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

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Budget 1
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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.

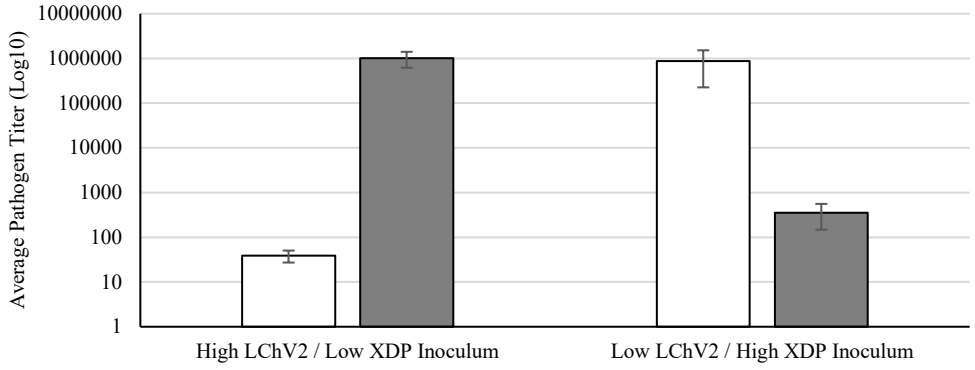

Figure 1. Average titer of populations of the X-disease phytoplasma (white) and little cherry virus 2 (grey) in *P. avium* cv. 'Mazzard' 14 months after infection from two different sources of inoculum.

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.