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

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Budget 1
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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.

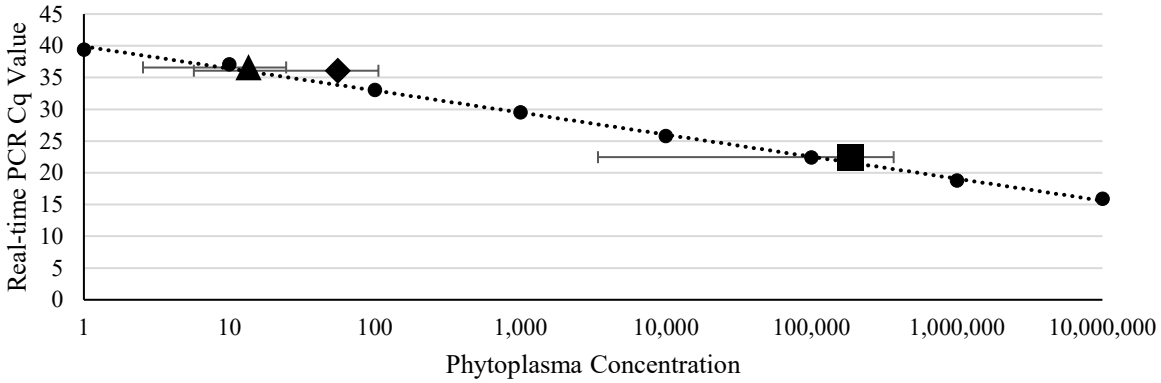

Figure 2. Concentration of X-disease phytoplasma present in asymptomatic (diamond), mild (triangle), and severely (square) symptomatic plants.

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.

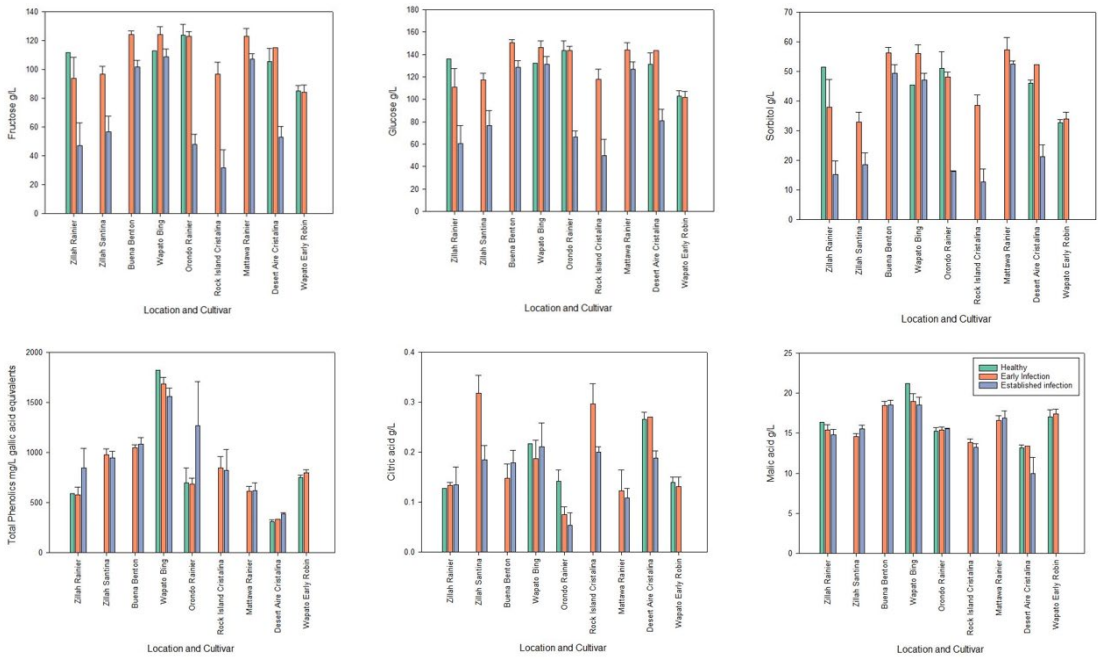

Figure 3. Fructose, glucose, sorbitol, total phenolics, citric acid, and malic acid content across cultivars and locations for healthy, early infections, and established infections.

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.

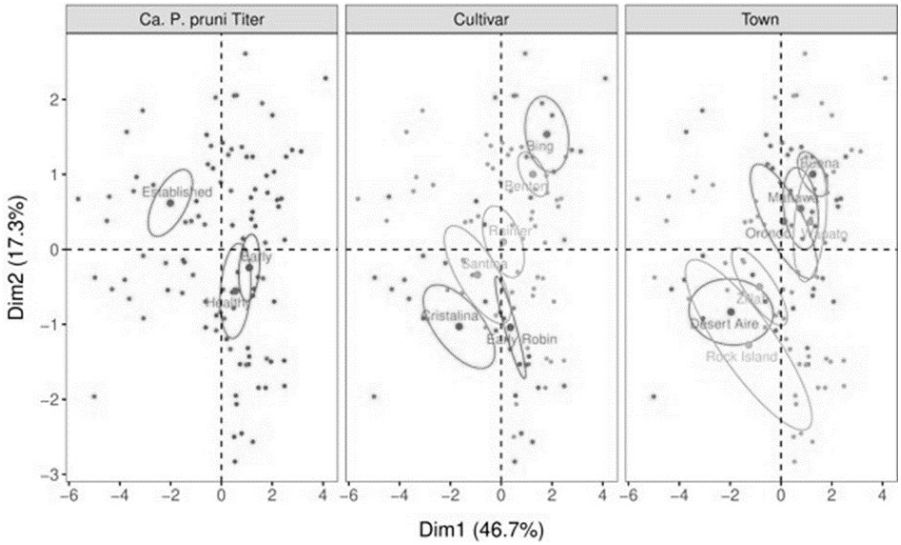

Figure 4. Principal components analysis (PCA) analysis of individual plots comparing pathogen titer, cultivar and location on disease severity.

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.

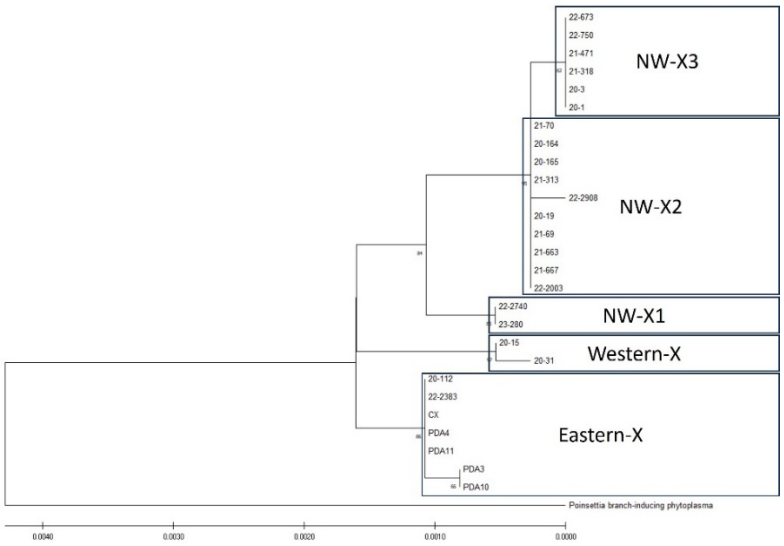

Figure 5. A phylogeny of sequences of the five strain groupings of X-disease-inducing '*Ca*. P. pruni' that have been identified to date.

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.

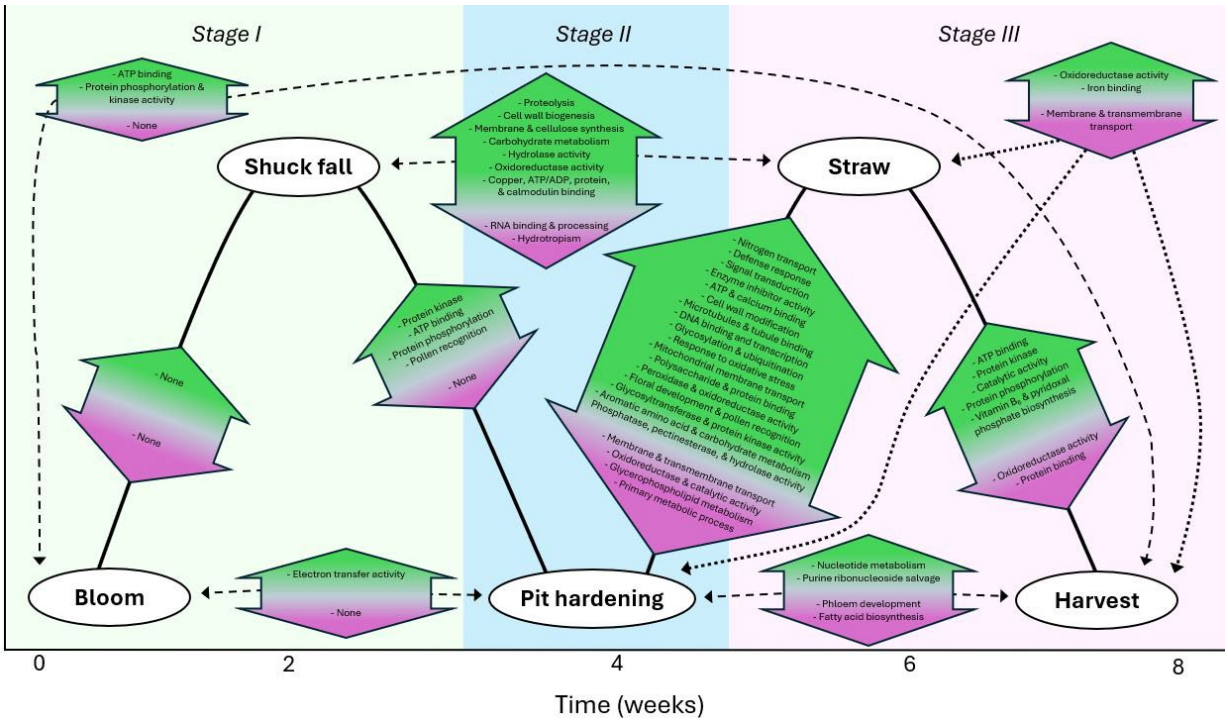

Figure 3. Differential expression of genes, grouped by gene ontology at the different stages of cherry fruit development. Green highlights represent groups of upregulated genes, and pink, groups of downregulated genes ($p \leq 0.01$, ±0.5 log2 fold change).

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.