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

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

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

Co-PI 2: Ashley Thompson
Organization: Oregon State University
Telephone: (541) 296-5494
Email: ashley.thompson@oregonstate.edu
Address: 400 E. Scenic Dr.
City/State/Zip: The Dalles, OR 97058

Co-PI 3: Corina Serban
Organization: Washington State University
Telephone: (509) 574-1600
Email: corina.serban@wsu.edu
Address: 2403 S 18th St
Address 2: Suite 100
City/State/Zip: Union Gap, WA 98903

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

Project Duration: 3 Year

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

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

Item	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.

Justification

X-disease is caused by the pathogenic phytoplasma, *Candidatus* Phytoplasma pruni, and in sweet cherry causes small, pale, unpalatable fruit.[1] Currently, potentially infected cherry trees are identified by visually inspecting fruit close to harvest. Vegetative material is then gathered and sent to a lab for PCR analysis in order to confirm presence of the phytoplasma.[2,3] This mode of identification presents a number of challenges for cherry growers. Symptom scouting must take place at the same time as harvest preparations, symptoms may be caused by a number of other diseases or disorders, and lab confirmation is expensive and results may be delayed. These issues highlight the need for more convenient and efficient disease detection. In this project, we aimed to take advantage of the documented physiological changes that have been shown to be caused by related phytoplasmas in related plant hosts, in order to develop a physiology-based assay capable of detecting X-disease infected sweet cherry trees.

Phytoplasmas colonize phloem tissues, where they trigger a Ca^{2+} influx that induces occlusion of the sieve tube elements.[4] This blockage is a defensive mechanism aimed at controlling the spread of the pathogen, but it also drastically alters the plant's ability to transport photoassimilates from the leaves to other areas.[5] This restriction of sugar transport has been shown to occur in phytoplasma infections of a variety of economically-important fruit species including apple, grape, coconut, papaya, jujube and citrus.[6–11] In all of these species, phytoplasma infections cause significant increases in starch content in the leaves, which may be visually undetectable or mimic symptoms of nutrient imbalance. In the most economically-devastating examples of phytoplasma infection, Huanglongbing (HLB) in citrus and Flavescence dorée (FD) in grape, a number of field-based assays have recently been developed to help identify infected trees, either by testing leaf starch content directly[12] or inferring starch content based on specific changes in spectral reflectance.[13–16] Spectral reflectance methods, particularly when combined with new technologies like UAVs, have the potential to be adapted to high-throughput, large scale X-disease identification approaches in the future.

Excess starch accumulation in aerial portions of the tree also has the potential to alter phenology, which has been shown in girdling experiments involving different tree species,[17,18] and to a limited extent in phytoplasma infected fruit trees like apricot and plum.[19,20] X-disease infected cherry trees have been reported to have early bud-break in the spring and late leaf senescence in the fall, indicating that a simple, visual, phenology-based observation may also be useful in identifying potentially infected trees. If this proves to be the case, this is another potential avenue for aerial imaging to be used in order to identify potential areas of outbreak in large orchards.

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

- Starch content varies by leaf, and is not statistically different between positive and negative trees. Starch content is not an indicator of infection.

- Iodine tests (which test for starch) do not show differences between positive and negative trees and are not a feasible method for diagnosing X-disease.

- Leaf spectrometry (with our specific method, see below) also does not show a difference between positive and negative tress, and is not a feasible method for diagnosing X-disease.

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.

Starch assays attempted using a Cell Biolabs kit were attempted in 2023, and were resumed in 2025 using positive trees identified at MCAREC. Unfortunately, the kit gave unreliable results, with standard controls not matching expected values. This led us to explore other methods. Eventually we were successful with a modified protocol originally used for quantifying starch in cherry floral primordia.[21] Sample preparation consisted of flash freezing leaf tissue, grinding it to a fine powder, weighing, and washing with ETOH to remove pigments. Samples were then digested, converting starch to glucose using enzymatic hydrolysis. Glucose was quantified using an enzyme-coupled assay, using a spectrometer to take readings at 340 nm before and after the addition of glucose-6-phosphate. Using the slope and intercept calculated from a standard curve, the volumes of reagents and the original weight of the leaf tissue, we calculated the final values for mg starch/g sample. This assay was performed on three trees in the field, located at MCAREC, that were verified as positive for X-disease in 2023. These trees are part of a breeding trial and are different accessions; for each positive tree a neighboring tree of the same accession was used as a control, resulting in three "sets".

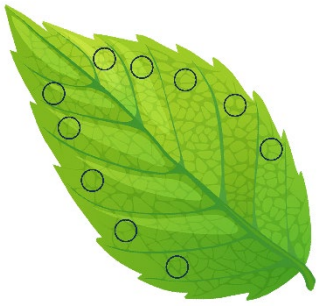


Figure 1. Leaf punches that were collected and pooled for starch assays.

Identification using trained canines (Ruff Country K9) was performed in 2025 – the three positive trees were alerted on, and the control trees were not. Q-PCR validation is pending. We also performed the assay on three infected and three non-infected Mazzard plants received from the Harper Lab (WSU). These small plants are from tissue culture (not seeds) but will be referred to as "seedlings" in the text to represent their small size. For field trees, four leaves were collected for starch analysis, one from the N, S, E and W side of each tree. For the seedlings, one leaf was collected. For each leaf, multiple punches were taken from the perimeter of the leaf blade (avoiding vascular tissue) and pooled (Fig 1).

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

For the iodine testing, a number of approaches were evaluated during the duration of this project. 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.

In 2025, the first protocol (sandpaper/bag) method) was repeated on the three positive field trees and the three positive lab trees (plus negative controls) used for starch analysis. Fresh Lugol's solution was used.

Spectral Methods

In order to determine whether a spectral signature could be used to identify X-disease infected trees, we used a handheld leaf spectrometer (C1-710, CID Biosciences, WA). This tool takes absorbance, transmittance, and reflectance data from a small portion of the scanned leaf, recording values for wavelengths from 360 to 1100 nm.

Figure 2. Handheld leaf spectrometer used for recording spectral data from infected and non-

In 2024, reflectance data taken from orchards in The Dalles, OR and at MCAREC from 2023-2024 was analyzed. In order to visualize the difference between infected and non-infected trees, Princial Component Analysis (PCA) was performed. 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 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 2025, we took multiple scans of leaves from the three infected field trees located at MCAREC that were used in starch testing and iodine testing. We also scanned lab seedlings. Numbers of leaves scanned matched those tested for starch. Individual values, average values, and PCAs were plotted for visualization.

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 and so this objective was not advanced.

Results and Discussion

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.

Three field trees and three lab seedlings with qPCR-verified X-disease infections were tested alongside negative controls, using a starch protocol optimized for cherry material. Starch content for individual leaves did not show an obvious pattern in either field trees (Fig. 3A) or in seedlings (Fig. 3D), with leaves from infected trees exhibiting both higher and lower levels of starch than leaves from uninfected trees. When values were averaged across leaves (Fig. 3B), there were no significant differences between infected and uninfected trees (ANOVA, Tukey HSD). This was true when tree values were averaged as well (Fig. 3C, 3E) with no significant differences between positive and negative categories (Student-t test). The lack of correlation between infection status of trees and starch levels in leaves indicates that this will not be a useful marker for X-disease detection.

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 Results

A number of methods using iodine staining to visualize starch were used throughout the duration of this project. Results are summarized in Table 1.

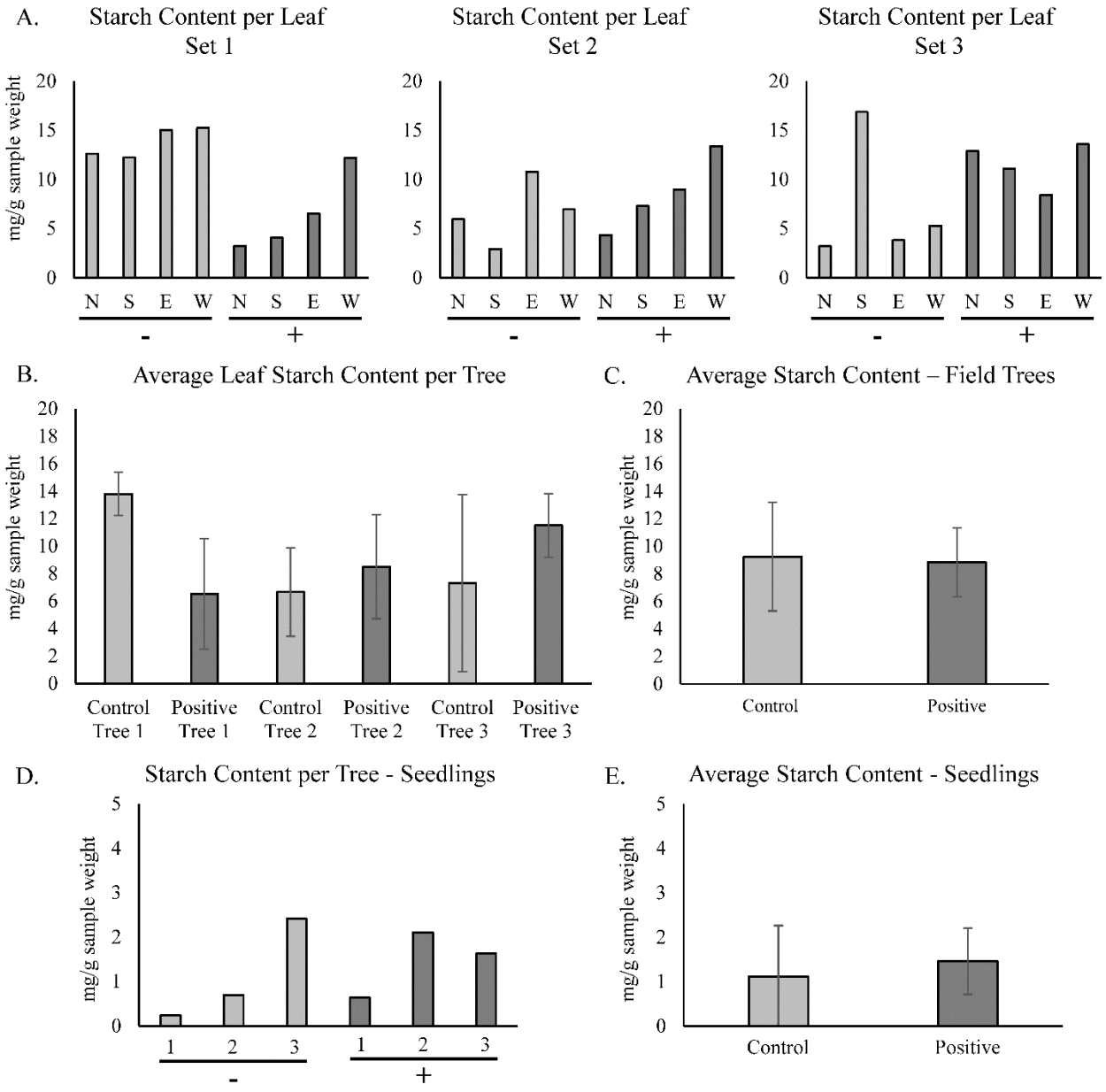


Figure 3. Amounts of starch in X-disease positive and negative trees of different ages. A. Starch content from three infected and three uninfected four-year-old trees in the field, located at MCAREC. For each tree, four leaves were tested. B. Starch content averaged between the four tested leaves for each tree. Values were statistically similar between all trees (ANOVA, Tukey HSD). C. Starch content averaged between positive and negative trees. Values are statistically similar (Student-t). D. Starch content from three infected and three uninfected one-year-old "seedlings". One leaf for each tree was tested. E. Averaged starch content between positive and negative trees. Values were statistically similar (Student-t).

In 2025, we repeated the sandpaper/bag method reported in 2023, on the same trees from the field and the same seedlings that were analyzed for starch content. There were no visual indications of the dark color that occurs when Lugol's reagent comes in contact with starch (Fig. 4). Both positive and negative leaves appeared to be similar in color. In combination with the lack of starch in lab tests, this indicates that iodine testing is not a feasible method for X-disease detection.

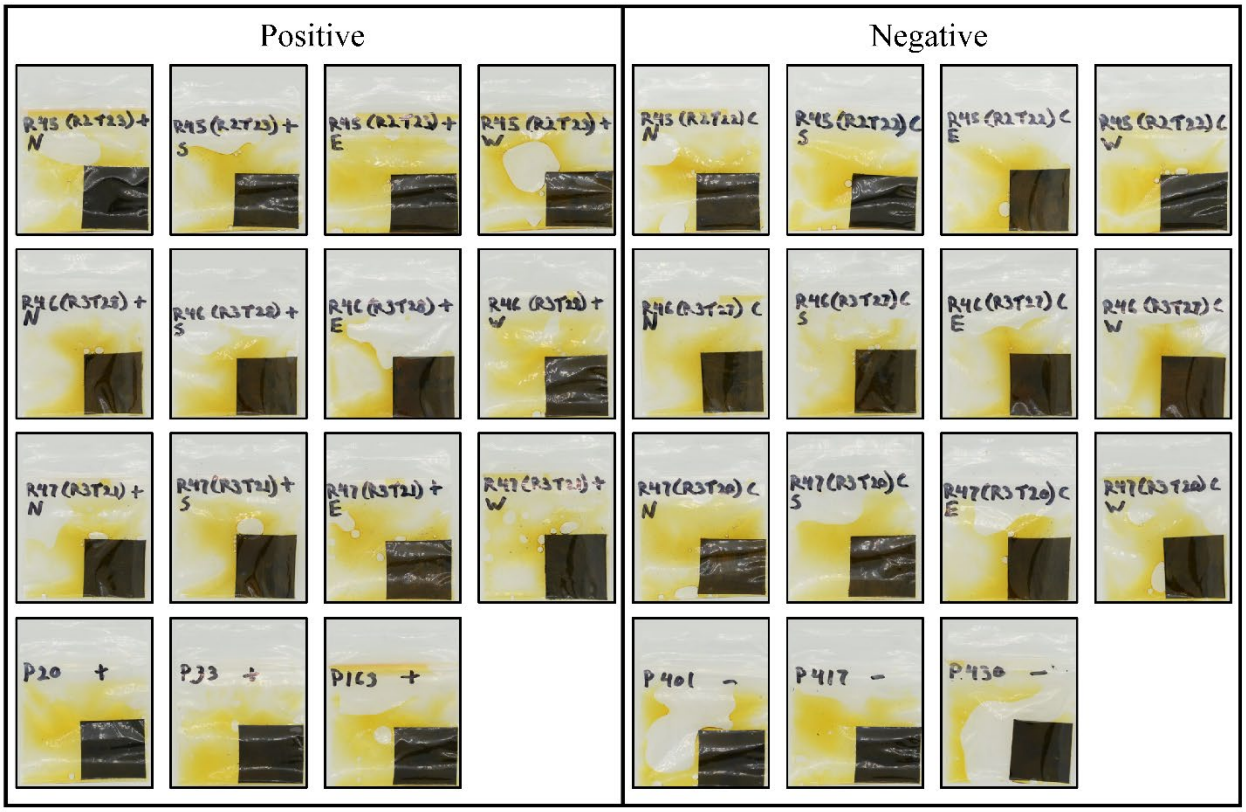


Figure 4. Iodine staining of leaf material to detect starch. One-square-inch pieces of sandpaper were rubbed on four leaves from three infected and three uninfected (top three rows) trees in the field at MCAREC, and on one leaf from three infected and uninfected seedlings. Once enough leaf material was gathered, it was immersed in Lugol's solution in plastic bags to observe darkly colored starch staining. No dark color was present in these tests, independent of X-disease status.

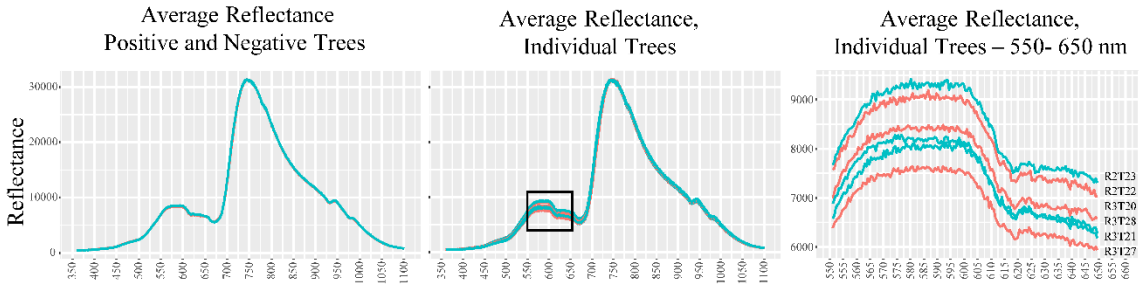
Spectral Results

Spectral analyses in past years did not result in any clear patterns that correlated with X-disease infection (Table 1). In one instance (one site in The Dalles, OR, 2024), a principal component analysis (PCA) indicated a difference between infected and uninfected leaves. In that biplot, we could see clear separation between the control and infected groups. However, on the other days and locations, there was much less dissimilarity between the control and infected trees. Ultimately, it was determined that the differences between infected and uninfected trees in that single analysis was due to differences in reflectance values outside of the wavelengths that

the CI-710s can reliably scan. Files from each scan contain data outside of the 360-1100 nm range, but communication with the company had made it clear this outside data is too "noisy" to be used reliably.

In 2025, we scanned leaves in July, August, and September from the field sets at MCAREC and the seedlings. Scans were done similarly to collections for starch analysis and for iodine testing. Data was trimmed to the appropriate range before analyzing. Reflectance values for each wavelength were averaged and plotted, with average values for infected and uninfected scans appearing very similar in both field and seedling trees (Fig. 5). When values associated with individual trees were plotted, some differences were observed in the 550-650 nm range. However, these differences did not correlate with infection status, making them unlikely to help with disease identification. PCA likewise, did not illustrate any grouping by infection status (Fig. 6). It is possible that scans using our spectrometer are too spatially limited to detect infection, so any further studies with similar objectives should focus on whole-tree scanning and/or wavelengths outside of the 360-1100 nm range.

A. Reflectance plots of qPCR positive and negative (control) field trees, August 2025.



B. Reflectance plots of qPCR positive and negative (control) seedlings, September 2025.

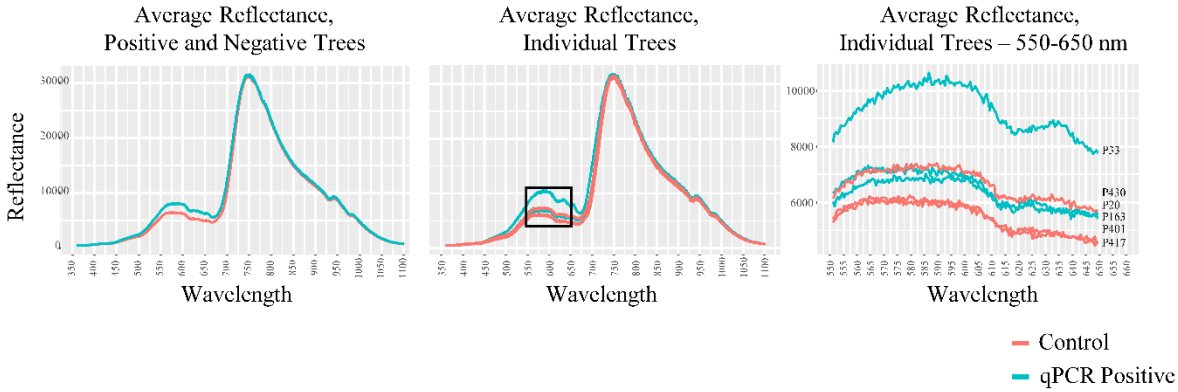


Figure 5. Reflectance values for wavelengths 360-1100 nm. A. Average values for infected and uninfected field trees, individual field trees, and zoomed into the 550-650 nm range for individual field trees. B. Average values for infected and uninfected seedlings, individual seedlings, and zoomed into the 550-650 nm range for individual seedlings. In both cases, infected (blue) and uninfected (red) values are apparently random, with no clear correlation between reflectance values and X-disease infection.

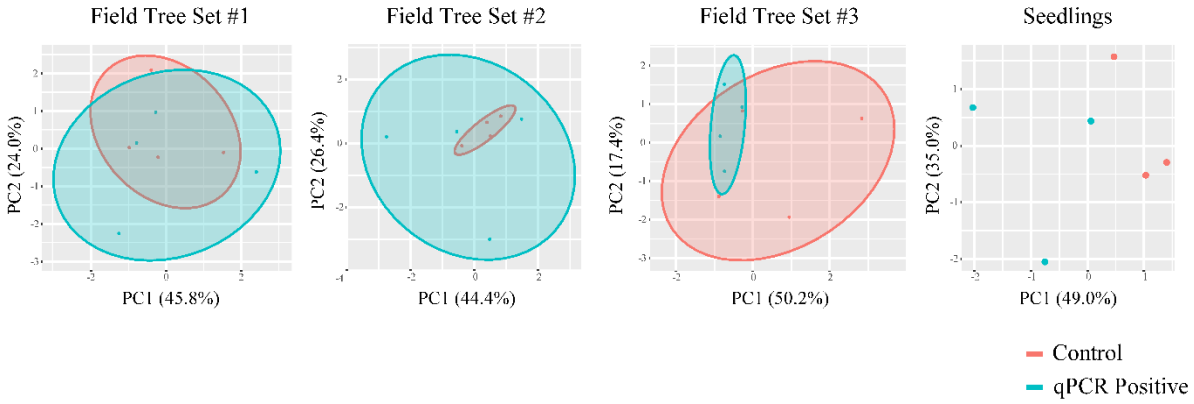


Figure 6. Principal Component Analysis (PCA) for September scans of three field sets (three infected and three uninfected trees, four leaves each) and seedlings (three infected, three uninfected, one leaf each). No clear division of positive/negative is present and PC scores are relatively low for all plots.

Table 1. Compilation of tests and results associated with this project.

Year Reported	Material	Method	Results	Comments
2023	qPCR positive trees control trees from three orchards in The Dalles, OR	Starch testing – Cell Biolab kit	N/A	Method didn't work
2025	qPCR positive and control trees from MCAREC and seedlings	Starch testing – Santolaria *et al.* 2025	No statistical difference in starch levels between +/- trees.	Method worked well.
2023	qPCR positive trees control trees from three orchards in The Dalles, OR	Iodine testing – sandpaper/bag	No difference between +/- trees.	Starch staining not visible from any samples.
2023	qPCR positive trees control trees from three orchards in The Dalles, OR	Iodine testing – clearing whole leaves	Variable starch staining – no difference between +/- trees	-
2023	Uninfected (not qPCR verified) trees from MCAREC – sun vs shade and different times through the day.	Iodine testing – clearing leaf squares	Variable starch staining – no obvious temporal or sun/shade patterns	-
2025	qPCR positive and control trees from MCAREC and seedlings	Iodine testing – sandpaper/bag	No difference between +/- trees.	Starch staining not visible from any samples.
2024	qPCR positive trees control trees from three orchards in The Dalles, OR	Spectrometer	Difference at one site, for one date in The Dalles	Differences only occurred outside of the acceptable wavelength range for this equipment.
2024	qPCR positive and control trees from MCAREC	Spectrometer	No difference between +/- trees.	-
2025	qPCR positive and control trees from MCAREC and seedlings	Spectrometer	No difference between +/- trees.	-

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Executive Summary

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

Keywords: X-disease, starch, spectral signature, iodine, diagnostic

Abstract:

The cherry industry is in need of more convenient and efficient detection methods for cherry X-disease, a phytoplasma-based disease that causes small, pale, unpalatable fruit. In this project, we aimed to take advantage of the documented physiological changes that have been shown to be caused by related phytoplasmas in related plant hosts, in order to develop a physiology-based assay capable of detecting X-disease infected sweet cherry trees. Phytoplasma infections have been shown to cause a build-up of starch in the leaves of apple, grape, coconut, papaya, jujube and citrus. To determine if this occurs in X-disease infected cherry as well, we tested starch levels in leaves, comparing both young and older infected and uninfected trees. Starch levels varied between leaves, but no correlation was present between infection and starch level. We also explored field-based methods to detect starch in the field. The first, an iodine-based assay, also did not result in a correlation between infected and uninfected leaves, which is expected given the lack of differences in starch levels. The second method, which relies on spectral readings and analysis, could potentially detect starch levels or some other difference independent of starch. However, our analyses did not reveal any signature that correlated with infection status of trees. This project explored methods that could have been valuable for X-disease detection, but it appears that the physiological changes present in infected cherry trees differ from other crop/disease changes and will likely not be useful tools in this situation. Further studies into spectral diagnostics should focus on different scanning methods and/or include wavelengths outside of the range we used.