CONTINUING PROJECT REPORT **YEAR**: 1 of 3

Project Title: Understanding little cherry disease pathogenicity

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Cooperators: Washington cherry growers and extension agents.

Total Project Request: $458,022 **Year 1:** $155,882 **Year 2:** $153,942 **Year 3:** $148,198

Other funding sources: **None**

WTFRC Collaborative Expenses: None

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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
RCA Room Rental	0	0	0
Shipping	0	0	0
Supplies	64,850	59,350	49,905
Travel	1,500	1,500	1,500
Plot Fees	575	575	575
Miscellaneous	0	0	0
Total	155,882	153,942	148,198

Footnotes:

OBJECTIVES

Objective 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.*

During 2021 grafting of selection scions will continue, with planting planned for May followed by inoculation in June when pathogen titer is highest.

Objective 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.*

In 2021 we aim to continue following the effects of LChV-2 and XDP on cherry cultivars in selected commercial orchards from bloom (March) through to harvest (June/July), collecting samples for diagnosis, phenotypic characterization, and for transcriptomic analysis. These data will be added to the 2020 results to separate seasonal trends from the effects of pathogen infection, and a) aid in producing a description of the effects of the two pathogens as infection progresses, and b) inform the transcriptomic studies.

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

In 2021, we intend to continue to collect samples on a biweekly timeframe from bloom through to harvest to examine transcriptomic changes occurring during fruit development that are affected by LChV-2 and DXP infection. As in 2020, we are targeting generative tissues (fruit buds and developing fruit) and comparing those to somatic tissues (stems and leaves) to define host response to infection from disease expression pathways.

SIGNIFICANT FINDINGS

- Fruit shows increasing severity of symptoms with a higher concentration of either XDP or LChV2.
- In LChV2 infected Rainiers, a decrease in fructose, glucose, and sorbitol content was observed while citric acid and total phenolic content increased.
- Flowers and immature fruit were observed near pruning cuts in infected trees of Benton, Skeena, Santina and Cristalina cultivars at harvest, suggesting a broader deregulation of signaling.

METHODS

Objective 1. We will establish a 1-acre test block at WSU-IAREC consisting of 32 different cherry varieties (Table 1). This list includes commercially grown varieties, as well as cherries reported to have some level of tolerance or resistance to LChV-2 or X-disease, and several accessions that represent more unique genetic backgrounds. For each variety, we will plant eight trees, three of which are to be inoculated with LChV-2 or XDP respectively, with two non-inoculated controls. To promote early fruiting, we will bud the trees on a precocious rootstock Gisela-6. Budding will take place in greenhouse conditions during late winter/early spring, and in May the budded trees will be transplanted to the field. Inoculation for both diseases will be via chip budding of infected material in June when pathogen titer is highest. Orchard maintenance, including pruning, fertilization, pesticide application, and weed control, will be conducted according to current horticultural practices.

Table 1. List of germplasm to be screened for tolerance/resistance to LChV-2 and X-disease phytoplasma

Variety Name	**Notes**
Benton	Commercial cultivar
Black Pearl	Commercial cultivar
Chelan	Commercial cultivar
Coral Champagne	Commercial cultivar
Early Robin	Commercial cultivar
Rainier	Commercial cultivar
Santina	Commercial cultivar
Skeena	Commercial cultivar
Sweetheart	Commercial cultivar
Tieton	Commercial cultivar
Brooks	Genetic diversity
Kristen	Genetic diversity
Moreau	Genetic diversity
Black Spanish	Genetic diversity
Walpurgus	Genetic diversity
Yellow Glass	Genetic diversity
Lambert	Genetic diversity
Van	Genetic diversity
Yellow Spanish	Genetic diversity
Schmidt	Genetic diversity
PMR-1	Genetic diversity
Ambrunes	Genetic diversity
Cristobalina	Genetic diversity
Attika	Genetic diversity/commercial cultivar
Regina	Genetic diversity/commercial cultivar
Bing	Possible resistance to LChV-2/X-disease
Black Tartarian	Possible resistance to LChV-2/X-disease
Napoleon	Possible resistance to LChV-2/X-disease
Angela	Reported resistance to X disease
Sweet Ann	Reported resistance to X disease
Utah Giant	Reported resistance to X disease
Windsor	Possible resistance to X disease

Objective 2. Knowing how different cultivars respond to both LChV-2 and X-disease phytoplasma is essential to developing an accurate field guide for growers. Therefore, we propose to collect symptom development observations and physiological data from both the controlled field experiments and grower fields throughout the state. To do so we will focus on two areas:

1) Observation and recording of symptoms present on known infected trees under controlled conditions as the fruit develop from fruit set to harvest, collecting data on fruit size, weight, color, and seed size/maturation. This data will be collated by cultivar type, and infected status.
2) Collecting and recording biochemical data present in maturing fruit at the fruit set, straw/yellow and harvest phases by collecting fruit from different varieties, reducing to pulp via blending and separating the liquid exudate through filtration. This liquid will then be used for sugar content and

metabolite analysis. Sugar, acid, and phenolic content analysis will be performed on cherry pulp using enzymatic and chemical assay kits.

Objective 3. The underlying genetic basis of LCD development will be examined in parallel with the physiological studies. Samples will be collected from different symptomatic and asymptomatic cultivars in the controlled field trial described in objective 1 as well as from field samples. From the trees in the new research block, three different tissue types (fruit, fruit stem, and leaf tissue) will be sampled at three time points (fruit set, straw/yellow, and harvest), macerated and total RNA extracted. Samples will be samples submitted for library preparation and deep sequencing. The resulting data will be analyzed to generate a transcriptome against which individual samples can be compared for differential gene expression analysis. This analysis will be performed to identify transcripts that are upregulated or downregulated between samples. Differentially expressed transcripts will be assigned a function, if possible, based on homology to sequences with known function. These transcripts will be examined to determine which pathways may be altered in cherry when infected with the X-disease phytoplasma or LChV-2, and associated with disease expression, particularly with reference to fruit development.

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

RESULTS AND DISCUSSION

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

Due to delays in obtaining rootstocks, the need to grow the rootstocks used to an adequate size for grafting, and scion material availability, preparation of the field block trees has been slower than anticipated with 7 of 25 cultivars completed. Furthermore, after consultation with participants in the RosBreed Project, 7 additional cultivars have been added to capture greater genetic diversity. Over the winter of 2020-2021 we are forcing growth of the budded plants and remaining stocks, and will collect budwood for the remaining trees during the winter to bud them in the greenhouse in early 2021.

Objective 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.*

In 2020 we focused on collecting symptom data from commercial orchards across central Washington. At each site, healthy or asymptomatic trees were compared to symptomatic trees for the purpose of sample collection; in select sites, trees were selected at random at bloom, and followed through to harvest for tissue collection for objective 3.

All samples were tested for the presence of XDP and LChV-2 by qPCR or RT-qPCR, and pathogen load quantified (Table 2). We assessed fruit symptom severity, fruit size, and fruit color as shown in table 2, and collated the data for each cultivar based on infecting pathogen and titer, to represent the different stages of the infection cycle.

Table 2. Effect of XDP and LChV-2 titer on symptom severity and fruit characteristics in different cherry cultivars.

Cultivar	Pathogen	Titer	N	Symptom rating[a]	Fruit Size[b]	Fruit color[c]
Benton	XDP	Low	8	1.375	2	4.5
		Medium	8	2.375	1.938	2.875
Bing	LChV-2	Low	1	2.5	2	4.25
	XDP	Low	14	1	2.5	4.678
		Medium	9	2.667	1.722	2.77
	Both (L/X)	L/L	15	1.277	2.5	4.767
		L/M	2	3	1.5	2.5
		L/H	1	3	1.5	2.5
		H/L	2	2.5	1.75	4
Cristalina	XDP	Low	9	0.333	2.667	4.667
		Medium	15	2.93	1.633	2.467
Lapins	LChV-2	Low	1	0	3	5
	XDP	Low	4	0.5	2.625	4.875
	Both (L/X)	L/L	4	0.25	2.75	4.875
Santina	XDP	Low	8	0.5	2.625	4.438
		Medium	5	3	1.8	3.3
		High	1	3	1	1.5
Skeena	LChV-2	Low	6	1.667	2	4.25
	XDP	Low	4	0.75	2.5	5
		Medium	1	3	2	1.5
	Both (L/X)	L/L	19	1.421	2.105	4.131
		L/M	3	3	1.667	4
		L/H	1	3	1.5	4
Sweetheart	LChV-2	Low	4	1.5	2	4.125
	XDP	Low	13	1.769	2	4.115
		Medium	5	3	1.6	3.1
	Both (L/X)	L/L	10	2.1	1.909	3.7
		L/M	1	3	1.5	3
Rainier	LChV-2	High	2	2.5	1.5	3.25
	XDP	Low	22	1.27	2.34	3.54
		Medium	7	2.86	1.71	2.78
		High	2	3	1.25	2.5
	Both (L/X)	L/L	6	1.167	2.16	4
		L/M	1	3	1.5	3.5
		H/L	10	2.5	1.75	3.45
Early Robin	XDP	Low	5	0.4	2.6	4.6
Sour Cherry	XDP	Low	5	0.4	2.6	3.8
		Medium	3	3	3	2

a. Symptom rating: 0 = asymptomatic, 1 = mild, 2 = medium, 3 = severe.
b. Fruit size: 1 = small (<50% of normal), 2 = medium (75% of normal), 3 = normal.
c. Fruit color for dark cherries: 1 = green/yellow, 2 = mottled/blush, 3 = pink/light red, 4 = red, 5 = dark red. For yellow cherries: 1 = green, 2 = white, 3 = yellow, 4 = pale blush, 5 = deep blush.

As can be seen in table 2, the stage of infection (low titer representing a new or early infection, and medium or high titer representing an established infection), has a significant effect on the type and severity of symptoms. Depending on the cultivar infected, fruit size was more severely impacted than fruit color. Also, while sample size for LChV-2 infected cherries is lower, due to general lower incidence in the field, Bing and Skeena are more severely impacted by LchV-2 than XDP at equivalent titers, whereas for Lapins and Rainiers, the opposite was observed. Sweetheart was comparable, regardless of pathogen. Interestingly, in Skeena, Sweetheart, and Rainier, infection with both pathogens produced slightly more severe symptoms on infected plants than single infection of either pathogen at equivalent titer.

Next, we began assessing the impact of pathogen infection on fruit quality though measuring the sugar and metabolite content of infected versus healthy or asymptomatic fruit at different stages of infection (determined as before, by pathogen titer). Due to reduced operating levels, these assays are ongoing, and will be completed over the winter. However, preliminary data from Rainier cherries infected with either LChV-2 or XDP compared to healthy fruit indicated that both LChV-2 and XDP reduced fructose, glucose, and sorbitol content in established, but not early, infections (Figure 1), whereas sucrose content increased in early-stage infections for both.

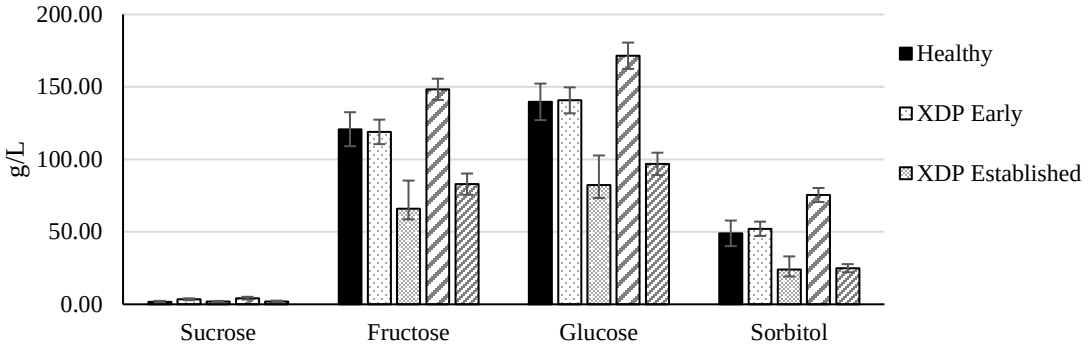

Figure 1. Sugar content of healthy rainier fruit compared to LChV-2 and XDP infected fruit at different stages of infection.

In contrast, citric acid content increased in LChV-2 infected fruit in established infections, whereas XDP fruit did not significantly differ from the healthy controls (Figure 2a). Total phenolic content increased in both LChV-2 and XDP fruit in established infections (Figure 2b).

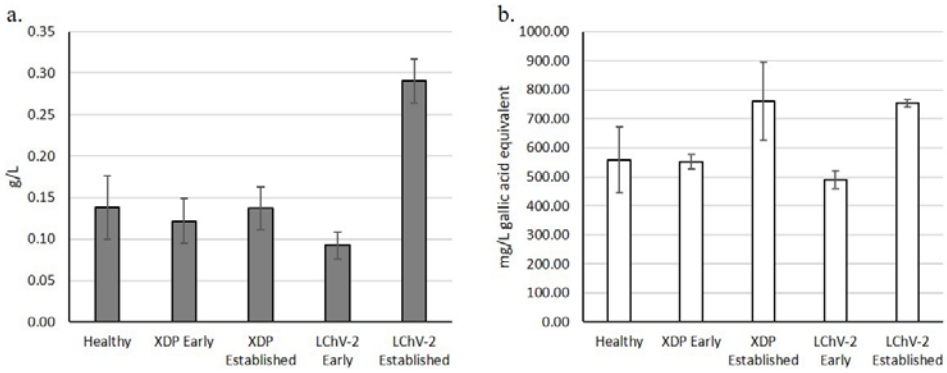

Figure 2. Citric acid (a) and total phenolic (b) content of healthy rainier fruit compared to LChV-2 and XDP infected fruit at different stages of infection.

Cumulatively these data suggest that there is a significant drop in fruit quality between the initial stages of an infection (the first 1-2 seasons), and when the infection of either pathogen becomes systemic and increases in titer (seasons 2-3 and beyond).

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

In early 2020 we performed RNA-seq analysis on diseased and asymptomatic samples collected during the 2019 season, and identified eight genes of interest. RT-qPCR assays were designed for these genes to verify the differential expression observed from sequencing (Figure 2).

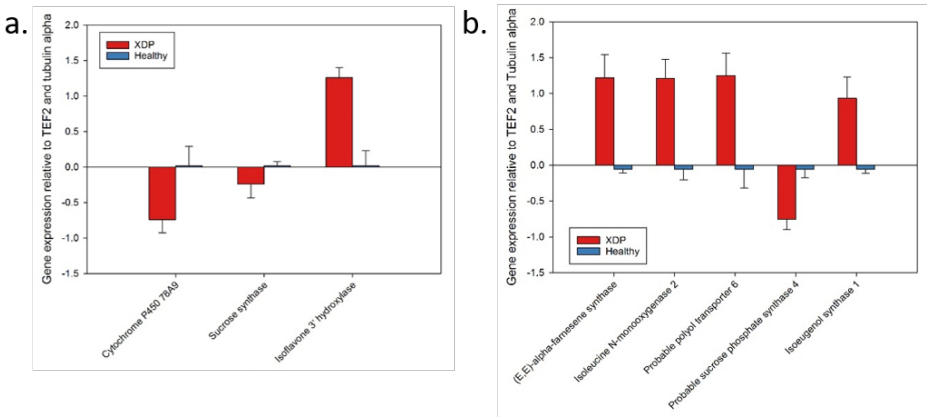

Figure 2. Expression of genes of interest in XDP positive trees compared to uninfected, healthy trees at shuckfall in a) developing fruit, and b) leaves.

We found that, interestingly, cytochrome P450 78A9, which regulates fruit size (Qi et al. 2017) and a sucrose synthase were downregulated in infected fruit. Isoflavone 3' hydroxylase, which is involved in isoflavonoid biosynthesis was upregulated. In leaves, a sugar transporter, polyol transporter 6, was upregulated in infected leaves while a sucrose phosphate synthase 4 was downregulated. (E,E)-alpha-farnesene synthase, which serves as chemoattractant for insects in apples (Bengtsson et al. 2001), was upregulated. Isoleucine N-monooxygenase 2 and isoeugenol synthase, which are involved in phenylpropene and cyanogenic glucoside biosynthesis, respectively, were upregulated. How differential expression of these genes may play into symptom development is not known and will need to be explored further.

Samples have been collected for RNA-seq during the 2020 season, although processing has been delayed due to covid-19 restrictions. Therefore, analysis of these samples will occur in late 2020 and early 2021.

REFERENCES

Bengtsson M, Backman A-C, Liblikas I, Ramirez MI, Borg-Karlson A-K, Ansebo L, Anderson P, Logqvist J, Witzgall P. 2001. Plant odor analysis of apple: antennal response of codling moth females to apple volatiles during phenological development. Journal of Agriculture and Food Chemistry. 49:3736-3741.

Qi X, Liu C, Song L, Li Y, and Li M. 2017. PaCYP78A9, a cytochrome P450, regulates fruit size in sweet cherry (*Prunus avium* L.). Frontiers in Plant Science. DOI: 10.3389/fpls2017.02076.