2015 Technology Research Review
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WA Cattlemen's Association, Ellensburg

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CONTINUING PROJECT REPORT **YEAR**: 2 of 2
WTFRC Project Number: TR-15-102A

Project Title: Genetic analysis of Western Cherry Fruit Fly to facilitate species ID

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Total Project Request: **Year 1:** $22,264 **Year 2: $20,181**

Percentage time per crop: Cherry: 70% Apple: 30%

Other funding sources: None

WTFRC Collaborative expenses: None

Budget 1
Organization Name: UC Davis **Contract Administrator:** Jessica Kaur
Telephone: (530) 752-5267 **Email address:** jeskaur@ucdavis.edu

Item	2015	2016
Salaries	$9,198	$9,566
Benefits	$3,716	$3,864
Wages	-	-
Benefits	-	-
Equipment	-	-
Supplies	$5,000	$5,000.00
Travel	-	-
Miscellaneous	$3,600	$1,000.00
Plot Fees	-	-
Total	$21,514	**$19,431**

Footnotes: Salary is to support a technician at 25% effort in both years 1 and 2 of the project. Benefits are calculated at UC Davis specified rate of 40.4%. Supplies include reagents for DNA/RNA extraction, Illumina sequencing library preparation, quality control of sequencing libraries, PCR enzymes, standard laboratory consumables and chemicals for molecular biology (PCR and agarose gel electrophoresis). Miscellaneous costs include transcriptome sequencing costs, which will be performed at BGI@UCD in Sacramento, CA, in Year 1 of the project. Miscellaneous costs for year 2 will be for publications and reporting costs to facilitate implementation of the diagnostic assay.

Budget 2

Organization Name: OSU-MCAREC **Contract Administrator:** L. J. Koong

Telephone: 541-737-4066 **Email address:** l.j.koong@oregonstate.edu

Item	2015	2016
Salaries	-	-
Benefits	-	-
Wages	-	-
Benefits	-	-
Equipment	-	-
Supplies	-	-
Travel	-	-
Plot Fees	-	-
Miscellaneous	$750	$750
Total	$750	$750

Footnotes: Miscellaneous costs include costs for collecting and shipping insect samples from Oregon to California for transcriptome sequencing (Year 1) and testing and validating of the molecular diagnostic (Year 2).

JUSTIFICATION

The Western Cherry Fruit Fly (WCFF), *Rhagoletis indifferens*, is a serious pest of cultivated cherries in the Western U.S. and British Columbia, Canada (Yee et al. 2011; Yee et al. 2014a; Yee 2014b; Kumar et al. 2014). It damages the crop directly, and more importantly, becomes a quarantine and quality issue if found in fruit by domestic or oversea inspectors. Once fly larvae that remotely resemble WCFF are found by inspectors at packing houses or export facilities, fruit shipments are halted until a positive or negative species ID is determined. Distinguishing larvae from other insect species that infest cherries can be difficult, and rearing to adulthood for more reliable ID is not practical if marketability of the shipment were to be maintained. When even one suspect larva is found, an entire load of fruit can be rejected, and all subsequent fruit shipments will undergo intensive inspection to uphold the zero tolerance policy.

In order to (1) speed up species ID and (2) ensure reliability of ID to prevent false positives, which can lead to unnecessary quarantine measures and intensive inspection, both leading to increased economic burden to the Cherry industries, we propose to develop a molecular diagnostic test that can be used to rapidly (less than 2 hours) identify WCFF and differentiate it from other insect larvae, including the apple maggot, *Rhagoletis pomonella* (Green et al. 2013), which is an occasional pest in cherries, as well as the Spotted Wing Drosophila (SWD; *Drosophila suzukii*) (Beers et al. 2011; Walsh et al. 2011). There are a number of commonly used PCR-based molecular diagnostics that have been used for species ID, but these approaches often vary in cost and duration to obtain the results (Behura 2006, Gariepy et al. 2007, Hebert et al. 2003, Williams et al. 1990, Wyman and White 1980). So far, only microsatellite markers have been designed for the molecular identification of WCFF (Maxwell et al. 2009; St. Jean et al. 2013), but results generated using microsatellites are generally difficult to interpret, even for trained scientists. The molecular diagnostic we propose to develop will be a simple, easy-to-interpret, one-step PCR amplification using WCFF-specific primers that is not dependent on sequencing or restriction enzyme digestion, procedures that add both cost and processing time. LAMP PCR approach, which will not require a thermocycler, will also be tested, and can potentially further reduce processing time. Our goal is to develop a molecular diagnostic that is easy to interpret, accurate, and require minimum processing time and equipment.

OBJECTIVES

Objective 1: Sequence the transcriptomes (all expressed genes) of WCFF and perform bioinformatic and comparative sequence analysis with other insect pests of cherries (common and occasional) in the Pacific Northwest as well as closely-related species to identify appropriate species-specific molecular diagnostic markers. Genetic analysis will also pave the way for future molecular analysis of WCFF to improve management strategies, e.g. evaluation of response to insecticide treatments and development of RNAi biopesticide.

Objective 2: Develop an accurate PCR-based molecular diagnostic test to identify WCFF at all life stages from limited starting materials, e.g. a single larva. Both conventional and LAMP PCR will be tested to design a user-friendly and economical diagnostic assay. The assay will be validated using WCFF and closely-related insect specimens.

SIGNIFICANT ACCOMPLISHMENTS

- We obtained WCFF larvae, pupae, and adult samples and sequenced their transcriptomes.
- We completed the bioinformatic analysis to assemble the first transcriptome for WCFF.
- We collected closely-related insect species as well as species that co-inhibit cherry hosts and preserved them in preparation for validation of our molecular diagnostic in Year 2 of the project (Table 1). Insect specimens include WCFF, apple maggot fly, olive fruit fly, walnut husk fly, blueberry maggot fly, and Spotted Wing Drosophila.

METHODS

Objective 1: Sequencing the transcriptome of WCFF

***Overview*:** In order to design an accurate and efficient one-step PCR diagnostic that can differentiate WCFF from other common and occasional cherry pest species, we would first need to obtain substantial sequence information of WCFF, which is not currently available. We therefore propose to sequence the transcriptomes of WCFF using different life stages as starting material. Sequencing the transcriptomes instead of full genomes will reduce cost of the project by at least 50%. Bioinformatic analysis will then be performed to compare WCFF sequences with sequence data of other pest species that infest cherries, e.g. the apple maggot *Rhagoletis pomonella,* (Schwarz et al. 2009) and *Drosophila suzukii* (SWD) (Chiu et al. 2013) to design molecular diagnostic markers that can be used to differentiate these species.

RNA extraction and Transcriptome Sequencing

Total RNA will be extracted from individual specimens collected from the Pacific Northwest using Tri-reagent (Sigma). Following polyA mRNA enrichment, which enriched for RNA from expressed genes, using the Next PolyA magnetic isolation module (New England Biolabs), paired-end sequencing libraries with an approximate average insert length of around 150 to 200 bp (standard for transcriptome analysis) will be created using the Next Ultra RNA library Prep Kit (New England Biolabs). Transcriptome libraries will be sequenced using 100bp paired-end Illumina HiSeq at the UC Davis Genome Center Sequencing facility.

Since the genome sequence of WCFF are not available, we will perform *de novo* transcriptome assembly using the de Brujin graph based assembler, Trinity release 2013-02-25 (Grabherr et al. 2011). Our experimental and bioinformatic pipeline will yield transcriptomes for different life stages of WCFF.

Bioinformatic analysis to design species-specific diagnostic PCR primers

To design species-specific diagnostic PCR primers from the transcriptome data, we will perform comparative sequence analysis of our WCFF against available genomes and transcriptomes in the public database for closely related insect species, especially those that are known to infest cherries and apples. Our laboratory has extensive experience in bioinformatic and comparative sequence analysis. We spearheaded the complete sequencing of the SWD genome (Chiu et al. 2013), and used a similar comparative genomic approach to design SWD species-specific diagnostic (Murphy et al. 2015).

Objective 2: Design a species-specific molecular diagnostic for WCFF

***Overview*:** Our goal is to develop a PCR diagnostic that is easy to interpret, accurate, and requires minimum processing time and equipment. Upon completion of the project, we will work with stakeholders and scientists to implement our species diagnostic into their monitoring or research programs.

Multiplex PCR primer design to identify WCFF

Our PCR diagnostic test design relies on the use of two primer sets in a multiplex PCR reaction. The first set of primers is designed to amplify a product that is conserved (high sequence identity) in all species of interest to confirm the presence and quality of DNA in the reaction and to verify that a negative test result is not due to an unsuccessful PCR reaction, thus providing quality assurance. The second set of primers in our multiplex reaction will be designed from a gene that is more divergent in sequence between the species of interest such that only the addition of WCFF DNA in the PCR reaction will result in successful PCR amplification.

Genomic DNA extraction and PCR reactions

To test the specificity of our multiplex PCR, we will extract genomic DNA from larvae or adult WCFF for use as template for PCR reactions. Adults or larvae will be homogenized by motorized pestle in DNA extraction buffer and processed using standard extraction protocol that is routine in our

laboratory. PCR will be performed using Taq DNA polymerase (Life Technologies, Grand Island, NY) in a Mastercycler Pro PCR machine (Eppendorf, Hauppauge, NY). The amplified DNA products will be resolved by agarose gel electrophoresis and visualized under UV light to assess (i) presence/absence of DNA bands, and (ii) size of DNA bands as compared to a size standard. To ensure utility of our WCFF diagnostic, we will validate the assay using specimens collected from a wide geographical range as genetic variations exist between populations from different collection sites.

Direct larval tissue PCR

Once PCR amplification using extracted genomic DNA is successful, we will proceed to test our molecular diagnostic using crude larval extract as starting material. Our goal is to optimize our diagnostic to enable use of crude extract isolated from as little as one WCFF larva. The use of crude extract as starting material for the PCR will also greatly reduce necessary chemical reagents as well as processing time to allow for SWD identification in less than 2 hours. Individual larvae will be cut in half with a sterile razor blade and incubated in PCR-grade water. The samples will be vortexed briefly and a small aliquot of the crude extract will be used directly as input to our PCR diagnostic. PCR amplification and DNA visualization will be the same as described above. We have previous success optimizing our species diagnostic for SWD (Murphy et al. 2015) to be performed using crude larval extract with DNA extraction.

Previous success in the development of species-specific molecular diagnostic

With the funding support of the Washington Tree Fruit Research Commissions (2014-15), we have successfully developed a molecular diagnostic to differentiate SWD (another serious cherry pest) from 8 other species of common Drosophilids, including *D. biarmipes*, *D. subpulchrella*, and *D. takahashii*, which are closely related to SWD and belong to the *suzukii-takahashii* subgroup (Murphy et al. 2015). The molecular diagnostic has been validated using 9 species of *Drosophila* species (SWD and non-SWD) and 19 populations of SWD collected from different geographical regions in the US and around the world (CA, OR, WA, MD, Hawaii, Japan, South Korea, Italy). The SWD molecular diagnostic protocol has been optimized to be fast, simple, and reliable (1 to 2 hours to completion). It does not require the procedure of DNA extraction, and requires minimal starting material (e.g. one single larva).

RESULTS AND DISCUSSION:

Sequencing and assembly of WCFF transcriptome

We collected WCFF larvae, pupae, and adults and generated three RNA sequencing libraries for each of the WCFF life stages. We then performed paired end sequencing on an Illumina HiSeq 3000 platform, and obtained a total of 778,742,672 100-bp reads. Trimmomatic v0.35 was used to trim adaptor sequences and low quality ends for quality control. 99.07% of nucleotide bases were retained after trimming indicating high quality sequence data, and subsequently passed on to Trinity 2.1.1 (Grabherr et al. 2011) for transcriptome assembly. To reduce runtime and computing resource requirements, *in silico* read normalization was performed as part of the Trinity assembly process. A total of 230,770 transcript sequences and 204,659,650 bases were assembled. The transcript contig N50 is 1,943, demonstrating good quality sequence assembly. The GC content is 38.81%. Paired reads were mapped back to the assembly using STAR v2.5.0c and passed to Corset v1.04 for clustering into genes. Corset generated 96,628 clusters, representing possible number of expressed genes in WCFF. We anticipated that the number of expressed genes to be lower. Future genome sequencing can likely further improve WCFF transcriptome assembly. Nevertheless, the transcriptome data we generated will be sufficient for the development of molecular diagnostic for WCFF and Apple Maggot Fly species ID.

Bioinformatic analysis to compare the WCFF transcriptome and sequence data from Apple Maggot Fly, Spotted Wing Drosophila, and other closely-related species is currently in progress, and will yield species-specific molecular diagnostic to differentiate WCFF from species that co-inhabit cherry host plants. Specimens of WCFF, apple maggot fly, olive fruit fly, walnut husk fly, blueberry maggot fly, spotted wing Drosophila have been collected in preparation for testing and validation of the molecular diagnostic in year 2 of the project.

Although not within the scope of this project, comparative transcriptome analysis from different stages of WCFF will also advance our understanding of WCFF biology at different life stages.

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Table 1: Specimens for validation of WCFF and Apple Maggot Fly molecular diagnostics

Common Name	Species Name	Source	Institution	Collector
Western Cherry Fruit Fly	*Rhagoletis indifferens*	Yakima, WA	USDA ARS Research Station	A. Abrams
Apple Maggot Fly	*Rhagoletis pomonella*	Hood River, OR	OSU	P. Shearer
Olive Fruit Fly	*Bactrocera oleae*	Davis, CA	Plant Pathology Field Station, UC Davis	N. Nicola
Walnut Husk Fly	*Rhagoletis completa*	CA	UC Berkeley	B. van Steenwyk
Blueberry Maggot Fly	*Rhagoletis mendax*	Benton Harbor, MI	Southwest Michigan Research and Extension Center	R. Isaacs
Spotted Wing Drosophila	*Drosophila suzukii*	Hood River, OR	OSU	P. Shearer

FINAL PROJECT REPORT

Project Title: Mechanical pollination for yield security

PI: Matthew Whiting
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City/State/Zip: Prosser/WA/99350

Cooperators: OnTarget Spray Systems, Firman Pollen Co., Olsen Brothers, Hayden Farms, Russ LeSage; Jason Matson; Allan Brothers

Total Project Request: **Year 1:** 30,123 **Year 2:** 31,086

Percentage time per crop: Apple: 25% Pear: 25% Cherry: 50% Stone Fruit: 0%

Other funding sources

Notes: In kind support is provided by Firman Pollen Co (pollen donations) and OnTarget Spray Systems (donation of sprayer and technician for support)

Budget 1
Organization Name: WSU **Contract Administrator: Carrie Johnston**
Telephone: 509 335-4564 **Email address: carriej@wsu.edu**

Item	**2014**	**2015**
Salaries	13,028	13,549
Benefits	1,173	1,219
Wages	9,360	9,734
Benefits	562	584
Equipment		
Supplies	3,000	3,000
Travel	2,500	2,500
Plot Fees	500	500
Miscellaneous		
Total	30,123	31,086

Footnotes:

OBJECTIVES:

1. Ensure consistent fruiting in sweet cherry, apple, and pear through the development of an effective mechanical pollination system.
2. Pursue further funding to build upon this project's outcomes

SIGNIFICANT FINDINGS

- Artificial pollination with pollen suspensions applied electrostatically is capable of setting fruit in apple, pear, and sweet cherry
- We documented the deposition of mechanically-applied pollen to the stigmatic surface in sweet cherry – increasing pollen density per stigma by about 3-fold compared to open pollinated
- A single supplementary application of a pollen suspension can improve fruit set
- Replacement pollination tests (i.e., no pollen applied other than via our system) are promising in apple and sweet cherry – tree yield was similar from two applications of pollen compared to open pollinated
- Inconsistent and very weak correlation found between flower density, fruit set, shoot length, shoot angle and shoot diameter
- Including sucrose and boron in pollen suspension media can improve pollen viability for more than 1 hr
- Pure pollen is required for our pollination system
- Pure pollen can be prepared on a commercial scale for artificial pollination systems
- Percentage of pollen germination varied for same sucrose concentration in medium, across the genotypes used in current study
- Minimal variation of pollen deposition variation by flower location in the tree was noticed, receiving electrostatic pollen suspension spray
- It is feasible to incorporate growth regulator ReTain® to aid against premature ovule senescence in sweet cherry, without further affecting satisfactory germination rate in pollen suspension
- Sweet cherry stigmas exhibit high receptivity on the second day after bloom

RESULTS AND DISCUSSION

This project has evaluated the potential to pollinate and fertilize tree fruit flowers with applications of liquid pollen suspensions through commercial electrostatic sprayers. This research project has combined small-scale lab/research plot trials with larger-scale field trials. Our proposed precision pollination system is comprised of 3 steps:

1. Collect and purify pollen
2. Suspend pollen in liquid
3. Apply pollen through electrostatic application system

This research project has addressed the second and third aspects directly, and we have worked in collaboration with Firman Pollen Company to address pollen collection and purification.

1. ***Pollen collection and purification***.
Through our field trials and lab analyses of suspension materials, we learned that it is necessary to utilize pure pollen in our pollination system. Field trials in 2014 revealed problems with rehydration of non-pollen floral parts (e.g., filament, anthers) that caused filters in the sprayer to clog rapidly. The standard pollen that is used commercially for hive inserts or to be applied through dusting means contains too many of these non-pollen materials to be useful for field application through a sprayer. We investigated the potential to modify filtration systems in the On Target Spray Systems sprayer but it became clear that further pollen purification would be necessary. Firman Pollen Company has developed an additional filtration step that yields pure pollen. Our field tests in 2015 utilized this purified pollen product, and we had no difficulties with filtration.

Pollen collection systems will need to be improved to keep up with demand. The current process of hand-harvesting flowers in commercial orchards is too laborious and time-consuming. This is largely due to the pickers' inability to harvest all available flowers – the process is selective because flower must remain for the grower to harvest a crop. This critical issue is addressed in our new proposal. Orchards should be planted strictly for pollen collection – this will require investigation of new training systems (or modification of existing systems) to optimize pollen yield.

2. ***Suspend pollen in liquid***
In 2014 we conducted lab studies on pollen viability and the role of suspension media components. From our tests of three sweet cherry pollen genotypes (Lapins, Rainier, Sweetheart), we found that we could improve pollen viability (i.e., germination) when pollen was suspended in a modified medium, compared to water. For each genotype, the following suspension mediums were evaluated: solution 1: 5% (w/v) sucrose + 0.15% (w/v) pollen grain, solution 2: 10% sucrose (w/v) + 0.15% (w/v) pollen grain, solution 3: 15% (w/v) sucrose + 0.15 % (w/v) pollen. Pollen germination declined over time in the control treatment with only water. This occurred for all cultivars similarly, losing about 20% germination from initial suspension to 60 minutes later. In contrast, the suspension solutions improved pollen germination and extended viability over time. There appears to be a benefit to higher rates of sucrose, with viability improved more than 600% after one hour in suspension. Suspension medium containing 15% sucrose (w/v) improved germination significantly in all pollen genotype irrespective of how long the pollen was in suspension. This is likely due to the favorable osmotic balance in the medium with sucrose. In contrast, we documented approximately a 15-20% decrease in the viability of pollen grains in control between the first and last sampling time. The highest pollen germination was observed with Sweetheart (67%) followed by Rainier (59%) after 40 minute suspension time in media enriched with 15% sucrose (w/v).

In 2015, pollen suspension development continued with multiple experiments carried out to investigate the role of candidate suspension components on pollen viability. For our in vitro pollen germination experiments, we utilized one sweet cherry, apple and pear genotype (Rainier, Red Delicious and Bartlett). Cultivar selections were based on most suitable and compatible pollen genotypes to source pollen for our field trial pollen receiving cultivars. In every case, pure pollen was utilized. Candidate suspension components were selected from a literature review of relevant scholarly articles. Initially we evaluated pollen viability after incubation of 1 hour at a concentration gradient of each the components. Afterwards, we assessed combinations of components on pollen viability. In each case we used 0.18% w/v of pollen for the experiments. Replicate tubes of suspension materials were created, pollen was added, and the tubes were agitated briefly (<5 sec) before incubated at room temperature. Suspension aliquots were withdrawn after 5, 30 , and 60 minutes and pollen germination was evaluated to assess viability. Following suspension, our modified medium maintained or significantly improved pollen germination percentage compared to only water, irrespective of pollen genotypes and cultivars. Recent findings revealed that, using sucrose at lower rate could maintain favorable osmotic balance retention with optimal

pollen viability, either alone or in combination of other components in suspension, across all three genotypes.

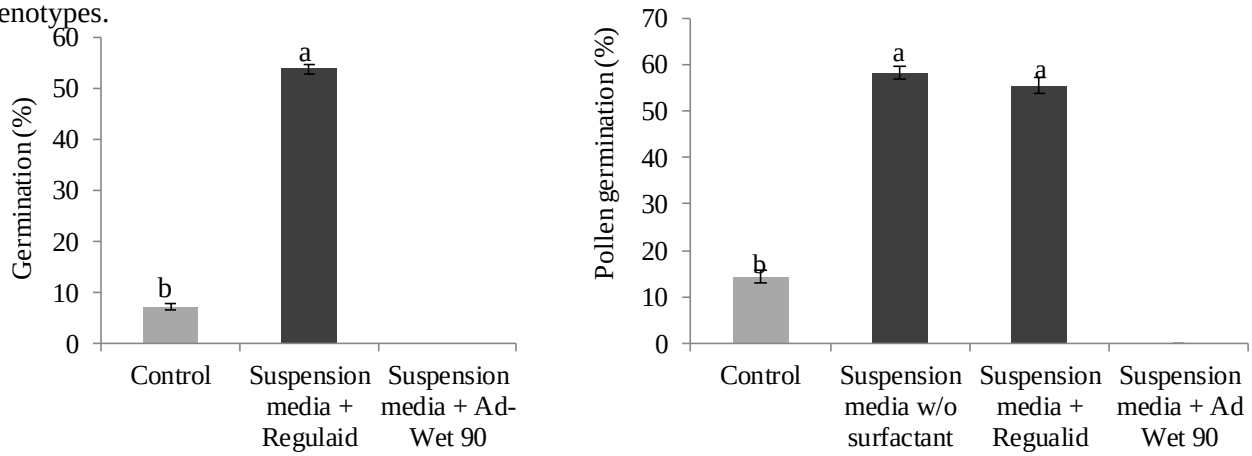

Figure 1: Effects of suspension media on sweet cherry (left) and apple (right) *in vitro* pollen germination after 60 minutes in suspension (both p-values: 0.000).

Pollen + water alone is not an effective combination. First, pollen is not easily wettable (i.e., difficult to suspend); secondly, pollen losses viability in water. Germination rate of pollen suspended in water consistently decreased over the incubation periods, and reached a minimum after 60 minutes. We evaluated candidate suspension components individually and in combination for their effect on pollen germination. After 5 minutes in suspension with water + a single suspension ingredient, several suspension components improved germination – rates were 151% and 144% of control for 'Red Delicious' pollen. Similar results were found with cherry and pear pollen. We then combined suspension constituents at their optimum concentration and found improved pollen germination (206% of control). After 30 minutes of pollen in suspension, we found great improvements in pollen germination. Several suspension components increased germination by 220% to 230% of control. The greatest improvements in pollen viability were seen after 60 minutes of pollen incubation, when our suspension improved pollen germination rates that were more than three-fold greater that the control of water + pollen. Our combined suspension media improve pollen germination by more than 4x compared to the control after 60 minutes (Figure 1).

Future work is needed to transition from lab-scale tests to large-scale suspensions in commercial application equipment. Lab tests need to be repeated in the field, collecting pollen that has been loaded in suspension in the spray tank, and sprayed through the system. We do not know how the application system will affect pollen viability, nor how easily suspensions will be maintained under constant agitation, nor the effect of agitation on the suspension and pollen viability. In addition, suspension creation in the lab is fairly straight forward using lab agitation tools (e.g., vortex system), in our field trials of 2015 we encountered some difficulty in creating a uniform suspension in the sprayer tank. This process needs further development.

***In vivo* pollen deposition**

We also evaluated pollen deposition to the stigma using cut limbs of 'Rainier' sweet cherry in the lab. *In vivo* pollen deposition tests were carried out on emasculated flowers was carried out with two pollen rates applied at 2 and 3 days after bloom (DAB) under greenhouse conditions. Flowering limbs were

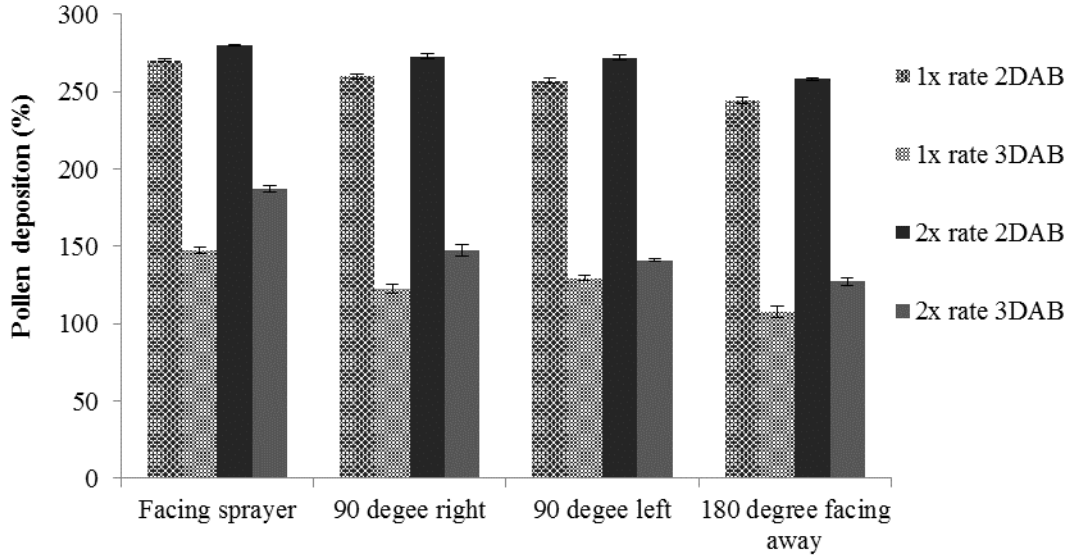

Figure 2: Percent pollen deposition after mechanical pollination only, according to the total number of pollen collected from emasculated sweet cherry flower stigma at 2 and 3 days after bloom under controlled condition. Pollen deposition represented as the average of 5 flowers from each of five replicated flowering limbs, *i. e.* for each treatment n=25/direction, per sampling day.

positioned vertically in buckets of water. Using a single-nozzle electrostatic application setup we applied two rates of pollen in suspension to replicate limbs moving the nozzle past the limbs at about 2 mph (to mimic application in the field). Post application, sample flowers from two different bloom dates were collected and the deposition of pollen on the stigmatic surfaces was assessed microscopically on flowers that faced the sprayer as well as those that were 90 degrees away, and those that were 180 degrees from the sprayer (i.e., on the 'back' of the limb). Pollen deposition was consistently higher when pollen was applied at 2DAB, at both pollen rates (Figure 2). Highest pollen deposition on flower stigmas facing nozzles was found from double rate (30g/acre) of pollen applied at 2DAB (279%), and lowest from single rate (15g/acre) applied at 3DAB at similar angle. Minimal differences observed in pollen deposition on flowers stigmas located at 90° from the direction of the spray at both 2DAB and 3DAB applications, separately, with either pollen rate. However, at this angle flowers from 2DAB has shown 125% higher deposition compared to 3DAB. Flowers located at straight angle (180° facing away from the spray) received lowest pollen deposition (108%), when single rate pollen applied at 3DAB. These results support our lab's previous studies of stigmatic receptivity which revealed maximum receptivity of sweet cherry stigmas on flowers that were open for two days.

Field trials

In 2014 we conducted several field trials to evaluate the potential for improving fruit set with supplemental applications of pollen as well as replacing the use of pollenizers and pollinators (i.e., replacement pollination). Cherry field trials were established in an Early Robin orchard (Pasco) and two Tieton orchards (Roosevelt and Benton City). We are unable to collect data from the Early Robin and Roosevelt Tieton orchards due to difficulties with the application system. We discovered that our pollen mixture was clogging the sprayer's filter and we had trouble with the nozzles getting plugged too. We attributed this to two factors: the quality of the pollen used and the characteristics of the pollen suspension media used. We have learned that only pure pollen will work with the current application

system because anthers and filament material commonly present in standard “off-the-shelf” pollen mixes will hydrate rapidly and plug the filters and nozzles. In addition, the pollen suspension media we used was too viscous and contributed to the clogging of the sprayer. This media was provided by PollenTech, a startup company trying to develop mechanical pollination systems. We did not use their proprietary slurry in 2015 trials. In our third field trial, we were able to use pure pollen, in the Tieton orchard in Benton City, and the system performed well. Our supplemental pollen applications at about 50% and 90% of full bloom increased fruit set by 15% compared to open-pollinated trees (Fig. 1). The application system was clearly effective at placing pollen in suspension on the stigmatic surface – we documented nearly a 3-fold increase in pollen deposition on treated stigmas compared to open-pollinated stigmas (Figure 3).

We also conducted a full replacement pollination (i.e., pollen applied through bee exclusion netting) trial, applying pollen suspension twice (about 25% and 75% full bloom) to ‘Bing’ trees. The artificial pollination system was as effective as natural open pollination (Fig. 2). Interestingly, yield was less variable among trees that were pollinated artificially compared to those open pollinated trees. Furthermore, in a ‘Gala’ apple trial of replacement pollination to limbs covered with netting, we recorded very high fruit set from electrostatic pollen application. We evaluated two pollen suspension solutions and recorded approximately 56% and 75% higher fruit set over natural pollination (see continuing report from 2015 for these results).

In 2015 we established four sweet cherry trials were designed at multiple locations including Tieton (Grandview), Early Robin (Pasco), Benton (Prosser) and Regina (Brewster). We also conducted one apple trial and one pear trial (D’Anjou near Naches). Each of these trials was supplemental pollination – we applied pollination treatments to trees or entire rows in orchards where both pollenizers and pollinators were present.

In a 9th leaf Tieton orchard trained to a vertical UFO architecture, supplemental pollen application using a single pollen rate of 15g/acre, with two applications (once at 50% and again at 100% bloom, increased fruit set by 10% compared to natural pollination. In this orchard we recorded a 2 to 3 fold increase in pollen deposition (Figure 1) on flower stigma sprayed with our liquid pollen suspension compared to naturally pollinated stigmas. We have also noticed that pollen deposition was superior in treated than non-treated stigmas, irrespective of flower position and training systems of cultivars under investigation.

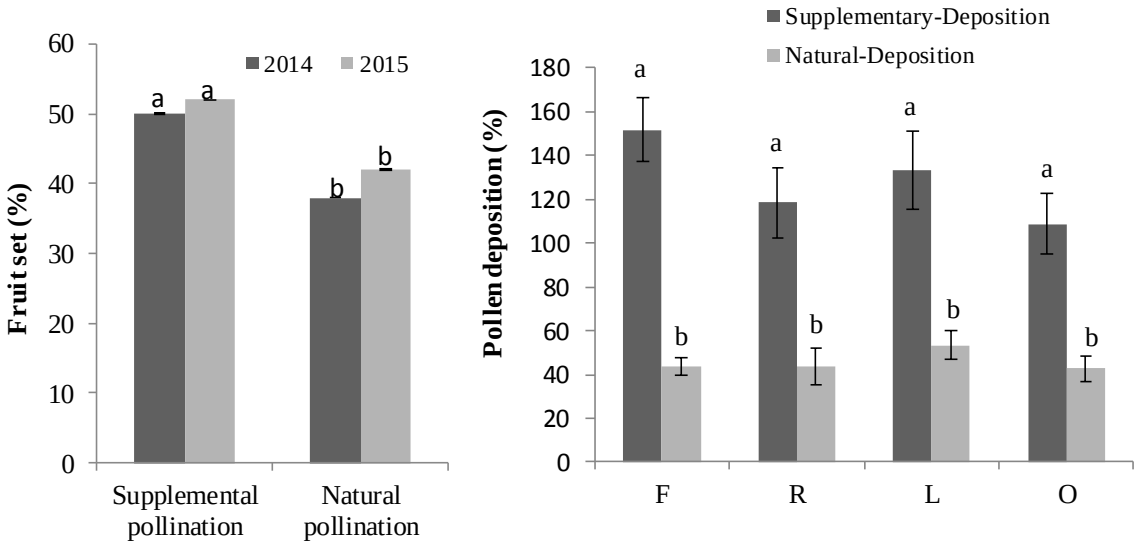

Figure 3. Effect of supplemental pollination (two applications) on fruit set in 'Tieton' sweet cherry (both year p-value: 0.041) (left) and pollen grain deposition on the stigma (year 2015; p-value: 0.000) (right). F=Facing sprayer, R and L= 90º from the direction of sprayer right and left, respectively, and O= 180º from the direction of sprayer.

In a Y-trellised 'Early Robin' orchard we evaluated pollination efficacy of two application timings and two rates of pollen. Supplemental pollination was made at about 50% and 100% full bloom. Each supplemental pollination treatment improved fruit set. The greatest response was in response to applying the high pollen rate (30g/acre) applied twice – this treatment increased fruit set by 65% compared to natural pollination. Including the growth regulator ReTain® in the spray did not improve fruit set (our lab tests revealed no toxic effect of ReTain® on pollen viability). Single applications of pollen were similarly effective – the timing of application and the rate of pollen made no difference. In each case, fruit set was increase by about 40%.

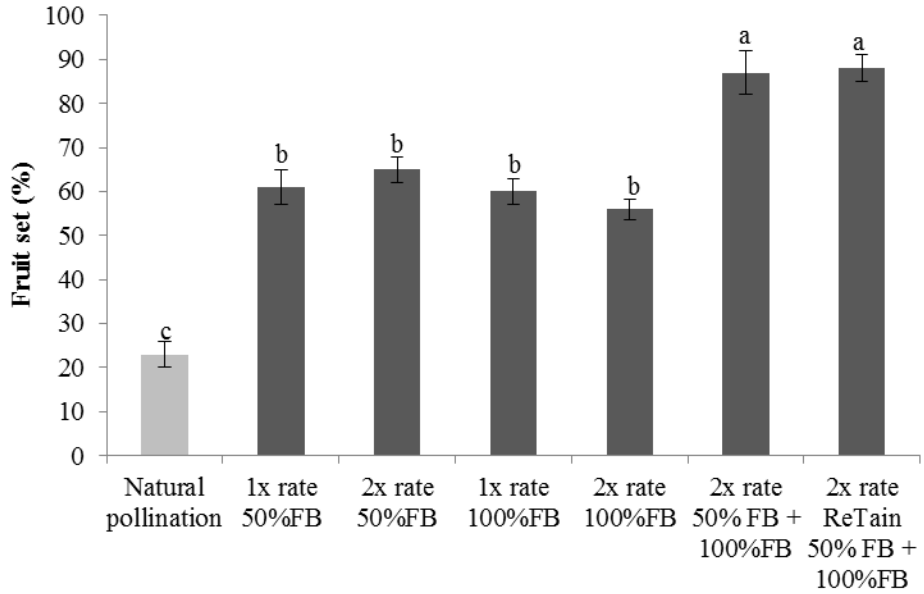

Figure 4. Fruit set in 'Early Robin' sweet cherry upon two supplemental pollen suspension applications (at 50% and 100% bloom) with single and double rate and natural pollination (non-supplemental) (p-value:0.000).

In a Regina orchard near Brewster a single application of 15g pollen was applied at about 75% full bloom. This supplemental pollination treatment improved fruit set by about 20% (Figure 5). The improvement in fruit set (and subsequently yield) from our supplemental pollination treatment was enough to convince the orchardist to keep the orchard (this block was set to be removed due to poor production).

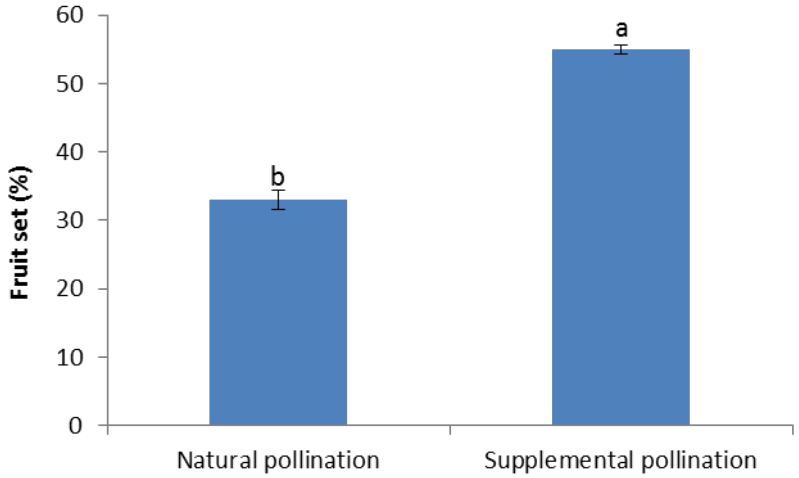

Figure 5. Fruit set (% of available flowers) for open-pollinated trees and those treated with supplemental pollination at ca. 75% full bloom and 15 g/acre of pollen.

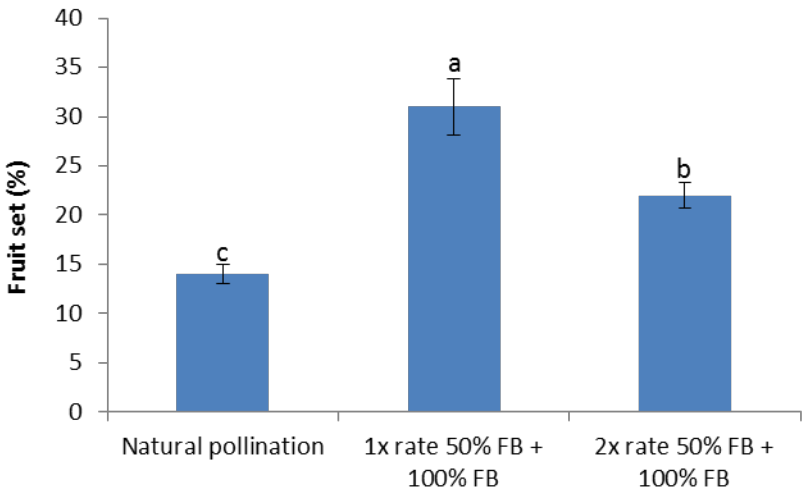

Figure 6. Fruit set (% of available flowers) in D'Anjou pear as affected by pollination treatment. 1x rate = 15 g/acre, 2x = 30 g/acre.

Supplemental pollination treatment to D'Anjou pear resulted in significant improvements in fruit set. Natural fruit set in this orchard was less than 15% of available flowers (Figure 6). Two applications of pollen at roughly 50% and 100% full bloom improved fruit set by 200% and 150% for single and double pollen rates, respectively. Interestingly, our application of a 1x rate (15 g/acre) was more effective than the double rate. This underscores the importance of further investigation into the pollen rate response. It will be prudent to utilize as little pollen as necessary to set the desired crop since pollen will be in short supply. We propose to evaluate fertilization rate response to pollen rate in sweet cherry and apple in a new proposal.

In a Jazz/M9 block we treated trees with two pollen rates (15 or 30 g/acre) at roughly 50% and 100% full bloom. We also treated rows where manual blossom thinning was complete, leaving only one flower per cluster in comparison to rows that were unthinned. Both unthinned and thinned fruiting limbs of Jazz apple receiving replacement pollination had fruit set despite the absence of any bee-mediated pollination (Figure 7). We documented no fruit set in untreated and bagged limbs whereas limbs treated with our pollination system exhibited fruit set rates between about 50% and 65% (unthinned limbs) or 40% and 55% (thinned limbs). The double rate induced 17% higher fruit set compared to single rate. Percent of open flower at pollen application was same in thinned and unthinned block. This is further evidence that we are able to fertilize flowers successfully with our pollination system. This may be particularly important when growers go to the expense of hand-thinning flowers. It will be critical to fertilize each remaining flower, and this may be accomplished with artificial pollination. Our vision of precision pollination systems in the absence of pollinators and pollenizers has the potential to revolutionize crop load management if we are able to successfully pollinate only a portion of the flowers.

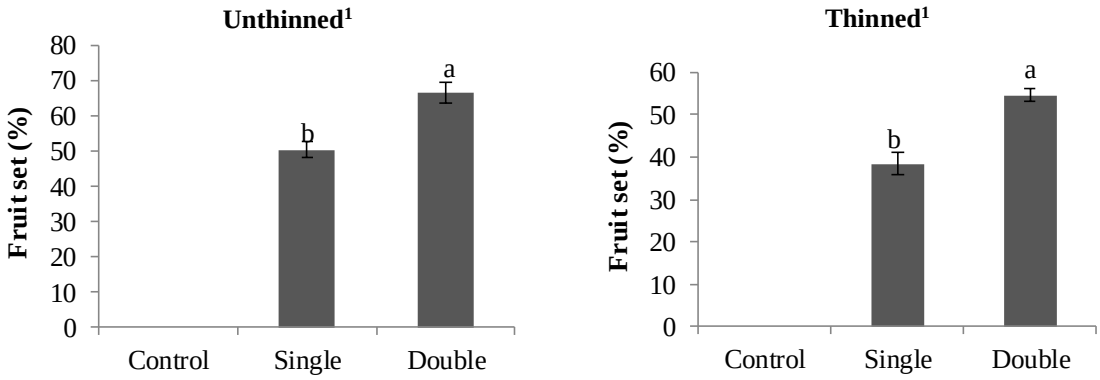

Figure 7. Fruit set in 'Jazz'/M®9 apple branches upon pollen suspension treatment with two pollen rates- single (15g/acre) and double (30g/acre), applied to unthinned (left) and thinned (right) flowering limbs at about 50% and 100% full bloom. All treated limbs including control were enclosed by bee boxes throughout flowering (p-value: 0.000). [1]Percent of open flower at pollen application was same in thinned and unthinned block.

EXECUTIVE SUMMARY

This research project has provided preliminary data and proof of concept data on the possibility to pollinate tree fruit crops artificially with pollen suspensions applied with commercially available electrostatic sprayers. Herein we report on successful pollination of apple, pear, and sweet cherry using a pollen suspension that is capable of maintaining or improving pollen viability for up to 1 hour. We have proven that one can take previously harvested pollen, incorporate it in a suspension, and spray it through commercially available sprayers to effect pollination in tree fruit crops. The implications are considerable – ensuring consistent fruiting in the face of declining bee populations, increasingly variable spring weather conditions, and the perennial challenges growers face with both pollinators and pollenizers. Further, the ability to artificially pollinate tree fruit holds the potential to revolutionize crop load management. Our vision for precision pollination systems that do not include pollenizers nor pollinators appears plausible. Clearly these results should be considered promising yet preliminary. More research is needed into the role of pollen rate, application timing, and suspension development to extend pollen viability. We have collaborated with interested growers across Washington, Firman Pollen Company, and On Target Spray Systems – all to facilitate the commercial adoption of our research. As a result of this research, there is significant interest in precision pollination systems. Every grower we worked with in 2015 is interested to collaborate further, seeing promising results – this underscores the importance of pollination/fertilization in commercial fruit production as well as the confidence our collaborators have in our research approach and vision.

FINAL PROJECT REPORT

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

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Cooperators: Dr. Shulu Zhang, Senior Research Scientist, Research & Development, Agdia, Inc.

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.

FINAL PROJECT REPORT

Project Title: Testing a portable, non-destructive measure of Ca, Mg and K in apple

PI: Lee Kalcsits
Organization: WSU Tree Fruit Research and Extension Center
Telephone: 509-663-8181
Email: Lee.kalcsits@wsu.edu
Address: 1100 Western Ave. N
City/State/Zip: Wenatchee, WA 98801

Cooperators: Stefano Musacchi, WSU TFREC, Glade Brosi, Stemilt Growers LLC, Rob Lynch, Redox Inc, Garrett Bishop, GS Long, Lav Khot, WSU BSYSE, Sindhuja Sankaran, WSU BSYSE

Percentage time per crop: Apple: 80% Pear: 15% Cherry: 5%

Other funding sources: None

Total Project Funding: $76,632

Budget History:

Item	**2014**	**2015**
Salaries[1]	$12,000	$12,480
Benefits[2]	$3,588	$3,732
Equipment[3]	$39,000	$0
Supplies[4]	$2,400	$2,400
Travel[5]	$508	$532
Total	$57,496	$19,136

OBJECTIVES

1. **Test if the concentration of calcium, potassium and magnesium measured non-destructively using handheld x-ray fluorescence (XRF) corresponds to traditional lab analysis of fruit.**
2. **Determine if there is a relationship between K+/Ca2+ ratios and the presence of bitter pit in apple**
3. **Explore applied applications of the instrument in the field and lab**

SIGNIFICANT FINDINGS

- The instrument arrived at the end of September, 2014 and was set up in the lab (Figure 1).
- Magnesium is too light of an element to detect with any precision using the handheld XRF. Calcium and potassium are measurable.
- There is a strong relationship between potassium and calcium measured using the XRF and calcium and potassium concentrations measured using traditional lab analysis (Figures 3-6)
- Presence of bitterpit is associated with high ratios of K/Ca measured using the XRF (Figure 7-9).
- There is a high variability in calcium and potassium within a tree and is likely related to the location on the tree (Figures 10 and 11).
- The effect of crop load on calcium and potassium ratios can be measured using the XRF (Figure 12)
- Calibrations need to be developed for different tissues (skin and flesh of different tree fruit) to be able to quantify potassium and calcium concentrations in ppm. Currently, values are semi-quantitative and allow for relative comparisons among treatments.

RESULTS & DISCUSSION

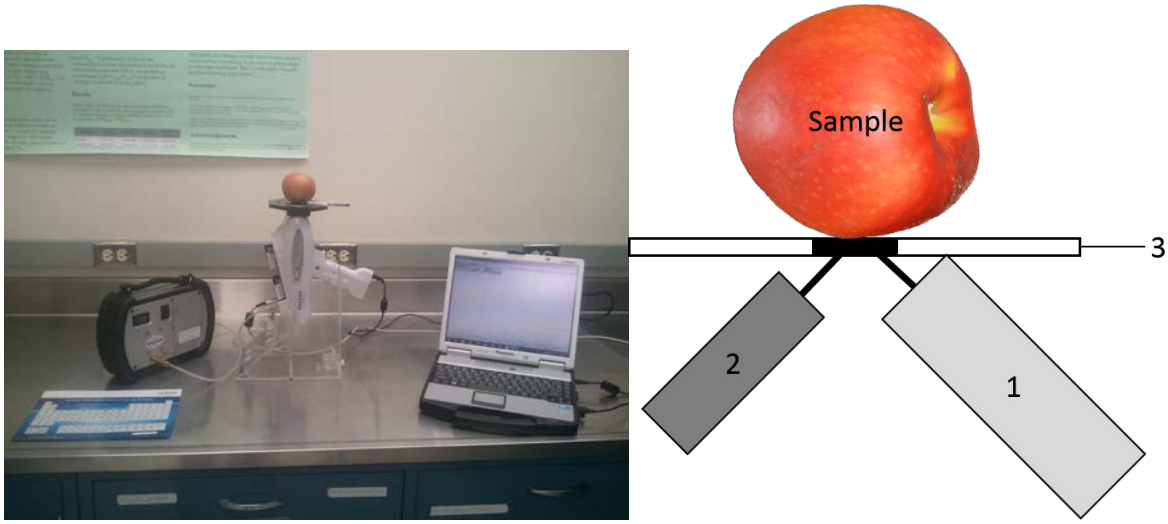

Figure 1. Non-destructive measurement of an apple fruit using a Bruker Tracer III SD portable x-ray fluorometer. 1. X-ray source (rhodium tube); 2. Detector (Si(Li) X-ray detector); 3. Platform
Semi-quantitative, non-destructive measurements using a hand-held PXRF are correlated with MP-AES analysis of Ca and K

Calcium concentrations in apple peel tissue ranged by a factor of 10 from approximately 150 mg kg-1 DW to 1500 mg kg-1 dw. Semi-quantitiative measurements ranged by equivalent orders of magnitude in the apple samples. Out of 54 samples, semi-quantitative measurements of calcium using the portable x-ray fluorometer measured on four spots around the equator of the fruit were significantly correlated with the calcium concentrations in the entire peel (See figure 2 for a description of sampling) surrounding the equator of the apple (Figure 3) ($r=0.941$, $P<0.001$). Similarly, when potassium concentrations were measured using MP-OES in apple tissue measured with the PXRF, there was a significant correlation (Figure 4) ($r=0.986$, $P<0.001$). For these 54 apples analyzed in this way, 15 showed symptoms of bitterpit, a calcium-related physiological disorder, on the calyx end of the fruit. Apples showing bitterpit had a higher potassium to calcium ratios than healthy apples (data not shown). High potassium:calcium ratios in fruit has been previously used as an indicator of bitterpit susceptibility (Ferguson and Watkins, 1983; Perring and Pearson, 1986). Individual fruit analyzed show high variations in the presence of potassium and calcium. Perring and Pearson (1986) demonstrated that calcium is lower on the calyx end of the fruit than the stem end. Furthermore, Xiaoyan and Chenglian, 2010 reported that calcium was greater on the sun-exposed portion of the apple and was again, higher on the stem-end than the calyx end of the fruit. In pears, calcium and potassium concentrations in individual fruit were strongly correlated with non-destructive PXRF measurements. The pearson correlation coefficients were 0.958 and 0.977 for PXRF measurements of calcium and potassium compared to digestion analysis using MP-AES (Figure 5 and 6).

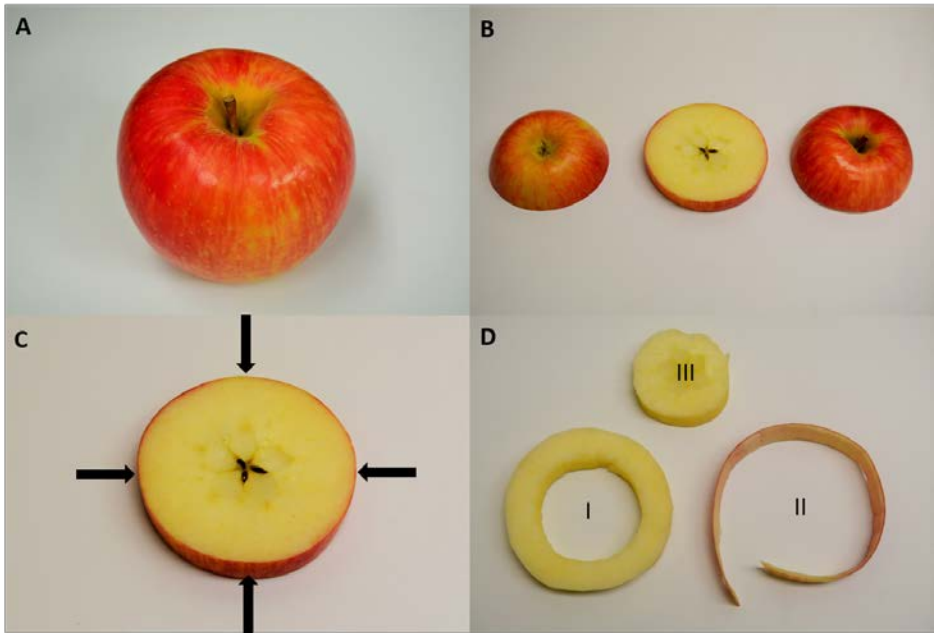

Figure 2. Sampling protocol of a 'Honeycrisp' apple for handheld x-ray fluorescence (XRF) and destructive lab sampling. A. Whole apple where 4 measurements were made with the handheld XRF along the equator of the fruit. B. To compare handheld XRF, an equatorial slice was removed from the apple. C. A representation of the sampling locations for handheld XRF analysis. D. Destructive sampling for elemental analysis of I) the outer cortex of the apple used for analysis of homogenized pellets using the handheld XRF and then digesting for lab elemental analysis, II) apple peel to compare with handheld XRF measurements made along the equatorial region of the fruit while it was whole, and III) the core of the apple which was discarded.

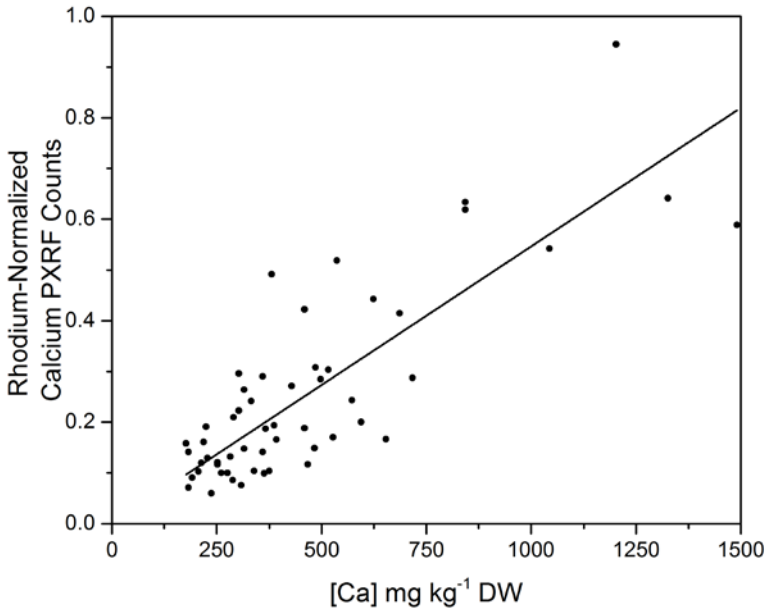

Figure 3. Correlation of non-destructive, semi-quantitative mean calcium content compared to corresponding MP-AES analysis of calcium in apple peel (N=54).

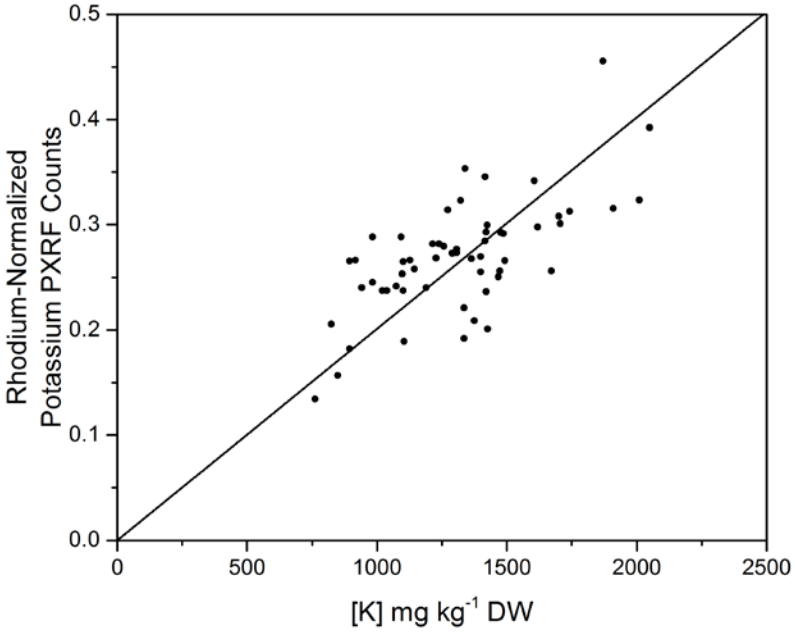

Figure 4. Correlation of non-destructive, semi-quantitative mean potassium content compared to corresponding MP-AES analysis of potassium in apple peel (N=54)

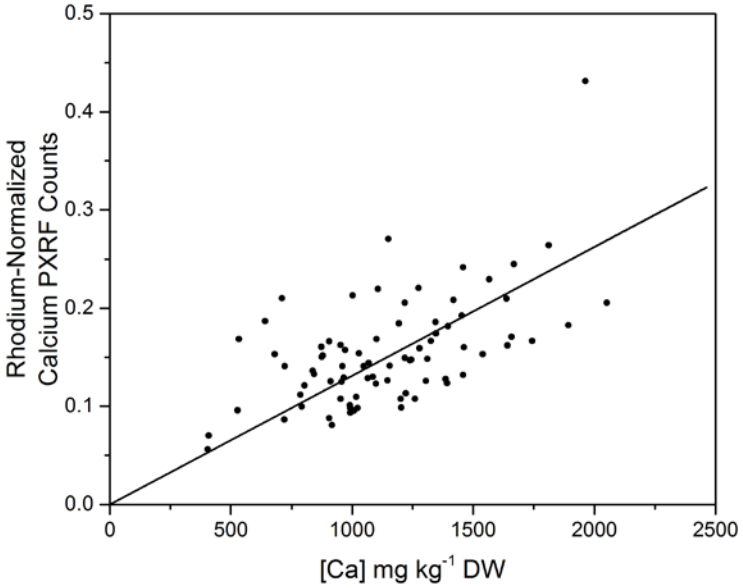

Figure 5. Correlation of non-destructive, semi-quantitative mean calcium content compared to corresponding MP-AES analysis of calcium in pear peel (N=80)

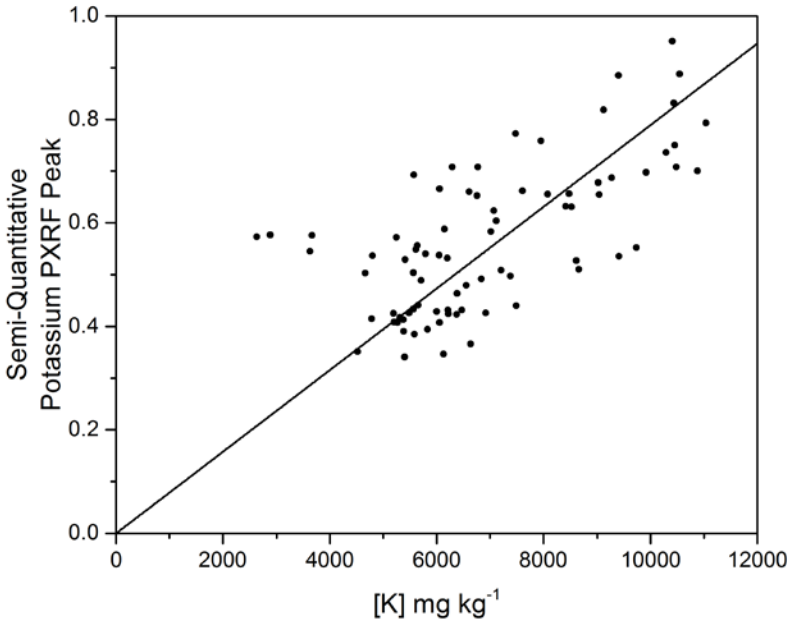

Figure 6. Correlation of non-destructive, semi-quantitative mean potassium content (n=4) compared to corresponding MP-AES analysis of potassium in pear peel (N=80)

Calibration development for quantitative analysis would represent an advancement in the capability to use handheld PXRF for non-destructive analysis. However, heterogeneity in cell type thickness and density among different cell types (i.e. epidermis versus cortex in an apple fruit) can lead to differences in x-ray penetration depth. Using epidermis and cortex as an example, if epidermal thickness was different among varieties of apple, the proportion of epidermis and cortex analyzed non-destructively using the handheld PXRF would also be different. Since the elemental concentrations are different between the epidermis and the cortex, differences in the proportions of each tissue analyzed using PXRF should produce different results. Therefore a calibration developed for one variety would likely be different for another. Calibration development will likely require species and possible even cultivar-specific calibrations for the instrument. Semi-quantitative analysis is still always a possibility but comparisons would have to be among biologically similar samples for valid relative comparisons.

Drying, grinding and pelleting of tissue is also suitable for measuring calcium and potassium concentration of apple flesh

As a more conventional comparison of apple and pear tissue among measurements and similar to Reidinger et al. (2012), homogenized tissue of apple from two different regions in Washington State were analyzed using the handheld XRF. Cortex samples had lower amounts of calcium and potassium than the peel for apple and were less variable than when analyzing the peel. In general, the region of the apple with the lowest concentrations of calcium is in the outer cortex (Wünsche and Ferguson 2005) and is similar to what was measured here using handheld XRF analysis of pelletized outer cortex tissue. Similar to the non-destructive measurements made on the peel, there was a significant relationship between the pelleted homogenized samples and MP-OES lab analysis (Figure 9 and 10). Pearson correlation coefficients were 0.787 and 0.89 for calcium and potassium, respectively. Reidinger et al. (2012) reported high precision in analyzing phosphorus and silicon using this approach. McLaren et al. (2012) also reported high correlations between homogenized samples and lab analysis of leaves from four different species of plants. However, homogenization still represents a destructive approach to sampling. In specific cases, such as calcium, which can be locally deficient within the plant, non-destructive analysis is more appropriate since variation between tissues and organs (in the case of non-destructive handheld XRF) may be more important than a pooled value from completely homogenized tissue. Handheld XRF measurements of in-tact fruit allows for repeated measures of the same spot over time. It also provides opportunities to address different biological questions related to nutrient uptake and mobility that are not possible using traditional lab analysis or destructive homogenization.

Calcium and potassium are unevenly distributed within an apple fruit

There is a high amount of variation in calcium and potassium in the peel of apple fruit that was measured using handheld PXRF. Figure 11 shows the potassium to calcium ratio on the surface of a Honeycrisp apple affected by bitter pit and a healthy bitter pit apple. The potassium to calcium ratios were almost twice as high in the bitter pit affected fruit and the variation between different regions of the fruit was also markedly different. The differences in these ratios were driven by both a higher presence of potassium and a lower presence of calcium (data not shown). Calcium and potassium were as much as an order of magnitude different in one location on the fruit compared to another. As concentration increases in fruit, the likelihood of large localized differences increased. The sun-exposed portion of the fruit often had larger concentrations of calcium than shaded portions of fruit (data not included). In the limited number of fruit analyzed here, there were no distinct gradients from the stem end to the calyx end of the fruit. However, Lewis and Martin (1973) reported a decrease in calcium along the longitudinal axis of the fruit. Initial observations on larger amounts of fruit indicate that this trend was also observed when using the PXRF as a semi-quantitative measure of calcium and potassium. Differences not only exist on the peel of an apple but also within the fruit. Ferguson and Watkins (1983) reported that flesh calcium concentrations

decrease with increasing distance from the core. This is related to the mode of distribution and proximity to xylem vessels in the fruit (de Freitas et al. 2012).

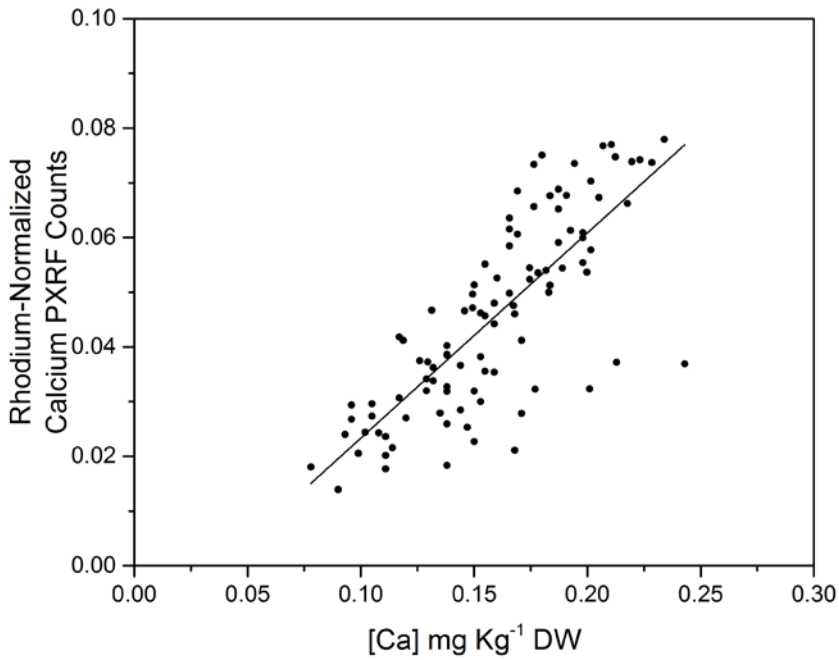

Figure 7. Correlation of non-destructive, semi-quantitative mean calcium content of pelletized, ground apple cortex tissue compared to corresponding MP-AES analysis of calcium. (N=104)

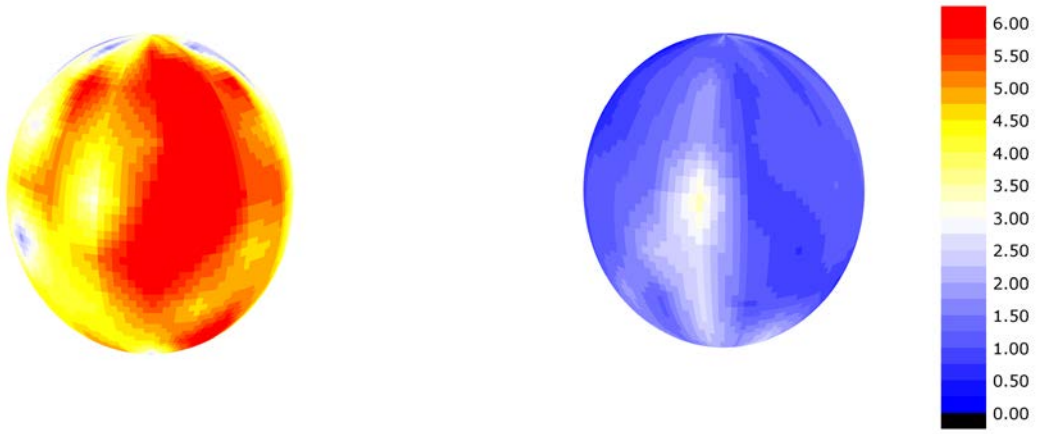

Figure 8. Smoothed semi-quantitative potassium:calcium ratio on the surface of a apple affected by bitter-pit (left) and a healthy apple (right) calculated as the rhodium-normalized PXRF counts for potassium divided by the rhodium-normalized PXRF counts for calcium.
Potassium/Calcium ratios are two times greater in fruit affected by bitter pit and is measurable using the handheld PXRF

In the fall of 2014, 20 apples that were either healthy or affected by bitterpit from three different culativars (‘Golden Delicious’, ‘Granny Smith’ and ‘Honeycrisp’ from two different locations) were analyzed using the handheld PXRF on four locations along the equator of the fruit. Calcium and

potassium was measured and then expressed as a potassium:calcium ratio. In all three cultivars, potassium:calcium ratios were more than two times higher in fruit affected by bitterpit. This was a function of both lower calcium concentrations and higher potassium concentrations. There appeared to be differences among cultivars in the threshold for bitterpit development. Granny Smith appeared to have a much higher threshold than Honeycrisp or Golden Delicious. This will need to be examined in more detail in the future.

Calcium and potassium:calcium ratios measured using the handheld PXRF varied depending on position within the canopy of 'Honeycrisp' apple on a M9 rootstock

As part of another WTFRC-funded project, 16 apples per tree were tagged in June in different regions of the tree. The distance from the ground and from the trunk was measured. At harvest, these fruit were picked and taken back to the lab for PXRF analysis to measure calcium and potassium concentrations. There was a high amount of variability in calcium and the potassium:calcium ratio in the canopy (Figures 9 and 10). In general, calcium was higher on the outer parts of the canopy in the upper half of the tree. Subsequently, the potassium:calcium ratios were lower in the upper and outer parts of the canopy. This implies that there may be differences in bitter pit susceptibility between fruit from the lower-interior parts of the tree and the upper-outer parts of the tree.

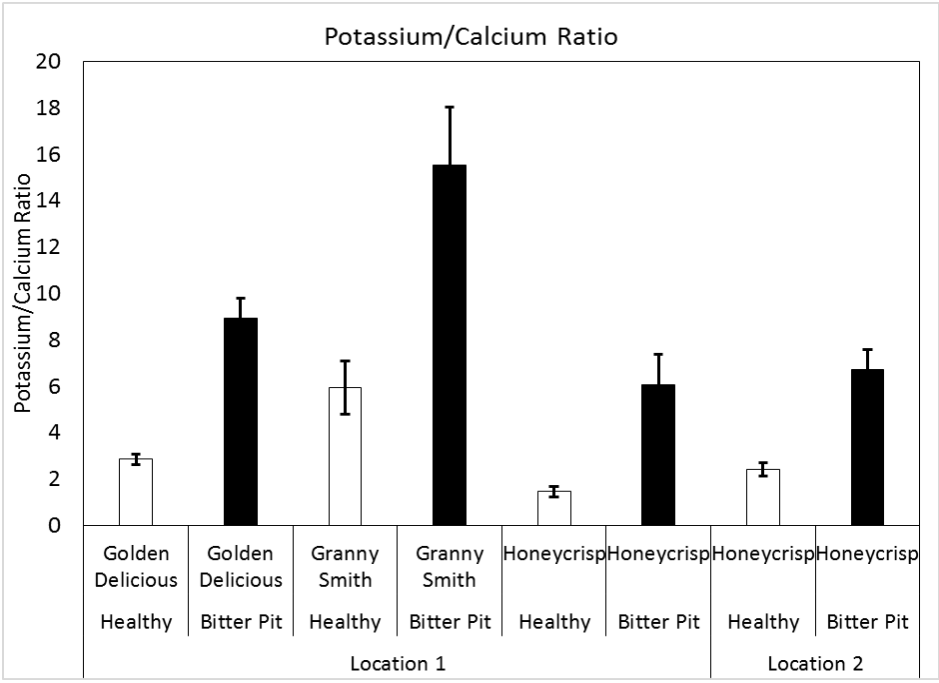

Figure 9. Mean potassium/calcium ratio of Golden Delicious, Granny Smith and Honeycrisp apples either showing bitterpit symptoms or not. Each mean is an average of 20 apples and error bars represent standard error.

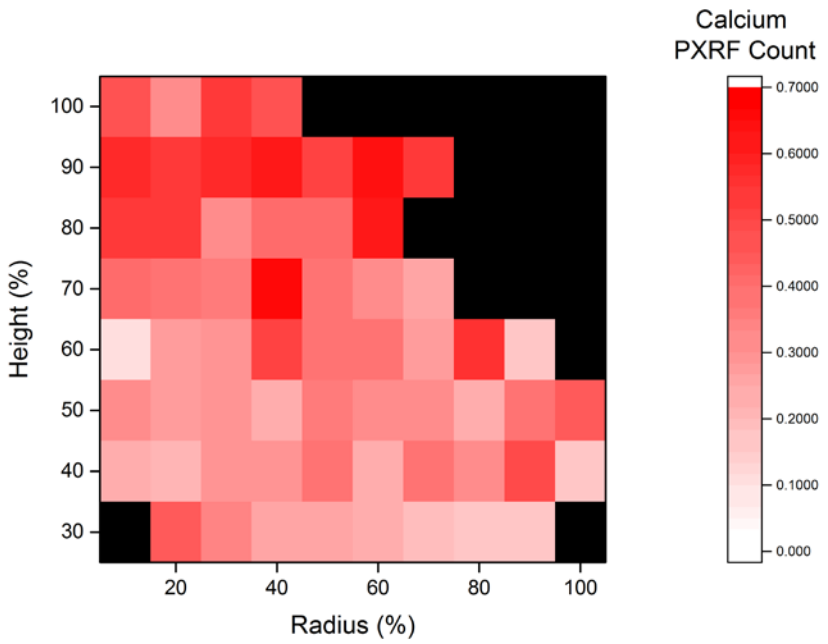

Figure 10. Calcium PXRF counts of apples measured using the PXRF at different heights (y-axis) and distances from the trunk (x-axis). Height and distance is expressed as a relative distance from the top of the tree or tip of the branch where 100% is equal to the top of the tree and tip of the longest branch. 16 Honeycrisp apples were measured from 9 trees at 9 different sites after being tagged in early June.

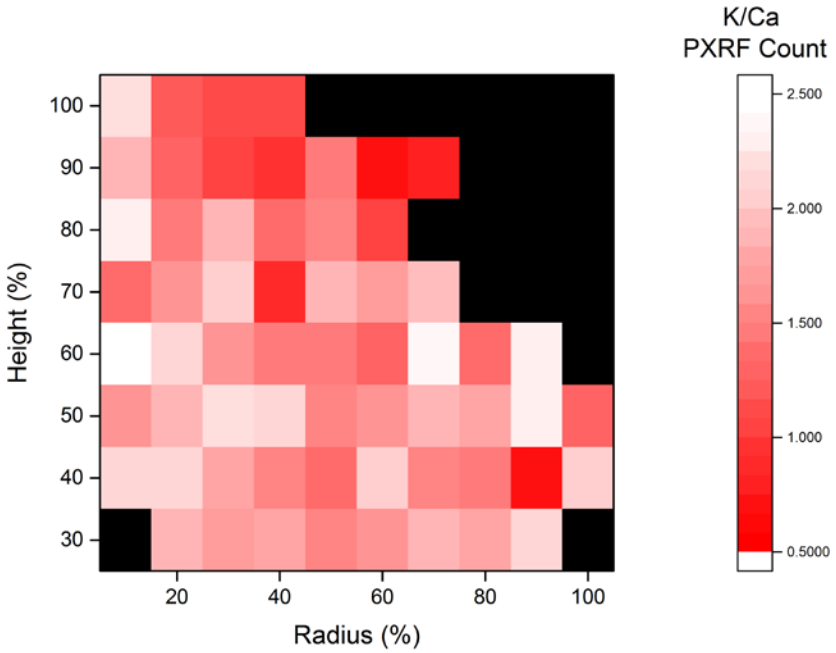

Figure 11. Potassium:calcium (K/Ca) ratios of apples measured using the PXRF at different heights (y-axis) and distances from the trunk (x-axis). Height and distance is expressed as a relative distance from the top of the tree or tip of the branch where 100% is equal to the top of the tree and tip of the longest branch. 16 Honeycrisp apples were measured from 9 trees at 9 different sites after being tagged in early June.

Crop load affects calcium and potassium concentrations in apples measured using the handheld PXRF.

Eight Honeycrisp apples were measured from 78 different trees from 9 different orchards across Washington State that were thinned to different target crop loads in early June. Calcium increased as crop load increased from less than 3 fruit per cm-2 to 5 to 7 fruit cm-2 then decreased as crop load increased further. However, potassium showed a linear decrease in concentration as crop load increased. As such, the potassium: calcium ratios were the highest at low crop loads then gradually decreased as crop load increased. The treatment with the greatest potassium to calcium ratio is also the treatment with the most optimum crop load for quality and storability (Wünsche and Ferguson, 2005; Serra et al. 2016 unpublished).

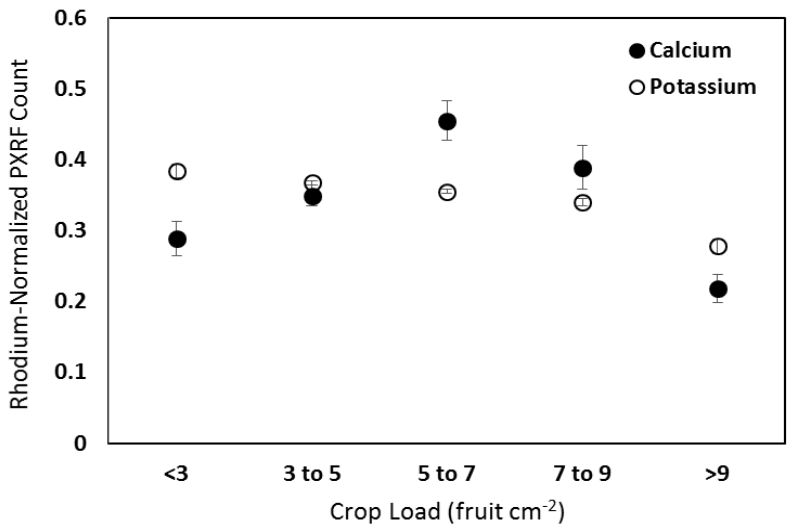

Figure 12. Rhodium-normalized portable x-ray fluorometer (PXRF) counts for calcium and potassium as a function of tree crop load measured on 78 trees across 9 different orchards.

CONCLUSIONS

Handheld PXRF has potential to be used as a semi-quantitative instrument that provides information to make relative comparisons on calcium and potassium concentrations amongst treatments with biologically similar samples. However, as a quantitative measure, there is still a need for the development of species-specific, or even cultivar-specific, calibrations. Even still, here we show the use of handheld PXRF for semi-quantitative, non-destructive measurements. Using this approach, repeated measures are possible on the same biological sample through time and it also permits greater replication, reduced sampling time and more complex data sets that are often not possible with traditional lab analysis techniques. Potential applications include improved precision in estimating changes in elemental concentration over time in plant tissue, analysis of the elemental distribution within an organ, within a tree or within a field when compared with traditional lab analysis. Handheld PXRF is a viable alternative to compliment traditional lab elemental analysis which can improve the understanding of calcium and potassium dynamics in plants and make *in-situ* non-destructive elemental measurements in the field.

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EXECUTIVE SUMMARY

Objectives

1. **Test if the concentration of calcium, potassium and magnesium measured non-destructively using handheld x-ray fluorescence (XRF) corresponds to traditional lab analysis of fruit.**
2. **Determine if there is a relationship between K+/Ca2+ ratios and the presence of bitter pit in apple**
3. **Explore applied applications of the instrument in the field and lab**

In the 16 months that the instrument has been active in the lab, we have been testing it against lab analysis in pears and apples. We have analyzed apples, pears and cherries. Non-destructively, the instrument measures calcium and potassium of a layer that is approximately 1 mm (~1/25 of an inch) thick. This is inclusive of the peel and a small portion of the flesh. Magnesium is too light to precisely measure using the PXRF in plant tissue. There is a strong relationship between potassium and calcium measured using the XRF and calcium and potassium concentrations measured using traditional lab analysis. This was evident in both non-destructive analysis at the surface of the fruit and destructive analysis using dried, ground and pelleted flesh tissue.

The presence of bitter pit is associated with high ratios of K/Ca measured using the XRF. In three apple cultivars, there were consistent trends where semi-quantitative K/Ca ratios measured using the PXRF were almost two times greater in fruit affected by bitter pit than healthy fruit. There is also a high degree of variability on the surface of the fruit for the K/Ca ratio. There is a high variability in calcium and potassium within a tree (Figures 10 and 11). The effect of crop load on calcium and potassium ratios can be measured using the XRF (Figure 12).

Handheld PXRF has potential to be used as a semi-quantitative instrument that provides information to make relative comparisons on calcium and potassium concentrations amongst treatments with biologically similar samples. In a productive day, a user can make approximately 200 measurements in the field or up to 500 measurements in the lab. With replication on individual fruit, a user could measure about 50 fruit in the field (4 measurements per fruit) and 120 fruit in the lab. Only in situations where changes in calcium need to be measured on the same fruit (i.e. calcium applications or change in calcium concentrations during fruit development) does measurements need to be made in the field.

As a quantitative measure, there is still a need for the development of species-specific, or even cultivar-specific, calibrations. Even still, here we show the use of handheld PXRF for semi-quantitative, non-destructive measurements. Using this approach, repeated measures are possible on the same biological sample through time and it also permits greater replication, reduced sampling time and more complex data sets that are often not possible with traditional lab analysis techniques. Potential applications include improved precision in estimating changes in elemental concentration over time in plant tissue, analysis of the elemental distribution within an organ, within a tree or within a field when compared with traditional lab analysis. Handheld PXRF is a viable alternative to compliment traditional lab elemental analysis which can improve the understanding of calcium and potassium dynamics in plants and make *in-situ* non-destructive elemental measurements in the field.

FINAL PROJECT REPORT

Project Title: Refinement/integration of vacuum-based end effector for fruit picking

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City/State/Zip: Menlo Park, CA 94025

Cooperators: N/A

Total Project Request: Year 1: $300,000 (ROM)

Percentage time per crop: Apple: 50% Pear: 30% Cherry: 10% Stone Fruit: 10%

Other funding sources

Agency Name: SRI International
Amt. awarded: $425,000
Notes: Internal Research and Development funds to support this effort

WTFRC Collaborative expenses: $15,000

Budget 1
Organization Name: SRI/Abundant **Contract Administrator: Dan Steere**
Telephone: 650 868 8467 **Email address: dan@abundantrobotics.com**

Item	2015
Salaries	$160,000
Benefits	
Wages	
Benefits	
Equipment	$120,000
Supplies	
Travel	$20,000
Miscellaneous	
Plot Fees	
Total	**$300,000**

ORIGINAL OBJECTIVES

The original objectives of the 2015 funded research were:

- **Refined the nozzle design**
    a. The first proposed refinement to the nozzle design was aimed at reducing stem pulls and spur pulls.
    b. The second refinement to the nozzle design focused on minimizing damage caused to the body of the apple by the nozzle.
- **Developed an ultra-compact decelerator**
    a. Because the flow rates needed to apply a pull force on an apple from a distance are high, the speed of the apple once it enters the nozzle is exceptionally high. We proposed to develop a mechanism to decelerate the apples without bruising them.
- **Integrated the End-Effector on a Commercial Robot Arm**
    a. We proposed to integrate our end-effector with a commercial robot arm to ensure that our end-effector design is compatible with a robot arm and to facilitate a demonstration of the manipulation subsystem.
- **Demonstrated Integrated Manipulation Solution**
    a. We proposed to take the integrated test platform into the fields during 2015 harvest to evaluate its performance and demonstrate the system to the commission and growers.

SIGNIFICANT FINDINGS

The significant findings from our 2015 activities were:

- **Refined the nozzle design**
    a. We commissioned a careful study of the effect of pulled stems on apple storage life.
    b. We moved the urethane-polycarbonate interface to a location that would not cut the apples.
- **Developed an ultra-compact decelerator**
    a. We identified memory foam as a material with preferred viscoelastic properties for decelerating apples without bruising them.
- **Integrated the End-Effector on a Commercial Robot Arm**
    a. We integrated our end-effector with a commercial robot arm and a 3D stereo sensor developed by Carnegie Melon University and showed that our end-effector design is compatible with a robot arm.
- **Demonstrated Integrated Manipulation Solution**
    a. We tested autonomous picking in 7 different orchards in 2015, demonstrated the system to growers and the commission, and gathered data on a subset of the autonomously picked apples.

RESULTS AND DISCUSSION

Refine the nozzle design

Stems

The first proposed refinement to the nozzle design was aimed at reducing stem pulls and spur pulls. Early in the performance phase, we discovered that a couple of small experiments had been conducted to determine the effect of stem pulls on apple decay in storage. The results suggested that there might be little to no difference in decay between apples with an intact stem and apples with pulled stems. Rather than invest resources in minimizing the machine-induced stem pulls, we instead took the preliminary step of commissioning a formal experiment across multiple varieties to determine the effect of pulled stems on fruit decay in storage.

A postharvest study is currently underway to evaluate the quality of stored apples that were harvested without stems. In the fall of 2015, five bins of Granny Smith, Jazz, and Pink Lady, and six bins of Fuji (three of first pick and three of second pick) were harvested with an approximate ratio of 50:50 stem-on to stem-off. Photos of the test groups are shown in Figure 1 below. The test bins were placed on trailers with 'normal' fruit, bound for storage facilities. Test bins were placed randomly on a trailer and multiple trailers were used for each variety. Test bins were drenched using Scholar fungicide. Drench cycle number was recorded. Currently, the apples are in CA storage and will be evaluated alongside 'normal' harvested bins of fruit. Each bin will be evaluated for stem bowl rot or other defects attributed to a stemless condition. Evaluation is expected to start in March after approximately six months of storage.

Figure 1 Photos of Fuji apples included in our experiment to determine the effect of pulled stems on the decay of apples in storage. Note apples with stems and apples without stems in the image on the right

Damage reduction

The second refinement to the nozzle design focused on minimizing damage caused to the body of the apple by the nozzle. The nozzle used during the 2014 field trials was a urethane extension on a polycarbonate tube. The transition between the urethane and the polycarbonate was abrupt and caused some cuts and indentations to the apple. We moved this transition to be just beneath the urethane orifice. This put the sharp edge transition in a place that the apple could not contact.

Develop an ultra-compact decelerator

We determined that the least expensive, most reliable, and most compact decelerator would be a monolithic piece of viscoelastic foam. The viscous property decelerates the apple without causing the apple to bounce back at a high rate of speed, and the elastic element enables the material to restore its shape before the next impact. It was important to find a material with the right balance between these two properties. We found that Memory foam had the right balance of viscous and elastic properties. Originally developed by NASA for improved seat cushions, this material does an excellent job of distributing contact forces across the surface of the apple, and quickly decelerating the apples without bruising them. Initial experiments were conducted by dropping apples from a 19 ft height.

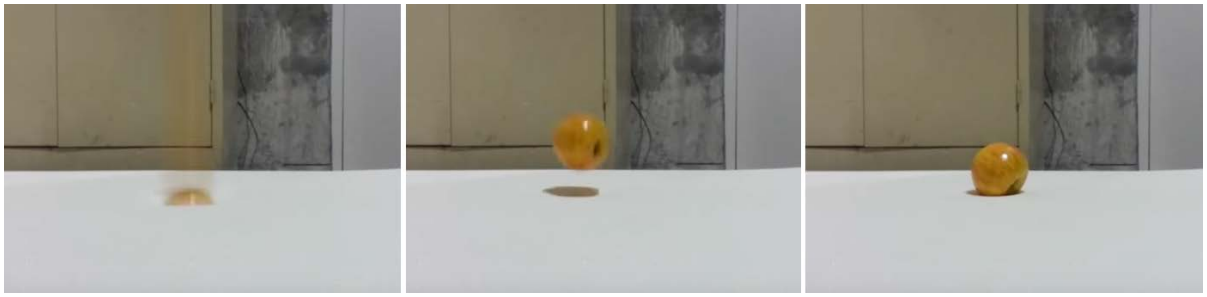

Figure 2 Drop testing of apples onto Memory foam. Left: Impact. Center: Rebound. Right: Resting Position.

Gravity accelerated the apples to a speed of 35 feet per second (approximately 24 miles per hour) upon impact and no bruising was observed across different apple varieties and sizes. Despite the drop height of 19 ft, the apples did not rebound more than approximately 3 inches (see Figure 2 above), but the memory foam would recover to near its original geometry within under a second. This is the optimum tradeoff between viscous and elastic behaviors. We then integrated the memory foam into our nozzle design and showed with lab testing that apples could be pulled into the vacuum nozzle, exit through check-valve doors and decelerate by colliding with the memory foam, all without bruising.

Integrate the End-Effector on a Commercial Robot Arm

We proposed to integrate our end-effector with a commercial robot arm to ensure that our end-effector design is compatible with a robot arm and to facilitate a demonstration of the manipulation subsystem. The integrated system is shown in Figure 3 below. The end-effector was mounted to the commercially available pick-and-place robot which was in turn mounted to the structure of a utility trailer. Also attached to the trailer structure was the vision system developed by Carnegie Melon University. We subcontracted a part of our grant funds to Carnegie Melon University to integrate their existing sensor with our picking platform in an effort to demonstrate fully autonomous apple picking.

Other significant hardware shown in the photo is the vacuum system, driven by the PTO of the tractor pulling the system. When an apple is not passing through the end-effector, the vacuum system draws 15 hp: 3 hp across the silencer, 6 horsepower across the pump, and 6 hp across the hoses and nozzle. The event of separating and apple from the tree and passing it through the end-effector has a duration of approximately 100 ms. At a picking rate of 1 per second, the average additional horsepower draw is approximately 2 hp.

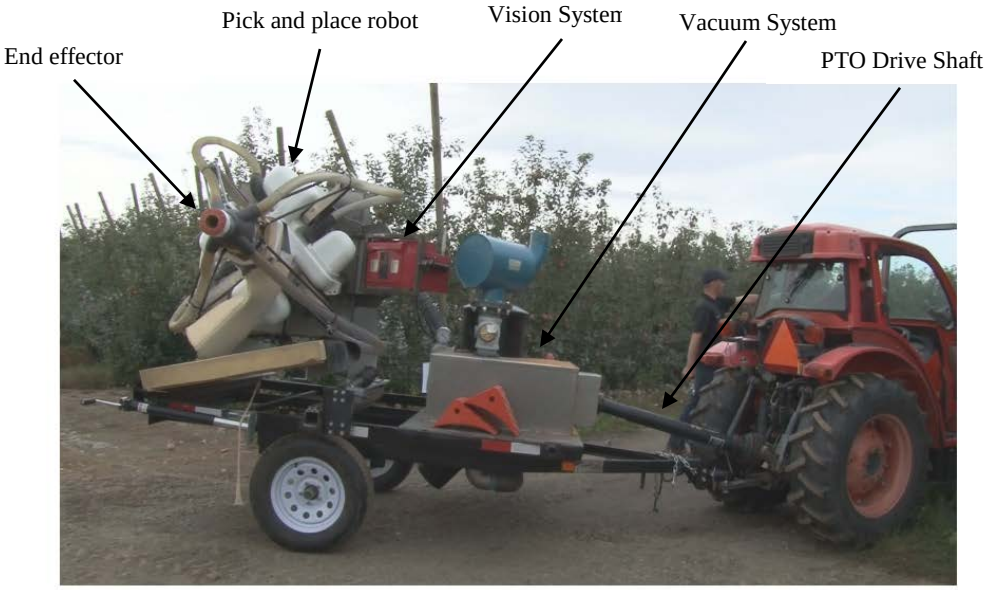

Figure 3 Photo of the demonstration and evaluation system

Demonstrate and Evaluate the Integrated Manipulation Solution

We brought the demonstration platform to Washington State for some initial testing September 8-11. Based upon those results, we returned the platform to SRI for some repairs and modifications. After the updates, the platform was sent again to Washington State for additional testing October 5-9. Our September testing was conducted at Yakima Valley Orchards' Glead ranch, Chiawana Orchards' Glead ranch and Doornink's Selah Ranch. Our October testing was conducted at Matson's Fruit Orchard in Wapato, McDougall and Sons' Gambler Ranch, Yakima Valley Orchards' Airport ranch and Flemming Farms in Quincy. We tested in multiple locations to enable us to evaluate our performance with a variety of different horticultural (pruning, training, etc) approaches. We also attempted to test in northern and southern geographies to enable as many growers to observe our evaluation and demonstration as possible. A map with the test locations is shown in Figure 4 below.

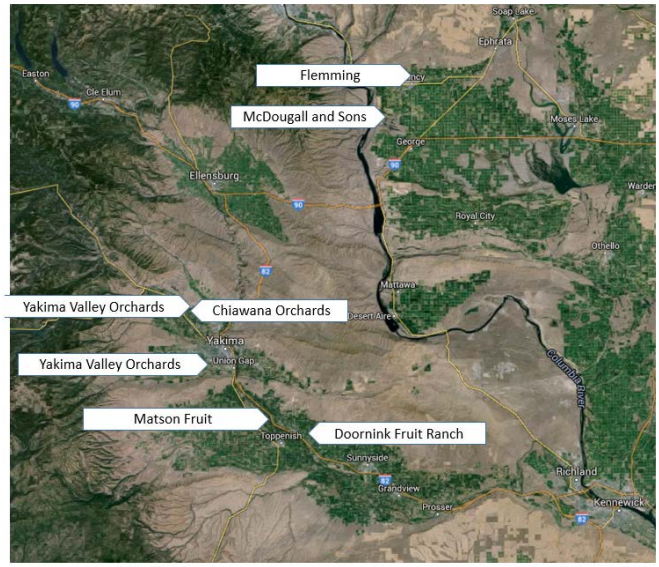

Figure 4 Map of Eastern Washington showing the 7 test locations

Our testing procedure was to park the system in an orchard row and press a key on the keyboard to initiate an autonomous picking sequence. Once initiated, the sensor would capture a photo of the trellis, recognize apples from leaves, determine the 3D coordinates of the recognized apples and then send those coordinates to the robot. The robot would then deploy the end effector to those coordinates and in the ideal case, the end effector would successfully pick the apples. A set of frames from one of our autonomous picking runs is shown in Figure 5 below. The image in the center shows the photo captured with the 3D sensor and includes circles drawn to indicate where the vision algorithm identified apples. The frames show the end effector moved to each of those locations. The system successfully picked all seven of the apples shown in the image in the center. This was one of hundreds of picking sequences we did throughout our field testing. We picked thousands of apples.

Figure 5 Frames of video capturing the pick events. The sensor output is shown in the center and the pick sequence is clockwise from the top left frame

As a part of this activity, the Washington Tree Fruit Research Commission collected 180 of the apples which we picked. The variety was Fuji. The results are shown in the table below.

SAMPLE SIZE		180	APPLES
BRUISING	DOWNGRADE	4	PERCENT
	CULL	2	PERCENT
PUNCTURE/CUT		12	PERCENT
TOTAL CULL		14	PERCENT

22% of the apples were found to have stem pulls, and none of the apples had spur pulls. The photos below show an example of the bruises and cuts in the experiment that were considered culls. An interesting finding was that the likely cause of nearly all of these culls was the apple rubbing across a branch during the picking event. If the canopy had long flexible branches, the branch would be sucked into the end-effector before the apple, and as the apple was subsequently sucked into the end effector it would slide along the branch causing either a bruise or a cut.

Figure 6 Left: Bruised Apple, Right: Cut Apple

CONCLUSIONS

Based upon our development and testing activities in 2015, we were able to demonstrate a vision system and end-effector solution that are capable of recognizing, localizing, and picking apples without bruising the apples. We demonstrated the ability of these systems to work together to support a picking rate of faster than 1 pick per second. We identified cuts as the principal cause of culls, and have a working hypothesis of the cause of these cuts. Specifically, it appears that the presence of long flexible branches near apples cause the end-effector to be prone to cutting the apples. We understand from growers that this issue can be reasonably addressed by pruning the long flexible branches from the tree.

EXECUTIVE SUMMARY

Refine the nozzle design

We took the preliminary step of commissioning a formal experiment across multiple varieties to determine the effect of pulled stems on fruit decay in storage. Currently, the apples are in CA storage and will be evaluated alongside 'normal' harvested bins of fruit. Each bin will be evaluated, beginning in March, for stem bowl rot or other defects attributed to a stemless condition. The second refinement to the nozzle design focused on minimizing damage caused to the body of the apple by the nozzle. The transition between the urethane and the polycarbonate was moved to be just beneath the urethane orifice. This put the sharp edge transition in a place that the apple could not contact.

Develop an ultra-compact decelerator

We determined that the least expensive, most reliable, and most compact decelerator would be a monolithic piece of viscoelastic foam. The viscous property decelerates the apple without causing the apple to bounce back at a high rate of speed, and the elastic element enables the material to restore its shape before the next impact We found that Memory foam had the right balance of viscous and elastic properties. We then integrated the memory foam into our nozzle design and showed with lab testing that apples could be pulled into the vacuum nozzle, exit through check-valve doors and decelerate by colliding with the memory foam, all without bruising.

Integrate the End-Effector on a Commercial Robot Arm

We integrated our end-effector with a commercial robot arm to ensure that our end-effector design is compatible with a robot arm and to facilitate a demonstration of the manipulation subsystem. We conducted some initial lab test to tune the integrated test platform.

Demonstrate and Evaluate the Integrated Manipulation Solution

We brought the demonstration platform to Washington State for demonstration and evaluation September 8-11 and October 5-9. The system successfully autonomously picked thousands of apples. As a part of this activity, the Washington Tree Fruit Research Commission collected 180 of the apples which we picked. The variety was Fuji. The results are shown in the table below.

SAMPLE SIZE		**180**	**APPLES**
BRUISING	**DOWNGRADE**	**4**	**PERCENT**
	CULL	**2**	**PERCENT**
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22% of the apples were found to have stem pulls, and none of the apples had spur pulls. An interesting finding was that the likely cause of nearly all of these culls was the apple rubbing across a branch during the picking event. If the canopy had long flexible branches, the branch would be sucked into the end-effector before the apple, and as the apple was subsequently sucked into the end effector it would slide along the branch causing either a bruise or a cut.

In conclusion, our development and testing activities in 2015 demonstrated a vision system and end-effector solution that are capable of recognizing, localizing, and picking apples without bruising the apples. We demonstrated the ability of these systems to work together to support a picking rate of faster than 1 pick per second. We identified cuts as the principal cause of culls, and have a working hypothesis of the cause of these cuts. Specifically, it appears that the presence of long flexible branches near apples cause the end-effector to be prone to cutting the apples. We understand from growers that this issue can be reasonably addressed by pruning the long flexible branches from the tree.

FINAL PROJECT REPORT

Project Title: Improving tools for early detection of brown marmorated stink bug

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Cooperators: Todd Murray, Skamania County Extension

Percentage time per crop: Apple: 65% Pear: 10% Cherry: 20% Stone Fruit: 5%

Other funding Sources

Agency Name: USDA SCRI awarded to Washington State University, Brunner
Amt. awarded: $67,693 over three years (2012-2014)
Notes: This SCRI grant provides funds to Washington State University to assess distribution of BMSB in WA and to evaluate pheromone technology. Some of the funding (≈ $40,000) from the WSU portion of the SCRI BMSB budget will be used to support the activities proposed here.

Agency Name: USDA SCRI awarded to Oregon State University, Shearer
Amt. awarded: $146,995 over three years (2012-2014)
Notes: This SCRI grant provides funds to Oregon State University to develop management strategies for BMSB across several crops. The funds requested here are not provided in the SCRI funding.

Agency Name: USDA SCRI awarded to USDA-ARS, Leskey
Amt. awarded: $559,072 over three years (2012-2014)

Total Project Funding: **Year 1**: $110,927 **Year 2:** $39,863

Budget 1 History WSU-TFREC

Item	2013	2014
Salaries	14,080	0
Benefits	5,562	0
Wages[1] (temporary labor)	11,520	7,200
Benefits [1]	1,118	698
Equipment	0	0
Supplies[2]	500	1,000
Travel[3]	5,032	1,680
Plot Fees	0	0
Miscellaneous	0	0
Total	37,812	10,578

Budget 2 History Oregon State Univ.

Item	2013	2014
Salaries	0	0
Benefits	0	0
Wages[1] (temporary labor)	11,250	7,830
Benefits (10%) [1]	1,125	4,385
Equipment	9,800	0
Supplies[2]	3,000	5,500
Travel[3]	1,000	1,000
Plot Fees	0	0
Miscellaneous	0	0
Total	26,175	18,715

Budget 3 History USDA-ARS-NAA

Item	2013	2014
Salaries	20,822	0
Benefits (35%)	7,288	0
Wages[1]	13,565	7,009
Benefits (8%) [1]	1,085	561
Equipment	0	0
Supplies[2]	4,180	2,000
Travel[3]	0	1,000
Plot Fees	0	0
Miscellaneous	0	0
Total	46,940	10,570

OBJECTIVES:

1. Compare a new BMSB light trap with standard pheromone-baited pyramid trap.
2. Determine the pheromone release rate that optimizes attraction to and capture of BMSB in traps.
3. Determine host-plant odors (kairomones) that enhance attraction/capture of BMSB in pheromone-baited and/or light traps.

SIGNIFICANT FINDINGS

Year one

1. BMSB were detected in sites considered to have low populations and in three sites considered to have medium populations. At three of the four positive catch sites, BMSB were captured in pheromone-baited traps, NOT in light traps.
2. The release rate of USDA#20 pheromone from a commercially produced lure was low and appeared to be depleted, or stopped releasing, after about one week.
3. The release rate of MDT (a synergist for USDA#20 pheromone) from a commercial were releasing after 21 days.
4. Two compounds known from English holly, which were also present in tree of heaven, hexyl formate (hex) and cis-3-hexen-1-ol (cis3), showed positive response by BMSB in Y-tube bioassays.
5. The kairomones hex and cis3 alone did not capture of BMSB late in 2013. However, when they were combined with MDT there was significant increase in BMSB capture.

Year two

1. BMSB were detected at three out of the nine sites with light or light+pheromone traps. The light+pheromone traps captured as many or more bugs than the light only trap. However, traps placed in the same general area as the light traps that were baited with a pheromone only consistently capture more BMSB (64%-93).
2. The release rate from a two-component commercial lure, Sterling Rescue, differed by component. While the release rates of each component (packet) differed they both lasted past the four weeks advertised by the company.
3. Eight potential chemical attractants evaluated for release rates in the laboratory showed short longevity, <20 days, to very extended longevity, >60 days.
4. Using a new olfactometer design one chemical, heptanol, showed promise as an attractant for BMSB. However, when heptanol and four other chemicals, including cis3 evaluated in 2013, all suppressed BMSB capture in traps when they were combined with USDA#20 and MDT.

Year three – no cost extension 2015

1. Three lures were evaluated using large black pyramid traps. The Rescue lure captured a large number of BMSB including nymphs and adults. An experimental two-component lure compared to the Rescue lure resulted in the former capturing more BMSB. Two experimental lures (Scentry) were tested with only one capturing of BMSB.
2. A 4-way olfactormeter was used to characterize the behavior of BMSB to selected kairomones. The main chemicals that showed promise were phenylethanol and heptanol.
3. Numerous potential host plants were monitored throughout the season for presence of BMSB. Maple was the host plant that was most commonly found to harbor BMSB followed by English holly, hawthone and hazelnut.
4. The egg parasitoid *Trissolcus japonicus* (Ashmead), native to Asia, was detected for the first time in the western US. This is an important natural enemy of BMSB and it has the potential to reduce populations of this pest in urban and native habitats.

RESULTS & DISCUSSION

OBJECTIVE 1

In 2013, light and pheromone traps were set up in nine locations, five in WA and four in OR. Two light and two pheromone-baited traps were established at each location (Fig 1), with light and pheromone-baited traps alternating. Two locations were in Hood River, OR and two were in the Willamette Valley (Aurora and Talent, OR). There were five locations in WA, one in Vancouver, one in Pringle and one in Underwood and two in the Yakima area near to where BMSB was detected in 2012, Wiley, WA and the Apple Tree Golf Course. Based on results in 2013 we changed our approach and paired a light+pheromone trap and pheromone only trap at each location.

Fig. 1. Arrangement of light and pheromone traps at one location.

Since our objective was to determine what traps might be best at detecting low levels of BMSB we focused on areas where we anticipated housed low to moderate populations. The anticipated BMSB population at each location based on previous detections was rated by the project participants and is shown in Table 1. The Vancouver location was at the WSU extension center on 78th street, which was known to have BMSB but not in high numbers. Traps were placed in an open field but near to habitats that would likely house BMSB. This location was not in the epicenter of BMSB in the area but it, along with the Aurora, OR site, was thought to be the most likely location to easily capture BMSB adults. Most other locations were considered to have low to very low BMSB populations. The low BMSB sites were known to have existing populations in the area but not necessarily at the location where traps were placed. The very low BMSB sites were where only one or two bugs had been previously detected.

In 2013, BMSB were captured at five of the nine sites (Table 1). The sites anticipated to have medium levels of BMSB captured the most bugs, with one site showing high captures. A few BMSB were captured at the Underwood site, but none were captured at the other five sites. The only site to capture BMSB in the light traps was Aurora and at this site 88% of bugs were captured in the pheromone-baited traps.

Table 1. The location, estimated population level and captures in pheromone and light traps in 2013.

Location	BMSB Population	Pheromone trap	Light trap
Aurora, OR	Medium-low	216	29
Vancouver, WA	Medium	18	0
Prindle, WA	Medium	13	0
Underwood, WA	Low	4	0
Hood River 1	Low	0	0
Hood River 2	Low	0	0
Talent, OR	Very Low	0	0
Yakima 1	Very Low	0	0
Yakima 2	Very Low	0	0

2013 results suggested that light traps might be drawing BMSB to the area of the traps at night but once in the area the bugs entered the pheromone-baited trap instead of the light trap. Based on these results, in 2014 we paired two traps at each locations, both with lights but one with pheromones lure (USDA#20 and MDT) (Fig. 2). There were also two other traps placed at a distance from the light and light+pheromone traps, one baited with pheromone lures and one with no lures.

Fig 2. Light trap with pheromone lure.

In 2014 there were nine locations, three in OR and six in WA (Table 2). In most locations no BMSB were captured. In Underwood where four BMSB were captured in 2013, none were captured in 2014. At sites were BMSB were captured the light+pheromone captured more than in the light-only trap, however, at these same locations more BMSB were captured in traps with a pheromone only. While these data indicate that adding pheromones to light traps enhances BMSB capture it does not appear to be an improvement over traps baited with only pheromones.

Table 2. BMSB captured in traps baited with light only, light+pheromone, pheromone only and a black trap with no pheromone, 2014.

Location	Light-NO pheromone	Light + pheromone	No lure	Pheromone
Pindle, WA	0	0		2
Underwood #1, WA	0	0	0	0
Underwood #2, WA	0	0	0	0
Yakima, WA	0	0	0	0
Talent, OR	0	0	0	0
Oak Creek, OR	0	2	0	0
Vancouver 2, WA	2	3		56
Hood River, OR	3	23	0	63
Vancouver 1, WA	5	172	0	310

Conclusions from two years of BMSB trapping indicates there is added value to adding pheromones to pyramid traps with lights, however, there was no evidence that a trap with lights and pheromones was better at detecting / capturing BMSB in locations where populations were low or moderate.

OBJECTIVE 2

In 2013, USDA #20 lures were obtained from the West Virginia ARS laboratory for use in field monitoring associated with the light trapping study (Obj. 1). In addition, ChemTica International provided *Plautia stali* (MDT) lures for use in the light trap study. Ten lures of each type were placed in a fume hood and weight loss was measured over time. The average weight loss in the #20 lures on day 4 was about 0.8 mg/day after which weight loss declined sharply and from day 7 through 25, 0.1 mg/day or less was released. Weight loss from the MDT lures averaged almost 3.5 mg/day between day 0 and day 3. Weight loss declined to about 1.5 mg/day between day 3 and 10 and declined only slightly to 1.3 mg/day between day 10 and day 21. These weight loss data agree with results of weight loss from a different MDT lure assessed in 2011. The #20 BMSB pheromone seemed to be released very quickly or it is not being released at all or at very low levels after only a few days bringing into question the longevity of these lures in the field.

In 2014 a commercial lure provided by the makers of the Rescue trap were evaluated for release rate based on lure weight loss. The lures came as two-component packets, a green and yellow. The company would not tell us what chemicals were in each packet, but one was thought to contain MDT and the other the USDA pheromone. Weight loss was assessed over 35 days. The average release rate of the yellow packet was 8.9 mg/day while the average release rate of the green packet was 3.0 mg/day. These lures were use in Rescue traps to monitor BMSB in Yakima and Wenatchee areas but no bugs were trapped in 2014.

While we have been able to evaluate the release rates of some commercial or USDA provided lures we have not been able to address the specific objective as stated. The main issue complicating achieving full completion of this objective has been access to technical USDA#20 BMSB pheromone in amounts that can be used to make polyethylene lures for release rate testing and then field evaluations. We have now received a small amount of technical USDA#20 BMSB pheromone and are evaluating release rates from polyethylene lures. We have been promised additional USDA#20 pheromone that can be made up into lures for trapping BMSB in 2015 at different locations throughout WA.

In 2015, two formulations of BMSB lures provided by Scentry were compared to unbaited traps. Lures were placed in black pyramid traps and checked weekly and replaced bi-weekly. Traps were located at various sites at the WSU Clark County Extension Center, Heritage Farm. Trapping started on June 8 and ended on July 6, 2015. Very few BMSB were captured in lure-baited traps. Six adult and 28 BMSB nymphs were captured in traps baited with formulation #1, none were captured in traps baited with formulation #2 and 4 BMSB nymphs were captured in the unbaited control traps. The Recue trap lure (Sterling two-component likely USDA+MDT) was evaluated at seven sites. Black pyramid traps were baited with Rescue lures on May 19 and trapping continued through August 4. An unbaited trap was used as a control. A total of 136 BMSB were captured accounting for 88% of all stink bugs collected. No stink bugs were collected in the unbaited traps. At the same location a short trial was initiated on August 10 and ended on August 24. The Rescue lure was compared to a two-component experimental lure. The experimental lure-baited traps captured a total of 69 BMSB (22 adults and 47 nymphs) while the Rescue lure-baited traps 25 BMSB (13 adults and 12 nymphs). This lure shows promise at least relative to the Rescue lure. Additional lure testing was conducted at other sites associated with the BSMB SCRI grant headed by Dr. Beers and is therefore not reported here.

OBJECTIVE 3

Olfactometer bioassays. In 2013, initial studies were conducted evaluating different host plant volatiles using a Y-tube bioassay. Two compounds, hexyl formate (hex) and cis-3-hexen-1-ol (cis3), showed greatest activity in Y-tube bioassays (Fig. 3). In 2014, additional studies were conducted using a four-tube olfactometer setup. Choices included heptanol, MDT, USDA#20 and a blank. Out of positive choices BMSB adult moved to the heptanol 57% of the time, compared to 21% to USDA#20 and 14% to MDT. The four-tube olfactometer appears to provide a good tool for continued assessment of chemicals or plants in the search for kairomones attractive to BMSB.

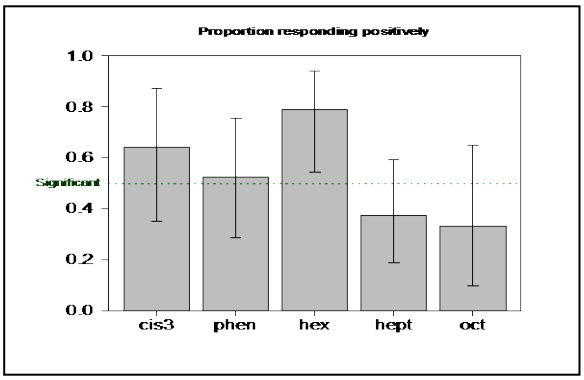

Fig. 3. Proportion response of BMSB in Y-tube bioassay to compounds derived from host plants.

Field evaluation of kairomones. At the end of the summer in 2013 some kairomone lures were deployed to the field to determine if they had any biological activity. Kairomone lures were combined with MDT and USDA#20 lures to examine potential synergy. Lures were placed into Rescue ® stink bug traps and hung in hazelnut trees in an abandoned orchard near Tualatin, OR. This orchard was located as a site of high BMSB activity in 2012. Traps were maintained at 50 ft spacing and were rotated weekly. Lures were changed every two weeks. Although the USDA#20+MDT (pheromone + synergist) captured the most BMSB, the kairomone lures cis3 (cis 3 hexen 1 ol) and hex (hexyl formate) enhanced trap capture when used in combination with MDT compared to MDT alone or un-baited controls (UBC). The compounds cis3 and hex were not attractive on their own (Fig. 4).

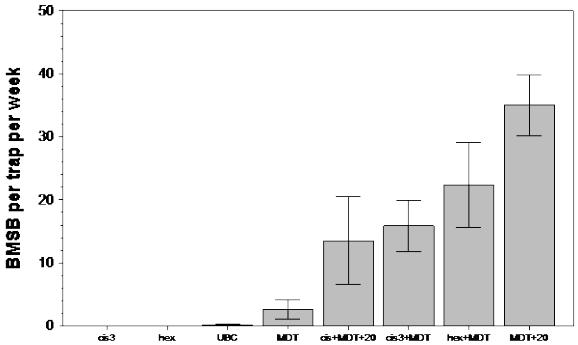

Fig. 4. Average capture of BMSB in traps baited with lures containing candidate plant volatile compounds, pheromones or combinations of volatiles and pheromones.

Table 3. Longevity of kairomones placed in polyethylene lures.

Chemical	Duration in days
hexyl formate	84
cis 3 hexen 1 ol	69
heptanol	82
octanol	95
phenylethanol	117
6 cis 3 hexen 1 ol	89
6 hexyl formate	13
hexyl formate	9

In 2014 a group of kairomones were placed in sealed polyethylene pouches and evaluated for their relative release rate based weight loss. Table 3 shows the average longevity of the different chemicals. Some like hexyl formate and 6-hexyl formate had fast release while most others had relative slow release. A sub-group of these kairomones were made up in lures and then combined with the BSMB pheromone (USDA#20) or the BMSB synergist (MDT) and evaluated in the field throughout the summer for capture of BMSB.

Fig. 5 showed the average BMSB capture in traps baited with different attractants. The USDA#20 plus MDT captured the most BMSB followed by traps baited with only MDT. The addition of any of the kairomone chemicals appeared to suppress captures of BMSB. The combination of cis3+MDT and hex+MDT that showed synergy of BMSB capture were not evaluated in 2014. In addition, heptanol (hept) that looked promising in olfactometry studies did not provide a synergy of BMSB capture when combined with USDA#20 and MDT.

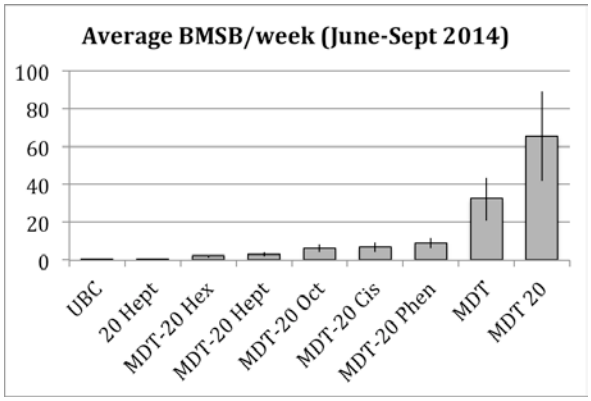

Fig. 5. Average capture of BMSB in traps baited with kairomone lures and USDA#20 and MDT.

In 2015 there was additional work conducted with a 4-way olfactormeter to characterize the behavior of BMSB to selected kairomones. The main chemicals that showed promise in these studies were phenylethanol and heptanol, especially the latter. Previous field tests did not show promise that these compounds would add to captures of BMSB, but these were conducted late in the season when BMSB might not have been optimally attractive to kairomones. Future field studies with these products in conjunction with the known BMSB aggregation pheromones are planned as part of the BMSB SCRI project, which runs through 2016.

Additional information

The individual, Josh Milnes, who was partially paid on this project's no-cost extension funding in 2015, was housed in Vancouver, WA. In addition to the trapping results reported here he worked closely with Dr. Nik Wiman (OSU) and Dr. E. Beers and Todd Murray (WSU), to assist with other BMSB research and extension activities. Mr. Milnes conducted monitoring of various host plants for presence of BMSB and other stink bug species. Results of this study showed that Maple was the host plant that was most commonly found to harbor BMSB followed by English holly, Hawthorne and hazelnut. BMSB was not common on apple, wild current, pear and bitter brush. These results contribute to a dataset of host information being collected by this and other projects on the west coast giving a better understanding of the ecology of BMSB.

In addition to host plant surveys Mr. Milnes conducted some surveys natural enemies that attack BMSB eggs. He placed sentinel egg masses on different hosts at several locations in the Vancouver area. Due to Mr. Milnes inquisitive nature and careful observation he identified three groups of predators visiting/feeding on BMSB egg masses; earwigs, sucking bugs (Hempitera) and lacewings, as well as a parasitic fly (Tachinidae). However, the most exciting result of his survey work was the discovery of a parasitic wasp attacking BMSB eggs. The parasite was collected and identified as *Trissolcus japonicus* (Ashmead). This is a known parasite of BMSB eggs in eastern Asia and is the main parasite introduced but under quarantine for possible release as a biological control agent of BSMB in the US. *T. japonicas* has been reported from the eastern US (2014) but this is the first detection of it in the western US. Mr. Milnes reared *T. japonicas* adults from three different BMSB egg masses, all in roughly the same area. Adult *T. japonicas* were submitted to USDA/ARS/Systematic Entomology Lab in Washington, D.C. to confirm identity of the species and other specimens were submitted for DNA analysis, which like the eastern *T. japonicas,* confirmed that the Washington population was different from colonies still in quarantine. This discovery is important because there is now no need to await permission from APHIS to approve the release of this parasite from quarantine and improves the possibility of enhanced biological control in urban and wild habitats.

EXECUTIVE SUMMARY

Detecting BMSB in an area where populations were low could help improve detection or estimates of this new pest in or near tree fruit crops. Studies in the eastern US pointed to the potential to increase capture of BMSB when including a light source in a pyramid trapping system. This premise was tested in 2013 in areas of OR and WA where BMSB were considered to be at low relative population densities. When pyramid traps with a light source were paired with traps baited with pheromones (USDA#20 plus MDT) the pheromone-baited traps capture more bugs and in a relative sense reflected the expected BMSB populations in a location. In 2014, pheromones were included with a light in one trap while the other trap remained with a light as the only attractant. In addition, other traps in the same area had pheromones as an attractant or were blank. This study showed that indeed when pheromone attractants were added to a trap with a light source more BMSB were captured that in the trap with only a light source. However, the traps with only the pheromone attractants captured more BSMB than the trap with both light and pheromone. The conclusion from this study is that adding a light source to a pyramid tapping system for BMSB did not enhance detection/captures of this pest over a two-year period and is, therefore, not considered a good approach for detecting low-level BMSB populations in an area.

Lures containing the USDA#20 BMSB pheromone and MDT, the pheromone of a closely related stink bug species, were evaluated for release rates by following weight loss. The lures containing USDA#20 pheromone had a low release rate and after about one week appeared to stop releasing pheromone. The lure containing the MDT lasted for at least 21 days. The release rate of a commercially produced two-component lure (Sterling Rescue) for BMSB showed good release of both chemicals over 35 days. Preliminary evaluation of BMSB pheromone USDA#10 using polyethylene packets suggested a high release rate but the amount of pheromone received was so small we were unable to repeat the study. Release rates from polyethylene packets of USDA#20 are being evaluated in the laboratory. These data will inform which lures will be used in 2015 to trap BMSB. One formulation of a Scentry experimental lure showed promise as a BMSB attractant as did a different experimental two-component lure that captured more BMSB than traps baited with the commercially available Rescue lure.

Chemicals identified from BMSB host plants were evaluated in an olfactometer. Initial results indicated two compounds might be attractive to BMSB, hexyl formate (hex) and cis-3-hexen-1-ol (cis3). When these chemicals were put into polyethylene lures and tested in the field in 2013 there was no attraction. However, when each chemical was included with MDT, capture of BMSB was enhanced over MDT alone. In 2014, heptanol was tested in a four-tube olfactometer and showed to be attractive to BMSB relative to MDT and USDA#20 pheromone. When heptanol and four other chemicals, including hex and cis3 evaluated in 2013, were evaluated in the field by combining them with MDT+USDA#20 all reduced capture of BMSB. It is possible that some of these chemicals could enhance capture of MDT but not USDA#20. A behavior study using a 4-way olfactomenter showed that phenylethanol and heptanol were kairomones attractive to BMSB adults.

Discovery of the egg parasitoid *Trissolcus japonicus* (Ashmead), near Vancouver, WA, was the first detection of this important natural enemy in the western US. While native to Asia, *T. japonicas* has been held in quarantine for several years pending approval by APHIS for release. This discovery demonstrated that *T. japonicas* was not an escape from quarantine colonies but represented an accidental introduction, similar to that of its host, BMSB.

CONTINUING PROJECT REPORT **YEAR:** 3 of 4 (No-cost extension)
WTFRC Project Number: TR-13-101

Project Title: Mechanical pruning in apple, pear and sweet cherry

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Cooperators: Olsen Brothers, Keith Oliver, McDougall & Sons, Brent Milne, Sara Serra (WSU-TFREC)

Total Project Request: **Year 1:** 77,536 **Year 2:** 47,959 **Year 3:** 50,210
Percentage time per crop: Apple: 60% Pear: 10% Cherry: 30% Stone Fruit: 0%
Other funding sources: None
WTFRC Collaborative expenses:

Item	2013	2014	2015
Wages	3,000	3,000	3,000
Travel	1,000	1,000	1,000
Total	4,000	4,000	4,000

Budget 1
Organization Name: WSU Contract Administrator: Carrie Johnston
Telephone: 509 335-4564 Email address: carriej@wsu.edu

Item	2013	2014	2015
Salaries[1]	26,295	26,307	27,359
Benefits[2]	2,183	2,271	3,135
Wages	7,214	7,503	7,803
Benefits	844	878	913
Equipment[3]	25,000		
Supplies	5,000	2,000	2,000
Travel	7,000	5,000	5,000
Miscellaneous			
Plot Fees			
Total	73,536	43,959	46,210

Footnotes: [1] Salary for student. [2]Medical costs include increase of 4% per year. [3] Purchase or lease of 1 sickle-bar pruner and 1 circular saw pruner and tractor attachments.

OBJECTIVES

The primary goal of this project is to establish best management practices for pruning PNW apple, pear and sweet cherry orchards mechanically.

(1) Understand equipment and orchard requirements for successful operation of both a circular saw and sickle bar mechanical pruning system
(2) Compare pruning technologies for their effects on fruit yield and quality
(3) Conduct a preliminary economic assessment of mechanical pruning systems
(4) Train an M.S. student in horticulture with extensive exposure to tree fruit horticulture, agricultural engineering and applied economics
(5) Conduct demonstration trials and associated outreach activities

APPLE: Fuji – September Wonder/Nic29, Spindle. Planted 2009

SIGNIFICANT FINDINGS

- Machine dormant pruning is faster than hand pruning.
- The least amount of wood was removed in the dormant hand/summer mechanical (T3) plots, with an average of 0.42 lb wood/tree and 0.20 lb wood removed/tree, respectively. The greatest amount of wood was removed from dormant hand (T1) and dormant mechanical (T2).
- The number fruit/tree from dormant mechanical (T2) was 31% higher than number fruit/tree from dormant hand /summer mechanical (T3), with an average of 70.2 apples/tree. (Tab. 1)
- Apples from dormant hand treatment (T1) had 10% lower °Brix than those from dormant hand/summer mechanical (T3), with an average of 12.4 °Brix.
- Apples from dormant mechanical/summer mechanical (T4) had 46% more sunburn than the apples from dormant mechanical (T2), with an average of 7.6 apples with some degree of sunburn per tree.

METHODS

Trial block: Fuji/Nic 29 block trained to Slender Spindle at McDougalls & Sons (Mattawa) as a Complete Randomized Block Design. Pruning treatments are coded as follows:

Tmt code	2014		2015	
	Dormant pruning	Summer pruning	Dormant pruning	Summer pruning
T1	Hand	-	Hand (hedging and topping)	-
T2	Mechanical	-	Mechanical + hand cleanup (hedging and topping)	-
T3	Hand	Mech. 12-15 leaves	Hand cleanup (hedging and topping)	Mech. 12-15 leaves
T4	Mechanical	Mech. 12-15 leaves	Hand cleanup (hedging and topping)	Mech. 12-15 leaves

Dormant pruning performed on 3/10/2015 with the LaGasse hedger. All rows were topped manually with use of a platform. Data collection included time to prune each plot and weight of wood pruned per plot. Total time/tree includes hedging and topping (s/tree/person). Summer pruning at 12 leaves was done on 5/29/2015. Data collection included time to prune and weight of wood. Wood that was pruned was taken to the lab to record fresh and dry weights.

RESULTS AND DISCUSSION

Times recorded for mechanical pruning were 68%, 62% and 40% faster than times recorded for hand pruning. The average speed for hand pruning was 34.6 s/tree while the average time for mechanical was 12.3 s/tree and 20.6 s/tree. The average time to prune a tree by hand at dormant timing was 34.6 s/tree. This was 40% slower than dormant mechanical (T2) and 60%, and 54% slower than dormant hand/summer mechanical (T3), and dormant mechanical/summer mechanical (T4), respectively. T3 and T4 were the fastest pruning treatments, and did not differ among themselves

Yield

- Number fruit/tree from dormant mechanical (T2) was 31% higher than dormant hand/summer mechanical (T3).
- The lowest yield (lb/tree) was observed in the plots from dormant hand/summer mechanical (T3)
- Yield (lb/tree) from T3 was 29% and 25% lower than yield from T2 and T1, respectively.
- Yield and yield efficiency from T1 and T2 did not differ from each other.
- Yield efficiency from T1 did not differ from T3, as it did in lb/tree.
- Yield efficiency from T3 was 25% lower than yield efficiency in T2.
- The highest lb/fruit was observed from T1, which was 15% higher than T2, T3 and T4.

Tab. 1 Effect of four pruning treatments on averaged harvest metrics for 'Fuji'

TMT	TMT	Number fruit/tree	Kg/Tree	lb/tree	Yield Efficiency (kg/cm^2)	Yield Efficiency (lb/cm^2)	Total kg/tree	Total lb/tree
T1	Dormant Hand	**54.47**	14.09	**31.06**	1.14	**2.51**	0.26	**.57**
T2	Dormant Mechanical	**70.27**	15.01	**33.09**	1.27	**2.80**	0.22	**.48**
T3	Dormant Hand + Summer Mechanical	**48.47**	10.63	**23.43**	0.95	**2.09**	0.22	**.48**
T4	Dormant mechanical + Summer Mechanical	**58.67**	12.90	**28.44**	1.25	**2.75**	0.22	**.48**

APPLE: Cripps Pink - Masilin /M9-337, Spindle. Planted 2012

SIGNIFICANT FINDINGS

- Trees mechanically pruned in summer and winter + summer showed the same pruning weight.
- Trees that were mechanically pruned in summer only had higher yields than trees pruned in winter only by hand or machine and those that were mechanically pruned in winter and summer.
- At harvest, the number of fruit per tree, net weight of fruit, and yield efficiency was significantly lower in the control than the other treatments. However, the weight of the fruit in the control was significantly higher than other treatments

METHODS

Four treatments: dormant mechanical pruning, summer mechanical pruning, dormant and summer mechanical pruning, and control (hand dormant pruning). All treatments trees were thinned to remove damaged and undersized fruit. Fruit was harvested on 9/28 for all the treatments; fruit from 9 representative trees per treatment were sized to assess the fruit size distribution at harvest. Some fruit in all treatments dropped a few days prior to harvest and fruit from 3 trees per winter and control treatments were sized. Weight, background and over-color, DA index, starch, firmness, and SSC (brix) were recorded.

RESULTS AND DISCUSSION

Hand pruning in the winter (control) removed more material than mechanical and hand pruning (Fig 1). Trees mechanically pruned in summer and winter + summer showed the same pruning weight, however significantly more fruits were removed when the trees were mechanically pruned in summer alone than in both seasons (Fig 2). Thinning in June, both total weight of fruit removed and weight of individual fruits per control tree were significantly heavier than other pruning treatments.

At harvest, the number of fruit per tree, net weight of fruit, and yield efficiency was significantly lower in the control than the other treatments. However, the weight of the fruit in the control was significantly higher than other treatments (Tab 1).

Tab. 1 - The effect of four pruning treatments on averaged harvest metrics for Cripps Pink

Treatment	**Count of fruit/tree**	Net weight fruit kg/tree	**Net weight fruit lb/tree**	**Fruit weight g**	**TCSA cm^2**	Yield eff kg/cm^2	**Yield eff lb/cm^2**	Metric ton/A	**US ton/A**
winter pruning	111.67 a	22.04 ab	48.59 ab	197.62 b	15.09	1.48 a	3.26	32.00 ab	35.27 ab
summer pruning	115.89 a	23.64 a	52.11 a	205.64 b	15.61	1.40 a	3.09	34.32 a	37.83 a
mechanical winter+summer	97.44 a	19.28 b	42.51 b	199.39 b	14.45	1.36 a	2.99	28.00 b	30.87 b
Control hand only	62.33 b	14.10 c	31.09 c	226.31 a	14.32	1.01 b	2.22	20.47 c	22.57 c
Significance	***	***	***	***	NS	**		***	***

p<0.05, *; p<0.01, **; p<0.001, *; ns, not significant for Type III sums of squares model significance.**
Arithmetic means are presented; post hoc tests were done with LSMEANS option and the Bonferonni adjustment provided letter.

PEAR: Bartlett/ OHF87, Spindle. Planted 2012

SIGNIFICANT FINDINGS

- Hand pruning (control) Bartlett trees resulted in the removal of less total wood per tree than mechanical + hand pruning. (0.88 lb/tree and 1.34 lb/tree respectively)
- No statistical difference in vigor, as determined by trunk cross sectional area (TCSA), was found between the two pruning treatments.
- Hand pruning resulted in a greater yield than mechanical + hand (8.8 lb fruit/tree and 6.6 lb fruit/tree), but this difference was not found to be statistically significant.
- Mechanical + hand pruning produced a greater proportion of large fruit (>70mm diameter) than the control treatment (11.9% and 5.7%, respectively). (Fig. 1)
- Colorimetric readings indicated significant differences between fruit harvested from each pruning treatment; fruit from hand pruned trees were lighter in color, less green, and more yellow than fruit harvested from mechanical + hand pruned trees.
- No statistical differences between pruning treatments were found in fresh weight, dry weight, and percent over color, or SSC (Brix).

METHODS

The experiment was designed to test the effect of mechanized pruning on fruit quality and yield. The trees were separated into two treatments: 1) hand pruning (4 plots), and 2) mechanical pruning (4 plots) with additional hand pruning (mechanical+hand).

Fruit was harvested August 7, 2015. Fruit were sized by diameter and separated into the following classes: <55mm, 55-60mm, 60-65mm, 65-70mm, and >70mm. Quality parameters investigated include fresh weight and weight loss after storage, I_{AD} values before and after storage, flesh color (L*a*b*, Minolta), percent red blushed overcolor, firmness (FTA, measured in kg), SSC (Brix).

RESULTS AND DISCUSSION

Pruning and vigor

In 2015, we found hand pruning Bartlett trees resulted in the removal of less total wood per tree than mechanical+hand pruning (0.88 lb/tree and 1.34 lb/tree respectively), but the difference was not statistically significant. No statistical difference in vigor, as determined by trunk cross sectional area (TCSA), was found between the two pruning treatments (18.9 cm^2 hand and 19.2 cm^2 mechanical+hand).

Yield and quality

Hand pruning resulted in a greater yield than mechanical+hand (8.8 lb fruit/tree and 6.6 lb fruit/tree), but this difference was not found to be statistically significant, while yield efficiency (yield per tree/average TCSA), differed significantly between the two pruning groups; hand pruning resulted in greater yield efficiency than mechanical+hand pruning (data not shown). In the comparison of secondary fruit harvested from each pruning treatment, the mechanical+hand treatment averaged 33% fewer secondary fruit than the hand pruning treatment, but the difference was not statistically significant.

Mechanical+hand pruning produced a greater proportion of large fruit (>70mm diameter) than the control treatment (11.9% and 5.7%, respectively) and lower incidence of fruit categorized in the 55mm diameter group (20.2% compared to 25.5, Fig. 1).

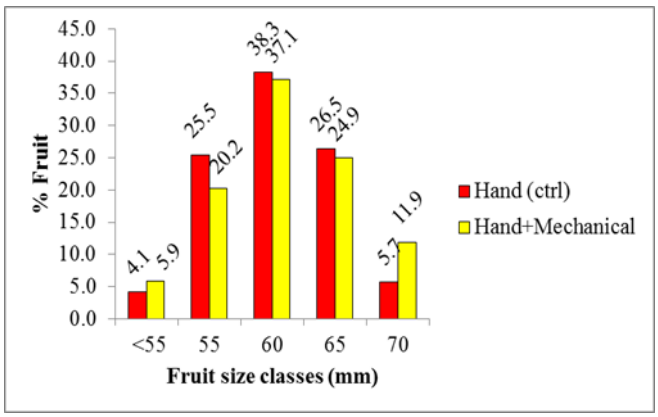


Figure 1: The effect of two pruning treatments on Bartlett fruit size distribution in 2015.

Fruit quality analysis revealed significant differences in measured I_{AD} values immediately after harvest, indicating fruit harvested from hand pruned trees (I_{AD} = 1.96) were more ripe than fruit harvested from mechanical+hand pruned trees (I_{AD} = 1.99). Differences in rate of I_{AD} change during ripening were statistically significant, suggesting fruit from hand pruned trees ripened quicker than fruit from mechanical+hand (data not shown). Comparison of firmness showed significant differences between fruit harvested from both pruning treatments (data not shown). Colorimetric readings indicated significant differences between fruit harvested from each pruning treatment; fruit from hand pruned trees were lighter in color, less green, and more yellow than fruit harvested from mechanical+hand pruned trees (Table 2). No statistical differences between pruning treatments were found in fresh weight, dry weight, percent over color, or SSC (Brix) (data not shown).

CHERRY*: Tieton / Gisela 5, UFO. Planted 2008*

SIGNIFICANT FINDINGS

<u>Sweet Cherry (Mechanical pruning vs/+ hand pruning)</u>

- Mechanical pruning was 29 times faster than hand pruning alone at a tractor speed of 1.3 m/h, and 17 times faster than the combination of both approaches.
- Hand pruning removed 2.6 times more wood/tree than mechanical pruning, and 1.2 times more wood/tree than the combination of mechanical and hand pruning.
- Mechanical pruning was 11 times more efficient than hand pruning, the combination of mechanical and hand pruning was 1.4 times more efficient than hand pruning alone.

- Mechanical pruning had no effect on yield or yield efficiency. There was no difference between treatments regarding yield efficiency and kg fruit/tree.

Sweet Cherry (Preharvest and postharvest topping)

- Total current season shoot length per upright was significantly greater from uprights pruned during the dormant season and full bloom compared to those topped 2 and 3 months after full bloom. Pruning performed 2 and 3 months after full bloom removed 73% more wood than full bloom pruning and 48% more than DP.
- Timing of pruning affected yield per upright but not yield efficiency in 2015.
- Yield was 35% higher on unpruned uprights compared to uprights pruned in the dormant season, and no different from the other treatments.
- Fruit quality traits were not affected by timing of topping, except for soluble solids content.

METHODS

Mechanical pruning vs/+ hand pruning: The experiment was designed to assess the effect of pruning over two years. The three treatments are as follows (2014/2015): 1) hand pruning/hand pruning, 2) mechanical pruning/mechanical pruning, and 3) mechanical pruning/mechanical pruning + hand pruning follow-up. Each treatment has 5 replications of 20-tree blocks (i.e., 100 trees/treatment).

Mechanical pruning was performed with the sickle bar (Gillison's Center Mount Topper and Hedger) with 3 passes of the machine (hedging on each side of the row and topping), topping was performed at 11 feet height. The motor of the sickle bar was positioned at the top to avoid hitting the lower branches of the trees, and the speed of the tractor was fixed at 1.2 m/h in 2014 and 1.3 m/h in 2015. Hand pruning was performed by the commercial crew (4 people) using ladders, and with the practices that they regularly perform at the orchard. The time to prune each plot and the weight of the wood pruned were recorded for each treatment. Additionally types of cuts and wood damage were observed, as well as the general performance of the machine. The block was picked at commercial harvest (6/4/2015), 3 trees/rep/treatment were randomly chosen for yield and fruit quality evaluation and samples of 25 cherries/tree. Fruit quality was evaluated in the laboratory with weight, firmness, soluble solids content, stem pull force and diameter measurements.

Preharvest and postharvest topping: this trial was also established at Olsen Brothers in Benton City with 'Tieton'/'Gisela®5'trained to the UFO system in a complete randomized design, consisting of 5 different timings of hand pruning (treatments) with 5 replications, and 3 trees/rep. Trials were initiated in 2014, the trees were topped by hand at 11-12' high at different timings: 1) dormant, 2) full bloom, 3) full bloom + 1 month, 4) full bloom + 2 months and 5) full bloom + 3 months. Fruit was picked at commercial harvest (6/11/2014) with samples of 25 cherries per upright. Data of the diameter at the cut site and the length of the removed branches were recorded. Fruit quality was evaluated in the laboratory with firmness, total soluble solids, titratable acidity, weight, and diameter measurements. Regrowth was measured during the winter 2015. The experiment was replicated in 2015 on 4 different rows, fruit was picked at commercial harvest (6/4/2015) and fruit quality was evaluated in the laboratory. Regrowth is being measured during the winter 2016.

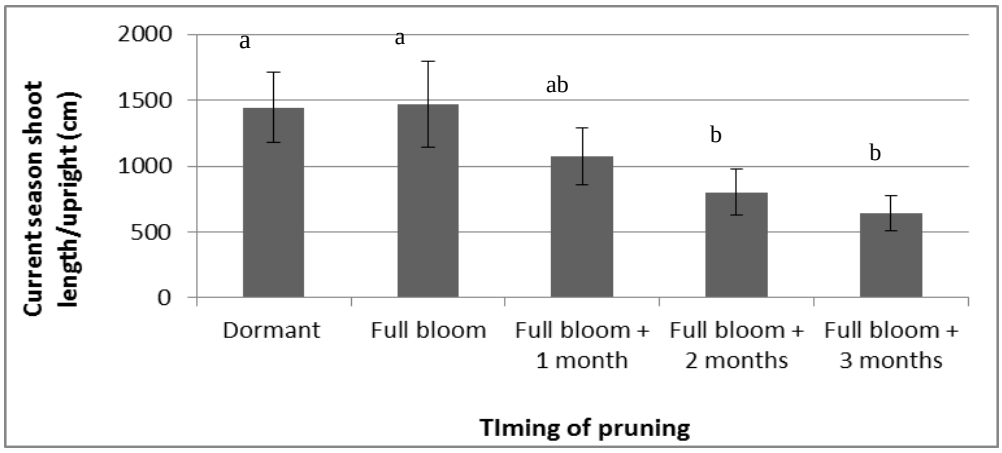
RESULTS AND DISCUSSION

Mechanical vs/+ hand pruning: Our 2015 results showed that mechanical pruning was 29 times faster than hand pruning alone and 17 times faster than the combination of both approaches, mechanical pruning followed by a hand cleanup was 1.6 times faster than hand pruning alone (Figure 1). Each tree was pruned approximately in 14 s with the sickle bar, 408 s per person by hand (6.8 min) and 245 s (4 min) with the sickle bar followed by a hand cleanup.

Hand pruning removed 2.6 times more wood/tree than mechanical pruning, and 1.2 times more wood/tree than the combination of mechanical and hand pruning, with 6.5 kg wood removed/tree approximately. With these results, we evaluated pruning efficiency as kg of wood removed/min/tree and mechanical

pruning was 11 times more efficient than hand pruning, and 8.2 times more efficient than mechanical pruning followed by a hand cleanup. The combination of mechanical and hand pruning was 1.4 times more efficient than hand pruning alone. It is believed that hand pruning removed more wood than mechanical pruning because it is more selective. Mechanical pruning had no effect on yield or yield efficiency. Yield efficiency ranged from 0.05 to 0.06 kg/cm2 TCSA, which represents 7.6 tons/acre for hand pruning, 9.1 tons/acre for mechanical pruning and 7.5 tons/acre for mechanical pruning 2 (Figure 2). Mechanical pruning had an effect on fruit diameter and weight but in a very small percentage. Fruit diameter from mechanical pruning1 and mechanical pruning2 was 3% (0.9 mm) and 2% (0.7 mm) lower than fruit from trees that were hand pruned, but row size was the same (9) for all treatments. Fruit weight from mechanical pruning was 7% lower (0.8 g) than fruit from trees than were hand pruned. We believe that these results might be due to the fruit yield of our trial, even though the difference was not significant, yield from mechanical pruning was 10% higher than yield from hand pruning.

Preharvest and postharvest topping: Our 2015 results showed that pruning performed full bloom + 2 months (FB2) and full bloom + 3 months (FB3) removed 73% more wood than full bloom (FB) pruning and 48% more than dormant pruning (DP). DP and FB were the treatments with the lowest amount of wood removed, but they were not different from each other. There was a positive correlation between length and caliper of wood removed at different timings of pruning, R2 coefficient was lower for 2015 than for 2014. Differently from our 2014 trial, timing of pruning affected yield per upright but not yield efficiency. Yield was 35% higher on unpruned uprights compared to uprights from DP, and no different from the other treatments. Yield efficiency ranged from 0.12 kg/cm^2 TCSA to 0.18 Kg fruit/cm^2 TCSA. Timing of pruning showed an effect on fruit SSC and stem pull force. Fruit SSC ranged from 14.1 °Brix to 15.5 °Brix. The lowest value was observed from full bloom + 1 month (FB1) , SSC from FB1 was 4%, 5% and 7% lower than fruit SSC from DP, FB and unpruned uprights, respectively. There was no difference between unpruned trees and DP and FB1. Fruit from all treatments were row size 9.



2016

Apple	2nd year of Kanzi trial, demonstration trials and outreach
Pear	2nd year of Bartlett, demonstration trials and outreach
Cherry	Demonstration trials and outreach

CONTINUING PROJECT REPORT **YEAR**: 2 of 3
WTFRC Project Number: TR-14-103

Project Title: Development and validation of pest and natural enemy models

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Collaborator: Betsy Beers, WSU-TFREC

Total Project Request: **Year 1:** $75,154 **Year 2:** $78,160 **Year 3: $81,306**

Percentage time per crop: Apple: 50% Pear: 20% Cherry: 20% Stone Fruit: 10%

Other funding sources

Agency Name: WSU-Extension
Amt. awarded: $266,344
Notes: The amount funded is the contribution that WSU-Extension provides for DAS support and maintenance + an additional 1 FTE for a second programmer for one year.

WTFRC Collaborative Expenses: None

Budget 1
Organization: WSU-TFREC **Contract Administrator:** Carrie Johnston/Joni Cartwright
Telephone: 509-335-4564/509-663-8181 x221 Email: carriej@wsu.edu / joni_cartwright@wsu.edu

Item	2014	2015	2016
Salaries[1]	42,129	43,814	46,354
Benefits[2]	14,983	15,582	16,668
Wages	12,480	12,979	12,480
Benefits[3]	262	273	300
Equipment	0	0	0
Supplies[4]	2,500	2,600	2,704
Travel[5]	2,800	2,912	2,800
Miscellaneous	0	0	0
Plot Fees	0	0	0
Total	75,154	78,160	**81,306**

Footnotes:
[1] U. Chambers Y1-3 (0.5 FTE); T. Melton Y1-3 (0.25 FTE)
[2] 33.5%
[3] 2.1%
[4] includes lab and field supplies
[5] w/in state travel

Objectives:

1. Develop models for mites and aphids using literature data and validate the information as needed.
2. Validate natural enemy models already developed in the SCRI biological control grant.
3. Re-evaluate the San Jose scale model and its biofix and accuracy.

Significant Findings:

- New models developed this year include the green apple aphid, rosy apple aphid, and western orchard predatory mite. Validation is underway on all models
- We were able to complete validation for the syrphid fly, *Eupeodes fumipennis*, which is a predator of aphids in all tree crops in WA.
- Two spotted spider mite diapause termination was quantified in two orchards in 2014 and 2015, these results show that diapause termination is based solely on photoperiod.
- San Jose Scale phenology in 2015 was similar to 2014, which suggests the current PETE model needs to be modified and that predictions of the second crawler generation can provide a second treatment window if populations are high.

Objective 1. Develop models for mites and aphids using literature data and validate the information as needed

Methods:

Model development: Last year, models for two spotted spider mite (TSSM), European red mite (ERM), and woolly apple aphid (WAA) were synthesized from literature data. This year the European red mite model was refined and developed into a life table format which better describes the overall development and population dynamics. In addition, we created models for the green apple aphid (*Aphis pomi*), rosy apple aphid (*Dysaphis plantaginea*), and the western orchard predatory mite (*Galendromus occidentalis*). We have developed completely synthesized life table data for the two aphid species, but have not yet developed the same for *G. occidentalis*. Our models currently provide upper and lower threshold information and population growth rates (Table 1), but the life table format is readily adaptable to the demographic degree day models that allow us to provide estimates of pesticide impacts throughout the season (similar to those we developed for CM, OBLR, and the two lacewings *Chrysoperla carnea* and *Chrysopa nigricornis*). Movement to the demographic models depends on the validation process – if we can accurately predict phenology (versus just growth rates), the demographic models will take just a few weeks to develop. For the aphid species, one essential component we need is when they move in and out of the orchard and to see if that is predictable. Data this year (see field validation) may provide us that information. We have been collecting data on mite and aphid abundance throughout the year, but until the data is processed and analyzed, we cannot estimate whether we will get the added benefit of the more comprehensive models until that time.

We have also begun the synthesis of the model for the parasitoid of the woolly apple aphid, *Aphelinus mali*. At this point, we have determined the upper and lower thresholds for development (Table 1), and accumulated some of the literature needed to develop understanding of its phenology. However, the data available does not seem to be suitable for a broader model; this may depend on three papers that have been ordered from inter-library loan.

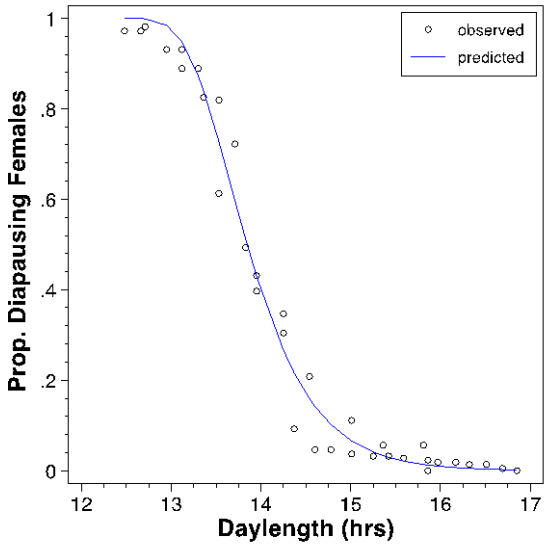
Table 1. Developmental thresholds and population growth rates for two aphid species, the western predatory mite, and *Aphelinus mali*

Model	Lower Threshold	Upper Threshold	Type	Pop. Growth Rate/DD
Green apple aphid	42.1	77.7	Vertical	0.0112
Rosy apple aphid	39.5	75.2	Vertical	0.0135
G. occidentalis	50.5	86	TBD	0.0141
Aphelinus mali	46.6	86	TBD	0.00963

Results and Discussion:

Field data for validation: Two spotted spider mite (TSSM) emergence from diapause was evaluated from two orchards in 2014 and 2015. We found that diapause termination was predictable based on day length (Fig. 1) which should simplify our understanding of TSSM population dynamics in the spring.

Fig. 7. Diapause termination for female two spotted spider mite in the spring in 2014 and 2015**.**



Woolly apple aphid: We collected data on WAA crawler movement on the trunks, shoot infestation levels, colony growth, and parasitism rates in four orchards in 2015 (two minimally managed research plots, one organic orchard, and one heavily infested conventional orchard). Initial analyses show that crawlers moved upwards and downwards along the trunk throughout the season with peaks in May/June, July, and September/October. Temperature appears to play a strong role in the onset of crawler movement on the trunk in spring. Crawler movement was observed when the average daily soil and air temperatures were above 47ºF. Hoyt and Madsen (1960) report a lower threshold for crawler movement of 50ºF. Initial analyses suggest that the onset and progress of crawler movement could be predictable by temperature and DD.

Increases in the number and size of WAA colonies were observed in June/July, followed by a decrease or plateau and another increase around mid-August/September. Parasitism followed this trend and peaked in July and increased again during September/October. The total number of parasitized WAA colonies strongly correlated with the total number of parasitoids that were observed. The number of earwigs was not correlated to the number of WAA colonies or crawlers.

Work next year: We should finish all the models next year and be able to evaluate the field data. We will focus this next year's data collection to get a better set of mite data and clarify when the different species of aphids move in and out of the field. We will use some liquid bait traps for the aphid species to allow us to better quantify the flight period of the different species.
For modeling crawler movement, we will collect more data starting early in the year from more orchards. More in-depth analyses will be conducted to determine how temperature, phenology, natural enemies, and potentially pesticide treatments affect crawler movement and colony growth. Acceleration and inhibition of colony growth is likely governed by temperature, but parasitism, predation and crawler migration modify these correlations and add complexity. Field data collection this coming year will be adjusted to determine those interactions.

Obj. 2. Validate natural enemy models already developed in the SCRI biological control grant.
Results and Discussion: Our data this year has not all been processed, so more information will be available later. However, evaluation of our data from the last two years allowed us to complete the model for the syrphid fly, *Eupeodes fumipennis*, which is one of the most common syrphids that eat aphids attacking apples and cherries. Our data showed that there were up to at least 5 generations per year and the model generally provided good fit to all the generations except the first summer generation where

error was about twice as high as in the other generations (Table 2). Examination of the apple data showed that in all cases, the first summer generation occurs during the period when the first codling moth sprays would be applied (using either a delayed first cover or the normal 425 DD larvicide treatments). This probably affected the model predictions because eliminating any part of the curve causes a shift in the observed numbers of syrphid flies. This timing also resulted in fewer numbers of orchards where an apparent flight would have occurred (note, if the number of flies captured in a flight at a particular orchard drops below 20 we drop the data because it doesn't allow us to accurately assess the flight curve for that orchard). Overall, we were able to perform validation using 41 orchards, although not all flights were found in each orchard.

Table 2. Average error in DD for the *E. fumipennis* model over 41 orchards.

	Average Error in DD per Flight					
Crop	Over wintering	1st summer	2nd summer	3rd summer	4th summer	Average
apple	39	104	42	51	39	47
cherry	31	43	68	39	26	37
walnut	–	–	43	–	19	30
Average	38	91	42	49	30	45

Work new year: We will continue sampling but focus more on *Deraeocoris brevis* (general predator of spider mites, aphids, leafhoppers and other soft-bodied insects) and *Aphelinus mali* (parasitoid of WAA). There is considerable information on life history of *Deraeocoris* that might allow us to develop the demographic models if we can get a reasonable phenology model developed. *A. mali* has less information available, but we should be able to get a phenology model for it at least based on the current data set we have and what we have collected this past season.

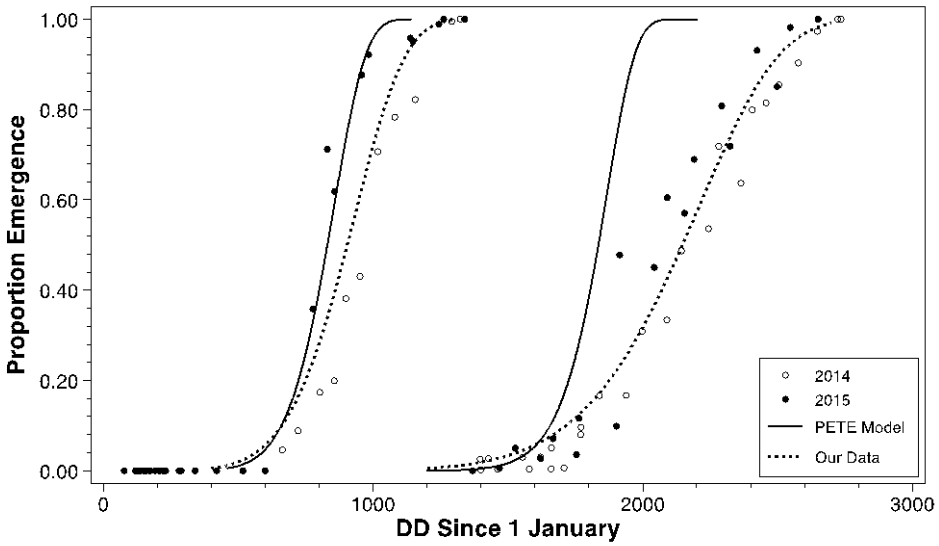
Objective 3. Re-evaluate the San Jose scale model and its biofix and accuracy.

Methods: As with the previous year, the pheromone trap catch is very low and erratic. This is likely caused by the males being very sensitive to even low wind speed. In addition, predicting the males are not that important, as treatments are never directed towards the males, but only against the crawler stages.

To evaluate phenology of the crawler stage, we placed 50 sticky tapes on branches that have SJS scales present. The sticky tapes were removed and replaced every week, with the tapes then applied to clear acetate sheets so that we could transport them to the lab and count them with a stereomicroscope.

Results: The sticky tapes again showed two crawler generations, with first crawlers of the first generation having nearly the same emergence time in 2014 (660 DD) and 2015 (685 DD). The second generation occurred about 100 DD later in 2015 (1498 DD) versus 2014 (1398 DD). Over both years and all locations, we found that the PETE model that has been used as the basis for DAS predicts the first generation marginally well, but the second crawler generation is completely wrong (Fig. 2). Our data shows the emergence of the second crawler generation occurs over a much longer period and could be a viable treatment window if the first crawler generation is missed. Evaluation of the normal timings for codling moth treatments would likely have no effect on either generation, but instead would require a specifically timed spray.

Fig. 2. Proportion emergence of the crawler stage of San Jose scale in 2014 and 2015, comparing the PETE model and our model.



Evaluation of the currently used biofix for codling moth to start the biofix for SJS shows that it is not a very useful tool. Although it can work in some years, like many "rules of thumb", there can be situations where it is wildly off. Our data suggests that we can use a no-biofix model, and we will use that model this coming year. Before any second generation timings are suggested, we will collect another year's data. However, overall it is clear that our timings will need to be changed over the currently used PETE model.

Work this coming year: We will continue monitoring the SJS crawlers in sticky tapes. We also have some additional sticky trap data taken from the base of the trunk (for monitoring movement of WAA up and down the tree trunk) that also showed high levels of SJS that we will process in the next month or two and determine which of the two locations provide better information.