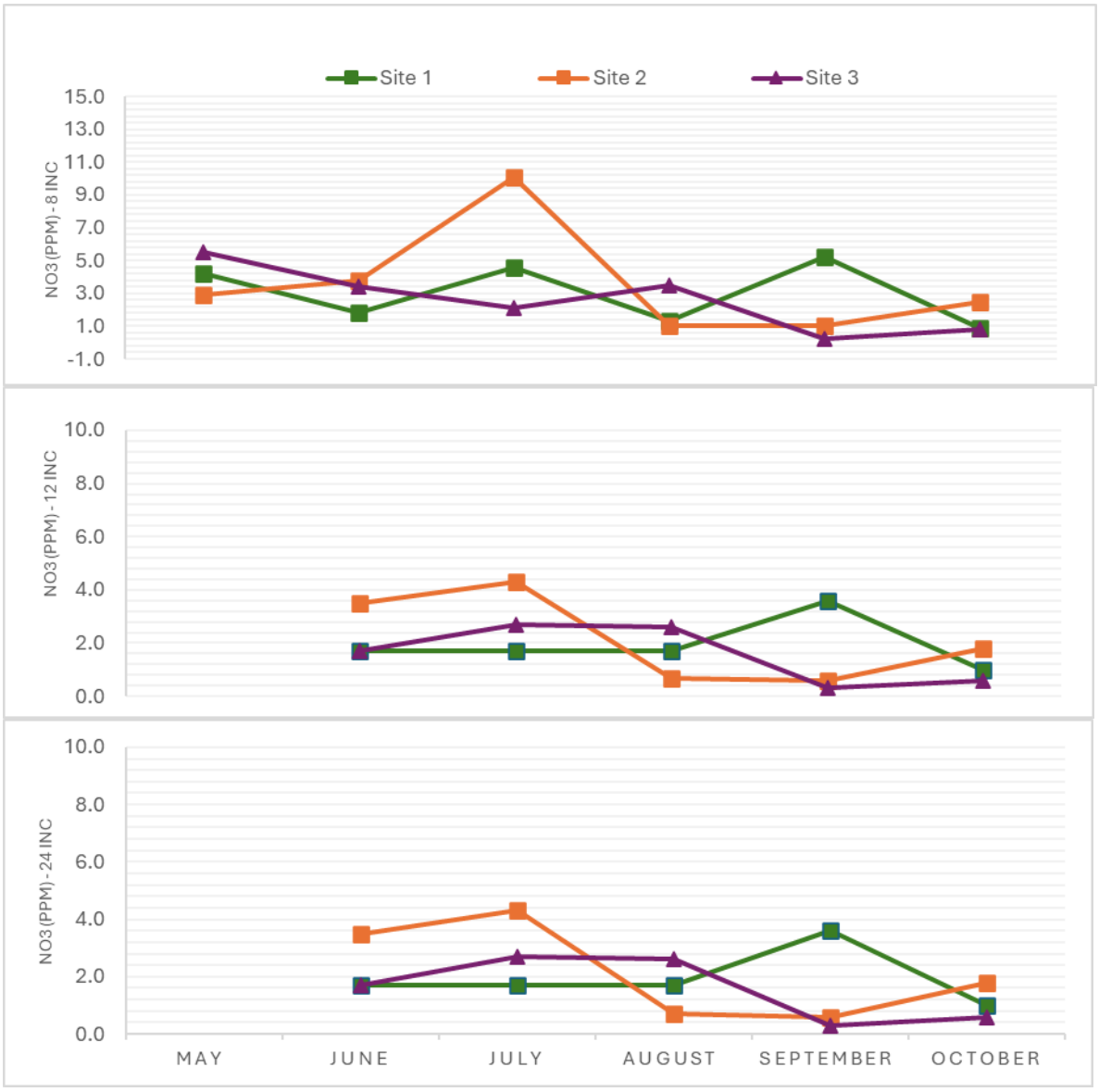

Figure 5. Soil nitrate (NO_3) throughout the season at three depths on site 1, Site 2 and Site 3.

Project Title: Targets and tools for post-bloom thinning

Report Type: Continuing Project Report

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Cooperators: Haydn Farms

Project Duration: 2 Year

Total Project Request for Year 1 Funding: $ 43,135
Total Project Request for Year 2 Funding: $ 45,310

Budget 1
Primary PI: Bernardita Sallato
Organization Name: Washington State University
Contract Administrator: Hollie Tuttle
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Item	2023	2024
Salaries	30,434	31,651
Benefits	4,678	4,865
Wages	6,146	6,392
Benefits	627	652
Equipment		
Supplies	250	250
Travel	1,000	1,500
Miscellaneous		
Plot Fees		
Total	43,135	45,310

Footnotes: salaries will fund M.S. student, wages for summer timeslip assistance with trial setup, fruit harvest, and fruit quality analyses; supplies for fruit quality testing and field trials, travel is to research plots in WA and to attend industry meetings for presenting results.

OBJECTIVES

1. Develop practical balanced cropping targets
2. Better understand the effects of timing of thinning
3. Investigate efficacy of Ethephon and Accede® as post-bloom thinners
4. Summarize and disseminate key findings to stakeholders

In the first year (2023) we were unable to recruit and hire a graduate student in time to lead the project. We did however complete three PGR trials, assessing the efficacy of Ethephon and Accede in 'Coral Champagne', 'Chelan', and 'Benton'. We received a no-cost extension in 2024 and are considering the 2024 season as year 1 and 2025 as year 2.

SIGNIFICANT FINDINGS

- Ethephon and ACC are variable in their efficacy as post-bloom thinners
- Thinning efficacy may be cultivar dependent – treatments were largely ineffective for 'Chelan' but nearly thinned all fruit in 'Benton'
- Timing of thinning affected fruit quality but the earliest thinning did not yield the best results
- There can be benefits to thinning as late as 6 weeks after full bloom

METHODS

Investigate efficacy of Ethephon and Accede® as post-bloom thinners

Based on our previous findings, we will focus efforts on the period between shuck fall and 7 – 10 days afterward. We will evaluate the effect of Ethephon 2 (21.7% 2-chloroethyl phosphonic acid) and Accede® applied at 100 and 200 ppm on fruit set and fruit quality. Treatments will be made to heavily-set trees at shuck fall or shuck fall + 7 – 10 days (orchard blocks to be identified each Spring). Applications will be made to entire trees or limbs, with a minimum of 10 replicate branches (2/tree) selected for determining fruit set/density and fruit quality.

Within a day of application, we will flag two limbs in every tree (1 east-facing and 1 west-facing) and count fruitlet density (fruitlets/limb cross-sectional area and length), measuring limb caliper at the same time. In addition, we will measure fruit diameter on 10 fruit per limb to record fruitlet size at the time of treatment – this will facilitate comparisons among cultivars with respect to timing and final fruit size. A photo journal will be collected as well to visually document application timings and crop densities. At commercial fruit maturity we will make fruit counts to the same limbs and assess thinning efficacy as % fruitlet removal. Fruit subsamples (minimum 100 fruit per replication) will be collected and analyzed for quality attributes including color, weight, diameter, and firmness, at the facility in Prosser.

RESULTS & DISCUSSION

'Coral Champagne'. This trial was conducted in a 'Coral Champagne'/Mazzard block north of Pasco. Trees were trained to a Y-trellised, Tatura-type architecture with 7 horizontal fruiting tiers. Treatments were made on 10 May by backpack sprayer just to drip. Fruit set in untreated control was just under 70%. Fruit set reported here is the number of fruit harvested from selected limbs compared to the number of fruit present at the time of treatment (i.e., not overall fruit set as a % of available flowers). The inverse represents fruit drop and was ca. 32% for the control. Fruit density of untreated control limbs was not particularly high at ca. 36 fruit per foot. By other metrics that we evaluated, fruit density in control was ca. 8.0 fruit/spur or 0.86 fruit/cm^2 branch cross-sectional area. Treatment with ACC did not reduce fruit set (i.e. did not increase fruit drop) though fruit density was reduced by ca 40% with ACC applied at 400 ppm (Table 1). Ethephon at 100 ppm was ineffective as

a thinner, however, Ethephon at 200 ppm reduced fruit set by about 36% (P=0.12) compared to the control. Fruit density was significantly reduced by applications of Ethephon 200 compared to the control – 14.7 vs. 35.5 fruit/foot, respectively.

Table 1. Effects of PGR treatments on fruit drop, fruit density, and fruit quality in 'Coral Champagne'. Data with different letters are significantly different at P>0.1.

Treatment	Fruit set (%)	Fruit/ft	Color (CTIFL)	Soluble solids	Firmness (g/mm)	Fruit weight (g)
Control	67.9 a	35.5 a	4.2 ab	13.4 a	246.8 bc	9.2 a
ACC 200	64.9 a	25.8 ab	3.6 b	12.7 a	255.3 abc	7.1 cd
ACC 400	58.2 a	20.8 b	3.8 ab	14.1 a	270.1 a	6.7 d
Ethephon 100	52.7 a	18.8 b	4.3 a	13.6 a	241.1 c	8.5 ab
Ethephon 200	43.7 a	14.7 b	4.0 ab	13.3 a	260.8 ab	7.9 bc
Pr > F	0.119	0.001	0.031	0.21	0.001	<0.0001

No treatment improved fruit quality, with the exception of an increase of about 10% of fruit firmness in response to treatment with ACC at 400 ppm (Table 1). Interestingly, treatment with ACC reduced fruit weight by about 25% compared to the control. The higher rate of Ethephon also reduced fruit weight by about 14%. Only Ethephon at 100 ppm did not have a negative effect on fruit weight. It is also interesting to see that no treatment had any significant effect on fruit color. Therefore, these treatments did not hasten maturity – a concern that growers have expressed.

*'**Chelan'***. This trial was conducted in a 'Chelan'/'Gisela6' block, in Tricities. Trees were trained to a Y-trellised architecture with 7 horizontal fruiting tiers. Treatments were applied at ca. 10 days after shuck fall. Fruit set in the untreated control was much lower than in 'Coral' at 37% (Table 2). This means that between the time of treatment and harvest, nearly 63% of the fruit dropped without any treatment. This is unusually high and affected this experiment. Ethephon treatments did not affect the drop significantly. Treatment with 100 or 200 ppm exhibited fruit drop rates of ca. 67% and 59%, respectively. ACC treatments were similarly ineffective at inducing fruit drop. Applications of 200 and 400 ppm ACC resulted in drop of 67% and 74%, respectively, statistically similar to the untreated control. Similarly, no treatment had any effect on fruit density as fruit/foot was similar among all treatments, and relatively low overall (only ca. 16 for control).

Table 2. Effects of PGR treatments on fruit drop, fruit density, and fruit quality in 'Chelan'. Data with different letters are significantly different at P>0.1.

Treatment	Fruit set (%)	Fruit/ft	Color (CTIFL)	Soluble solids	Firmness (g/mm)	Fruit weight (g)
Control	36.7 ns	15.9 ns	4.04 ab	12.1 ab	269.1 ns	7.0 ns
ACC 200	32.6	15.5	4.13 ab	12.7 a	262.4	6.9
ACC 400	26.3	9.9	3.84 b	11.5 b	262.8	7.0
Ethephon 100	33.9	11.6	4.93 a	11.9 ab	286.6	7.7
Ethephon 200	41.4	14.6	3.93 ab	12.0 ab	275.8	7.3
Pr > F	0.64	0.59	0.088	0.12	0.38	0.31

Fruit quality was largely unaffected by treatment. Soluble solids were reduced slightly with ACC at 400 ppm, and treatment with Ethephon at 100 ppm increased fruit color (though treatment with Ethephon at 200 ppm did not). Firmness and weight were similar across all treatments.

'Benton'. This trial was setup at the WSU-Roza experimental farm in a 'Benton'/'Gisela5' block. Trees were trained to a modified steep leader architecture. Treatments were applied about 12 days after shuck fall.

Table 3. Effects of PGR treatments on fruit drop, fruit density, and fruit quality in 'Benton'. Data with different letters are significantly different at P>0.1.

Treatment	Fruit set (%)	Fruit/ft	Color (CTIFL)	Soluble solids	Firmness (g/mm)	Fruit weight (g)
Control	40.3 a	17.2 a	5.0 ns	20.0 ns	213.2 a	9.2 a
ACC 200	17.8 b	6.6 b	5.3	20.2	196.4 ab	8.4 ab
ACC 400	14.1 b	2.8 b	5.4	19.4	194.5 ab	9.2a
Ethephon 100	12.5 b	3.2 b	5.1	19.5	219.4 a	7.5 b
Ethephon 200	0.31 b	0.6 b	5.5	19.7	187.3 b	7.4 b
Pr > F	<0.0001	<0.0001	0.19	0.74	0.047	0.003

For 'Benton', fruit set of control limbs was 40% (i.e., about 60% of the fruit present on application of treatments dropped by harvest time). This translated to about 17 fruit/foot at harvest, which again is not a very high crop load. Control limbs had ca. 3 fruit/spur on average. In this trial each thinning treatment was effective (Table 3). Application of ACC at 200 and 400 ppm reduced fruit set by 56% and 66%, respectively. These treatments reduced fruit density to 6.6 and 2.8 fruit per foot. Treatment with ACC200 and ACC400 reduced crop load to 1 and 0.5 fruit/spur, respectively (data not shown). Ethephon applications were even more effective at reducing crop load. Fruit set was reduced by 69% and nearly 100% with applications of Ethephon at 100 and 200 ppm, respectively. This translated to 3.2 and 0.6 fruit/foot. Despite the significant reductions in crop load with all treatments, there was no increase in fruit size nor soluble solids. This is likely due to the low crop load in untreated limbs – fruit had sufficient carbohydrate resources. ACC treatments were similar to the control in fruit weight, but the Ethephon treatments reduced fruit weight (Table 3). This is similar to the reduction in 'Coral Champagne' fruit weight we observed under treatment with 200 ppm Ethephon.

We assessed the relationships between fruit quality attributes and fruit density (as fruit/foot, fruit/limb cross-sectional area, fruit/spur) and found little to no correlations. For example, in 'Coral Champagne', there was no clear relationship between fruit/foot and any key fruit quality attribute (Fig. 1). These experiments need to be conducted in blocks that have higher crop load in order to better assess these relationships.

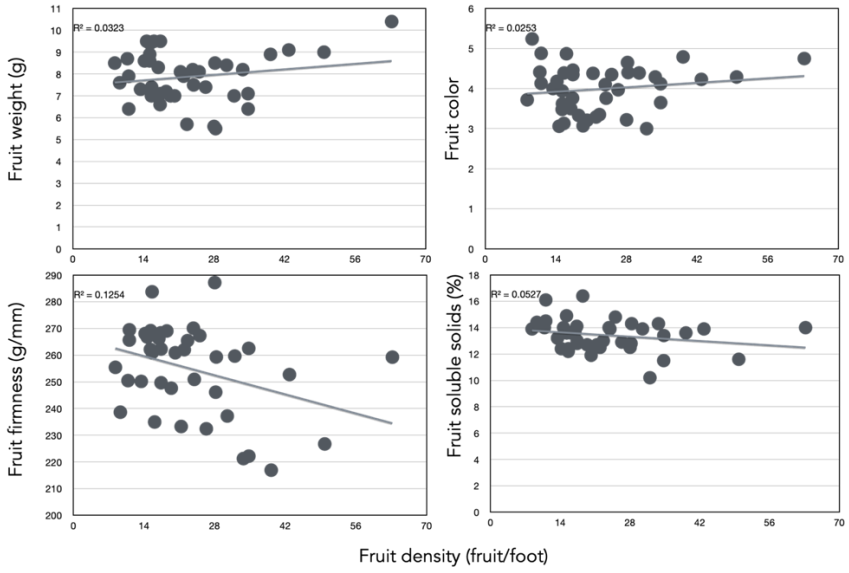

Figure 1. The relationships between fruit quality and fruit density (fruit/foot) for 'Coral Champagne'.

PGR Trials 2024

We conducted trials in 4th-leaf 'Black Pearl' and 'Santina' blocks. Both blocks were trained to a Y-trellised UFO architecture on 'Gisela 12' rootstock. Each treatment was applied to 12 replicate upright branches. Fruit counts were made on each branch on the day of treatment and again, at harvest. At harvest all fruit were harvested, weighed, and brought to IAREC for quality analyses. The basal caliper of each upright was measured at harvest.

Table 4. Effect of thinning treatments applied to 'Santina' on 29 April. Data with different letters are significantly different at 95% confidence. N=12.

Treatment	Fruit drop (%)	Fruit density (g/mm^2)	Weight (g)	Firmness (g/mm)	Color (1-7)	Sol. Solids (%)
Control	32.2 b	6.58 a	6.8 b	229 ns	5.7 ns	14.7 b
ETH100	72.4 a	2.56 b	6.9 b	240	5.8	17.7 a
ETH200	82.9 a	1.00 c	7.6 ab	238	6.2	17.9 a
ACC200	89.3 a	0.81 c	8.3 ab	241	5.6	15.7 ab
ACC400	86.1 a	0.97 c	9.3 a	236	5.9	14.6 b
Pr > F	<0.0001	<0.0001	0.011	0.637	0.141	0.011

Thinning treatments were applied on 29 April, about 2 weeks after full bloom. At this stage nearly all fruit were past shuck fall. Natural drop in 'Santina' was about 32%, meaning nearly one of every 3 fruitlets dropped between the date of treatment and harvest. All PGR treatments were effective as thinners, inducing significant drop compared to untreated control (Table 4). The drop induced by thinning treatments was ca. 2 – 2.5 times greater than the control, and there was no difference among thinners. As a result, fruit density (number of fruit per mm^2 of branch cross-sectional area) and fruit density (fruit weight per mm^2 branch cross-sectional area) were reduced by all thinning treatments. Without any treatment, fruit density was about $6.6 g/mm^2$ bcsa. This was reduced by nearly 90% with Ethephon at 200 ppm and both Accede treatments. For reference, unthinned control limbs had an average of 215 fruit and ca. 3.1 lbs of fruit. In contrast, ETH100, ETH200, ACC200 and ACC400 had branch yield of ca. 1.0, 0.85, 0.5, and 0.7 lbs, respectively. Yield of unthinned trees in this block was ca. 11 tons/acre so thinning treatments would have reduced this to about 3.5, 2.9, 1.7, and 2.4 tons/acre, respectively.

Thinning treatments improved fruit size (weight) and soluble solids, but had no effect on fruit firmness nor exocarp color. The only treatment that improved fruit weight was Accede at 400 ppm – this increased weight by 37%. This thinning was excessive however, as most branches dropped almost 90% of the fruit. At the time of treatment, there was an average of 320 fruit per branch in the ACC400 limbs. This was reduced to fewer than 40 at harvest, with several branches having only a few fruit left. Accede at 200 ppm was similarly effective as a thinner, inducing ca. 90% drop. Interestingly, Accede treatments did not improve fruit soluble solids but both Ethephon treatments did, by ca. 3%. This improvement would improve consumer appeal – previous research has shown the importance of fruit sweetness for consumers, and an increase from 14.7 to 17.7 would be easily discernible to consumers' palates.

There has been concern over use of ethylene-related PGRs in sweet cherry and their potential negative effect on fruit firmness. We did not document any influence of our treatments on either fruit firmness or color (indicative of maturity). This is likely due to these treatments being applied relatively early in fruit development. In other work we have demonstrated the potential for Ethephon treatments to reduce firmness and advance maturity (via darker fruit color) when applied within weeks of harvest.

Table 5. Effect of thinning treatments applied to 'Black Pearl' on 26 April. Data with different letters are significantly different at 95% confidence. N=12

Treatment	Fruit drop (%)	Fruit density (g/mm^2)	Weight (g)	Firmness (g/mm)	Color (1-7)	Sol. Solids (%)
Control	21.0 c	4.6 b	6.0 ns	265 c	5.0 b	16.9 b
ETH100	20.0 c	5.9 a	6.2	276 bc	5.7 a	18.4 ab
ETH200	44.4 b	3.1 b	6.6	284 ab	5.8 a	18.7 a
ACC200	37.3 bc	3.9 b	5.9	277 abc	5.4 ab	17.1 ab
ACC400	67.9 a	2.3 c	6.1	293 a	5.3 b	17.8 ab
Pr > F	<0.0001	0.01	0.383	0.002	0.0001	0.057

The thinning effects of Ethephon and Accede were variable in 'Black Pearl', and the response was different from that of 'Santina'. Natural fruit drop was less for 'Black Pearl' than it was for 'Santina', with about one in every 5 fruit dropping between 26 April and harvest (i.e., 21% drop). Treatments with Ethephon at 100 ppm and Accede at 200 ppm were ineffective at inducing drop. In contrast, 200 ppm of Ethephon was effective, inducing twice as much drop as the control. In addition, Accede at 400 ppm was the most effective at inducing drop with more than two of three fruit dropping (ca. 68%) before harvest. Fruit density was highest in ETH100 and lowest in ACC400 but similar for all other treatments. Yield per branch was about 3.1 lbs in control compared to 3.0, 1.7, 1.6, and 1.0 lbs for ETH100, ETH200, ACC200, and ACC400, respectively. At a proportional reduction, yield would be about 10.2, 5.8, 5.4, and 3.4 tons/acre for these treatments (from ca. 10.5 t/ac in unthinned). Interestingly, the thinning treatments were less effective overall in 'Black Pearl' compared to 'Santina', despite being applied at a very similar timing.

In contrast to the results with 'Santina', no treatment improved 'Black Pearl' fruit size/weight despite the higher rates of both Ethephon and Accede inducing significant drop. Clearly this is an unfavorable, and unexpected response – we don't yet understand how a PGR could induce drop (i.e., reduce crop load) at this timing without improving fruit quality. Trials will be repeated in 2025 to determine whether this response is consistent for 'Black Pearl'. In contrast, fruit firmness was improved ca. 7 and 11% with ETH200 and ACC400 treatments, respectively. In addition, the ETH200 treatment improved fruit soluble solids by about 11% - no other treatment was different from untreated control. Interestingly, for 'Black Pearl' the treatments with Ethephon advanced fruit color development – fruit were nearly a full grade darker at harvest compared to control. This change in fruit color was not associated with any reduction in firmness.

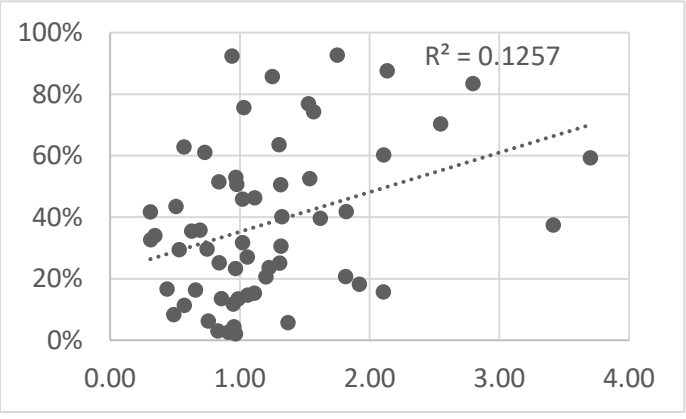

Figure 2. Relationship between thinning efficacy (% drop) and fruitlet density at the time of treatment.

Because we analyzed each limb separately we can assess relationships that may have influenced thinning efficacy. We found no strong role for initial crop load nor branch caliper on the extent of drop induced by treatment (Fig. 2).

Timing of thinning trials

Trials were established in the same 'Santina' and 'Black Pearl' blocks where PGRs were assessed. To determine the effect of timing of thinning, entire trees were thinned by hand at 6 distinct timings including dormant, full bloom (FB), and at two-week intervals subsequently. Treatments were carried out on 8 whole-tree replicates, though we collected data on each upright separately. At harvest, each upright limb was harvested separately and its basal caliper was measured. Branch yield was determined in the field and fruit were transported to IAREC for quality assessments.

'Santina'. Yield per tree was 12.0 kg (ca. 11 tons/acre) in the unthinned treatment. This is high considering trees were in their 4th leaf and reflects the excellent precocity of the UFO system. Yield was similar for trees thinned at all timings except the latest (FB+8 weeks) for which yield was ca. 3.5 tons/acre (70% lower than unthinned; Table 6). The significant yield reduction from the final thinning timing may reflect some unintended damage to fruit during the thinning process. Thinning treatments had little effect on fruit density – similar to yield, all timings except the last were similar to the control. Our goal was to isolate the timing effect as much as possible so it is useful that yield and fruit density were similar for most of the timings.

Table 6. Effect of timing of whole-tree thinning treatments on 'Santina' yield and fruit quality. Data with different letters are significantly different at 95% confidence.

Timing of thinning	Tree yield (kg)	Fruit density (g/mm^2)	Fruit weight (g)	Firmness (g/mm)	Color (1-7)	Sol. Solids (%)
Unthinned	12.0 a	5.0 ab	7.0 b	231.9 b	5.6 a	15.0 d
Dormant	12.1 a	5.3 ab	7.6 b	225.4 b	5.7 a	16.4 cd
Full bloom	12.0 a	5.9 a	8.3 ab	235.9 ab	5.6 a	17.4 bc
FB+ 2 weeks	10.5 a	4.8 ab	7.9 b	232.4 b	5.6 a	16.3 cd
FB+ 4 weeks	9.1 a	3.8 b	9.7 a	248.6 a	5.7 a	18.2 ab
FB+ 6 weeks	8.2 a	3.5 bc	9.7 a	247.3 a	5.6 a	19.2 a
FB+ 8 weeks	3.7 b	1.8 c	7.7 b	233.8 b	5.7 a	17.1 bc
Pr > F	<0.001	<0.001	<0.001	<0.001	0.9	<0.001

Fruit weight was improved by nearly 40% from thinning treatments at FB+4 weeks and FB+6 weeks (Table 6). These improvements in fruit weight clearly offset the reduction in crop load since yield and fruit density were similar for these two timings and control. The earliest thinning treatments did not improve fruit size significantly. Nor did thinning at FB+8 weeks. It isn't clear why the period of FB+4 – 6 weeks should be beneficial compared to earlier timings. Final fruit size is determined largely by cell size, but also by cell numbers in the mesocarp. Thinning at 4 to 6 weeks after full bloom coincides roughly with the end of phase II of stone fruit growth and therefore largely precedes cell expansion. We did not see a similar response for 'Black Pearl' (see below) so we will repeat the trials in 2025 to determine if this is unusual to 'Santina' and consistent across years.

Fruit firmness responded similarly – it was improved only by thinning treatments imposed between FB+4 and FB+6 weeks. These treatments increased firmness by ca. 7% compared to the control. Color was unaffected by timing of thinning. Soluble solids was improved by most timings of thinning, with the greatest improvements for trees thinned at FB+4 and FB+6 weeks. These treatments improved soluble solids by ca. 21% and 28%, respectively. Both thinning treatments led to improvements in soluble solids of more than 3^{o}brix – a level that would significantly improve the eating experience of the fruit. This is especially important for early-season cultivars as repeat purchases of sweet cherries are driven by eating experience and consumers routinely rank sweetness as the most important attribute.

Combined, it appears that thinning at FB+4 weeks provided the best response – yield of 9.1 tons/ac (est.) with significant improvements in fruit size/weight (+39%), firmness (+7%), and soluble solids (+ 21%). Again, this work will be repeated in 2025 to characterize the thinning response across years.

'Black Pearl'. Yield per tree was 11.6 kg (ca. 10.5 tons/acre) for unthinned. Importantly, yield of trees thinned at dormant, full bloom, and FB+2 weeks was statistically similar despite thinning – this is reflected in the improvement in fruit weight (Table 7.). The later thinning treatments reduced yield. Estimated yield for thinning at FB+4 and 6 weeks is similar at ca. 6.5 tons/acre (ca. 38% reduction compared to unthinned). The latest thinning treatment had the lowest yield at 4.7 kg/tree or, ca. 4.3 tons/acre, less than half of unthinned. In these cases, treatment effects may not reflect the timing of thinning but the reduction in overall crop load. Fruit density was assessed as the yield of fruit per branch basal cross-sectional area. Overall this varied between ca. 5.5 g/bsca and 3 g/bcsa, and was highest in the unthinned trees and trees thinned at dormant, FB, and FB+2 weeks treatments. The fruit density was lower than control only for the final three timings, which were similar.

Table 7. Effect of timing of whole-tree thinning treatments on 'Black Pearl' yield and fruit quality. Data with different letters are significantly different at 90% confidence.

Timing of thinning	Tree yield (kg)	Fruit density (g/mm^2)	Fruit weight (g)	Firmness (g/mm)	Color (1-7)	Sol. Solids (%)
Unthinned	11.59 a	5.6 a	6.3 b	257 b	5.4 ab	16.9 cd
Dormant	8.72 ab	4.7 ab	8.5 a	279 ab	5.7 a	18.4 bc
Full bloom	9.12 ab	4.6 ab	7.5 a	294 ab	5.4 ab	18.9 b
FB+ 2 weeks	8.97 ab	4.8 ab	7.6 a	293 ab	5.3 b	16.6 d
FB+ 4 weeks	7.19 bc	4.0 bc	7.8 a	293 ab	5.6 ab	20.7 a
FB+ 6 weeks	7.27 bc	3.6 bc	8.2 a	292 ab	5.3 b	18.2 bcd
FB+ 8 weeks	4.70 c	2.9 c	8.0 a	355 a	5.5 ab	18.1 bcd
Pr > F	<0.001	<0.001	<0.001	0.1	0.01	<0.001

Fruit quality was affected by the timing of thinning. Fruit weight was improved similarly (by 20 – 35%) from all timings (Table 7). This is a different response from what we observed with 'Santina' where the earliest and latest timings were ineffective for improving size. Fruit firmness by contrast was improved only with the final timing of thinning. In addition, we recorded subtle effects of the timing of thinning on fruit color. Fruit from trees thinned as dormant buds were darker that those thinned at FB+2 weeks and FB+6 weeks. The response of fruit soluble solids to timing of thinning was odd, with the greatest improvements seen at FB+4 weeks (a 22% improvement). For 'Black Pearl', it appears that the best timing of thinning was full bloom – yield would have been about 9.2 tons/acre with a 20% increase in fruit weight and 12% improvement in soluble solids.

By harvesting fruit from each upright individually we are able to look for relationships between fruit density/crop load and quality. We analyzed 540 'Santina' samples and 470 'Black Pearl' samples (i.e., uprights), determining branch basal caliper, fruit yield, and fruit quality for each. Our analyses revealed little to no relationships between the crop load/fruit density (g of fruit/branch cross-sectional area) for key fruit attributes (Fig. 3). Our analyses are now focused on determining common elements for the highest quality fruit.

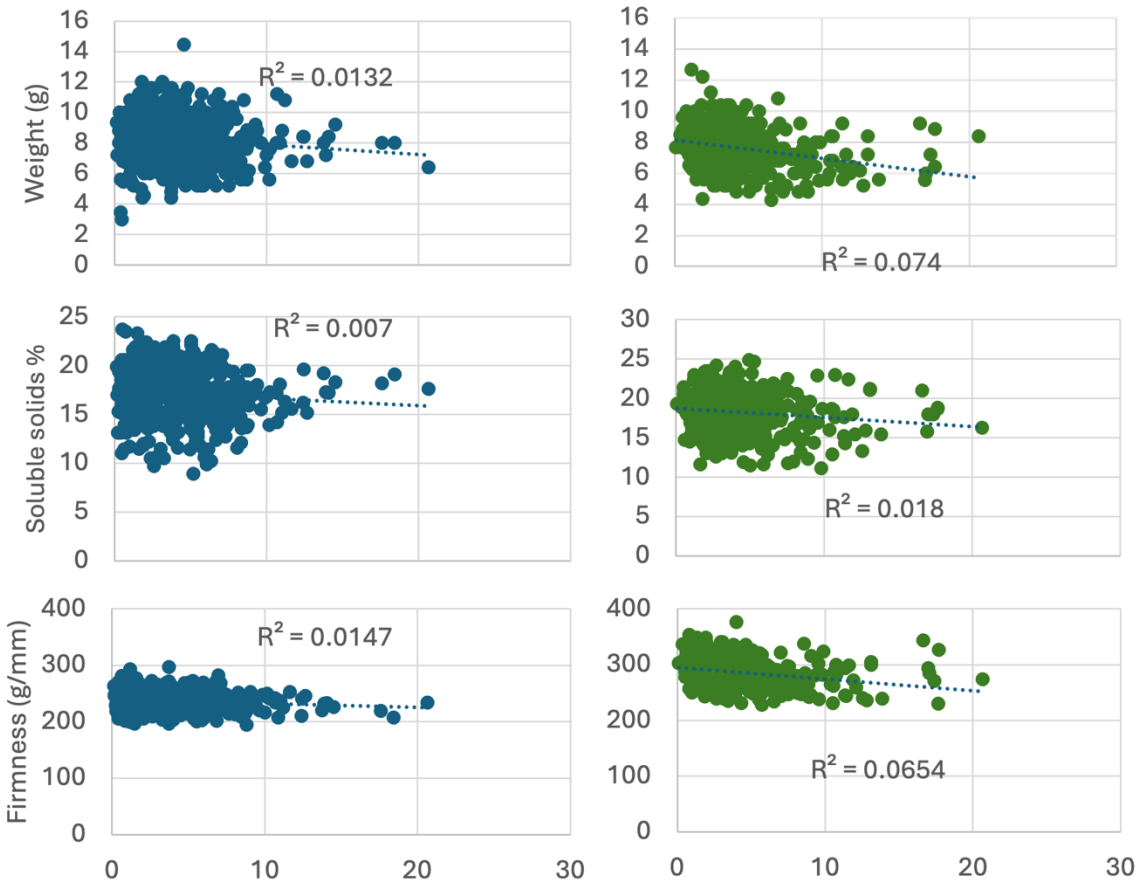

Figure 3. Relationships between fruit density (g/mm2 bcsa) and fruit weight, soluble solids, and firmness for 'Santina' (left) and 'Black Pearl' (right).

The effects of Ethephon and Accede can be compared with our hand thinning treatments imposed at FB+2 weeks since this timing coincided closely with the application of chemical thinners. Hand thinning 'Santina' at FB+2 weeks did not affect yield or fruit quality significantly (Table 6). No chemical post-bloom thinning treatment was especially beneficial since the reductions in yield were not offset by improvements in fruit quality (Table 4). In 'Black Pearl', hand thinning at FB+2 weeks did improve fruit weight/size without reductions in fruit yield (Table 7). Unfortunately, treatments with Ethephon and Accede at this timing were not effective for improving fruit size despite showing efficacy as thinners (Table 5).

Executive Summary
Project Title: Targets and tools for post-bloom thinning

Abstract: This project has evaluated the effect of timing of thinning as well as the efficacy of EthephonII and Accede as post-bloom thinning agents in several cultivars. We found that, when crop load is high, there are benefits from thinning as late as 6 weeks after full bloom (ca. straw timing – the transition from stage II to stage III of fruit growth). The best response in 'Santina' occurred from thinning at ca. 4 weeks after full bloom whereas the best response to thinning in 'Black Pearl' was at full bloom. It is not clear why there would be differences between cultivars in their response to thinning – this will be investigated further in 2025. Both EthephonII and Accede can induce fruit drop when applied about shuck fall (ca. 2 weeks after full bloom). The response may vary among cultivars though this has yet to be determined across years. These treatments improved fruit soluble solids but did not always improve size. At this stage, we recommend further testing (which we will do in 2025) before recommending either PGR for post-bloom thinning.

Key words: fruit quality, crop load management, fruit density, thinning

Project Title: Sweet cherry cultivar-specific export suitability
Report Type: Final Report

Primary PI: Rachel Leisso
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Cooperators: Sweet cherry packinghouse personnel and other industry members in the Pacific Northwest; Dr. Ashley Thompson

Project Duration: 2-year*
* Due to ability to complete the 2024 season with 2023's budget, the 2024 budget was not disbursed.
Total Project Request for Year 1 Funding: $54,944

Other related/associated funding sources: Awarded
Funding duration: 2023-2024
Amount:
Agency Name: USDA-ARS base funds
Notes: Support for base-funded research assistants and travel.

Budget 1
Primary PI: Dr. Rachel Leisso
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	Item	2023
1	Salaries	$25,329
2	Benefits	$9,042
3	Wages	$7,208
4	Benefits	$551
5	RCA Room Rental	$1,319
6	Shipping	
7	Supplies	$4,000
8	Travel	$2,000
9	Plot Fees	
10	Miscellaneous	
11	Indirect cost	$5,494
	Total	$54,944

Footnotes:
1. **Salary for part-time research assistant pay grade GS-8 (0.425 full-time equivalent [FTE])**

2. **Benefits for part-time research assistant pay grade GS-8 (0.425 full-time equivalent [FTE])**
3. **Wages for summer research assistant pay grade GS-5 (approximately 0.5 FTE for 4 months each year)**
4. **Benefits for pay grade GS-5 (approximately 0.5 FTE for 4 months each year)**
5. **Cold room rental is per Oregon State University-Mid Columbia Research and Extension Center (OSU-MCAREC) rates.**
6.
7. **Supplies include data loggers and sensors, reagents, packing supplies, shipping costs, laboratory consumables.**
8. **Travel is for collecting samples.**
9. **NA**
10. **NA**
11. **Indirect cost at 10%.**

Objectives

1. **Postharvest sweet cherry cultivar comparison under potential export conditions.** Project goals were to evaluate stem quality, fruit quality, and physiology before and after simulated export handling (4-week hold) for present or potential Pacific Northwest export cultivars, in both air and modified atmosphere (MA) bags and in optimal (31 °F) and high (40 °F) temperatures, with the latter representing a break in cold chain. Based on discussion with industry stakeholders, a mix of both traditional cultivars and newer sweet cherry cultivars were selected for year 1: **Coral Champagne, Black Pearl, Chelan, Bing, Santina, Skeena, and Regina**, and in year 2**: Coral Champagne, Black Pearl, Chelan, Santina, Bing, Burgundy Pearl, Regina, Skeena, and Sweetheart**. There were four collaborating packinghouses in 2023 and five in 2024 representing major Washington and Oregon cherry production districts.

2. **The influence of transport stresses on sweet cherry quality**. Primary potential transport stresses evaluated in Year 1 were temperature (40 °F) and duration of holding (4 weeks). The effects of regular and MA bags were also contrasted in year 1, 2023.

Significant Findings

- For fruit commercially sized and packed, and combining data from 2023 and 2024, at pickup (shortly after packing) and after a 4-wk hold in a modified atmosphere (MA) bag at either 31 °F or 40 °F, sweet cherry cultivar specific differences relevant for export are detailed in Tables 1, 2, and 3 (below). No single cultivar outperformed all other cultivars on all fruit and stem characteristics; optimal export cultivars may vary based on supply chain or market priorities for particular quality attributes.
- Although cultivars differ in retention of quality over a 4-wk hold and in response to storage temperature, there is no evidence suggesting that all early season cultivars are less suitable for export than later season cultivars.
- Modified atmosphere bags prolonged stem and fruit quality longevity irrespective of cultivar or temperature, and in high temperature storage (e.g. cold chain breaks), retained quality better than non-MA bags (data from 2023). Only MA bags were utilized in 2024.
- Postharvest espiration rates differed among cultivars (measured at 1 wk and 4 wk into storage). Respiration rates (carbon dioxide [CO_2] production) varied with lot and over the course of storage and were higher at higher temperatures. While higher relative respiration indicates fruit are using stored carbohydrates at a higher relative rate, likely decreasing quality potential, in an all-lots, all-cultivars statistical correlation analysis no single measured fruit or stem characteristic consistently strongly correlated with respiration rate, suggesting a potential cultivar-specific effect of high respiration on gross indicators of quality. At present, respiration rate at 1 wk cannot be used an indicator of potential storage longevity.

- Stem width-to-length ratio (an indication of stem thickness) differences post-storage were influenced primarily by cultivar; storage temperature did not statistically influence outcomes. In addition, relative losses from pickup to 4-wk post-storage were not statistically significant according to experimental factors, suggesting additional unevaluated factors influencing outcomes. Previous studies indicate that humidity is an important factor in stem desiccation as is in-field temperature management. Humidity was not specifically evaluated in the present study but presumably well maintained in the tightly closed MA bags.
- Pedicel retention force (force required to separate stem from fruit) differed according to cultivar and post-pickup storage temperature (with higher temperatures leading to more loss in pedicel retention force).
- Stem retention/stem loss was primarily influenced by storage temperature (more loss at higher temperature) and "year" (possibly pre-harvest environment), and not by cultivar.
- Fruit firmness was affected by both cultivar and post-pickup storage temperature, with colder storage (31 °F) optimal. Fruit gained firmness in storage at 31 °F in MA bags.
- Lightness of color (colorimeter) and cracking incidence were too variable lot-to-lot to indicate statistically significant cultivar differences; neither storage temperature nor year (pre-harvest management/environment) affected outcomes.
- Pitting and pebbling (a fruit surface defect indicating desiccation) differed among cultivars; present results summarize incidence, which does not indicate affected fruit were necessarily in unmarketable condition. Since fruit were obtained post-commercial packing, fruit were sized and graded prior to project inception, which affects results at pickup and throughout. Changes in pitting incidence between pickup and 4-wks post-storage were too variable lot-to-lot to be statistically significant. We rated fruit marketability in 2024, but results were not statistically significant, perhaps due to an inability to have the same person rate fruit throughout the experiments.
- All °Brix levels were in acceptable ranges for cultivars at harvest. °Brix lost between pickup and 4-wk post-storage differed among cultivars, potentially suggesting cultivar-specific postharvest utilization rates.
- High temperature storage (40 °F) most clearly negatively affected pedicel retention force, fruit firmness, and titratable acidity. Cultivar-specific interactions with high temperature storage were most definitive for pedicel retention force (loss of pedicel retention force).
- Packinghouse (which, statistically speaking, was inclusive of all aspects of pre- and postharvest management) did not have a consistent influence on postharvest outcomes.
- Stem quality is an important visual attribute of cherry fruit quality that can quickly be negatively affected by suboptimal postharvest conditions, yet information on specific stem quality attributes affecting shelf-life longevity, marketing and consumer preference is limited; placing present results in the context of these considerations is challenging.
- Published research-based comparison of practices supporting maintenance of postharvest stem quality in the context of commercial handling is an area of research need.

Tables summarizing cultivar comparison at pickup (shortly after packing) and after 4-wk MA bag storage are on the following pages.

Table 1. Sweet cherry cultivar-specific stem and fruit summary shortly after commercial packing ("pickup") as well as after a 4 wk hold in modified atmosphere bags at either 31 °F (optimal) or 40 °F (simulating cold chain break), with the latter intended to assess cultivar-specific fruit and stem resilience. All fruit were sorted and packed before evaluation; no information on fruit size is discussed.

Early season	**Early season cultivars 'Black Pearl' and 'Chelan'** 'Chelan' had a higher stem weight-to-length ratio (stem thickness) at pickup, but differences were not significant after 4 weeks storage. Pedicel retention force (force required to separate stem from the fruit) was similar for both cultivars at either optimal or high storage temperatures and were the highest of all the cultivars evaluated; fruit firmness was also the highest of all the cultivars evaluated, both at pickup, and at either optimal or high temperature postharvest. Results indicate 'Black Pearl' is an unusually low titratable acidity (TA) cultivar; values were lowest among the varieties evaluated, both at pickup and post-storage. Post-storage percentage loss of TA (relative to other cultivars) for 'Black Pearl' was mid-range relative to other cultivars. Levels of pitting or pebbling were statistically similar.
Early mid-season	**Early mid-season cultivars 'Coral Champagne' and 'Santina'**. Of all the varieties evaluated, 'Coral Champagne' had the nominally lowest pedicel retention force post-storage, although this was not statistically different from 'Bing', Regina', or 'Santina'. 'Santina' had more pitting at pickup than 'Coral Champagne', but post-storage, 'Coral Champagne' was lowest of the cultivars evaluated. Note that fruit were graded on commercial lines prior to storage; the portion of pitting developing post-packing is unknown. 'Coral Champagne' (and 'Bing') had the lowest pebbling incidence of the cultivars evaluated. Both 'Coral Champagne' and 'Santina' had relatively lower loss of titratable acidity than many of the other cultivars evaluated. 'Santina' had mid-range respiration at 31 °F but the highest respiration at 1 week and 4 weeks storage at 40 °F.
Mid-season	**For mid-season cultivars, 'Bing' is the traditional standard. Only one lot for each of 'Cristalina' and 'Burgundy Pearl' were evaluated so no statistical analyses were performed, and results could be lot-specific.** 'Bing' stem characteristics at pickup and post-storage were mid-range relative to other varieties. The single lot of 'Cristalina' had low stem retention, but nominally lower cracking and pitting than either 'Bing' or 'Burgundy Pearl'. Firmness was nominally higher and color darker for both 'Cristalina' and 'Burgandy Pearl' relative to 'Bing' shortly after packing, but 'Bing' had higher firmness than both after a 4 week hold, both at optimal and high temperatures. 'Bing also had the highest titratable acidity of all the cultivars evaluated, and one of the lowest incidence rates of pebbling.
Late-season	**Late-season cultivars 'Regina' and 'Skeena'** After 4 weeks, late season cultivars 'Regina' and 'Skeena' had the highest stem thickness (potentially indicating resistance to desiccation, although percent loss of stem thickness between pickup and post-storage had weak statistical significance according to cultivar) and mid-range pedicel retention force. 'Regina' had the lowest firmness of the cultivars evaluated at pickup; 'Skeena' had higher fruit firmness post-storage than 'Regina', although postharvest 'Regina' retained firmness better than 'Skeena' (e.g. lost relatively less firmness). 'Skeena' and 'Santina' lost more firmness than other varieties postharvest. Pitting and pebbling where statistically similar for 'Regina' and 'Skeena', although values for 'Regina' were nominally lower. Fruit were graded on commercial packing lines prior to storage; the percentage of pitting attributed to harvest, transport, or packing, and the portion developing in storage is unknown.
Extra-late	**'Sweetheart'** is harvested so much later than other cultivars that it deserves a category of its own. We had challenges evaluating sufficient 'Sweetheart' due to overlap with pear harvest and staffing limitations. The one lot evaluated indicated that 'Sweetheart' stems are relatively resilient to high temperature provoked changes in stem weight-to-length and pedicel retention force. 'Sweetheart' had high firmness at pickup, but also high relatively high cracking and pitting incidence. With just one lot evaluated, this data should be viewed as preliminary and lot-specific.

Table 2a (continued on following page). Sweet cherry stem and fruit characteristics at pickup (shortly after packing by commercial entities). Size is not included as fruit were sized and graded prior to pickup. We requested export quality fruit from cooperators. 'Burgundy Pearl', 'Cristalina' and 'Sweetheart' only had one lot in the study; results should be considered preliminary.

Cultivar	Year	Notes	Stem retention (%)		Stem weight-to-length ratio		Stem pull (kg force)		Stem length (mm)		Cut stems (%)		Firmness (mm g^{-1})		Lightness (colorimeter)	
Bing			80	ns[1]	2.6	bc[2]	0.58	b	48	bc	41	ns	280	abc	28	ns
Black Pearl			90	ns	2.5	c	0.78	ab	48	b	28	ns	319	a	26	ns
Chelan			89	ns	3.1	a	0.80	ab	42	bc	20	ns	324	a	26	ns
Coral Champagne			78	ns	3.0	ab	0.60	b	39	c	0	ns	282	abc	27	ns
Regina			87	ns	3.0	a	0.95	a	56	a	39	ns	243	c	26	ns
Santina			85	ns	2.9	ab	0.70	ab	44	bc	37	ns	256	bc	25	ns
Skeena			89	ns	3.0	ab	0.56	b	43	bc	27	ns	304	ab	26	ns
Burgundy Pearl		one lot, preliminary	97		3.4		0.98		43		.		259		26	
Cristalina		one lot, preliminary	.		3.0		0.20		49		.		340		22	
Sweetheart		one lot, preliminary	89		2.9		0.94		42		.		334		26	
	2023		78	ns	2.8	a	0.40		47	ns	40	ns	280	b	26	b
	2024		93	ns	3.0	a	1.02		46	ns	30	ns	293	a	27	a
Pr < F[3]			0.0506		0.0298		<0.0001		0.0500		0.4237		0.0154		0.1937	
Cultivar			0.5036		0.0183		0.0326		0.0070		0.2177		0.0060		0.1965	
Year			0.0003		0.0213		<0.0001		0.3328		0.7605		0.3378		0.0170	
Cultivar*Year			0.7767		0.4190		0.3961		0.7814		0.6096		0.1801		0.6694	

1. ns, not statistically significant, that is, based on data analyses, numerical differences in a column are not indicative of true cultivar-specific differences.
2. Values in a column followed by different letters are statistically different per Fishers LSD *post hoc*, that is, differing letters indicate cultivar-specific differences.
3. The overall model (Pr < F) must have a value lower that 0.0500 to consider contributions of experimental factors such as cultivar and year, whose relative contributions to explaining differences in dependent variables (characteristics measured) must also have a value less than 0.0500 to be statistically significant.

Table 2b (continued from previous page). Sweet cherry stem and fruit characteristics at pickup (shortly after packing by commercial entities). Size is not included as fruit were sized and graded prior to pickup. We requested "export quality" fruit from cooperators. 'Burgundy Pearl', 'Cristalina' and 'Sweetheart' only had one lot in the study; results should be considered preliminary.

Cultivar	Year	Notes	Cracking incidence (%)		Cracking severity[4]		Pitting incidence (%)		Pitting severity[5]		Pebbling incidence (%)		°Brix		Titratable acidity (%)		Brix-to-acdity ratio	
Bing			15	ns	0.62	ns	61	a	1.06	ns	27	cd	22	a	0.96	a	23	cd
Black Pearl			16	ns	0.63	ns	40	b	0.81	ns	55	ab	20	ab	0.60	d	34	a
Chelan			14	ns	0.61	ns	54	ab	0.92	ns	76	a	19	ab	0.91	ab	22	d
Coral Champagne			6	ns	0.58	ns	31	b	0.78	ns	8	d	20	ab	0.77	bc	27	bc
Regina			16	ns	0.60	ns	51	ab	0.93	ns	37	abcd	21	b	0.81	b	26	bc
Santina			24	ns	0.72	ns	66	a	1.13	ns	47	abcd	18	c	0.64	cd	29	b
Skeena			20	ns	0.68	ns	54	ab	1.00	ns	53	ab	20	ab	0.80	b	25	cd
Burgundy Pearl		one lot, preliminary	8		0.13		4		0.36		1		19		0.63		31	
Cristalina		one lot, preliminary	13		1.25		16		1.00		30		19		0.71		27	
Sweetheart		one lot, preliminary	12		0.16		25		0.71		8		21		0.86		25	
	2023		14	ns	1.05	a	46	ns	0.71	b	51		20	ns	0.84	a	24	a
	2024		17	ns	0.12	b	55	ns	1.18	a	35		20	ns	0.73	b	28	b
Pr < F			0.4978		<0.0001		0.0122		<0.001		0.0172		0.0148		<0.0001		<0.0001	
Cultivar			0.6039		0.6352		0.0977		0.2645		0.0142		0.0026		<0.0001		<0.0001	
Year			0.7214		<0.001		0.5190		<0.0001		0.0857		0.9836		0.0041		0.0002	
Cultivar*Year			0.2827		0.2024		0.0054		0.0136		0.0917		0.3026		0.9437		0.1031	

4. Cracking severity: 0, none; 1, pitting present but marketable; 2, pitting renders fruit unmarketable
5. Pitting severity: 0, none; 1, pitting present but marketable; 2, pitting renders fruit unmarketable

Table 3a (continued on following page). Sweet cherry quality characteristics following 4 wk storage in a MA bag at either 31 °F (optimal) or 40 °F (high) temperature storage. 'Burgundy Pearl', 'Cristalina' and 'Sweetheart' only had one lot in the study; results should be considered preliminary.

Storage temperature		Notes	4-wk stem weight-to-length ratio[1]		% loss, 4-wk stem weight-to-length ratio relative to pickup		4-wk pedicel retention force (kg force)[1]		% loss, 4-wk pedicel retention force (kg force) relative to pick-up		4-wk stem retention (%)		4-wk firmness (mm/g)[1]		firmness % loss, 4-week vs pickup[1]		4-wk L (colorimeter "lightness")	
31 °F	Bing		2.1	B	14	ns	0.48	BC	2	c	87	ns	332	B	-20	B	26	ns
	Black Pearl		2.2	B	11	ns	0.68	A	2	c	92	ns	413	A	-31	B	24	ns
	Chelan		2.5	AB	18	ns	0.77	A	4	bc	94	ns	410	A	-27	B	25	ns
	Coral Champagne		3.0	A	-1	ns	0.38	C	29	abc	83	ns	376	B	-33	B	25	ns
	Regina		2.8	A	6	ns	0.61	BC	39	ab	93	ns	295	C	-23	B	25	ns
	Santina		2.4	AB	16	ns	0.63	ABC	9	abc	85	ns	291	C	-14	A	25	ns
	Skeena		2.6	A	13	ns	0.50	BC	1	c	93	ns	351	B	-15	A	25	ns
	Burgundy Pearl	preliminary data (one lot)	2.8		17		0.70		.		97		264.92		-2		26	
	Cristalina	preliminary data (one lot)	2.9		15		0.16		.		73		259.44		-39		26	
	Sweetheart	preliminary data (one lot)	2.1		29		0.57		.		88		375.47		-10		27	
40 °F	Bing		2.2	B	13	ns	0.36	BC	26	abc	77	ns	296	B	-6	B	26	ns
	Black Pearl		2.1	B	14	ns	0.59	A	19	anc	89	ns	349	A	-10	B	24	ns
	Chelan		2.3	AB	23	ns	0.51	A	35	ab	84	ns	343	A	-7	B	25	ns
	Coral Champagne		2.5	A	15	ns	0.2	C	65	a	68	ns	279	B	1	B	25	ns
	Regina		2.7	A	10	ns	0.42	BC	59	a	75	ns	267	C	-11	B	25	ns
	Santina		2.3	AB	20	ns	0.34	ABC	57	a	83	ns	213	C	17	A	25	ns
	Skeena		2.5	A	15	ns	0.28	BC	46	a	85	ns	278	B	9	A	25	ns
	Burgundy Pearl	preliminary data (one lot)	2.5		17		0.30		.		95		225.32		34		27	
	Cristalina	preliminary data (one lot)	3.3		.		0.09		.		10		238.08		22		26	
	Sweetheart	preliminary data (one lot)	2.1		26		0.51		.		80		321.79		4		26	
	Year	2023	2.4	ns	12	ns	0.28	B	22	ns	77	B	320	ns	-9.72	ns	25	ns
		2024	2.5	ns	15	ns	0.69	A	33	ns	93	A	321	ns	-14.74	ns	25	ns
	Temperature	31 °F	2.5	ns	11	ns	0.58	A	11	B	90	A	352	A	-23.37	B	25	ns
		40 °F	2.4	ns	16	ns	0.39	B	44	A	80	B	289	B	-1.07	A	25	ns
	Pr < F		0.0339		0.4004		<0.0001		0.0342		0.0289		<0.0001		<0.0001		0.1420	
	Cultivar		0.0019		0.5435		0.0004		0.0449		0.3439		<0.0001		0.0007		0.1574	
	Year		0.5358		0.3663		<0.0001		0.1435		0.0001		0.6083		0.0277		0.0690	
	Temperature		0.1605		0.2408		<0.0001		0.0003		0.0190		<0.0001		<0.0001		0.5840	
	Cultivar*Year		0.1844		0.0979		0.0145		0.3704		0.4793		0.0585		0.0665		0.1213	
	Cultivar*Temperature		0.9657		0.9831		0.7067		0.0337		0.9121		0.4970		0.4046		0.9705	
	Cultivar*Year*Temperature		0.8547		0.9204		0.06445		0.9597		0.7525		0.9132		0.6639		0.9949	

1. Where the interaction of cultivar*temperature is not statistically significant, *post hoc* statistically significant separations are according to cultivar (capitol letters). Significance of other main effects (year and temperature) are also indicated. Abbreviations: ns, not statistically significant, that is, based on data analyses, numerical differences in a column are not indicative of true cultivar-specific differences. Values in a column followed by different letters are statistically different per Fishers LSD *post hoc*, that is, differing letters indicate cultivar-specific differences.

Table 3b (continued from previous page). Sweet cherry quality characteristics following 4 wk storage in a MA bag at either 31 °F (optimal) or 40 °F (high). 'Burgundy Pearl', 'Cristalina' and 'Sweetheart' only had one lot in the study; results should be considered preliminary.

Storage temperature		Notes	Cracking incidence (%)[2,3]		Pitting incidence (%)[3]		Pebbling incidence (%)		Brix		Brix % loss, 4 wk vs. pickup		Titratable acidity (%)		Titratable acidity % loss, 4 wk vs.		Brix-to-Acdity ratio		Brix-to-acidity ratio % loss 4 wk vs.	
31 °F	Bing		12	ns	72	A	23	C	22	A	1.7	ABC	0.80	A	18	AB	28	DE	-20	ns
	Black Pearl		21	ns	49	BC	53	AB	19	CD	3.8	AB	0.50	D	17	AB	39	A	-17	ns
	Chelan		11	ns	57	ABC	87	A	19	D	3.1	A	0.70	B	22	AB	27	E	-26	ns
	Coral Champagne		8	ns	39	C	5	C	20	BC	0.0	BC	0.67	BC	13	BC	31	BC	-15	ns
	Regina		10	ns	58	AB	41	BC	21	B	-0.2	C	0.61	C	24	A	34	C	-35	ns
	Santina		33	ns	74	A	47	ABC	18	D	0.8	BC	0.60	C	6	C	31	B	-6	ns
	Skeena		17	ns	68	A	62	AB	19	CD	1.5	ABC	0.66	BC	18	AB	29	CD	-20	ns
	Burgundy Pearl	preliminary data (one lot)	70		74		8		17		1.7		0.39		9		43		-7	
	Cristalina	preliminary data (one lot)	9		15		48		18		11.3		0.48		38		37		-40	
	Sweetheart	preliminary data (one lot)	7		91		12		21		4.8		0.78		32		27		-1	
40 °F	Bing		13	ns	60	A	22	C	22	A	3.0	ABC	0.73	A	25	AB	30	DE	-29	ns
	Black Pearl		21	ns	48	BC	68	AB	19	CD	2.8	AB	0.46	D	24	AB	43	A	-28	ns
	Chelan		15	ns	54	ABC	81	A	18	D	8.3	A	0.68	B	25	AB	27	E	-23	ns
	Coral Champagne		6	ns	31	C	21	C	20	BC	-0.1	BC	0.59	BC	22	BC	34	BC	-29	ns
	Regina		21	ns	61	AB	58	BC	20	B	-0.7	C	0.60	C	27	A	34	C	-37	ns
	Santina		28	ns	69	A	58	ABC	18	D	-0.9	BC	0.52	C	19	C	36	B	-24	ns
	Skeena		20	ns	62	A	78	AB	19	CD	3.2	ABC	0.60	BC	25	AB	32	CD	-28	ns
	Burgundy Pearl	preliminary data (one lot)	66		81		0		18		-1.4		0.37		16		49		-12	
	Cristalina	preliminary data (one lot)	6		27		43		18		5.6		0.40		41		43		-59	
	Sweetheart	preliminary data (one lot)	4		93		3		22		-15.2		0.77		-8		28		-5	
	Year	2023	12	ns	45	B	51	ns	19	B	3.81	A	0.66	A	21	ns	35	ns	-24	ns
		2024	21	ns	69	A	49	ns	20	A	-0.04	B	0.59	B	19	ns	30	ns	-24	ns
	Temperature	31 °F	16	ns	50	ns	45	ns	20	ns	1.53	ns	0.65	A	4	B	31	B	-20	ns
		40 °F	18	ns	54	ns	55	ns	20	ns	2.24	ns	0.60	B	2	A	34	A	-28	ns
	Pr < F		0.5493		0.0003		0.0066		0.0005		0.0200		<0.0001		0.0070		<0.0001		0.3192	
	Cultivar		0.3493		0.0308		0.0008		<0.0001		0.0227		<0.0001		0.0089		<0.0001		0.0501	
	Year		0.0447		<0.0001		0.8109		0.0491		0.0007		0.0010		0.2352		<0.0001		0.8715	
	Temperature		0.7477		0.3937		0.1843		0.5568		0.4432		0.0223		<0.0001		0.8453		0.0095	
	Cultivar*Year		0.1756		0.0001		0.0189		0.1334		0.2914		0.4797		0.3275		0.0154		0.4173	
	Cultivar*Temperature		0.9890		0.9894		0.9845		0.9784		0.6432		0.9860		0.8055		0.6552		0.6350	
	Cultivar*Year*Temperature		0.8599		0.9979		0.9282		0.9891		0.5804		0.9890		0.8830		0.9779		0.8874	

Methods

This 2-year project evaluated cultivar-specific sweet cherry storage longevity, cultivar-specific resilience to higher than optimal storage temperatures, and related physiological indicators. In 2024, storage conditions were 4-weeks in modified atmosphere (MA) bags at optimal temperature (31 °F) and higher than optimal temperature (40 °F), with the latter simulating an extended cold chain break during transport. In 2023, regular polyethylene bags were also evaluated; MA led to superior quality outcomes and this data is not revisited in this report. Per stakeholder request, sweet cherries were obtained shortly after packing from commercial packinghouses (number of lots by year are listed in **Table 4**). Upon receipt, fruit were transferred from packinghouse-specific materials to modified atmosphere (MA) bags (LifeSpan, Amcor, Inc.) according to manufacturer's specifications regarding weight per bag. Respiration rates and bag atmosphere were evaluated at 1 wk storage for a subset of fruit stored in polyethylene bags (opening MA bags would alter the atmosphere), as well as after 4 wk storage (from MA bags). At pick-up and after a 4-week storage, sweet cherry fruit and stem quality were evaluated (attributes listed in **Table 5)** with 30 fruit evaluated per lot for continuous data and 100 fruit for binary data for each treatment combination. To avoid pseudoreplication, prior to statistical analysis, data for each attribute were averaged within each lot and treatment combination. Statistics were performed in a statistical software program (SAS, SAS Institute Inc, Cary, NC) with the programs PROC GLM (continuous data) and PROC FREQ or PROC LOGISTIC (binary data) or PROC CORR (Spearmans r) for correlations.

***Table 4**. Sweet cherry cultivars evaluated, harvest timing, and corresponding quantity of lots evaluated, 2023-2024. Lots were obtained from four packinghouses in 2023 and from five packinghouses in 2024.*

CULTIVAR[1]	HARVEST TIMING (DAYS RELATIVE TO 'BING'	NUMBER OF LOTS IN 2023	NUMBER OF LOTS IN 2024
CHELAN	-11	2	3
BLACK PEARL	-8	3	3
CORAL CHAMPAGNE	-5	1	2
SANTINA	-6	1	2
BURGUNDY PEARL	-4	-	1
CRISTALINA	-3	1	-
BING	0	3	3
SKEENA	+11	3	3
REGINA	+10	3	3
SWEETHEART	+20	-	1

1. *In addition to evaluated cultivars, 'Staccato' (-8 d), 'Suite Note' (-5 d), 'Royal Hazel' (-10 d), 'Royal Helen' (+10 d), 'Royal Edie' (+11 d) were cultivars of interest but were not evaluated due to trademark issues, young trees, or inability to source fruit.*

***Table 5**. Sweet cherry characteristics evaluated in this study.*

VISUAL QUALITY		INDIRECT SENSORY/PHYSIOLOGICAL	PHYSIOLOGICAL
Stem browning	Pitting	Firmness	Respiration
Stem presence	Pebbling	Brix (soluble solids) (sweetness)	Bag atmosphere
Stem quality	Cracking	Titratable acidity (TA)	
Cut stems	Rot	Pedicel fruit retention force (PRF)	
Color (lightness, hue, chroma)			

Results and Discussion

"New sweet cherry cultivar suitability for export" was a 2023 Oregon Sweet Cherry Commission research priority. Exporting cherries can involve a multi-week transport period, and therefore cultivars suitable for export 1) maintain fruit and stem quality for relatively longer periods postharvest and are 2) resilient to breaks in cold chain temperature, e.g. maintain quality through high temperature. Critical to this study, therefore, was defining what measurable characteristics of sweet cherries could indicate export suitability, e.g. what characteristics of fruit and stem quality that packers, buyers, and consumers utilize when choosing sweet cherries.

Determining characteristics to experimentally evaluate as indicators of export suitability was challenging as published information on supply-chain preferences and product factors influencing purchase-decision for sweet cherry are limited. A study by Gallardo et al. (2014) indicated that the potential for a longer shelf-life is a key characteristic influencing decision makers in the supply chain but defining "shelf-life" was beyond the scope of that study. Research often has emphasized consumer preference only (excluding other players in the supply chain) and typically includes emphases on fruit attributes, including flavor (for example, Ye, 2023; Zheng et al., 2016). Considering that many consumers do not taste fruit before they purchase and sweet cherries are typically marketed as "red" or "blush" both domestically and internationally, not according to cultivar, with "red" encompassing 10+ main cultivars, and "blush" 2-3 primary cultivars (e.g. 'Rainier'), research emphases on cultivar-specific flavor leaves knowledge gaps. The consumer is not choosing among an array of sweet cherry varieties based prior experiences with certain cultivars (as is possible with apples or pears), they are simply deciding – to buy or not to buy?

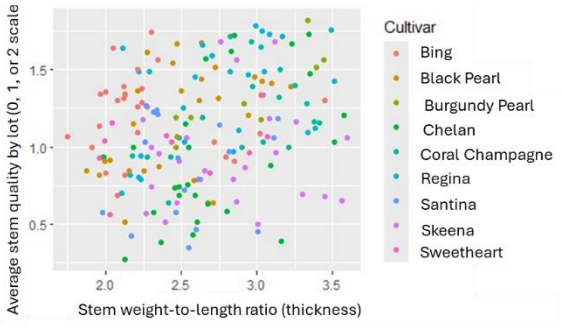
The information we gathered from informal interviews with industry members indicated that greater attention to postharvest post-storage stem quality is also necessary. Where published research is available, it has often emphasized fruit characteristics of size, flavor, and texture. There is sparse information on stem appearance or quality, although stem quality is, when results are considered as percent of change, potentially more quickly affected by at-harvest handling (Golding et al., 2017) and are more affected by postharvest packaging than fruit (Wang and Long, 2015; Zhi et al., 2023) and are thereby arguably a better tell of fruit "freshness" than the fruit itself. **In summary, based on the formal and informal information available, when determining cultivars-specific export suitability this study emphasized retention of visual quality and firmness, greater inclusion of stem characteristics than typical, as well as physical and physiological characteristics that could influence longevity of these qualities.**

Post-packing and post-storage stem quality and resilience to high temperature storage

Stem retention and pedicel retention force were affected by year and storage temperature (**Tables 1, 2, and 3**), indicating an environmental component to these characteristics, while stem weight-to-length ratio (an indicator of thickness and/or relative desiccation) and stem length did not significantly differ according to year, indicating a relatively stronger fixed genetic component; all of these parameters are objective data, e.g. not influenced by technician. Pedicel retention force (force to separate stem from fruit) was affected by cultivar and post-pickup storage temperature (with higher temperatures leading to more loss in pedicel retention force). Stem width-to-length ratio (thickness/desiccation) differences post-storage were influenced primarily by cultivar; storage temperature did not statistically influence outcomes. In addition, relative losses from pickup to 4-wk post-storage were not statistically significant according to experimental factors, suggesting additional unevaluated factors influencing outcomes. Previous studies indicate that humidity is an important factor in stem desiccation (Golding, 2017) as is in-field post-harvest temperature management (unpublished data by the authors in a separate study). Humidity was not specifically evaluated in the present study but presumably well maintained in the tightly closed MA bags. Stem retention/stem loss was primarily influenced by storage temperature (more loss at higher temperature) and "year" (possibly pre-harvest environment), and not by cultivar.

Overall stem quality, which is a subjective determination, and, despite detailed descriptors for numerical ratings, could be influenced by differing technician judgment, was recorded only in 2024, while stem browning **(**which is also subjective and confounded by abrasions from the cluster-cutter) was reported on more extensively in 2023 and is not reported here, due to inclusion of browning in overall "stem quality". Furthermore, neither were statistically significant according to cultivar, potentially due to confounding by relative portion of cut stems contributing to browning. Correlations between stem quality rating and stem weight-to-length ratio were statistically significant but very weak (**Figure 1**).

Figure 1. There was a significant ($p < 0.0003$) but not strong ($r = 0.26$) correlation (Spearman) between overall stem quality (subjective) and stem thickness (objective); the lack of strong correlative relationship is demonstrated in the plot below (2024 data only).



When a stem is cut on the cluster-cutter during packing, losing its fat distal end, stem weight-to-length values indicate that cut stems desiccate more relative to uncut stems (**Table 6**).

Table 6*. A cut stem results in lower postharvest stem weight-to-length, presumably due to desiccation. Data includes all cultivars, all lots.*

STEM CONDITION	STEM WEIGHT-TO-LENGTH (THICKNESS/DESICCATION)[1]
Not cut	2.74 a
Cut	2.38 b

1. Values in a column followed by differing letters differ statistically at $p < 0.0001$.

Statistically, there were no differences in cut stems according to cultivar (**Table 2**). As cluster-cutting could be influenced by packinghouse equipment or practices, the influence of packinghouse was specifically examined in more detail for subset of cultivars in 2024; there was no consistent influence of packinghouse apparent based on present data (**Table 7**).

Table 7. *Packinghouse did not consistently influence percentage of cut stems within a cultivar in 2024.*

Packinghouse	Chelan	Black Pearl	Bing	Skeena	Regina
1	23	-	-	48	42
2	29	34	26	42	32
3	-	32	-	40	32
4	-	-	29	-	-
5	9	32	37	-	-
	*	ns	ns	ns	ns

1. Wald's chi-square; ns, not statistically significant; *, significant at $p < 0.05$

Post-packing and post-storage fruit quality and resilience to high temperature

Post-storage firmness, pitting, pebbling, °Brix, and titratable acidity differed among cultivars and all of these except firmness differed according to year as well, indicating an environmental or

management component for these characteristics (**Tables 1, 2, and 3**). Fruit firmness was affected by both cultivar and post-pickup storage temperature, with colder storage retaining higher firmness. Mean °Brix values at pickup were within recommended ranges for harvest maturity (Long et al., 2021). °Brix lost between pickup and 4-wk post-storage differed among cultivars. Post-storage titratable acidity and percent lost relative to initial values at pickup were influenced by temperature, with higher temperature leading to a greater reduction in titratable acidity. Color and cracking did not differ among cultivars, reflecting a high lot-to-lot variability for these characteristics; neither storage temperature nor year (pre-harvest management/environment) affected outcomes. Cracking and pitting are reported simply as incidence; even small cracks or pits that may not be market-limiting are included in presented results. Severity data for cracking and pitting was not evaluated post-storage as results were not statistically significant at pickup according to cultivar. Rot is not reported here due to overall low incidence and lack of statistical significance according to cultivar. All cherries darkened with 4-wk storage (data not shown), as has been previously documented in 2023 and by other research (Wang and Long, 2014).

Physiology and physical characteristics influencing storage longevity

Carbon dioxide production is an indicator of respiration rate and may reflect how quickly fruit may be using carbohydrate reserves. Researchers have suggested that a higher respiration rate could indicate decreased storage potential as fruit that are respiring more quickly may utilize reserves more quickly (Tapia Garci-a, 2017; Wang, 2014). After about 1 wk in storage, respiration rates were typically at their lowest and would begin to increase thereafter (data from 2023). Holding temperature influenced respiration rates, with an increase in respiration rate at 40 °F relative to 31 °F. At ideal holding temperatures (31 °F) 'Black Pearl', 'Chelan', 'Coral Champagne', and 'Santina' had the lowest respiration rates, and 'Coral Champagne', 'Black Pearl', 'Chelan' remained among the lowest at 40 °F. 'Santina' was the highest at 40 °F both at 1 week and 4 weeks (**Table 8**).

Table 8. *Sweet cherry respiration at 1 wk (air) and 4 wk (immediately after removal from MA).*

Cultivar	CO_2 ppm g^{-1} h^{-1}, 1 wk at 31 °F		CO_2 ppm g^{-1} h^{-1}, 1 wk at 40 °F		CO_2 ppm g^{-1} h^{-1}, 4 wk at 31 °F		CO_2 ppm g^{-1} h^{-1}, 4 wk at 40 °F	
Bing	60	ab	66	c	499	ab	607	abc
Black Pearl	45	c	58	cd	550	a	667	ab
Chelan	47	bc	56	cd	532	a	594	bc
Coral Champagne	46	bc	47	d	453	bc	627	abc
Regina	71	a	89	a	499	ab	541	c
Santina	48	bc	91	a	406	c	705	a
Skeena	68	a	73	b	434	bc	603	abc
Burgundy Pearl	130		126		662		638	
Cristalina	37		45		no data		no data	
Sweetheart	113		114		294		395	
Pr < F	<0.0001		<0.0001		<0.0001		<0.0001	
Cultivar	<0.0001		<0.0001		<0.0001		<0.0001	
Year	<0.0001		<0.0001		no data, 2024 only		no data, 2024 only	
Cultivar*Year	0.0012		<0.0001		no data, 2024 only		no data, 2024 only	

Values in a column followed by differing letters differ statistically at p <0.0001. 'Burgundy Pearl', 'Cristalina', and 'Sweetheart' represent one lot only; results could be lot specific and should be viewed as preliminary.

Correlation between respiration and fruit and stem quality

In an all-lots, all-cultivars statistical correlation analysis no single measured fruit or stem characteristic consistently strongly correlated with respiration rate (data not shown). Correlation or multivariate analysis of characteristics within a cultivar and respiration rate remain to be explored.

Bag atmosphere

Average oxygen and carbon dioxide levels in modified atmosphere bags were higher for fruit held at higher temperatures and increased with storage duration (**Table 9**). Values were in line with optimal atmospheres per Wang and Long (2014).

Table 9. *Average MA bag atmosphere according to storage duration and temperature.*

Timepoint	Storage temperature	% CO_2	%O_2
1 wk	31 °F	9	13
	40 °F	11	11
4 wk	31 °F	11	12
	40 °F	15	7

Conclusion and research directions

This report details cultivar-specific sweet cherry characteristics post-long-term storage to provide guidance for decisions involving export or for orchard planning. In this two-year study containing multiple lots from multiple years for each cultivar (and data averaged within lot to impose more stringent comparison), there were cultivar-specific differences in both stem and fruit quality following storage and in response to storage temperature. Results further indicate that some sweet cherry quality attributes are more readily influenced by cultivar, whereas others are more greatly affected by storage conditions (such as temperature and packaging) and pre-packing factors beyond the scope of this study, likely including environmental conditions and management strategies. Knowledge of specific quality attributes more sensitive to postharvest management practices, whether cultivar specific or general challenges, can enable proactive planning or treatment to mitigate potential problems.

In addition, while the topics below may be considered common knowledge in industry, further study or publicly available documentation would expand the capacity to do informed research surrounding postharvest sweet cherry quality and export considerations:

1. Stem physical, biochemical characteristics, or stem management connected to general retention of stem quality postharvest, including post-cluster-cutter, e.g. attributes or management leading to minimal abrasion and browning of abrasions and retention of fully intact stems
2. Documentation of propensity of cultivars to produce fruit singly vs. in clusters, combined with stem dimensionality, to determine whether these characteristics in combination influence the percentage of fruit that retain fully intact stem through packing, given that this report documents a decrease in stem thickness (indicating desiccation) during storage for cut stems

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Executive Summary

Project Title: Sweet cherry cultivar-specific export suitability

Key words: sweet cherry, postharvest, export, cultivars, varieties, cold chain, modified atmosphere, Bing, Black Pearl, Burgundy Pearl, Chelan, Coral Champagne, Cristalina, Santina, Skeena, Sweetheart, Regina

Abstract: Sweet cherries destined for certain export markets must retain fruit and stem quality for 2 to 6 weeks postharvest, and be resilient to breaks in the cold chain. The goal of this two-year study was to contrast traditional sweet cherry cultivars with newer sweet cherry cultivars to determine relative export suitability. Cultivars selected for evaluation were based on stakeholder input and included Bing, Black Pearl, Burgundy Pearl, Chelan, Coral Champagne, Cristalina, Santina, Skeena, Sweetheart, and Regina. Export-quality sorted and packed fruit were obtained from commercial packinghouses and evaluated at pickup (shortly post-packing) as well as after 4 wk storage, where fruit were held at optimal (31 °F) or a high (40 °F) temperature in modified atmosphere bags, with the latter temperature simulating an extended break in cold chain. No single cultivar outperformed all other cultivars on all fruit and stem characteristics and post-storage contrasts in quality between cultivars maturing in similar harvest timeframes are detailed in this report. To inform cultivar selection and postharvest management practices, the influence of cultivar versus storage factors on quality outcomes were also compared. Some stem and fruit quality attributes, including stem weight-to-length ratio (an indicator of stem thickness and/or relative desiccation) and °Brix had definitive cultivar-specific differences irrespective of storage conditions, while others, like stem retention, were more greatly affected by storage temperature and pre-packing factors and no cultivar-specific influence was apparent, while additional characteristics such as stem pedicel retention force, firmness, and titratable acidity were influenced by both cultivar and storage conditions. In addition, respiration rates at 1 wk into storage were not meaningfully predictive of post-storage quality outcomes at 4 wk storage. Knowledge of specific quality attributes more sensitive to management practices postharvest, whether cultivar specific or general challenges, can enable choosing particular cultivars, proactive planning, or treatment to mitigate potential problems.