FINAL PROJECT REPORT

Project Title: Efficient strategy to diagnose important virus disease of fruit trees

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Percentage time per crop: Apple: 33% Pear: 0% Cherry: 67% Stone Fruit: 0%

Other funding sources: None

Total Project Funding: **$109, 351**

Budget History:

Item	2013	2014	2015
Salaries	$17,717[1]	$18,426[1]	$19,187[1]
Benefits	$7,025[2]	$7,306[2]	$7,669[2]
Wages	$0	$0	$0
Benefits	$0	$0	$0
Equipment	$0	$0	$0
Supplies	$10,258[3]	$10,668[3]	$11,095[3]
Travel	$0	$0	$0
Miscellaneous	$0	$0	$0
Plot Fees	$0	$0	$0
Total	$35,000	$36,400	$37,951

Footnotes:

1. A Post Doctoral Research Associated and a Research Associate at 20% of full time each.
2. Benefits calculates at the state standard rate.
3. Purchase of enzymes and primers; deep sequencing of virus isolates.

OBJECTIVES:

The overall objective of this research program is to develop detection methods for a number of significant fruit tree diseases caused by virus-like agents. The availability of these detection methods is critical for implementation of effective management practices. Because of its cost effectivity, the detection platform emphasized in this project is the recombinase polymerase amplification (RPA) assay. Priority is also given to the agents responsible for little cherry disease due to its recent escalation in Washington orchards. Specific objectives are:

1. Validate use of RPA for the detection of *Little cherry virus 2*.
2. Develop RPA for the rusty mottle group of cherry viruses, a complex group of viruses that moves into cherry orchards from surrounding native vegetation;
3. Develop RPA for the identification of apple stem pitting virus associated with green crinkle disease.

SIGNIFICANT FINDINGS:

- A detection kit, based on RT-RPA format, was developed for *Little cherry virus 2*. Similarly, a reliable RPA detection system for Western X phytoplasma is also available and an RT-RPA kit for *Little cherry virus 1* was successfully developed.
    - The LChV2 RT-RPA kit is available commercially and detects different genetic variants of the virus. The kit is reliable but offers more accurate virus detection if used during the latter part of the growing season (after harvest).
    - The individual components of the RPA and RT-RPA detection system for WX phytoplasma and LChV1, respectively, can be purchased individually and used in a similar manner as the LChV2 RT-RPA kit. For LChV1 assay, validation using LChV1 samples from commercial orchards is further needed.
- An RT-RPA assay to detect viruses associated with the cherry rusty mottle group and cherry twisted leaf diseases that would discriminate from *Cherry green ring mottle virus* was developed.
    - The reliability of this RT-RPA assay system in detecting samples from commercial orchards as well as its capability to discriminate other cherry viruses other than CGRMV is further needed before it can be used widely for routine detection.

RESULTS AND DISCUSSION

Validation of Little cherry virus 2 field kits:

The initiation of this research project coincided with the heightened awareness of the resurgence of little cherry disease in Washington orchards. Data compiled from our laboratory from 2010 to 2012 demonstrated that the outbreak is associated primarily with *Little cherry virus 2*, hence, development of rapid detection kit focusing primarily on this virus was initiated.

A rapid detection kit for *Little cherry virus 2* (LChV2), based on the reverse transcription recombinase-polymerase amplification (RT-RPA) technology, was made available during the 2014 growing season. Samples from symptomatic trees showing little cherry disease symptoms (e.g., small fruits) were tested for the presence of LChV2 by RT-RPA assays during the May to June period. Many of the samples from initial selection of symptomatic trees did not give positive results in the RT-RPA assay. These apparent discrepancies were investigated for 13 symptomatic samples. Reverse transcription polymerase chain reaction (RT-PCR) confirmed that each was infected with LChV2. However, RT-RPA assays were positive for only 7 of the 13 samples when crude plant sap extracts were prepared and tested following the method recommended by the kit manufacturers. When RT-RPA was repeated using purified RNA, 12 out of 13 samples yielded positive reactions for LChV2. The RNA purification process concentrates the RNA relative to crude extracts and removes many enzyme inhibitors from the sample. These results suggest that the RT-RPA test components

recognized the presence of LChV2, but the system was not sufficiently sensitive to detect the virus in these samples. Although RNA purification greatly enhanced sensitivity of LChV2 detection by RT-RPA, this approach adds significantly to the cost of the assay and requires specialized equipment that would not be amenable for use in field offices. Other parameters were investigated to increase sensitivity of the RT-RPA assay. Increasing the reaction incubation time from 15 minutes to 30 minutes increased signal strength, making the results easier to interpret, but it did not increase the sensitivity of the assay in terms of the number of positive samples detected by RT-RPA. Extending the incubation time further to 45 minutes resulted in a marked decrease in sensitivity. Increasing the sample volume added per reaction from 1 µl to 5 µl inhibited RT-RPA reactions, and positive reactions were lost for all but the strongest positives.

Studies of other viruses suggested that storing sample extracts may increase the level of detectable virus in extracts. To test this possibility, ten trees with confirmed LChV2 infection were selected. Leaves or buds were extracted separately from each tree and tested by RT-RPA either the same day or after storage at 39°F for 11 days. Storing extracts for a period of 11 days did not improve the detection of LChV2 (Table 1). Extracting buds versus leaf tissue also failed to improve the correspondence between the RT-PCR results and RT-RPA results. A parallel experiment with five different trees was conducted using petioles as a potential sample source. In this trial, the buds and leaves yielded positive RT-RPA results for 3/5 samples (the same ratio obtained in the experiment reported in Table 1), but only 1/5 petiole samples yielded a positive result. Therefore, petioles were not used in further studies.

Table 1. Samples were prepared from trees that were infected with *Little cherry virus 2* as determined by reverse transcription polymerase chain reaction assays. The samples were tested by reverse transcription recombinase polymerase amplification either on the same day or after 11 days storage.

Sample tested	**Test results performed on day 1 (no storage)** (# positive / # tested)	**Test results performed on day 12 (storage at 39°F)** (# positive / # tested)
Leaf extracts	6/10	6/10
Bud extracts	6/10	6/10

Previous studies had suggested that levels of LChV2 in infected trees might increase as the growing season progresses. As shown in Table 2, testing symptomatic trees by RT-RPA using crude sap extracts during the latter part of the growing season (after harvest) resulted in much more accurate virus detection without the need to purify RNA. This improved detection is presumably due to decreased carbohydrate inhibitors present in crude sap and the increase in concentration of LChV2 in infected tissue.

Table 2. Trees were tested by reverse transcription recombinase polymerase amplification (RT-RPA) technology before and after harvest. The results are compared to results obtained by reverse transcription polymerase chain reaction (RT-PCR) assays

Trial	**# trees tested**	**# positive by RT-PCR**	**# positive by RT-RPA (pre-harvest)**	**# positive by RT-RPA (post-harvest)**
Experiment 1	11	10	2	9
Experiment 2	22	22	0	15
TOTAL	**33**	**32**	**2**	**24★**

★Of the eight discrepancies, three corresponded to samples that were only weakly positive by RT-PCR indicating a very low virus concentration in these samples.

The revision of the sampling and assay protocols based on the above observations resulted in significant improvement in the reliability of the RT-RPA. However, the correspondence between RT-PCR and RT-RPA reached only 75% (Table 2). Other reasons for the apparent inconsistencies were explored.

Two other pathogens, namely, *Little cherry virus 1* (LChV1) and Western X phytoplasma (WX) can also be associated with little cherry disease. In 2014, with funding from the WTFRC Cherry Research Review, a total of 145 samples from symptomatic trees showing small fruit were tested by RT-PCR for the presence of all three pathogens. Analysis of these data (Table 3) revealed three important elements relating to the management of little cherry disease.

Table 3. Trees displaying symptoms typical of little cherry disease were tested by reverse transcription polymerase chain reaction for three pathogens associated with the disease (*Little cherry virus 1* (LChV1), *Little cherry virus 2* (LChV2) and Western X phytoplasma (WX)).

Pathogens	Number of positives per county				Total
	Chelan	Yakima	Grant	Benton	
LChV1	0	0	0	0	0
LChV2	38	23	0	10	71
WX	29	6	15	1	51
LChV1/LChV2	1	7	0	0	8
LChV1/WX	4	0	0	0	4
LChV2/WX	6	1	0	2	9
LChV1/LChV2/WX	2	0	0	0	2
TOTAL					**145**

1. It was previously presumed that WX was primarily associated with little cherry disease in the southern growing regions of WA State (e.g., Yakima, Benton and Grant counties) whereas LChV2 is predominantly located in the northern counties of the state (e.g., Chelan and Douglas counties). The results of the 2014 testing revealed a significant presence of WX in Chelan County and LChV2 in Benton and Yakima Counties. The high percentage of samples infected only with WX is one of the contributing factors to the seemingly failed detection of LChV2 in symptomatic samples; many negative samples were infected with WX rather than LChV2.
2. None of the symptomatic trees identified by growers were infected with LChV1 alone. This suggests that the symptoms induced by LChV1 are relatively minor. Consequently, it will be extremely difficult to detect LChV1-infected trees in the orchard setting based on symptoms alone. A reliable diagnostic will be required to identify singly infected trees.
3. Despite the improved efficacy of the RT-RPA-based LChV2 detection method, one symptomatic sample still remained negative for LChV2 by RT-RPA but positive by RT-PCR. This inconsistency occurred even with a purified RNA sample. The RT-PCR product was sequenced to confirm that the product represented LChV2. The possibility of a different genetic variant of LChV2 was examined by testing more symptomatic samples by both RT-PCR and RT-RPA. A total of four symptomatic trees tested negative by RT-RPA but positive for RT-PCR. Sequencing of the amplification products revealed the presence of a genotypic variant of LChV2 in these four trees. Close examination of the nucleotide sequences from this variants showed that the currently employed probe in the RT-RPA kit would fail to detect it. Therefore, additional sequence information was gathered from WA State LChV2 isolates and combined with sequence data available from public databases. Together, these sequences were used to design new probes and primers for RT-RPA. These data were made available to Agdia Inc. in late fall 2014 to aid in the

re-tooling of the RT-RPA kit for LChV2. In early summer 2015, a prototype of the re-tooled kit was evaluated in the laboratory of Clean Plant Center Northwest, CPCNW (WSU-IAREC Prosser, WA). Using LChV2 infected trees maintained in the greenhouse of CPCNW, the re-tooled kit successfully detected the unique LChV2 variant as well as common LChV2 strains. The redesigned test kit still discriminated between LChV2 and other agents associated with little cherry disease including *Little cherry virus 1* (LChV1) and Western X (WX) phytoplasma. The re-tooled kit was made commercially available in late October of 2015.

Development of an RPA test for WX phytoplasma and LChV1*:*

The results from the 2014 LChV2 RT-RPA kit validation study emphasized the need to have access to reliable diagnostic methods for all three pathogens associated with little cherry disease. More importantly, the relative importance of WX phytoplasma in Washington orchards was highlighted; therefore, resources for the development of rapid detection assay were prioritized for WX phytoplasma over LChV1.

Current PCR assays for WX are based on the ribosomal and intergenic regions. These sequences were used for analysis because they are highly conserved so there is minimal genetic variation between isolates. However, these sequences are also conserved in several other bacterial genera. As a consequence, assays targeting this region have a propensity to produce numerous false positive results. A region in the WX phytoplasma genome coding for two immunodominant proteins (imp and idpA) is highly specific for WX. Therefore, they were selected as candidate regions for the development of an RPA detection assay (note that because the genetic material of WX phytoplasma is DNA, reverse transcription is not necessary, hence the assay is called RPA instead of RT-RPA). Nucleotide sequence information for this region of the WX phytoplasma genome is limited to one isolate that is not known to occur in WA State. Using the sequence information generated by high throughput sequencing of three isolates of WX phytoplasma from WA, primers and probes were selected from the imp and idpA regions of the genome. In initial PCR test, both sets of primer pairs detected WX phytoplasma from 26 samples collected in 2014; these samples originated from orchards in Benton, Yakima, Grant and Chelan counties. Direct sequencing of these 26 WX phytoplasma PCR amplicons revealed conservation of nucleotide sequences corresponding to probe sequences. However, when crude extracts were used, one primer pair and probe combination yielded background reactions from trees not infected with WX. The other primer pair and probe combination (idpA) yielded results comparable to PCR. The idpA primer and probe combination was further demonstrated to be specific for WX. Infection by LChV1, LChV2 or bacterial canker did not affect the WX test results. A pear decline phytoplasma maintained in periwinkle also yielded negative reaction. These results demonstrated the reliability of the primers in detecting wide spectrum of isolates of WX phytoplasma and evidence of the suitability of the designed probes for WX phytoplasma detection by RPA.

In 2015, 25 samples originating from orchards in Benton, Chelan, Grant, Okanogan and Yakima counties tested positive for WX by PCR; crude sap preparations of these samples also tested positive for WX using the RPA assay (Table 4). The reliability RPA to detect WX, in comparison with PCR, was also monitored throughout the 2015 growing season. Both assay formats were unreliable in detecting WX phytoplasma during the earliest part of the season (mid-March: full bloom) but gave consistent positive detection a month after full bloom (starting on mid-Apr). Taken together, a reliable RPA assay for the WX phytoplasma targeting the idpA region of the pathogen was developed that is suitable for use in crude sap extracts.

Table 4. Trees were tested by polymerase chain reaction for Western X phytoplasma (WX). The results are compared to results obtained by recombinase polymerase amplification assay (RPA) assays.

Pathogens	Number of WX positives / number tested		Total
	WX PCR test	WX RPA assay	
Benton	4/4	4/4	4
Chelan	2/2	2/2	2
Grant	6/6	6/6	6
Yakima	7/7	7/7	7
Okanogan	5/5	5/5	5
TOTAL			**25**

The development of an RT-RPA assay for LChV1 was initiated by identifying conserved regions from full to near full genome sequences, obtained by high throughput sequencing, from ten different LChV1 isolates. Using this information along with sequences available in public databases, conserved target regions in the LChV1 genome were identified for potential use in a RT-RPA assay. A total of six DNA primer pairs (three of each designed from the LChV1 replicase and coat protein target regions) were evaluated initially by RT-PCR to detect the presence of the virus from seven LChV1 isolates maintained at CPCNW. One primer pair designed from the coat protein region yielded the most consistent result and was therefore chosen as the primer for RT-RPA; a corresponding probe was also designed. In initial trials, a real time RT-RPA platform was tested using crude extracts from three LChV1 isolates; the results yielded promising results, however, this type of RT-RPA was de-emphasized and replaced by the lateral flow platform, which is amenable for use in field offices. Using crude sap preparations, the RT-RPA assay detected all nine LChV1 isolates (maintained in the greenhouse of CPCNW) in the same manner as RT-PCR. The low incidence of LChV1 in Washington orchards hindered further validations of the RT-RPA detection system using LChV1 field infected trees (i.e. none of the symptomatic samples with small fruits tested positive for LChV1 at CPCNW laboratory during the 2015 growing season). Although not commercially available, the individual components for the WX RPA and LChV1 RT-RPA assays can be purchased individually (lyophilized enzyme, primers and probe) and used in a similar manner as the LChV2 RT-RPA kit.

Assays for other fruit tree viruses

Development of an RT-RPA assay system for the rusty mottle group of cherry viruses and for the identification of apple stem pitting virus associated with apple green crinkle disease were also included in the proposal. However, the rapidly unfolding situation with little cherry disease in Washington State prompted redirection of resources to develop rapid detection assays for WX phytoplasma and LChV1. This has usurped much of the effort towards these remaining objectives. Nevertheless, significant progress has been made.

Previous study was completed that characterized the viruses associated with a group of diseases including cherry rusty mottle, cherry necrotic rusty mottle and cherry twisted leaf. The results yielded a defined genetic entity (virus species) associated with each of these diseases. Moreover, upon examination of these virus species in context of known viruses (including *Cherry green ring mottle virus* or CGRMV), they form a distinct population with unique genomic organizations, genome sequences, and host range. Based on these criteria, a new virus genus was proposed. The association of these diseases with specific viruses permits accurate and rapid diagnosis of orchard samples for the first time. Primers for RT-PCR were developed that allow detection of each of the viruses separately, as well as broad spectrum primers that detect multiple components of this complex group. This baseline information is a necessary precursor to the development of an RT-RPA assay. Initially, this information was used in the development of an RT-RPA assay for this group of viruses. However, suitable primers and probes for the RT-RPA assay could not be selected using this information; the

information consisted of sequence data of the whole coat protein region of the viruses within this group. Therefore, other regions of the virus genome were examined. After thorough inspection of sequences from 24 whole genomes of viruses belonging to this group, a region within the virus replication protein encoding gene showed potential for use as target region for the RT-RPA assay system. It is important to note that all viruses that belong to this group induce acute symptoms in sweet cherry except for CGRMV (which is only latent in sweet cherry). Therefore, the path employed in the development of RT-RPA assay for this group of viruses was a broad spectrum assay that would detect viruses associated with cherry rusty mottle, cherry necrotic rusty mottle and cherry twisted leaf but not CGRMV. In initial trial using crude sap preparations, the RT-RPA showed positive reactions only in trees (eight trees) that are infected with viruses associated with the cherry rusty mottle group and cherry twisted leaf diseases (Table 5) but not in trees that are infected only with CGRMV (two trees). These results show promising potential of the RT-RPA assay for this group of viruses. However, there are immediate experiments that need to be done before this RT-RPA assay system can be used widely for routine detection. A validation trial using infected trees from commercial orchards in Washington State is necessary to check the reliability of the RT-RPA assay system with actual field samples. The second experiment is a cross reaction test of trees infected with other cherry viruses that are known to be present in Washington orchards. These viruses include LChV1, LChV2, *Cherry leafroll virus*, *Cherry raspleaf virus*, *Cherry virus A*, *Prune dwarf virus* and *Prunus necrotic virus*; this trial determines the specificity of the RT-RPA detection assay. In the same manner as the WX RPA and LChV1 RT-RPA assays, the RT-RPA detection assay for the viruses associated with cherry rusty mottle group and cherry twisted leaf diseases is not commercially available but individual components of the assay system can be individually purchased.

Table 5. Trees were tested by reverse transcription polymerase chain reaction for each of the viruses in the cherry rusty mottle group, cherry twisted leaf associated virus and *Cherry green ring mottle virus* (CGRMV). The results are compared to results obtained by recombinase polymerase amplification assay (RPA) assays.

Source trees	RT-PCR test★				RT-RPA test
	CNRMV	CTLaV	CRMaV	CGRMV	
8242-3	-	+	-	+	+
8265	+	+	-	+	+
95CI205P3	-	+	-	+	+
98CI194	-	-	+	-	+
8804	-	-	+	-	+
B48C	-	-	+	-	+
WD 01E3R2T7	-	-	-	+	-
02F23rD	-	-	-	+	-
04E36	+	-	-	-	+
103-13	+	+	-	-	+
P. avium 'Mazzard' (seedling roostock)	-	-	-	-	-
Water control	-	-	-	-	-

★CNRMV (*Cherry necrotic rusty mottle virus*) and CRMaV (Cherry rusty mottle associated virus) are viruses associated with cherry rusty mottle group of diseases whereas CTLaV (Cherry twisted leaf associated virus) is the virus associated with cherry twisted leaf disease.

Previous study that was completed on symptom expression of green crinkle disease showed association of *Apple stem pitting virus* (ASPV) with this disease. In order verify whether there are new viruses (or previously unidentified viruses) that might be further associated with apple green

crinkle disease, high throughput sequencing was pursued for three trees that consistently express green crinkle symptoms for over a period of 12 years. Results did not reveal new viruses in three green crinkle trees other than ASPV. Further genetic analysis of ASPV populations in trees expressing apple green crinkle disease and non green crinkle disease showed two candidate variants of ASPV that may be directly responsible for this troublesome disease of apple production. The target region used in the genetic analysis was the full coat protein region of several isolates of ASPV. After a thorough examination, suitable primers and probes for the RT-RPA assay within the coat protein region, as well as other regions within the ASPV genome, could not be selected that would not discriminate ASPV green crinkle variants from other variants of ASPV (non green crinkle associated variants). To further advance our understanding of green crinkle disease, a practical path is to determine conclusively which of the two variants are responsible for apple green crinkle disease. With the availability of full genome sequence information of different ASPV variants that we have obtained, single genotypes of different genotypic variants of ASPV can be produced and introduced individually or in combination into fruit bearing trees to demonstrate which combinations of ASPV variants are directly responsible for inducing green crinkle disease. Once a specific causal agent is identified through this process, diagnostic procedures can be reviewed and re-evaluated.

Project Title: Efficient strategy to diagnose important virus disease of fruit trees

EXECUTIVE SUMMARY:

Little cherry disease agents

A rapid detection kit for *Little cherry virus 2* (LChV2), based on the reverse transcription recombinase-polymerase amplification (RT-RPA) technology, was made available during the 2014 growing season. Subsequent validation trials using symptomatic samples from trees exhibiting small fruits from commercial orchards initially revealed unreliability of the kit in detecting LChV2. Overall, factors that contributed to the poor performance of the kit included inappropriate time of testing, presence of a unique genetic variant of LChV2 that could not be detected by the kit and the increased incidence of WX phytoplasma in little cherry affected trees. Consequently, the probe component in the LChV2 RT-RPA kit was redesigned to accommodate detection of the unique variant of the virus and the re-tooled LChV2 kit was commercially released in late October 2015. The re-tooled kit offers more accurate virus detection if used during the latter part of the growing season (after harvest). The results from the 2014 LChV2 RT-RPA kit validation study emphasized the need to have access to reliable diagnostic methods for the two other pathogens associated with little cherry disease. As a result, an RPA detection assay system was developed for WX phytoplasma and *Little cherry virus 1* (LChV1). The RPA detection system for Western X phytoplasma is reliable but the RT-RPA kit for LChV1 needs to be further validated using infected samples from commercial orchards. Both assay systems are not commercially available but individual components can be purchased individually and used in a similar manner as the LChV2 RT-RPA kit.

Assays for other fruit tree viruses

An RT-RPA assay to detect viruses associated with the cherry rusty mottle group and cherry twisted leaf diseases and discriminate from *Cherry green ring mottle virus* was developed. The foundation for the development of a group specific test that would discriminate CGRMV was based on the fact the all viruses in this group induce acute symptoms in sweet cherry except for CGRMV (which causes only latent infection). However, the reliability of this RT-RPA assay system in detecting samples from commercial orchards as well as its capability to discriminate other cherry viruses other than CGRMV is further needed before it can be used widely for routine detection.

Analysis of virus profiles of apple green crinkle trees revealed association of *Apple stem pitting virus* (ASPV) with the disease. Further genetic analysis of ASPV populations in trees expressing apple green crinkle disease showed two candidate variants of ASPV that may be directly responsible for this periodic but persistent disease of apple production. Efforts to develop an RT-RPA assay that would not discriminate ASPV green crinkle variants from other variants of ASPV (non green crinkle associated variants) were not successful. This was due to the fact that suitable primers and probes for the RT-RPA assay within the coat protein region of ASPV, as well as other regions within the virus genome, could not be selected. A future direction to advance our understanding of green crinkle disease is to determine conclusively which of the two variants are responsible for apple green crinkle disease. With the availability of full genome sequence information of different ASPV variants that we have obtained, single genotypes of different genotypic variants of ASPV can be produced and introduced individually or in combination into fruit bearing trees to demonstrate which combinations of ASPV variants are directly responsible for inducing green crinkle disease.