FINAL PROJECT REPORT **YEAR**: 2 of 2

Project Title: Understanding decline on select apple scion-rootstock combinations

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Cooperators: Washington apple growers.

Total Project Request: $116,180 **Year 1:** $60,200 **Year 2:** $55,979

Other funding sources

None

Budget

Organization Name: Washington State University **Contract Administrator:** Katy Roberts
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Item	2018	2019
Salaries	24,585	25,568
Benefits	17,415	18,511
Wages	-	-
Benefits	-	-
Equipment	-	-
Supplies	16,700	11,900
Travel	1500	-
Miscellaneous	-	-
Plot Fees	-	-
Total	60,200	55,979

Footnotes: Salaries and Benefits include a postdoc at 0.10 FTE, and an MS student. Tuition for the student is not included in this proposal.

OBJECTIVES

The objective of this project was to *determine whether a virus or viral-like pathogens are associated with decline and dieback on G.935 rootstock*. At present there is no clear association of a virus or virus-like pathogen with the expression of decline and/or dieback, only inconsistent findings of endemic viruses. Therefore we proposed to take a systematic approach to clearly identifying what pathogens are present in declining plants. This project looked to the future of the apple industry in the U.S., for understanding the cause of today's problems is key to ensuring that they do not reoccur.

SIGNIFICANT FINDINGS

- Plants exhibiting decline symptoms have reduced roots systems with the cortex and phloem of the root tissue showing necrosis. Necrotic streaking is, in severe cases visible up to the graft union, but not above. The scions die back due to the root loss.

- Similar disease symptoms have been observed on other rootstocks; while not as severe, the same viruses were found to be present, suggesting that this disease is not isolated to G.935.

- 17 new viral species have been identified from some of the symptomatic trees.

- While many viruses have been identified as being present in symptomatic trees, there is no clear association between virus species and the onset of decline symptoms. This does not exclude a secondary role for viruses in this disease, perhaps weakening the plant for subsequent infection/damage by an unknown agent.

METHODS

Determine whether a virus or viral-like pathogens are associated with decline and dieback on G.935

The goal of this project was to identify candidate viruses or viral-like organisms present in apple cultivars on G.935 rootstock that are exhibiting decline and dieback symptoms. Diseased plants underwent a brief physiological examination to determine whether disease symptoms are consistent, and/or whether they can be attributed to other, non-viral causes. Following this, disease and asymptomatic plants were first screened by RT-PCR for common endemic and recently discovered viruses, then representative samples submitted for high-throughput sequencing. The resulting reads were passed through a data analysis pipeline, and candidate viruses identified. Non-diseased trees, and trees on other apple rootstocks, were examined using the same methodology to identify which viruses are likely pathogens, versus those which are present but not a direct cause of the disease.

RESULTS AND DISCUSSION

Objective: Determine whether a virus or viral-like pathogens are associated with decline and dieback on G.935 rootstock

Throughout 2018 and 2019, trees exhibiting decline symptoms were collected from growers and nurseries in north-central Washington State. Honeycrisp cultivars and/or cultivars with Honeycrisp parentage on G.935 rootstock were the focus of collection efforts, as these have been found to most commonly exhibit the decline and dieback symptoms in the second, and sometimes first, leaf stages (Figure 1a).

Figure 1. A) A tree with decline symptoms next to a healthy tree. B) Necrosis of the phloem tissue at the graft union. C) Cross section of a root showing necrosis (brown areas).

From observation of the diseased samples we found that the root systems were much smaller than healthy plants of the same age, sometimes severely so. Feeder roots were sparse, with soft, flexible tissue rather than expected 'carrot-like' texture of an asymptomatic G.935. External necrosis was visible on the taproot and secondary roots, which was evident up to the graft union when the bark was removed (Figure 1b). Sections of these tissues revealed necrosis in the cortex, phloem, and phloem fibers (Figure 1c). Several plants showed stem pitting/grooving symptoms characteristic of Apple stem grooving virus or Apple stem pitting virus infection, and three showed foliar chlorosis typical of Apple mosaic virus infection. None of the above symptoms were evident on asymptomatic Honeycrisp variants on G.935, although interestingly milder necrosis and poor root development was observed on one Honeycrisp on Nic-29 and three on Pajam2 rootstock, as well as on one Fuji cultivar on M.9 rootstock. In Washington State the pathology appears to be consistent, although it should be noted that in Pennsylvania symptoms were observed above the graft union rather than below, with sucker formation on the rootstock; indicative perhaps, of a different causal agent.

Screening of 52 symptomatic Honeycrisp cultivars on G.935 rootstocks by RT-PCR and/or high throughput sequencing (HTS) revealed the presence of nine endemic and newly-reported apple-infecting viruses (Table 1). No single virus species was present in all plants, which is to be expected given differences in titer, distribution within the plant, and sampling time. Individual symptomatic plants had between one to eight distinct viral species infecting them, with an average of 3-4 viruses per plant. In only one diseased plant were no viruses detected. In contrast, the 18 asymptomatic Honeycrisp cultivars on G.935 tested had fewer viruses infecting them, with an average of 1-2 viruses per plant; seven had no viruses, although there was one asymptomatic outlier with a total of six viruses detected (Table 2).

While one could propose that symptomatic Honeycrisp cultivars on G.935 are more heavily infected than asymptomatic plants, there is no obvious correlation between the viral species present and the onset of disease. Examination of the frequency of viruses identified (Table 3) indicated that Apple mosaic virus (ApMV), Apple rubbery wood associated virus 2 (ARWaV2), Apple stem grooving virus (ASGV) and, Apple stem pitting virus (ASPV) were all found in over 50% of the symptomatic samples, but were also at high frequencies in asymptomatic plants. The only virus detected that showed significant differences in frequency between symptomatic and asymptomatic plants was Apple rubbery wood-associated virus-2 (ARWaV-2), however this was only in 54% of symptomatic plants so no clear conclusion can be drawn.

Table 1. Pooled results of the RT-PCR and HTS screening of disease-expressing samples of Honeycrisp cultivars on G.935 rootstock from Washington State. Viruses are as follows: Apple chlorotic leaf spot virus (ACLSV), Apple green crinkle associated virus (AGCaV), Apple mosaic virus (ApMV), Apple rubbery wood associated virus 1 and 2 (ARWaV-1 and ARWaV-2), Apple stem grooving virus (ASGV), Apple stem pitting virus (ASPV), Citrus concave gum associated virus (CCGaV), and Apple hammerhead viroid (AhVd).

Sample	ACLSV	AGCaV	ApMV	ARWaV-1	ARWaV-2	ASGV	ASPV	CCGaV	AhVd
1	+	+	+	-	-	+	+	-	-
2	+	+	+	-	+	+	+	-	+
3	+	+	+	-	-	-	+	-	+
4	+	+	+	-	-	-	+	-	+
5	+	-	+	-	+	-	+	-	+
6	+	-	+	-	+	-	+	-	+
7	+	+	+	-	+	+	+	+	+
8	+	-	-	-	+	+	+	+	-
9	+	-	-	-	+	+	+	+	-
10	-	-	-	-	-	+	-	-	-
11	+	+	-	-	+	+	+	+	-
12	-	-	+	-	-	+	-	-	-
13	+	-	+	-	+	+	+	-	+
14	+	-	+	-	+	+	+	-	+
15	+	-	+	-	+	-	+	-	+
16	-	-	+	-	-	-	-	+	+
17	+	-	+	-	-	-	+	-	+
18	-	-	+	-	-	-	-	-	+
19	-	-	+	-	-	+	-	-	-
20	+	-	+	-	-	+	+	-	+
21	-	-	+	-	-	+	-	-	+
22	+	-	+	-	+	+	+	+	-
23	+	-	+	-	+	+	+	-	-
24	+	-	+	-	+	+	+	+	-
25	+	-	+	-	+	+	+	-	+
26	-	-	+	-	+	-	-	-	+
27	-	-	+	-	+	-	-	-	+
28	-	-	+	-	+	+	+	-	-
29	-	-	+	-	+	+	+	+	-
30	-	-	-	-	+	+	+	+	-
31	-	+	+	-	+	+	+	+	+
32	-	-	-	-	-	+	-	-	-
33	-	-	-	-	-	+	+	-	-
34	-	-	-	-	-	+	+	-	-
35	-	-	-	-	-	+	+	-	-
36	-	-	+	+	+	+	+	-	+
37	+	-	+	-	+	+	-	-	-
38	+	-	-	-	+	+	-	-	-
39	+	-	-	-	+	+	+	-	-
40	-	-	-	-	+	-	-	-	-
41	-	-	-	-		-	+	-	+
42	+	-	-	-	+	+	+	-	-
43	+	-	-	-	+	+	+	-	-
44	+	-	-	-	+	+	+	+	-
45	+	-	-	-	-	-	-	-	-
46	+	-	-	-	-	-	-	-	-
47	-	-	-	-	-	+	-	+	-
48	-	-	-	-	-	+	-	-	-
49	-	-	-	-	-	+	-	-	+
50	-	-	-	-	-	-	-	-	-
51	-	-	-	-	-	+	-	-	+
52	-	-	-	-	-	-	-	-	+

Table 2. Pooled results of the RT-PCR and HTS screening of asymptomatic samples of Honeycrisp cultivars on G.935 rootstock from Washington State.

Sample	ACLSV	AGCaV	ApMV	ARWaV-1	ARWaV-2	ASGV	ASPV	CCGaV	AhVd
1	+	-	+	-	+	+	+	+	-
2	-	-	+	-	-	+	-	+	+
3	-	-	+	-	-	+	-	-	+
4	-	-	+	-	-	+	-	-	-
5	-	-	+	-	-	+	-	-	-
6	-	-	+	-	-	+	-	-	+
7	-	-	-	-	-	-	+	-	-
8	-	-	-	-	-	+	+	+	-
9	-	-	-	-	-	+	+	+	-
10	-	-	-	-	-	-	+	-	+
11	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	+	-	-
17	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-

It should be noted here that nearly all of these viruses, with the possible exception of Apple rubbery wood-associated virus-1 (AWRaV-1) are endemic and widespread in commercial apple orchards in Washington State, and indeed, across the country (Harper, unpublished data). One caveat to be observed with a study of this nature is that asymptomatic samples may not truly be healthy and free of disease, but may be expressing mild or initial stages of the disease.

Table 3. Frequency table for viruses detected in symptomatic and asymptomatic Honeycrisp cultivars grown on G.935 rootstock.

Disease	ACLSV	AGCaV	ApMV	ARWaV-1	ARWaV-2	ASGV	ASPV	CCGaV	AhVd
Symptomatic	48%	13%	54%	2%	54%	62%	62%	21%	38%
Asymptomatic	6%	0%	33%	0%	6%	44%	33%	22%	22%

Next, we compared these detection rates to viruses present in Honeycrisp on other rootstocks, both symptomatic and asymptomatic, as well as one symptomatic Fuji on M.9 and two G.935 samples (Table 4). Again, there was no correlation between the expression of disease and virus presence and/or load; indeed, symptomatic Honeycrisp on Pajam2 were less infected that asymptomatic plants. Cumulatively, these data suggest that there is no obvious correlation between presence of known, named viruses, and the expression of Apple Decline disease.

Table 4. Results of the RT-PCR screening of asymptomatic samples of Honeycrisp cultivars on different rootstocks from Washington State

Sample	Cultivar/Rootstock	Symptomatic	ACLSV	AGCaV	ApMV	ARWaV-1	ARWaV-2	ASGV	ASPV	CCGaV	AhVd
1	Honeycrisp / Nic29	Yes	+	-	+	-	+	+	+	-	-
2	Honeycrisp / Pajam2	Yes	+	-	-	-	-	-	-	+	-
3	Honeycrisp / Pajam2	Yes	-	-	-	-	-	-	-	+	-
4	Honeycrisp / Pajam2	Yes	+	-	-	-	-	-	-	-	-
5	Honeycrisp / Pajam2	No	-	-	+	-	-	+	+	+	-
6	Honeycrisp / Pajam2	No	+	-	-	-		+	+	+	-
7	Honeycrisp / Pajam2	No	+	-	+	-	+	+	+	+	-
8	Honeycrisp / G.41	No	-	-	-	-	-	+	+	-	+
9	Fuji / M.9 (T337)	Yes	+	+	-	-	+	+	+	+	+
10	G.935 Rootstock	No	-	-	+	-	-	-	-	-	+
11	G.935 Rootstock	No	-	-	-	+	-	+	+	-	+

Finally, we sequenced six disease expressing samples using either root or shoot tissue, as well as two asymptomatic samples by HTS and searched for novel or putatively new viruses. From these plants we found seventeen putative novel virus-like sequences (Table 5). Each one of the contigs has a low percentage coverage and amino acid identity (25% to 61%) to named viruses, indicating that these are distinct and novel viral species. These viruses included an ilarvirus, two tombus-like viruses, a barna-like virus, a picorna-like virus, three ourmia-like viruses, three partiti-like viruses, and two narna-like viruses; four additional viruses could not be classified. The presence of these viruses in these samples was confirmed by RT-PCR using primers designed against the detected sequences. These data further indicate that while novel, these viruses are also rare, being found in only a small number of samples and so cannot be correlated presence with disease; it is likely that they are incidental and not related to the onset of apple decline.

Table 5. Putative novel viruses detected in Honeycrisp cultivars on G. 935 rootstock exhibiting apple decline from Washington State.

Name	Sample No.	Sequence Coverage	Contig length	BLASTx results
Apple barna-like virus 1	4	40.78	4099	Riboviria sp RdRp (QDH90348)
Apple ilarvirus 1 RNA2	5	10.71	1058	Blackberry chlorotic ringspot virus replicase P2a (ARS65724.1)
Apple ilarvirus 1 RNA3	5	91.49	2124	Parietaria mottle virus movement protein (CAJ58667.1)
Apple narna-like virus 1	3	12.26	2511	Wenzhou narna-like virus 1 RdRp (APG77283.1)
Apple narna-like virus 2	3	29.05	2668	Wenzhou narna-like virus 1 RdRp (APG77283.1)
Apple ourmia-like virus 1	3	42.71	1856	Pyricularia oryzae ourmia-like virus 2 RdRp (BBF90577.1)
Apple ourmia-like virus 2	5	15.56	2570	Phomopsis longicolla RNA virus 1 RdRp (YP_009345044.1)
Apple ourmia-like virus 3	3, 6	93.05	3067	Cladosporium cladosporioides ourmia-like virus 1 RdRp (QDB74999)
Apple partiti-like virus 1	1, 2, 3	234.3	2010	Partitiviridae sp. RdRp (QDH87388)
Apple partiti-like virus 2	1, 2, 3	374.44	1825	Partitiviridae sp. RdRp (QDH87090)
Apple partiti-like virus 3	2, 3	783.33	1930	Partitiviridae sp. RdRp (QDH87090)
Apple picorna-like virus 1	4	39.05	11885	Polycipiviridae sp. RdRp (AZL87720.1)
Apple tombus-like virus 1	3, 5	36.81	2971	Sanxia tombus-like virus 3 hypothetical protein 1 (YP_009337434.1)
Apple tombus-like virus 2	6	914.6	4340	Cowpea tombusvirid 1 RdRp (APA23091.1)
Apple virus A	3	18.32	5751	Rhizoctonia solani putative virus 1 hypthetical protein (QDW81310)
Apple virus B	5	41.19	9234	Penicillium glabrum negative-stranded RNA virus 1 RdRp (QDB75014)
Apple virus C	1, 3	73.9	4242	Hubei narna-like virus 8 RdRp (APG77202.1)
Apple virus D	2	26.87	7966	Riboviria sp RdRp (QDH87729)

Of the putatively novel viruses detected, the ilarvirus and two tombusviruses are likely plant infecting, whilst the barna, ourmia, and narnaviruses may be. The partitiviruses and the picornavirus could be plant, fungal or insect infecting, as members from those genera are found in all three types of host. Given how distinct apple viruses A through D are, it is not possible to give an assessment of their putative host range. This illustrates one of the limitations of HTS as a diagnostic tool, for while it is effective at indicating whether viruses are present, it provides no information on their biological activity or relevance. Although the hosts of these viruses, apple or fungal, are suspected, they cannot be confirmed with the existing data.

How the viruses are transmitted is also unknown. Lastly, it is unknown if any of these viruses are pathogenic in any apple cultivar or if they have a synergistic effect when co-infecting. With 9 known viruses and viroids just in these trees and seventeen additional putative viruses, the number of combinations when addressing synergistic effects becomes very large (and even greater if multiple cultivars or rootstock/scion combinations are included). It should also be considered that the viruses themselves are not causing the symptoms observed directly, but are instead weakening the tree by downregulating host defenses or interfering with signaling pathways, and a secondary pathogen such as a bacteria or fungus is actively killing the tree.

To this end, sequencing of tissue from the roots of infected trees also revealed the presence of *Fusarium oxysporum*, *Leptosphairea biglobosa*, *Leptosphairea macculans*, *Nectria haematococca*, and *Rhizoctonia solani*, pathogens that are known to cause root rot in other species. At this time, it is not known if these pathogens are simply present or if they are responsible to some degree for the damage observed in the roots. Furthermore, during the 2019 season we observed several trees that showed symptoms of back cracking and necrosis above the graft union. After further study we found that this was likely due to winter injury given the severe weather in February, combined with an opportunistic infection by *Valsa ceratospora*. Growers should be cautioned that there are many pathogens and agents that could cause decline symptoms on a tree.

In conclusion, until Koch's postulates are performed, identifying whether any virus or group of viruses are responsible for the decline and death of trees, notably Honeycrisp, on G.395 rootstock will require further research. However, based on the data collected in this study, there is no one virus or group of viruses that we can say with any degree of confidence to be associated with apple decline. This syndrome has, from reports and observations across the country, potentially multiple causes and variable pathology, such as necrosis above versus below the graft union depending on location and cultivar / rootstock combination. Our observations in this study do indicate however, that cultivars with a Honeycrisp genetic parentage do seem to be susceptible to whatever the causal agent is, when grown on G.935 rootstock.

EXECUTIVE SUMMARY

Project title: Understanding decline on select apple scion-rootstock combinations

Key words: Apple decline, Virus, Disease

Abstract: The causal agent(s) of apple decline, particularly of Honeycrisp variants on G.935 rootstock are unknown. This project explored the possibility that one or more viruses could be the cause. While diseased plants were heavily infected with viruses, no clear association was found between specific species and disease.

Executive Summary: This project examined whether viruses are a potential cause of apple decline, specifically that of Honeycrisp variants on G.935 rootstock, a combination that has exhibited the most severe decline-like symptoms in Washington State. Our first step in this project was to examine the physiology of the disease, and determine what apple decline of Honeycrisp on G.935 really was. We found that the most obvious symptom was dieback of the limbs and upper parts of the tree, usually at the second leaf stage, and visible necrosis of the trunk below the graft union. The rootstock was more severely affected, with necrosis of the cortex and phloem. Necrotic streaking was also observed throughout the primary and feeder roots. Our conclusion is that decline of Honeycrisp on G.935 is due to dieback of the rootstock, rather than direct damage to the scion.

Next we examined whether viruses were the causal organism of this disease. We collected and tested a total of 52 symptomatic and 18 asymptomatic trees over the course of the 2018 and 2019 growing seasons, and found that both disease and healthy trees were infected with known, named viral species. As a general observation, diseased trees had more viruses than the healthy, although there was no correlation between the presence of specific viral species and disease, nor groups of viruses and disease. From this we suggest that while infection with known viruses may be generally detrimental to tree health, there is no link between these apple-infecting viruses, and no specific management recommendation can be made at this time. We further examined the potential viruses present by high-throughput sequencing (HTS), and found the named viruses detected by PCR, as well as a total of 17 new viral species in some of the symptomatic trees. These new viruses are not common or widespread in the diseased plants, and are not likely to be involved in pathogenesis but are endemic or environmental viruses.

In summary, while many viruses have been identified as being present in symptomatic trees, there is no clear association between virus species and the onset of decline symptoms. This does not exclude a secondary role for viruses in this disease, perhaps weakening the plant for subsequent infection/damage by an unknown agent. However, the data does not support active management of these viruses for the purpose of preventing apple decline, rather management to promote general plant health, and the prevention of the diseases that they do cause.