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

Report Type: Final Project Report

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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.

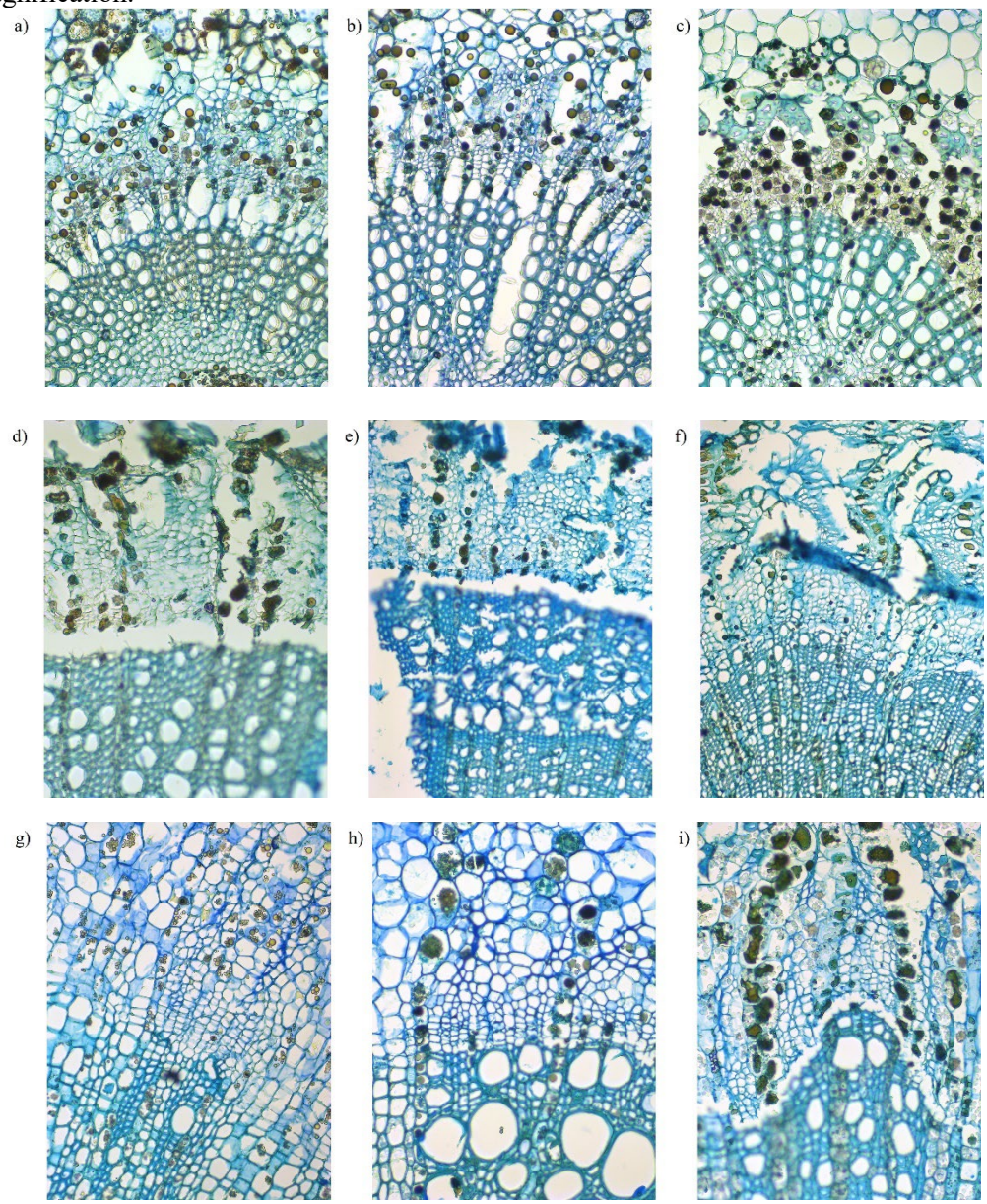
Results and Discussion

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.

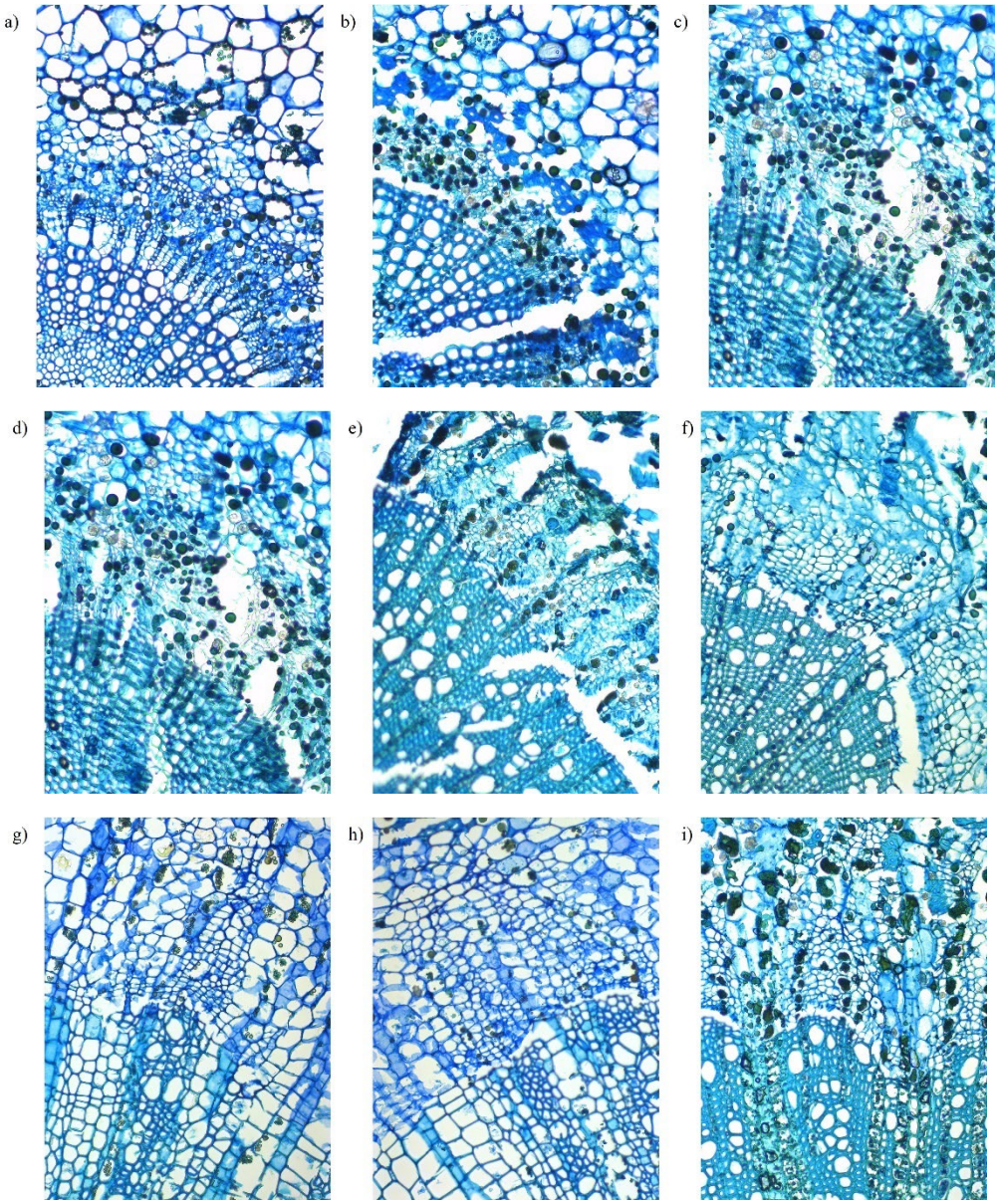
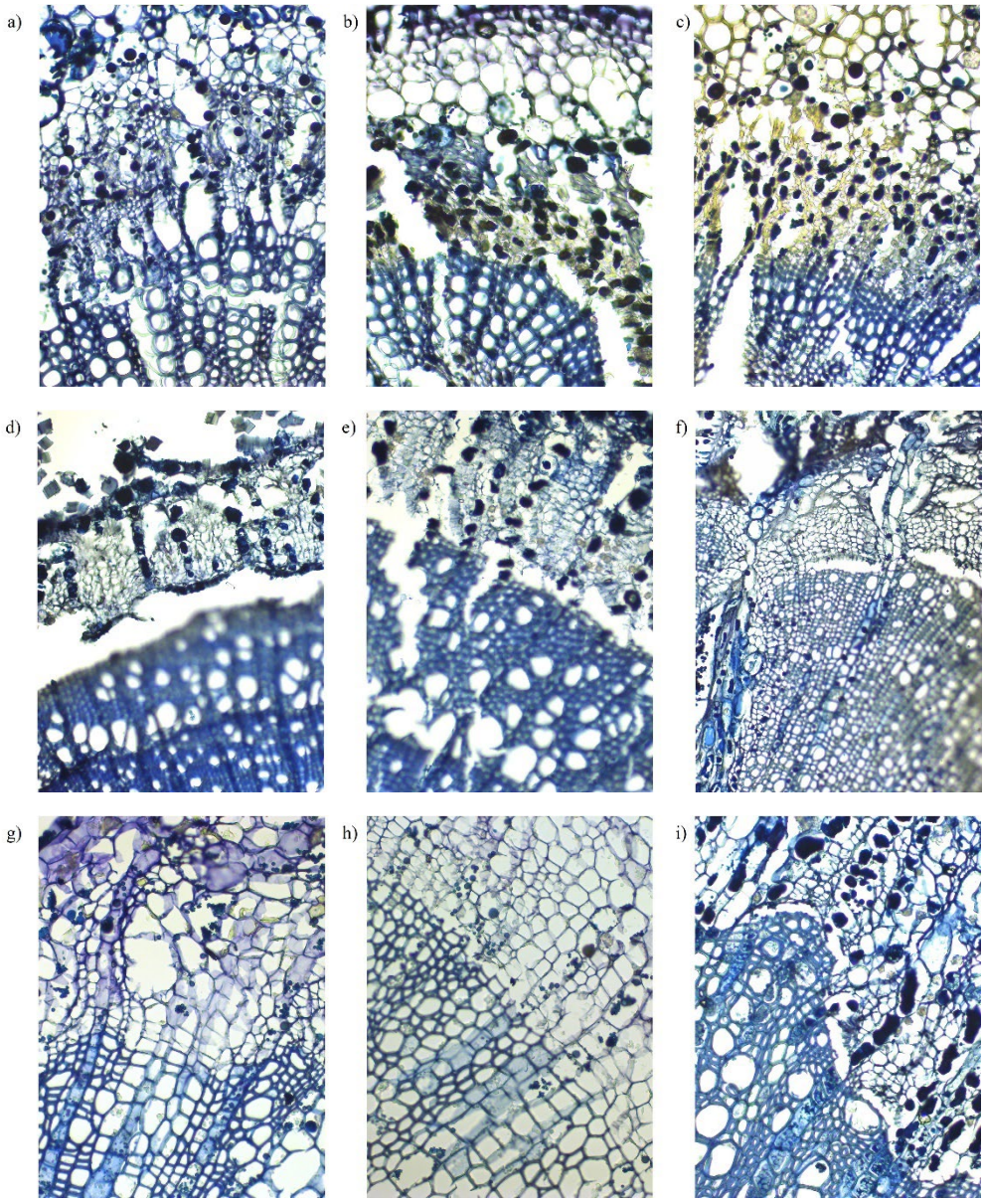
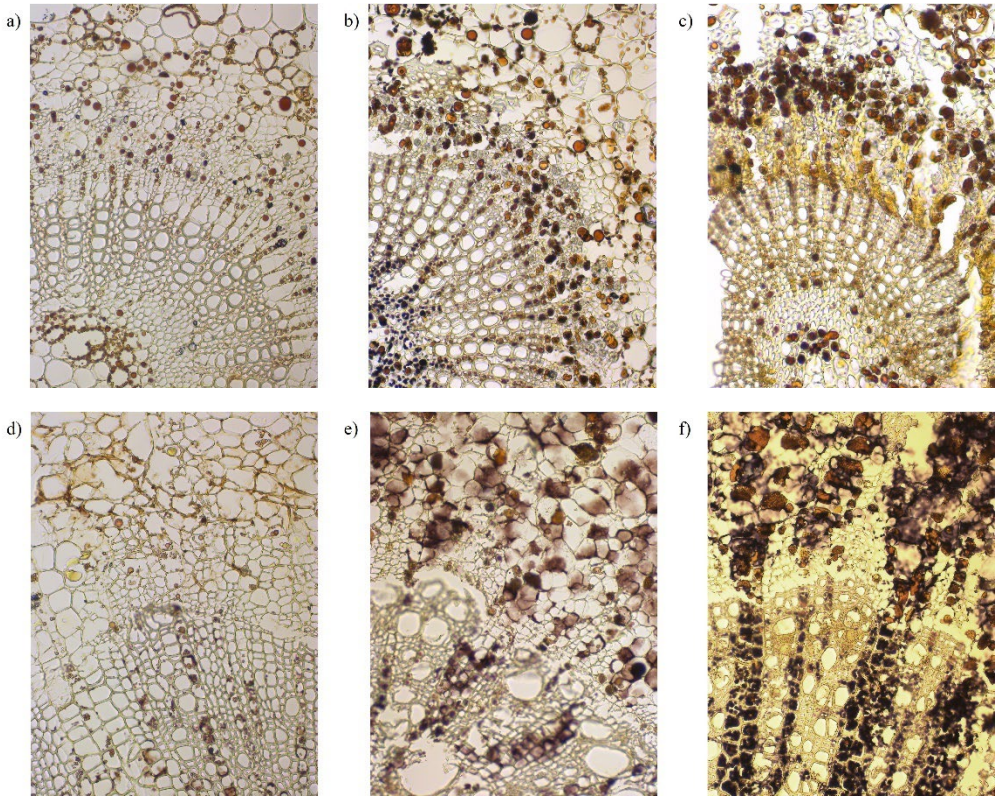


Figure 3. Thionin-Acridine Orange stained leaf (a-c), stem (d-f), and root (g-i) tissues from plants that were negative (a, d, & g), weakly infected (b ,e, & h), or heavily infected (c, f, & i) with '*Ca*. P. pruni' at 400x magnification.



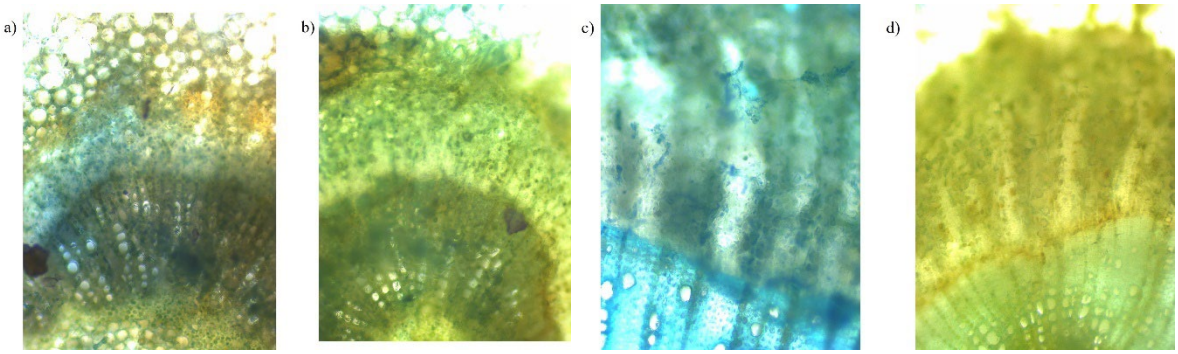
Finally, staining with iodine (Figure 4) was also not effective as an indicator of phytoplasma presence. While uninfected plants had less staining than infected plants, we observed that this was influenced by plant age and the time when samples were collected. Data from other research projects (i.e. 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.