2015 Technology Research Review
February 12, 2015
Hamilton Hall Large Conference Room, WSU - Prosser

Page No.	Time	PI	Project Title	Yrs
			Final Reports	
	8:00	McFerson	Introduction	
1	8:15	Brunner	Improving tools for early detection of brown marmorated stink bug	13-14
9	8:30	Chiu	Develop a PCR diagnostic for all life stages of *Drosophila suzukii*	14
19	8:45	Salazar	Effect of early spring temperature on apple and sweet cherry blooms	12-14
	9:00		Continuing Reports	
29	9:00	Ashmore	Technology roadmap implementation: *Teleconference*	13-15
31	9:10	Whiting	Mechnical pollination for yield security	14-15
38	9:20	Gordon (Lewis	Mechanical pruning in apple, pear and sweet cherry	13-15
45	9:30	Jones	Development and validation of pest and natural enemy models	14-16
50	9:40	Eastwell	Efficient strategy to diagnose important virus diseases	13-15
56	9:50	Kalcsits	Testing a portable, non-destructive measure of Ca, Mg and K in apple	14-15

FINAL PROJECT REPORT

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

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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

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

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

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.

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. There is residual funding in the commission funded project and we are requesting use of those funds to complete this objective and submit an amended final project report in 2016.

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.

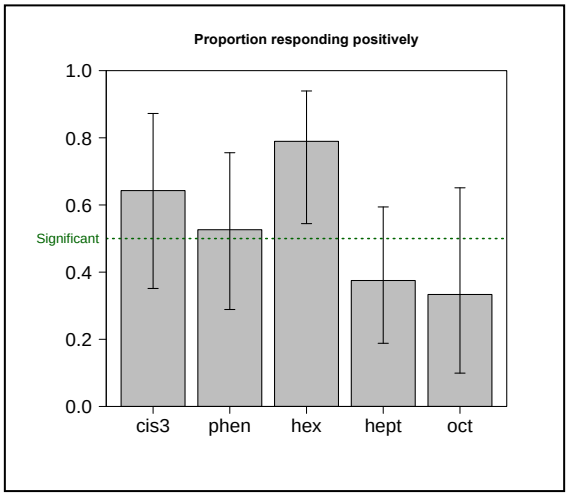

Fig. 3. Proportion response of BMSB in Y-tube bioassay to compounds derived from host plants.

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

and hex 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).

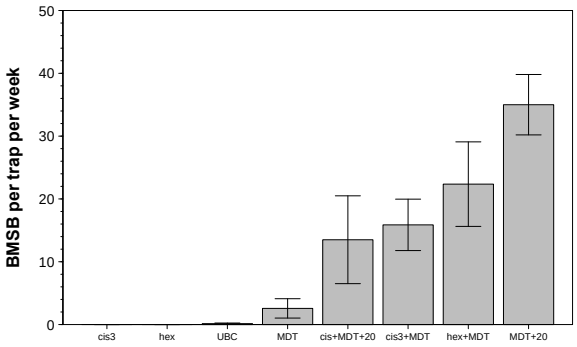

Fig. 4. Average capture of BMSB in traps baited with lures containing candidate plant volatile compounds, pheromones or combinations of volatiles and pheromones.

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 an synergy of BMSB capture when combined with USDA#20 and MDT.

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

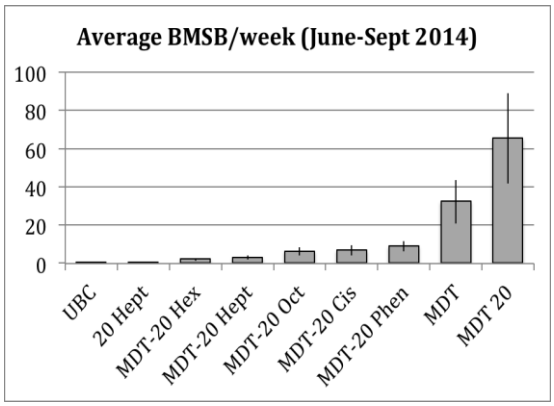

Fig. 5. Average capture of BMSB in traps baited with kairomone lures and USDA#20 and MDT.

The portion of objective 3 associated with the USDA laboratory (Dr. Ashot) was not addressed in this proposal. There was an issue with funding arriving in his laboratory until 2014 when he was fully involved in other aspects of the SCRI national project so he was not able to address his portion of the objective, analyze organic compounds produced in three of the most important early-season host plants for BMSB from the northwest: English holly (*Ilex aquifolium* L.), tree of heaven (*Ailanthus altissima* (Mill.)), and various maple species (*Acer* spp.). Dr. Ashot has assured us that his responsibility for objective 3 will be addressed in 2015 and his will findings will be submitted as part of an amended report to the Technology committee in 2016.

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.

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.

FINAL PROJECT REPORT

Project Title: Develop a PCR diagnostic for all life stages of *Drosophila suzukii*

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Percentage time per crop: Apple: 0% Pear: 0% Cherry: 60% Stone Fruit: 40%

Other funding sources: None

Total Project Funding: $5,500

Budget History:

Item	2014
Salaries	$2,800
Benefits	$84
Wages	-
Benefits	-
Equipment	-
Supplies	$2,516
Travel	-
Plot Fees	$100
Miscellaneous	-
Total	$5,500

Footnotes: Salaries and Benefits are for one Undergraduate research assistant
Supplies include DNA oligos, reagents for DNA extraction, PCR, agarose gel electrophoresis and lab consumables
Miscellaneous item represent shipping costs for flies from different geographical locations

JUSTIFICATION

Drosophila suzukii Matsumura (Spotted Wing Drosophila [SWD]) is a recent invader and has become a serious economic pest of a wide variety of fruit crops in the United States, Canada, and Europe, leading to substantial yearly crop losses that are in the tens of millions of dollars (Hauser 2011, Lee et al. 2011, Walsh et al. 2011, Calabria et al. 2012). Unlike other cosmopolitan *Drosophilids* that oviposit in overripe and blemished fruits with no commercial value, SWD female has evolved a serrated ovipositor that enables this pest species to oviposit in ripe or ripening berry fruits, e.g. caneberry, soft-skinned fruits, e.g. cherry, and stone fruits, thus allowing the larvae to develop within the fruit. Although adult SWD flies have morphological characters, i.e. serrated ovipositors in females and spotted wings in males, that facilitate identification and differentiation from other common, non-pest *Drosophilids* that co-inhabit the same geographical regions, identification of SWD from larval stages or from poorly preserved specimens is difficult. Identification of SWD larvae requires researchers or fruit inspectors to rear the larvae to adulthood. This may require a week or more and such delays can result in substantial economic losses for exporters of these perishable fruits as well as delays to research on this important pest. Australia and New Zealand currently have a quarantine safeguarding against entry of SWD-infested fruits and such quarantines may be erected by other countries. Shipment delays due to identification can reduce fruit quality and increase handling costs. A method to identify the immature stage of *Drosophila* species, particularly to specifically identify *D. suzukii* is therefore warranted. We therefore set out to develop an efficient PCR-based molecular diagnostic test that can be used to quickly (i.e. 1 to 2 hours) identify SWD of all life stages and differentiate it from other common *Drosophilids* using minimal molecular biology equipments.

Since this project has been funded and initiated, there have been two published diagnostic designed for SWD (Dhami and Kumarasinghe 2014; Kim et al. 2014). However, the PCR-RFLP diagnostic designed by Kim et al. (2014) cannot differentiate between SWD and the closely-related *D. subpulchrella*, which also has a serrated ovipositor and has the potential to become a pest (Atallah et al. 2014). Whereas the diagnostic designed by Dhami and Kumarasinghe (2014) can differentiate between SWD and *D. subpulchrella*, it is based on a method (high-resolution melt analysis) that requires more sophisticated and expensive instrumentation, e.g. quantitative real-time PCR, which is not available in most common laboratories and export sorting facilities.

ORIGINAL OBJECTIVE OF PROJECT:

Objective 1: **Develop a reliable PCR-based molecular diagnostic test to identify SWD at all life stages**

Using the *Drosophila suzukii* (SWD) genome we recently published (G3: Genes, Genomes, Genetics; Chiu et al., December 2013), we will design SWD specific primers to differentiate SWD from other common *Drosophilids*. This one-step PCR molecular diagnostic can be completed within 2 hours and can be performed from limited starting materials, e.g. a single larva, and the most basic molecular biology equipments.

SIGNIFICANT FINDINGS:

- We have successfully developed a molecular diagnostic to differentiate SWD 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.
- 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).

- A manuscript describing this PCR diagnostic is in revision in the Bulletin of Entomological Research. It was also presented in the annual Entomological Society of America meeting (Portland, OR) in November 2014 as part of a symposium talk.

RESULTS & DISCUSSION:

Comparative genomics analysis facilities primer design

The multiplex PCR diagnostic test for SWD relies on the use of two primer sets in a multiplex PCR reaction. The first set of primers was designed to amplify a product from any Drosophilid to confirm the presence of good quality DNA in the reaction and to verify the success of the PCR reaction. This internal control primer pair was designed from the coding region of a gene (*D. suzukii* ortholog [SpottedWingFlybase ID: DS10_00001395] of *sec61alpha* in *D. melanogaster* [FlyBase ID: FBgn0086357 and FBpp0078896]) that is highly conserved among the fifteen *Drosophila* species we used for our comparative genomic analysis to yield a 1,248 base pair (bp) product (forward primer: 5'- ATCCCTTCTACTGGATCCGTG-3' and reverse primer: 5'-ACAGCAGCGTGCCCATG-3') (Figure 1A).

The second set of primers was designed from a gene (*D. suzukii* ortholog [SpottedWingFlybase ID: DS10_00004458] of *D. melanogaster* FBgn0035268 and FBpp0072657) that is more divergent. Since there is currently no publicly available genome or transcriptome data available for the closely related species *D. subpulchrella,* for which females also have serrated ovipositors (Atallah *et al.*, 2014), we sequenced a gene region within the *D. subpulchrella* ortholog of *D. suzukii* DS10_00004458 (*D. melanogaster* FBgn0035268) to locate primer sequences that are sufficiently diverged between *D. suzukii* and *D. subpulchrella* to differentiate these two species in addition to the other *Drosophila* species included in our comparative genomics analysis. To obtain the sequence for the *D. subpulchrella* ortholog of *D. suzukii* DS10_00004458, PCR was performed using *D. subpulchrella* genomic DNA as a template with forward primer 5'-AGTTTTGCGTCAGCGGATCC -3' and reverse primer 5'-TCGTCGTCGGAGCTGTTG -3'. These primer sequences were designed based on *D. suzukii* sequences, but were sufficiently conserved to amplify *D. subpulchrella* genomic DNA. Sanger sequencing of the amplified gene region was performed by the UC Davis sequencing facility. Alignment of the resulting *D. subpulchrella* gene region with its orthologs from other *Drosophila* species were performed to identify primer sequences with sufficient sequence polymorphisms to yield a species-specific primer set (Figure 1B). We chose a primer pair that amplifies a 263 bp product. The forward primer (5'-ACTTGTGTCTTGTCCCTCACATAC -3') is located within an intron and the reverse primer (5'-TCCAGATCTTTACGTCATGCTCC -3') is located within the coding region.

Comparative genomic analysis provides a more robust framework for primer design and the theoretical foundation for the reliability of our PCR test. More importantly, since fly populations are continuously evolving, our comprehensive genomics analysis provides the basis for the design of additional diagnostic markers in the case that DNA sequence changes render our diagnostic marker ineffective for specific fly populations.

Multiplex PCR diagnostic differentiates *Drosophila suzukii* from other *Drosophila* species

The internal control primer pair amplifies a 1248 bp product that is conserved in all Drosophilids for quality control purposes. The presence of the control band in non-SWD samples signals that DNA quality and amplification condition are acceptable, and the lack of amplification for the species-specific primers is not due to suboptimal PCR conditions. The SWD species-specific primer pair amplifies a 263 bp product. Thus, a positive test should result in the amplification of two DNA fragments, one at 263 bp, and another at 1248 bp, visible upon DNA gel electrophoresis.

As we aim to develop an efficient assay that can be performed using limited starting materials, we tested our PCR diagnostic using crude extract isolated from either single larva or preserved adult specimen (when larva is not available) without any prior genomic extraction steps. To ensure reproducibility, at least five biological replicates were performed for each SWD and non-SWD

population. A total of eight non-SWD *Drosophila* species were tested (Figure 2A and Table 1). There were no false positives in that all non-SWD larvae showed robust amplification only for the control 1248 bp product. The size of the internal control band in the different species showed slight variation indicating possible in/del within the regions spanned by the control primer set. As sequence polymorphisms might have accumulated over time as *D. suzukii* populations spread, we tested the utility of our PCR diagnostic using larvae or preserved adult specimens from nineteen *D. suzukii* populations (Figure 2B and 2C, Table 1). Freshly sacrificed larvae were used to assay *D. suzukii* populations from the continental U.S.A. including California, Oregon, Washington and Maryland, as well as populations from Hawaii and Japan, as live cultures of these populations were available. On the other hand, preserved adult specimens were used to assay *D. suzukii* populations from Italy and South Korea because we only have access to RNAlater-preserved specimens for these sites. All of the *D. suzukii* larvae originated from different geographical regions in the U.S.A. as well as from Hawaii and Japan tested positive and showed amplification at the 263 bp (SWD-specific band) (Figure 2B and 2C). Moreover, adult *D. suzukii* specimens collected in South Korea and Italy and preserved in RNA*later* also tested positive (Figure 2C). This represents a 100% success rate.

As our diagnostic relies on multiplex PCR, there is competition between the primer sets for reaction components such as ATP and deoxynucleotide triphosphates (dNTPs). When one product is favored, it can outcompete the other reaction resulting in uneven amplification of the two products. Short amplicons, e.g. the 263 bp SWD-specific products, are often amplified with higher efficiency than long amplicons, e.g. the 1248 bp control product, because the polymerase is more likely to fully extend a larger percentage of the short products as the reaction proceeds. Thus, we designed the control primers to produce a longer amplicon than the SWD-specific primers so that when *D. suzukii* DNA is provided as the template, the SWD-specific product will be favored. This design increases the sensitivity of our assay, yet retain the advantage of having an internal control to illustrate the difference between robust vs. weak non-specific amplification. As shown in Figure 2B, the SWD-specific 263 bp product is clearly more robust than the 1248 bp control amplified fragment in all SWD samples. In a few of our test samples, the SWD-specific primers even outcompete the internal control primers, resulting in a single band at 263 bp (e.g. Figure 2B, lane 12; Figure 2C, lane 1). When the 263bp SWD-specific product is present and 1248bp control product is absent, a single band at 263bp can be interpreted as a positive test result.

This assay was also performed using crude extract from ethanol preserved larvae, pupae, and adult samples because this is a common collection and storage condition and produced amplification results and conclusions identical to those in reactions using live samples (data not shown). This direct larval tissue PCR method is economical and practical for rapid identification of single larva because the PCR reaction can be assembled in minutes, while the use of extracted genomic DNA typically requires at least an hour as well as additional equipment and reagents. Finally, we verified that our multiplex PCR diagnostic assay also performs well with genomic DNA extracted from all nine *Drosophila* species and observed that the SWD-specific primers only amplified *D. suzukii* DNA (data not shown).

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A

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Dsuz  ATCCCTTCTACTGGATCCGTG ---------- CATGGGCACGCTGCTGT
Dsec  ATCCCTTCTACTGGATCCGTG ---------- CATGGGCACGCTGCTGT
Dtak  ATCCCTTCTACTGGATCCGTG ---------- CATGGGCACGCTGCTGT
Dgri  ATCCCTTCTACTGGATACGTG ---------- CATGGGCACGCTGCTGT
Dana  ATCCCTTCTACTGGATCCGTG ---------- TATGGGCACGCTGCTGT
Dvir  ATCCCTTCTACTGGATCCGTG ---------- CATGGGCACGCTGCTGT
Dsim  ATCCCTTCTACTGGATCCGTG ---------- CATGGGCACGCTGCTGT
Dmoj  ATCCCTTCTACTGGATACGTG ---------- CATGGGCACGCTGCTGT
Dyak  ATCCCTTCTACTGGATCCGTG ---------- CATGGGCACGCTGCTGT
Dwil  ATCCCTTCTACTGGATTCGTG ---------- CATGGGCACACTGCTGT
Dper  ATCCCTTCTACTGGATTCGTG ---------- CATGGGCACGCTGCTGT
Dmel  ATCCCTTCTACTGGATCCGTG ---------- CATGGGCACGCTGCTGT
Dbia  ATCCCTTCTACTGGATCCGTG ---------- CATGGGCACGCTGCTGT
Dere  ATCCCTTCTACTGGATCCGTG ---------- CATGGGCACGCTGCTGT
Dpse  ATCCTTTCTACTGGATTCGTG ---------- CATGGGCACGCTGCTGT
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B

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Dsuz  TCGCAACACCAAGGATGGCGAGTACTTGCATTGCTACGAGGGACACAT|GTAAGTCCACGT
Dmel  TCGGAACTACAACAGAGGCGACTATTTGCACTGCTATAAGGGTCACAT|GTAAGTAAGCCC
Dsim  TCAGAACTACAACAGAGGCGACTACTTGCACTGCTATAAGGGTCACAT|GTAAGTTCACCA
Dsec  TCAGAACTACAACAGAGGCGACTACTTGCACTGCTATAAGGGTCACAT|GTAAGTTCACCA
Dyak  TCGGAACAACAAGGACGGCGAGTACTTGCATTGCTACCAGGGTCACAT|GTTAGTACTGCC
Dere  TCGGAACAACCAGCCCGGCGAGTACTTGCACTGCTACCAGGGTCACAT|GTAAGTACTGCC
Dbia  TCGCAGCACCAAGGATGGCGAGTACTTGCATTGCTACGAGGGACACAT|GTAAGTCCACGT
Dsub  TCGCAACACCAAGGATGGCGAGTACTTGCATTGCTACGAGGGACACAT|GTAAGTCCATGT
Dtak  TCGGAATACCAAAGACGGCGAGTATCTGCACTGCTACGAGGGGCACAT|GTAGGTTTATAG
Dana  CCGCAACTACACGGATGGCGAGACCCTGCACAGCTACAGGGGACATGT|GTAAG------G
Dwil  TCGCAATTGTA---ATGGCGGCATTCTGCGTACCTATCGAGGACACTT|GTAAGTTGTTAA

Dsuz  TGCATCACTTG--TGTCTTGTCCCT-----------CACATACCCCT-CCT-GTCGCCCA
Dmel  TGTTTCCCCCA-ATGTTCTGACACTGA---------CAAATATCATCTCCT-CTTTCCCA
Dsim  TGCCTCCCCCA-ATGTTCTGATATT-----------------TATCATCTCCT-CTTTTCCA
Dsec  TGCCTCCCCCA-ATGTTCTGATATT-----------------TATCATCTCCTCTTTTTCCA
Dyak  TGCAGCCCAGA-GTGTTCTGGCACT-----------CAAATATCATCTCCT-GTTACCCA
Dere  TGCAGCACCGA-GTGTTCTGGCACT-----------CACATATCATCTCCT-GTCCCTCA
Dbia  TGCAGCGCTAC--TGTGCTAGCAGT-----------CACATACCCCTGCTT-GTCGCCCA
Dsub  TGCATCGCGTG--TGTCTTGGCACT-----------CACATACCCCT-CTT-GTCGCCCA
Dtak  AGCATCACTAGTATGTCCTGGTACT-----------CACATTTCTCC-----TTCTACCT
Dana  TGCAAGTAAAA-ATGTTCAGCCTTC-----------TACTAACTTAGAACTTTGGTCATT
Dwil  TCCCACCAAAG-ATATTGGCCTAGTTAAATCTATCGCACACACACAC------ACACACA

Dsuz  CAG|CAATAGCCGCACCATTAAGGGAGTG-------- GGAGCATGACGTAAAGATCTGGA
Dmel  CAG|CAACAGCCGCACCATAAAGGGTGTG-------- AGAGCACGACGTCAAGATCTGGA
Dsim  TAG|CAACAGCCGCACCATCAAGGGTGTG-------- AGAGCACGACGTCAAGATCTGGA
Dsec  TAG|CAACAGCCGCACCATCAAGGGTGTG-------- AGAGCACGACGTCAAGATCTGGA
Dyak  CAG|CAACAGCCGCACCATCAAGGGAGTG-------- GGAGCACGATGTCAAGATCTGGA
Dere  CAG|CAACAGCCGCACCATCAAGGGAGTG-------- TGAGCACGACGTCAAGATCTGGA
Dbia  CAG|CAATAGCCGCACCATCAAGGGAGTG-------- GGAGCACGACGTTAAGATCTGGA
Dsub  CAG|CAATAGCCGCACCATCAAGGGAGTG-------- GGAGCACGACGTTAAAATCTGGA
Dtak  TAG|CAATAGCCGCACCATCAAGGGAGTG-------- GGAGCATGACGTCAAGATCTGGA
Dana  TAG|TAACAGCCGCACCATCAAGGGTGTG-------- GGAACACGACGTTAAAATTTGGG
Dwil  CAG|GAATAGTCGTACCATCAAGGGTGTT-------- GGAGCATGACGTTAAATTATGGG
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Figure 1. Alignments for the gene regions in which the conserved and diverged primer sets are located. (A) Alignment of *D. suzukii sec61alpha* (DS10_00001395; annotation of SpottedWingFlybase (Chiu *et al.*, 2013)) to orthologs from 14 other *Drosophila* species spanning multiple groups in the subgenus *Sophophora* (*D. melanogaster* (FBgn0086357), *D. simulans* (FBgn0193973), *D. sechellia* (FBgn0172841), *D. yakuba* (FBgn0235854), *D. erecta* (FBgn0115759), *D. ananassae* (FBgn0092730), *D. pseudoobscura* (FBgn0081850), *D. persimilis* (FBgn0163685), *D. willistoni* (FBgn0220690), *D. takahashii (*KB461656.1), *D. biarmipes (*KB462833.1)) and subgenus *Drosophila* (*D. virillis* (FBgn0205065), *D. mojavensis* (FBgn0140032), *D. grimshawi* (FBgn0120726)). Only the gene sequences corresponding to the location of the forward and reverse primers were shown, and sequence polymorphisms as compared to *D. suzukii sec61alpha* were indicated in grey. *D. suzukii* primer sequences are shaded in grey. (B) Alignment of *D. suzukii* DS10_00004458 (annotation in SpottedWingFlybase (Chiu *et al.*, 2013)) to orthologs from 10 other *Drosophila* species: *D. melanogaster* (FBgn0035268), *D. simulans* (FBgn0185353), *D. sechellia* (FBgn0169366), *D.ananassae* (FBgn0101826), *D. yakuba* (FBgn0238459), *D. erecta* (FBgn0107076), *D. takahashii (*KB461143.1), *D. biarmipes (*KB462838.1), *D. willistoni* (FBgn0218640), and *D. subpulchrella* (KM208658). *Drosophila* species including *D. grimshawi*, *D. virillis*, *D. mojavensis*, and *D. persimilis* have a larger and more diverged intron and were excluded from this figure. 66-bp are shown upstream of the forward primer and 52-bp are shown downstream to anchor the alignment and indicate the polymorphic nature of the intronic region used for the forward primer. Vertical lines mark exon-intron boundaries. The 8 dashes immediately before the reverse primer sequence indicate the portion of the sequence that is not shown in the alignment. Grey color highlights polymorphic base pairs or deletions in the other *Drosophila* sequences as compared to *D. suzukii* primer sequences, which are shaded in grey.

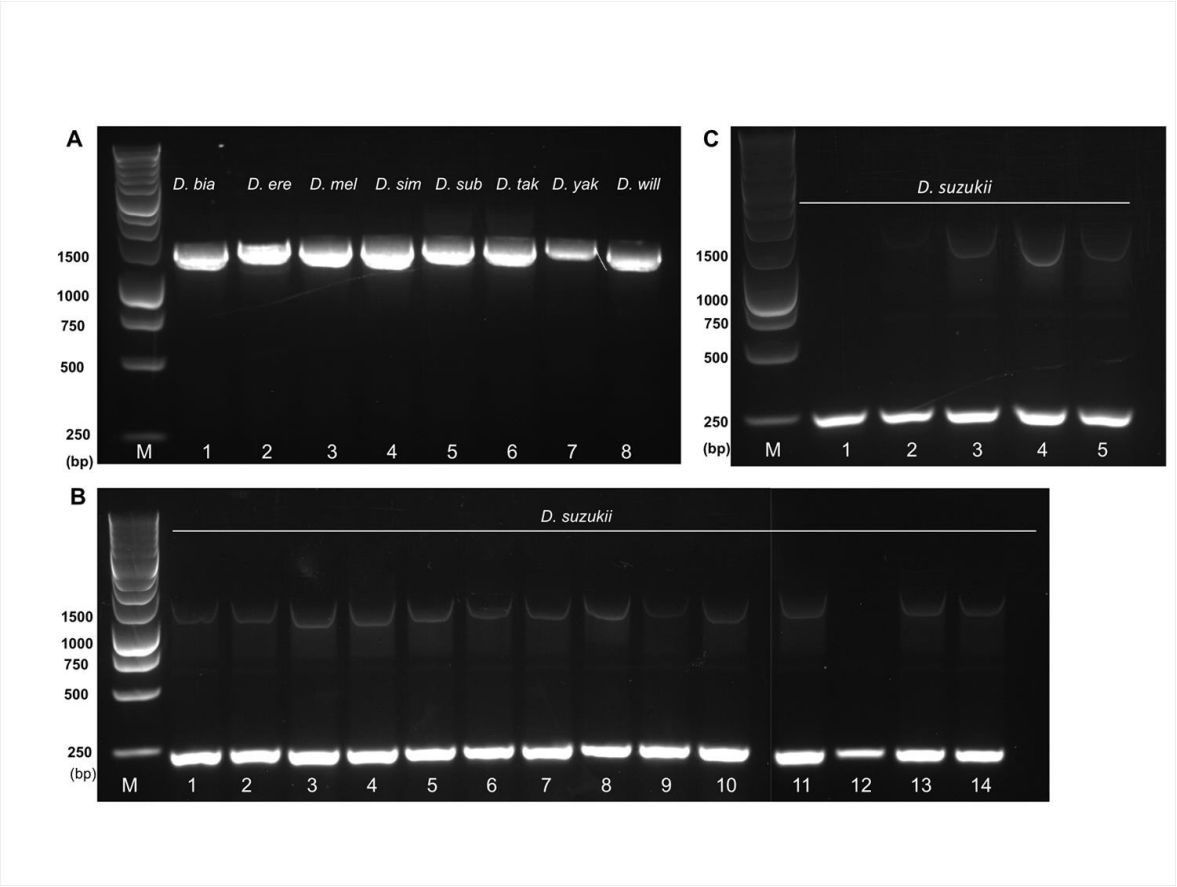

Figure 2: **DNA agarose gel electrophoresis showing results of multiplex PCR.** Multiplex PCR was performed using (A) multiple *Drosophila* species (see Table 1 for strain information) including *D. suzukii* (genome strain WT3 F10) and (B) *D. suzukii* collected from different regions in the Western United States (Lanes 1-14: genome strain WT3 F10, lime, grape, Mark Bolda WAT, Wolfskill IFL WO-2 [from CA], HR3 F4, TD3 F5, PD3 F5, ARS, OS1 [from OR], colony #8, colony #9, colony #10, and colony #11 [from WA]). PCR amplification for (A) and (B) was performed using crude larval extract isolated from a single larva that was freshly sacrificed. All crude DNA extractions and corresponding PCR reactions for (A) and (B) were repeated at least five times using biological replicate samples. Representative results are shown here. (C) Multiplex PCR was performed using crude extract from *D. suzukii* specimens collected from regions outside of the western United States. Adult flies were collected in South Korea and Italy (Table 1) and preserved in RNA*later* for storage (Lane 1: South Korea, Lane 2: Italy). Freshly sacrificed larva from strains collected in Japan, Hawaii, and Maryland, U.S.A. (Lane 1: MTY3, Japan, Lane 2: Oahu, Hawaii, Lane 5: Maryland).

Table 1. Species and strains used for testing the *Drosophila suzukii* molecular diagnostic.

Species	**Strain**	**Location of collection**	**Collector/Source**
D. suzukii	colony #8	Bray's Landing, WA, USA N47.738425 W120.167644	Beers lab, WSU
D. suzukii	colony #9	Daroga Park, WA, USA N47.705933 W120.19128	Beers lab, WSU
D. suzukii	colony #10	Royal City, WA, USA N46.837533 W119.5099	Beers lab, WSU
D. suzukii	colony #11	Rock Island, WA, USA N47.2350 W120.0727	Beers lab, WSU
D. suzukii	lime	Davis, CA, USA N38.55 W121.78	Begun Lab, UCD
D. suzukii	grape	Davis, CA, USA N38.55 W121.78	Begun Lab, UCD
D. suzukii	genome strain, WT3 F10	Watsonville, CA, USA N36.94 W121.76	Begun Lab, UCD
D. suzukii	Mark Bolda, WAT	Watsonville, CA, USA N36.94 W121.76	Zalom Lab, UCD
D. suzukii	Wolfskill IFL WO-2	Winters, CA, USA N38.49 W121.98	Begun Lab, UCD
D. suzukii	HR3 F4	Hood River, OR, USA N45.410860 W121.321011	Shearer Lab, OSU
D. suzukii	TD3 F5	The Dalles, OR, USA N45.351738 W121.131167	Shearer Lab, OSU
D. suzukii	PD3 F5	Parkdale, OR, USA N45.310333 W121.351362	Shearer Lab, OSU
D. suzukii	ARS	Corvallis, OR, USA N45.010035 W122.564377	Shearer Lab, OSU
D. suzukii	OS1	Corvallis, OR, USA N45.010035 W122.564377	Shearer Lab, OSU
D. suzukii	wild population	South Korea	Betsy Miller, OSU
D. suzukii	wild population	Scurelle, Trentino, Italy	Claudio Loriatti, FEM-IASMA
D. suzukii	MTY	Ehime, Japan	Kopp Lab, UCD
D. suzukii	wild population	Oahu, Hawaii	Kopp Lab, UCD
D. suzukii	wild population	MD, USA	Hamby Lab, UM
D. biarmipes	genome strain, 361.0-isol e-11	Ari Ksatr, Cambodia	Kopp Lab, UCD
D. erecta	14021-0224.01	Tucson Stock Center	Begun Lab, UCD
D. melanogaster	Oregon R	Roseburg, Oregon	Zalom Lab, UCD
D. subpulchrella	NGN5	Nagano, Japan	Begun Lab, UCD
D. simulans	W501	Genome strain	Begun Lab, UCD
D takahashi	genome strain, 311.5-iso4	Yun Shui, Taiwan	Kopp Lab, UCD
D. willistoni	14030-0814-10	Guadeloupe Island, France	Begun Lab, UCD
D. yakuba	CY28	Cameroon, Africa	Begun Lab, UCD

EXECUTIVE SUMMARY:

We have designed an accurate PCR diagnostic that can unambiguously differentiate *D. suzukii* from other common *Drosophila* species using the crude homogenate of a single larva as the DNA source without the need for additional steps such as genomic DNA extraction, sequencing, or restriction digestion. The use of the recently sequenced *D. suzukii* genome (Chiu et al. 2013) and a comparative genomic approach facilitated the discovery of the diagnostic marker we presented here. In order to increase the utility of this PCR diagnostic, we are actively working with researchers (e.g. in USDA), growers, and other interested parties to optimize this diagnostic for use with SWD samples of various conditions, e.g. flies trapped and soaked in apple cider vinegar, and larvae embedded in fruit samples.

FINAL PROJECT REPORT

Project Title: Effect of early spring temperature on apple and sweet cherry blooms

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Cooperators: John Ferguson and Markus Keller, IAREC-WSU

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

Other funding sources

Indirect support through the existing infrastructure of AgWeatherNet and its 159 weather stations.

Total Project Funding: **Year 1:** $95,000 **Year 2: $**80,000 **Year 3:** $80,000

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

Item	2012	2013	2014
Salaries	14,040	38,646	37,661
Benefits	5,616	7,803	7,102
Wages	42,400	20,860	21,694
Benefits	4,240	2,086	2,169
RCA Room Rental	0	0	0
Equipment	10,000	0	0
Supplies	10,204	2,605	2,874
Travel	8,500	8,000	8,500
Plot fees	0	0	0
Miscellaneous	0	0	0
Total	95,000	80,000	80,000

Footnotes: Salary for an Assistant Research Professor (Dr. Melba Salazar) for four months. Dr. Salazar will be supported by a graduate student, budgeted for two years of the project. One year of 0.5 FTE technical support to build the automated sampler system. The automated sampler will be integrated with a freezer, which is budgeted at $10,000. Additional budget items include part-time hourly labor to help with sample collection and sample analysis for all three years, goods and services for the parts associated with the automated sampler and travel for collection of the samples in the region.

Goal and Objectives
The overall goal of this project was to investigate the effect of early spring temperature on apples and sweet cherries at different developmental stages and to determine the hardiness. We used a traditional methodology through exposure to freezing temperatures, and automated part of this procedure. One of the general goals was also to develop a updated hardiness charts for apples and sweet cherries.

The following were the specific objectives:
1. To determine the effect of early spring temperature on bloom development for different apple and sweet cherry cultivars.
2. To develop a cold resistance curve from dormancy to bloom for apples and sweet cherry.
3. To update the charts for the different stages of blossom buds of apples and sweet cherry cultivars for local weather conditions in the Pacific Northwest.

Significant Findings
- Differences in hardiness and lethal temperature were found during different phenological stages for the same cultivar as well as among the sweet cherry and apple cultivars.
- We developed an automated sampler machines referred to as the "vending machine" to determine the hardiness of the crops when DTA was snot effective. The results indicated differences between apples and sweet cherries and among cultivars.
- The results from dissection indicate that there was a variation in cold hardiness for the different bud sizes of apples for the same sampling date and differences among phenological stages.
- A decision support tool was developed on the AgWeatherNet portal (www.weather.wsu.edu) were the cold hardiness information for cherries and apples is being posted.
- One alert was distributed during the early winter season in 2013 and one alert was distributed during the early winter season of 2014 with the respect to risk of damage in cherry buds.

Methods
Bud samples were collected throughout late winter and early spring in 2013 season to determine the effect of temperature on bloom development for apple and sweet cherry cultivars. We started our measurements in October 2012 and ended them around early bloom. For apples we evaluated the varieties Gala, Red Delicious and Fuji. For cherries we evaluated the varieties Bing, Chelan and Sweetheart. The sweet cherry and apple cultivars at different bud development stages were sampled from the field and tested in the laboratory. We restarted our sampling on October 2013 and ended in April for the 2013-2014 winter season. For the 2014-2015 growing season sampling was started again during October 2014 and will continue through the end of April, 2015.

Cold hardiness was assessed using differential thermal analysis (DTA) for the first phenological stages. When the DTA was not effective, beyond open cluster, a new automated sampling device was developed and used. For the new device we load the tissue samples into color coded cans and expose the material to different durations and controlled cold temperatures combinations in a freezer. After the cold temperature treatment has been completed each tissue sample is dissected to determine frost damage based on browning of the tissue.

Simultaneously to the process described above we collected dormant apple and cherry shoots that were 6 to 10 inches long with terminal flower buds. The shoots were kept in containers filled with water. The base of the shoots was recut every week and water was replaced every other day and forced in 3 different growth chambers with days/nights at a controlled temperature each one (54/39°F;

64/43°F; 75/54°F) similar to the procedures of Proebsting and Mills (1978), to simulate tree different spring environmental conditions. The samples were processed at three-day intervals and classified accordingly with its hardiness.

Digital pictures were taken for the different growth stages to illustrate, identify, and define the key growth stages for apple and sweet cherry to update the charts, these pictures were combined with the data obtained from the cold hardiness exposure described previously. All information was integrated to develop a digital system that can be accessed via the AgWeatherNet (www.weather.wsu.edu) portal.

Results and Discussion

Critical injury temperatures for apple buds and flowers of Fuji, Gala, and Red delicious and cherry buds and flowers of Bing, Chelan and Sweetheart were evaluated during the winter spring seasons of 2012-2013, 2013-2014, and 2014-2015. The relationship of the cumulative percentage of dead buds and the temperature was modeled using a logistic function (Fig 1). The following equation represents the fitted model:

$$CDF = c + \frac{(d-c)}{1+e^{-K(t-G)}} \qquad (1)$$

where CDF is the cumulative dead bud flower, in a logistic growth curve (Eq. 1), *c* and *d* represent the lower and the upper asymptote respectively which means the percentage of mortality presented already in the field (*c*) and the maximum percentage of mortality (*d*), *K* is the so called 'slope parameter', t is the gradient of temperature in the freezer and *G* is the temperature where the inflexion point of the curve occurs.

Significant logistic curves ($p < 0.01$) were adjusted for each of the cultivars and for different sampling dates. An example is shown for apple for three different dates in 2013 (Fig 1). The estimated parameter values of the model and the a few corresponding dates are presented in Table 1. As the confidence intervals for the *G* parameter are different, the overlapping curves are different. This means that the cultivars are different with respect to their resistance to lethal temperature (Table 1.)

The Probit procedure was used to calculate the percent of mortality (LT) for 10, 50 and 90. The resulting LT_{10}, LT_{50}, and LT_{90} values for each cultivar and each date of sampling were then used to model the behavior over time. A quadratic function was initially developed. However, it will be necessary to complete the measurements until bloom to develop the full model. The comparison among cultivars shows that there are variations in the temperatures at which injury occurs for each of the cultivars. The pattern of the injury is different at 10, 50 and 90 for each cultivar (Fig. 2).

The cold hardiness is greatly affected by bud development, since the temperature at which the buds become injured changes over time. These results support the earlier report that changes in hardiness were observed for different dates of sampling among cultivars and size of the buds. Buds from the first two sampling dates were less sensitive to cold temperature as compared to the latest sampling dates (Fig. 3). This shows that plants at the latest dates had less hardiness and that the deacclimation process has begun.

Until now there is a quadratic relationship between LT and the day when the sampling was conducted. We found this relationship for both apples (Fig. 3) and for cherries (Fig. 4). However, more data are required for model development. Each point represents the value of the temperature where the buds was frozen and dead on that date.

We also developed a simple decision support tool to present the cold hardiness data on the AgWeatherNet portal. For both sweet cherries and apples the user can select one of the three cultivars that are sampled, the two locations where the samples are collected and either the current growing season or past growing season for which data are available. These tools are currently still under developed and require evaluation by stakeholders. An example for apples for Fuji for the complete 2013-2014 growing season is shown in Fig. 5 and for cherries for Bing is shown in Fig. 6. In addition, we provided several early warnings during both the 2013-2014 and 2014-2015 growing season using the alert system of AgWeatherNet.

The initial results obtained from this project are very promising, although so far we only have one season of complete data for the 2013-2014 growing and limited late winter-early spring data for the 2012-2013 growing season and late fall-early winter for the 2014-2015 growing season due the funding cycle of this grant. The results confirm earlier cold hardiness observation obtained with the DTA system by others. However, our results have been collected at a weekly basis during the entire fall-winter-spring season, showing the change in acclimation and deacclimation over time of the reproductive buds and flowers. Our results also confirm that the DTA system cannot be very well used for apples. Therefore, we developed an automated sampling system that has worked well during the past growing season. The cold hardiness results obtained with the vending system are similar to those obtained with the DTA system. The initial data sets collected are insufficient for model development, but have shown a trend that can be easily reproduced during the growing seasons of this project. However, further data collection is needed for model development and evaluation. We already implemented the initial observations on the AgWeatherNet portal. Once the model is complete, hopefully during the second phase of this project, both the current season observed data as well as the modeled cold-hardiness data will be disseminated via the AgWeatherNet portal. The decision support system can then also be linked to an automated alert system to inform growers when conditions are favorable for potential damage to occur.

Table 1. Estimated parameters values of the logit model fitted for selected sampling dates for the three apple cultivars that are being evaluated.

Cultivar	Sample Date	*d*	*C*	*K*	*G*	95% Confidence Limits (G)	
Fuji	10/23/2013	1	0.1	-1.0	11.8	10.6	13.0
	11/22/2013	1	0.0	-0.6	-6.1	-7.1	-5.0
	12/16/2013	1	0.0	0.1	-25.1	-26.2	-23.9
	01/06/2014	1	0.0	0.1	-12.5	-13.3	-11.6
Gala	10/23/2013	1	0.0	-1.0	12.3	11.6	13.1
	11/22/2013	1	0.0	-0.5	-4.8	-5.3	-4.2
	12/16/2013	1	0.1	-0.6	-18.3	-19.4	-17.2
	01/06/2014	1	0.0	-0.5	-8.9	-11.1	-6.6
Red Delicious	10/23/2013	1	0.0	-1.5	11.2	10.6	11.8
	11/22/2013	1	0.0	-0.5	-4.9	-6.5	-3.3
	12/16/2013	1	0.0	-0.6	-13.8	-14.3	-13.2

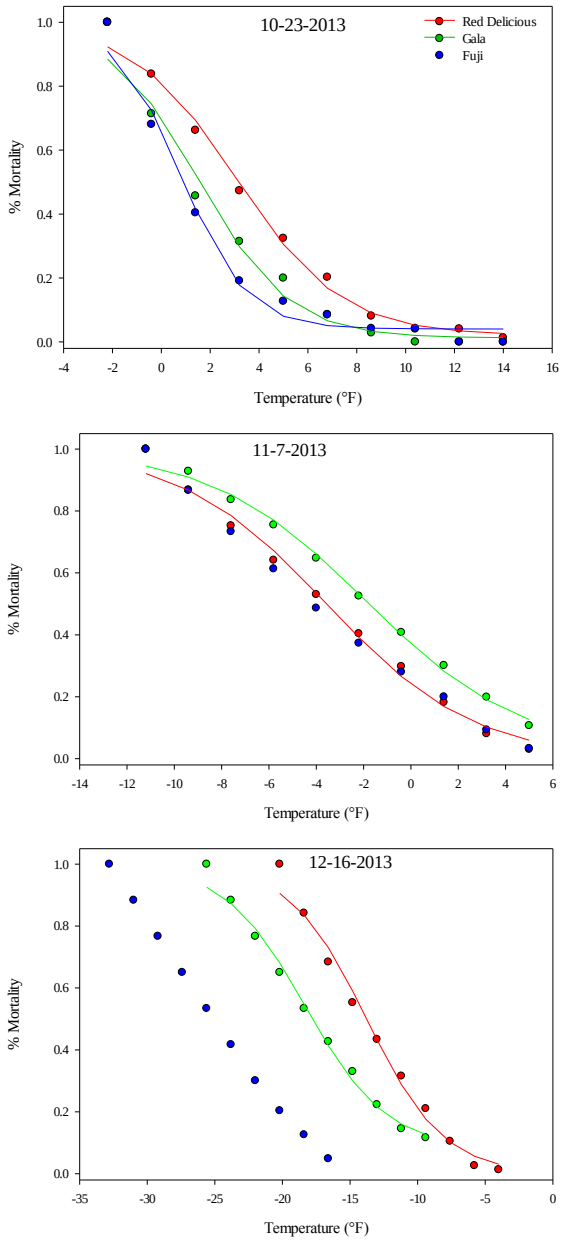

Figure 1. Probability of injured buds as function of temperature for apple cultivars at different evaluation dates.

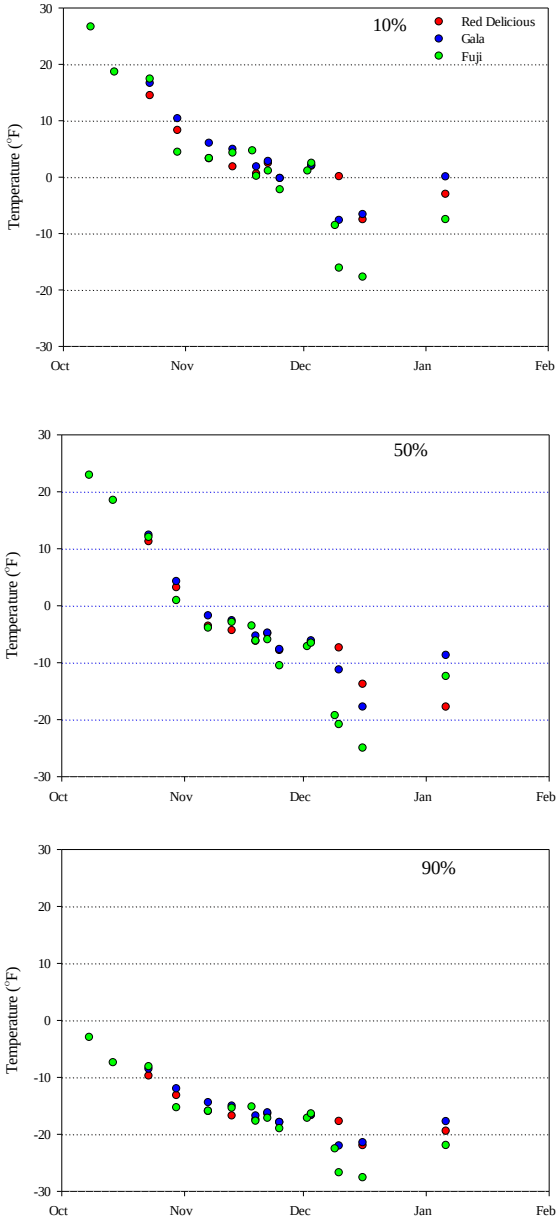

Figure 2. Seasonal pattern comparison of the LT temperatures (10, 50, and 90%) for the three apple cultivars evaluated on different dates in 2013

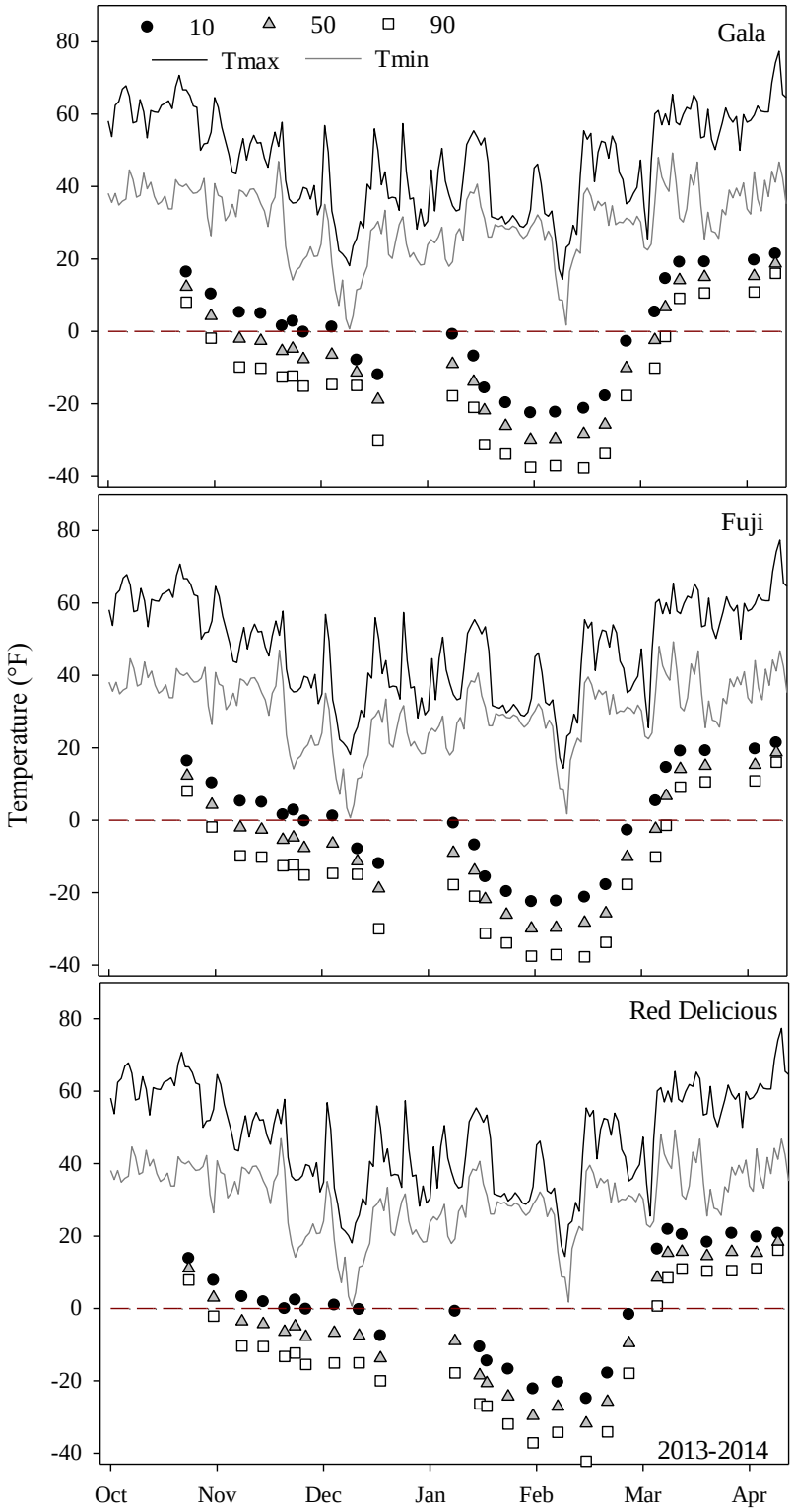

Figure 3. Daily maximum (Tmax) and minimum (Tmin) temperature for the 2013-2014 winter season and early spring for Prosser, WA, and progression of the critical injury temperatures for Gala, Fuji and Red Delicious. 10, 50 and 90 represent the lethal temperature (LTs) for each cultivar.

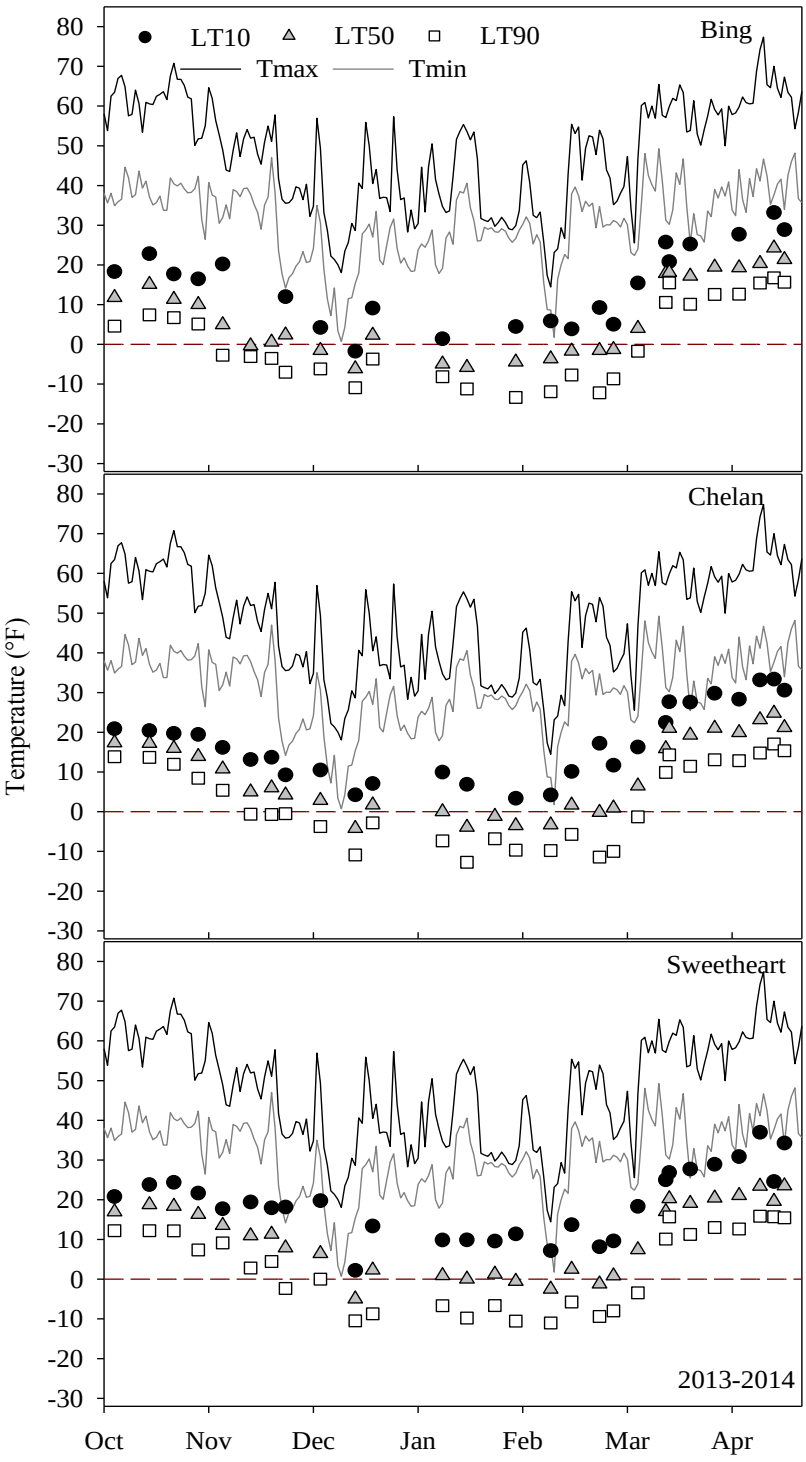

Figure 4. Daily maximum (Tmax) and minimum (Tmin) temperature for the 2013-2014 winter season and early spring for Prosser, WA, and progression of the critical injury temperatures for Bing, Chelan and Sweetheart. 10, 50 and 90 represent the lethal temperature (LTs) for each cultivar.

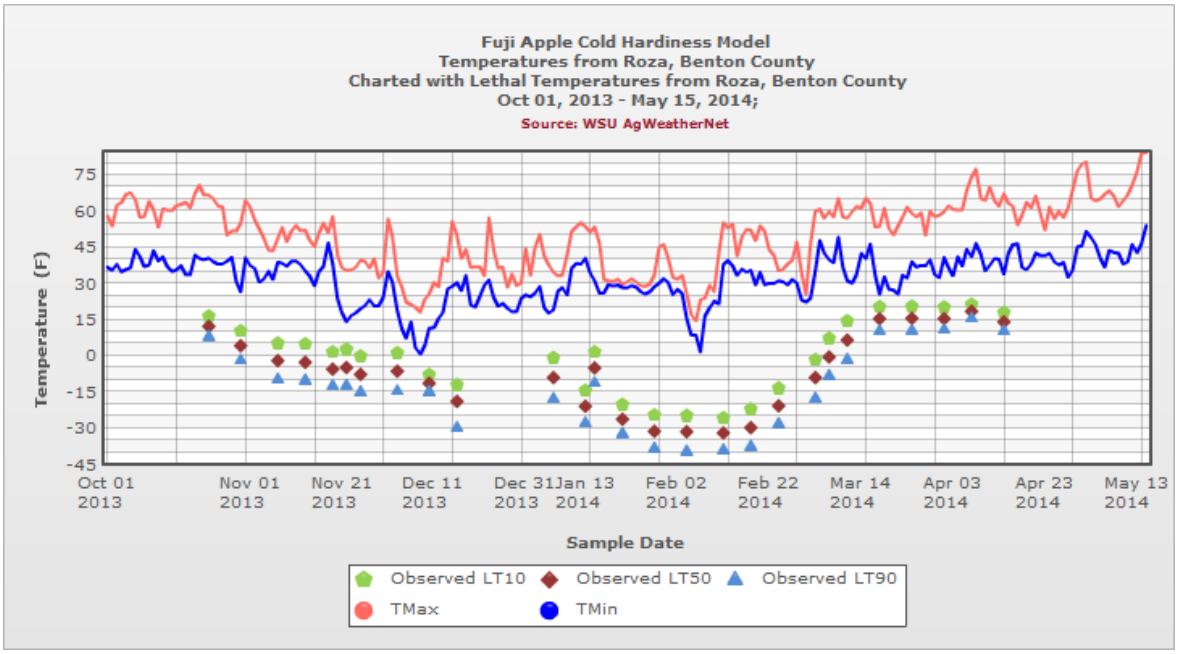

Figure 5. Data locally collected available through the AgWeatherNet web site at http://www.weather.wsu.edu/awn.php?page=cherrycoldhardness during 2013-2014 winter season and early spring for Prosser, WA, and progression of the critical injury temperatures for Fuji.

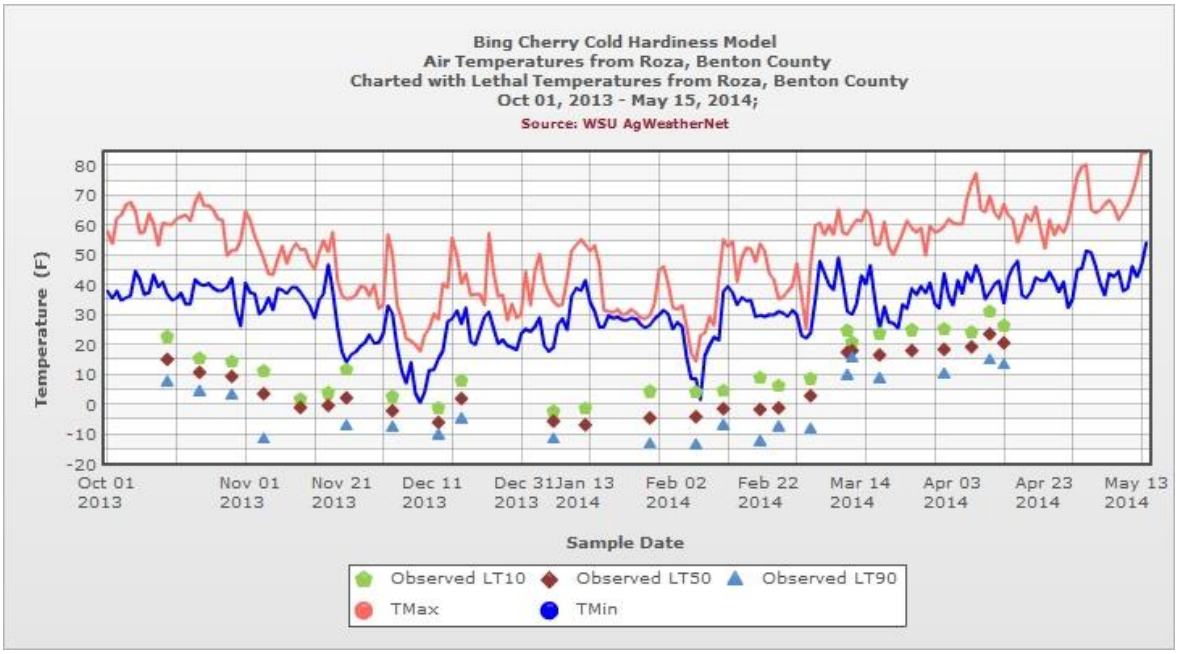

Figure 6. Data locally collected available through the AgWeatherNet web site at http://www.weather.wsu.edu/awn.php?page=cherrycoldhardness during 2013-2014 winter season and early spring for Prosser, WA, and progression of the critical injury temperatures for Bing.

EXECUTIVE SUMMARY

Temperature swings during the fall and early spring are common in the Pacific Northwest, but they can have a major impact on tree fruit crops such as apples in cherries. Low temperatures during early fall can damage buds that have not been exposed yet to low temperatures and are thus not cold hardy. During the spring early bud development or bloom can also be impacted by low night temperatures, especially during clear sky conditions at night. The overall goal of this project was to investigate the effect of early spring temperature on apples and sweet cherries at different developmental stages and to determine the hardiness. We used a traditional DTA methodology through exposure to freezing temperatures. For apple buds, apple flowers and cherry flowers we designed and built an automated sampling system that was installed in a controlled freezer, referred to as the "vending" machine.

- We found that there are differences in hardiness and lethal temperature during different phenological stages for the same cultivar as well as among the sweet cherry and apple cultivars.
- We found that there was a variation in cold hardiness for the different bud sizes of apples for the same sampling date and that there were differences among phenological stages.
- We found that the "vending" machine can be used for determining cold hardiness of samples that are not suitable for the DTA technology.
- We developed a decision support tool for the AgWeatherNet portal (www.weather.wsu.edu) where the cold hardiness information for cherries and apples can be posted.
- We distributed several alerts using the AgWeatherNet portal and alert distribution system during the 2013-2014 and 2014-2015 growing seasons based on the data we collected and the predicted low air temperatures.

This project was the first phase where we emphasized the development of appropriate methodologies for the determination of cold hardiness of apple and cherry flower buds and flowers. Further work is need for collection of additional data representing different growing seasons and this different weather conditions. Further work is also need to determine the impact of relative humidity and dewpoint temperature on cold hardiness. Additional data are also needed for the development and evaluation of robust models that can be implemented on the AgWeatherNet portal. The project could also be expanded to add other cultivars for apples and sweet cherries or to add other tree fruit crops, such as peaches.

CONTINUING PROJECT REPORT **YEAR**: 2014 (2 of 3)

WTFRC Project Number: TR – 13 - 100

Project Title: Technology Roadmap Implementation
PI: James Nicholas Ashmore
Organization: James Nicholas Ashmore & Associates
Telephone: (703) 517 5439
Email: nickashmore@cox.net:
Address: 9094 Blue Jug Landing
Burke, VA 22015-2106

Cooperators: NONE

Total Project Request: **Year 1: $**36,000 **Year 2:** $36,000 **Year 3: $36,000**

Percentage time per crop: Across Crops
(Efforts focused on policy, programs and procedures, and precedents for all crops)

Other funding sources: None

WTFRC Collaborative expenses: None

Budget
Organization Name: James Nicholas Ashmore & Associates
Contract Administrator: James N. Ashmore
Telephone: (703) 517 5439 **Email address:** nickashmore@cox.net

Item	2013	2014	**2015**
Salaries	$36,000	$36,000	$36,000
Benefits			
Wages			
Benefits			
Equipment			
Supplies			
Travel			
Plot Fees			
Miscellaneous			
Total	$36,000	$36,000	**$36,000**

Consultant activities in the Fourth Quarter (October, November, December), 2014 included but were not limited to the following:

- Continued efforts to explore possible administrative routes that would lead to the enhancement of pear breeding research in the Pacific Northwest;
- Continued to work with the Commission Manager and leaders of the Northwest Horticultural Council to explore possible options/strategies for involvement in the implementation of the Agricultural Act of 2014 (H. R. 2462, P. L. 113-79), focusing especially on the specialty crops provisions of the new statute and on how best to become involved in the FFAR process to move the USDA further in embracing the roadmap concept and enhancing how USDA displays research proposals and results in a roadmap format;
- Continued monitoring of efforts relative to the Clean Plant Network and worked with Dr. Mike Willett of the Northwest Horticultural Council on this issue to insure that there is no significant disruption in funding for this important program and to explore how that program might be affected by sequester in the future;
- Continued to monitor developments of appropriations legislation by the House and Senate to determine how those developments would impact on programs of importance to the Washington producers;
- Continued to monitor developments of the budget and impacts of sequestration on funding for relevant agencies and programs;
- Continued to maintain contact with Hill offices to review and discuss political developments as they occur in this election cycle;
- Continued to strengthen our relationship with our industry partners, the Congress, and the Administration as we work together on the implementation process of the Agricultural Act of 2014;
- Monitored other issues of possible concern to the state growers, including but not limited to on-going discussions/developments relative to pollinators and colony collapse disorder, water issues, and other complex environmental issues that could impact operations of tree fruit production in the Northwest;
- Continued efforts and discussions with Hill staff relative to the importance of establishing a process to expedite the movement of research results into commercial use; and,
- Took such other action, as appropriate, to advance research initiatives for the tree fruit research industry, especially as they relate to producers in the State of Washington.

CONTINUING PROJECT REPORT **YEAR: 1 of 2**
WTFRC Project Number: TR-14-100

Project Title: Mechanical pollination for yield security

PI: Matthew Whiting
Organization: WSU
Telephone: 509 786-9260
Email: mdwhiting@wsu.edu
Address: 24106 N. Bunn Road
City/State/Zip: Prosser/WA/99350

Cooperators: OnTarget Spray Systems, Firman Pollen Co., Olsen Brothers, Hayden Farms

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**

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 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

METHODS:

This project will evaluate the potential to pollinate and fertilize tree fruit flowers with applications of liquid pollen suspensions through commercial electrostatic sprayers. This research project will combine small-scale lab/greenhouse trials with larger-scale field trials. In the first year we will carry out lab/greenhouse trials to evaluate the following key components of the mechanical pollination system:

1. The effects of the application system on pollen viability
2. The deposition of pollen on the stigma
3. The density of pollen in the suspension
4. Suspension formulation to extend pollen viability

Experiments will address each issue separately.

1. ***The effects of the application system on pollen viability***
   Using a small ATV sprayer (Figure 1) loaned from On Target Spray Systems we will pass a pollen-water mixture through the system and collect pollen 3 feet from the nozzles. A series of experiments will be setup for 3 pollen genotypes for sweet cherry, apple, and pear. Pollen will be collected on petri dishes that will be oriented vertically and half-filled with our agar-based media. We will setup a tower with three petri dishes at three heights (3, 6, and 9 feet off the ground) and pull the sprayer past the tower at recommended application speed. The plates will be collected and we will evaluate pollen deposition density (i.e., pollen grains/cm^2). The petri dishes with pollen will be left at room temperature and the germination of pollen will be evaluated after 6 hours and 24 hours in the same dishes. Data

will be recorded as pollen viability (%) and will be compared to viability of pollen that has not passed through the system (control). This process will be repeated at 10-minute intervals. More specifically, we will load the sprayer with the pollen mix and run the application system past a new tower of petri dishes every 10 minutes. This will reveal the viability of pollen in the sprayer, determining the length of time that pollen will remain viable in the tank. As we improve the suspension formulation (section 4), we will re-evaluate the improved suspension by the process outlined above. In addition, if pollen viability is low, we will work with On Target Spray Systems to develop means for minimizing the effect of the application system on viability.
Outcome: determine the viability of pollen that has passed through an electrostatic application system; better understand the application window for applying pollen

2. ***The deposition of pollen on the stigma***
Using the same application system and process outlined above we will determine the efficacy of the deposition to the stigmatic surface of apple, pear, and sweet cherry flowers. To accomplish this we will use flowering cut branches of these three species placed in buckets of water and oriented vertically to the passing application system. Branches will be cut from commercial orchards and forced to bloom in controlled climate chambers. At ca. 50% bloom, the limbs will be held in a vertical position (i.e., parallel to tower of nozzles) at 3 feet from the passing sprayer. As above, the sprayer will be pulled past the flowering branches at the recommended application speed. Flowers will be collected post application and the deposition of pollen on the stigmatic surfaces will be assessed microscopically. Flowers will be collected from 3 orientations: (1) facing the sprayer, (2) at 90^{o} from the direction of the spray, and (3) 180^{o} from the direction of the spray (i.e., facing away from the sprayer).

3. ***Pollen density***
We will also evaluate the role of pollen density in the suspension (i.e., 'rate' of pollen) on the deposition on the stigma. We will use a rate of 30 g/acre (per Firman Pollen Co. recommendation for dry application) to begin and adjust accordingly if necessary. The goal is to ensure adequate pollen deposition – our evaluations of deposition outlined above will reveal whether it will be necessary to adjust pollen 'rate' to get sufficient pollination. If 30 g/acre is insufficient, we will conduct a rate trial up to 4x (as outlined above, to flowering branches) and assess pollen deposition.
Outcome: improve the efficiency of the system by optimizing the rate of pollen

4. ***Pollen suspension formulation***
We have preliminary evidence that a well-mixed pollen + water suspension maintains pollen viability for up to 30 mins. We will investigate the possibility of improving pollen viability (for apple, pear, and sweet cherry) in the suspension and uniformity of the suspension by including additional compatible solutes/solutions, a surfactant (e.g., Triton X-100) as well as utilizing polyethylene glycol as the carrier liquid. These will be lab-based tests in which a fixed quantity of pollen (1.0% weight:volume) will be dispersed into the liquid and agitated briefly. Aliquots will be removed at 10-minute intervals and transferred to germination media in petri dishes. Pollen hydration and germination will be evaluated after 6 hours at room temperature.
Outcome: improved application efficiency by optimizing pollen viability

Field trials
We will complement the lab tests outlined above with field trials in commercial orchards. We propose to evaluate the effects of four pollination treatments:

1. Untreated control (standard grower practice, bee-pollinated)
2. Supplemental mechanical pollination applied once at 50% bloom
3. Supplemental mechanical pollination applied once at full bloom
4. Supplemental mechanical pollination applied at 50% and full bloom

These treatments will be applied with grower collaborators using On Target Spray Systems application systems and pollen donated by Firman Pollen Company. We will work in year one with one apple grower, one pear grower, and two cherry growers (one in Yakima Valley, one in Wenatchee). Each trial will be conducted similarly with 3 replications of each treatment. Each replication will require treatment to two entire rows, leaving at least two guard rows between treatments. This will facilitate the generation of large-scale yield data (i.e., bins/acre). We will also collect yield data on individual tree basis and collect fruit samples to assess fruit quality. In addition, in each orchard we will collect data on fruit set by counting flowers (or clusters in apple/pear) on a minimum of 10 limbs per rep.

In addition, to evaluate the potential to entirely replace bee-mediated pollination we will use bee exclusion bags on a minimum of 5 limbs in each of the experiments above. Just prior to flowering the bee exclusion bags will be utilized to cover flowering limbs. The bags will be removed immediately prior to the mechanical pollination treatments and replaced immediately thereafter. By doing so, we will be able to estimate fruit set achieved strictly from the mechanical pollination treatments.

Trials in year two will build from our first year's results. In year two we will conduct the field trials with the best outcomes from the lab and greenhouse trials of year one. We anticipate the need to modify the spray system and have a commitment from On Target Spray Systems to do so, as needed, with their collaboration.

RESULTS AND DISCUSSION

Field trials

In 2014 we conducted several field trials as well as lab studies. 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 (these were our first two trials). We discovered that our pollen mixture was clogging the sprayer's filter and we had trouble with the nozzles getting plugged too. We believe this was due 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 (www.pollen-tech.com). We will not use their proprietary slurry in future 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 (Fig. 1).

We 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. 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 (Fig. 3).

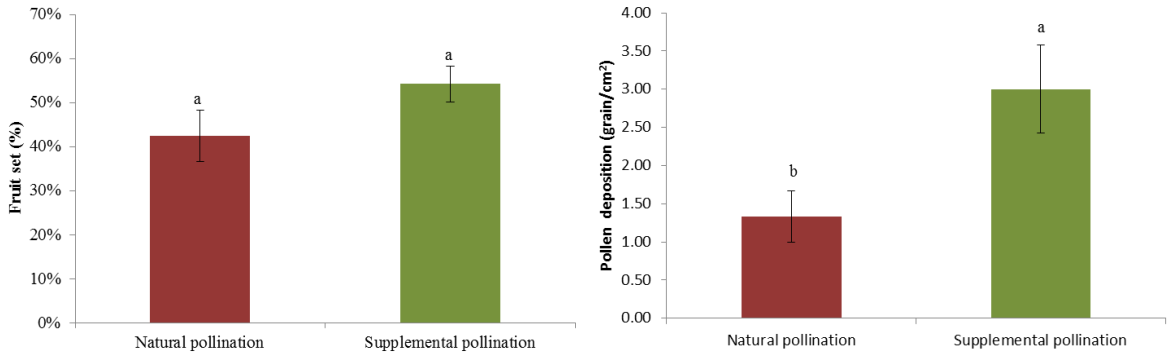

Figure 1: Effect of supplemental pollination (two applications) on fruit set in 'Tieton' sweet cherry (p-value: 0.120) and pollen grain deposition on the stigma (p-value: 0.067).

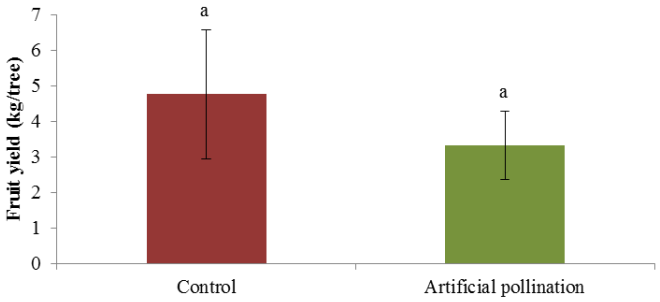

Figure 2: Yield of sweet cherry 'Bing'/ 'Gisela®6' (p-value: 0.449) under natural pollination (control) and artificial pollination (applied at 25%FB+75%FB under bee exclusion netting).

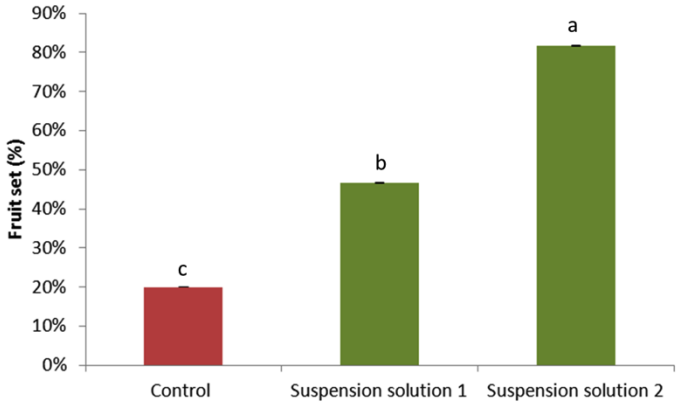

Figure 3: Fruit set in 'Gala' apple branches upon pollen suspension treatment applied to flowering limbs at about 50% and 90% full bloom. Treated limbs were enclosed by bee boxes throughout flowering. Control was natural, open pollination (p-value: 0.000).

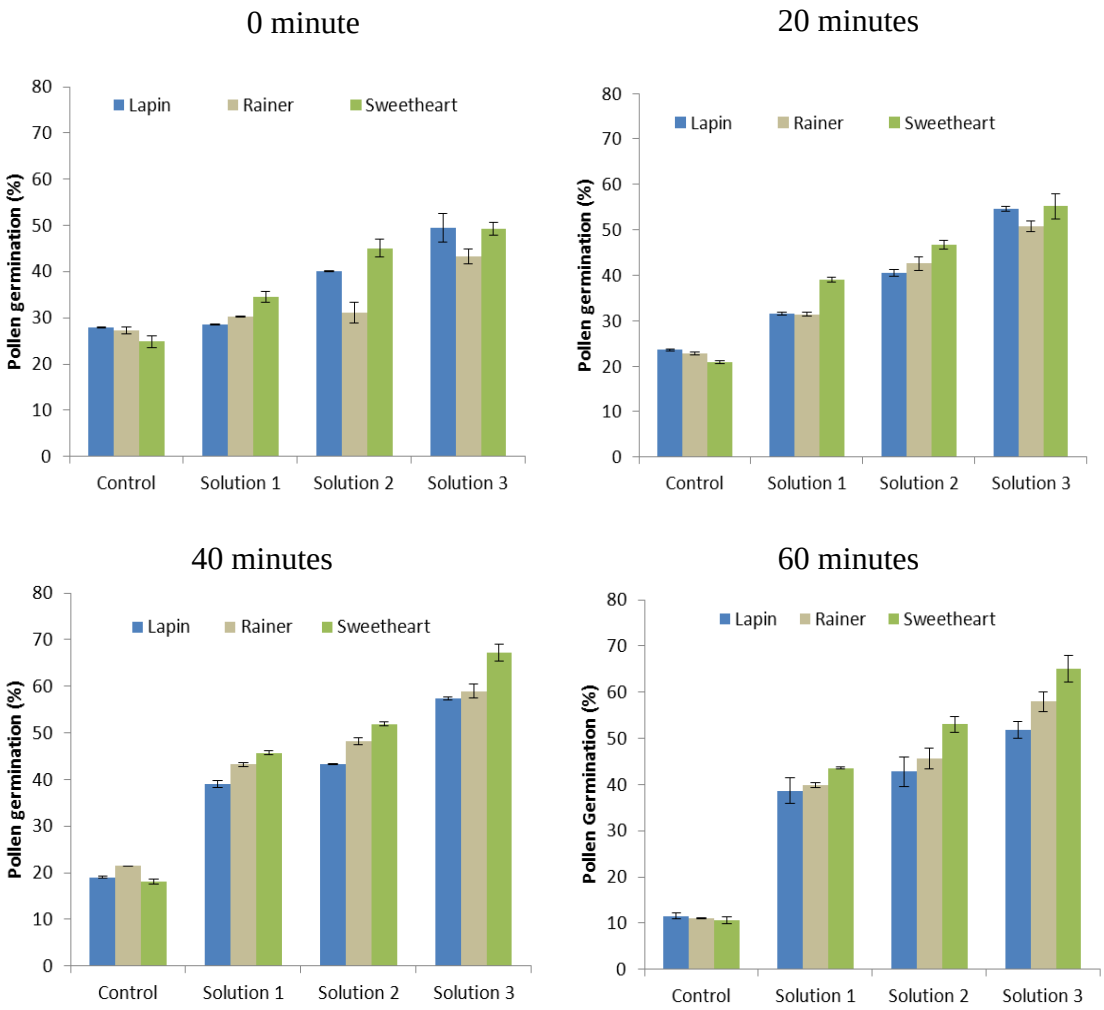

Figure 4: Effects of suspension media on three sweet cherry *in vitro* pollen germination after 0 minute (upper left), 20 minutes (upper right), 40 minutes (lower left) and 60 minutes (lower right) in suspension (all p-values: 0.000).

Lab studies

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 grain. 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 (Fig. 4). 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 (Fig. 4). 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). Our continued studies are evaluating additional suspension components, assessing pollen viability over time. These studies are currently underway for apple, pear, and sweet cherry and will yield a new pollen suspension medium that we will use for field trials in the spring of 2015.

Overall, our preliminary studies into the development of an artificial pollination system are promising. We have shown that pollen can be suspended in solution, maintaining viability for at least an hour; can be applied through a commercially-available electrostatic application system with only minimal modification; and that this pollen will reach the stigmatic surface and supplement standard pollination practices but also potentially replace the current system that relies on pollenizers and pollinators.

Figure 5. Bee exclusion netting applied to whole trees (left) at the WSU Roza orchards and single limbs (right) for replacement pollination studies. Pollen was applied through the netting to flowers opening inside the cages.

CONTINUING PROJECT REPORT **YEAR:** 2 of 3
WTFRC Project Number: TR-13-101A

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

PI: Karen M. Lewis
Organization: Washington State University
Telephone: 509-754-2011 X 412
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Address: POB 37 Courthouse
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Co-PI (2): Matthew Whiting
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City/State/Zip: Prosser, WA. 99350

Co-PI (3): Stefano Musacchi
Organization: TFREC, Wenatchee
Telephone: 509-663-8181
Email: stefano.musacchi@wsu.edu
Address: 1100 N. Western Ave.
City/State/Zip: Wenatchee, WA. 98801

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. We will follow four steps to achieving this goal:

(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

SIGNIFICANT FINDINGS

- Hand pruning removed twice as much wood as mechanical pruning in 'Tieton' sweet cherry trained to the UFO system (postharvest pruning)
- Mechanical pruning was 23 times faster than hand pruning (hedging and topping combined) in sweet cherry
- Hand dormant pruning removed 3 times more wood than mechanical dormant pruning in Fuji trained to a slender spindle system
- Mechanical pruning was 2 times faster than hand pruning (hedging alone - dormant) in Fuji apple
- A maximum of 6.5% of fruit were damaged by mechanical pruning
- The motor has to be positioned above the cutting bar; to avoid hitting the branches in the lower parts of the tree
- The machine is not suitable for Y-trellis UFO systems because of the motor that has the side bar.
- Wind can affect the stability of the bar and pruning might not be completely efficient with windy conditions.
- A high speed of the tractor causes that the bar hits the branches instead of cutting them. Probably due to the equipment that was not the most efficient to prune apple orchards.

METHODS

Sweet cherry

Mechanical pruning vs/+ hand pruning: the experiment was initiated in 2014 at an Olsen Brothers orchard in Benton City, WA. The test plot consists of 5 rows of 'Tieton' sweet cherry cultivar on 'Gisela 5' rootstock in their 8th year, trained to the 'UFO' system. The experiment is 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) 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/hr. 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. Pictures were taken before and after pruning for each treatment, and several videos were

also recorded. Return bloom, fruit quality, yield, and insect and disease presence or absence will be evaluated this year.

Preharvest and postharvest topping: this trial was also established at Olsen Brothers orchard in Benton City with 'Tieton'/'Gisela®5' in a complete randomized design, consisting of 5 different timings of hand pruning (treatments) with 5 replications, and 3 trees/rep. 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 with samples of 25 cherries/branch. 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 will be measured during the winter 2015, and later in the year fruit quality and yield will be analyzed again.

Apple

Fuji/NIC29

This trial was initiated in 2014 in a commercial orchard near Mattawa, WA. The test plot consists of 5 rows of Fuji apple cultivar on 'Nic 29' rootstock planted in 2011, trained to the 'Slender Spindle' system (planting distance 10ft x 3 ft). Five treatments were assigned as a Complete Randomized Design with 3 repetitions; each repetition comprises 2 plots with a total of 24-26 trees. Trees in the test plots were hedged mechanically or by hand at different timings (1) mechanical dormant pruning, (2) dormant hand pruning, (3) dormant hand pruning plus mechanical summer pruning at 12-15 leaves stage, (4) mechanical dormant pruning plus mechanical summer pruning at 12-15 leaves stage, and (5) dormant hand pruning plus mechanical dormant pruning at 20 leaves stage. Mechanical pruning was performed with the sickle bar at 10 cm of the canopy and a tractor speed of 1.2 m/h. Hand pruning was performed by Dr. Stefano Musacchi in the trial plots. Time to prune each plot and weight of the wood pruned were recorded for each treatment. Data were collected only in the three middle rows and the others were used to set the tractor speed and the sickle bar orientation. Pictures were taken before and after pruning for each treatment.

During the summer, green thinning was performed and the total number of fruit damaged per plot by the pruning was counted. The trunk circumference of all the trees in the three middle rows was measured and trunk cross sectional area was calculated for each tree. For the summer pruning treatments, the total weight of wood and leaves removed was recorded for each repetition, plus the weight of the wood alone and the separated leaves of 3 repetitions randomly selected. A subsample of 10% of the total leaves' weight was separated for each repetition and a leaf area meter was used to assess the total leaf are per repetition. Then, the samples were put in an oven at 62°C until they reached a constant weight, to determine dry weight.

Re-growth, return bloom, fruit quality and yield will be measured in 2015 season. Observations will be made concerning wood damage, and insect and disease incidence.

Pink Lady/M9-337

A demonstrative trial on Pink Lady/M9-337 planted in 2012 (planting distance 10ft x 3 ft) was set up in the same farm to assess the mechanical pruning also for this variety.

Three treatments were tested in three rows: winter mechanical pruning (March 2014), summer mechanical pruning (beginning of June 2014) and control (traditional hand winter pruning).

Only in summer we started to collect some data since the response seemed promising for this variety.

Fruit were harvested on October 2nd for all the treatment (3 plots with 10-11 trees each); fruit from 3 representative trees per plot were sized to assess the fruit size distribution at harvest. Trunk diameters were measured for all the harvested trees to calculate the trunk cross sectional area (TCSA).

A sample of 60 fruit (size 85 mm) per treatment was collected for quality analysis. Weight, background and over-color, DA index, starch, firmness, SSC (brix), acidity were estimated.

RESULTS AND DISCUSSION

Pruning is a labor- and time-demanding operation that generally represents the second greatest annual expense for tree fruit growers worldwide. Previous research into mechanized pruning in traditional orchard architectures yielded negative results. As orchardists adopt new two-dimensional, planar systems there is improved potential for the adoption of automation and mechanization technologies, including pruning. To date, there are few studies on the effectiveness of mechanical pruning in the top two tree fruit crops in the PNW - apple (*Malus domestica* Borkh.) and sweet cherry (*Prunus avium* L.). The goal of this project is to determine preliminary best management practices for pruning PNW apples and sweet cherry with a commercial sickle bar mechanical pruner.

Sweet cherry

Mechanical vs/+ hand pruning: Our preliminary results reveal that mechanical pruning was 23 times more efficient (s/tree) than hand pruning for sweet cherry (Figure 1). Each tree was pruned approximately in 16 s with the sickle bar, whereas by hand in 374 s per tree per person (6 min). The greatest variations in time of mechanical pruning within repetitions occurred because of the presence of sprinklers in the rows for evaporative cooling; however, these variations were not significant. This improvement in pruning efficiency occurred despite the need to pass each tree 3 times with the machine (hedging the east and west sides plus topping). The efficiency of mechanical pruning will be directly related to the tractor speed.

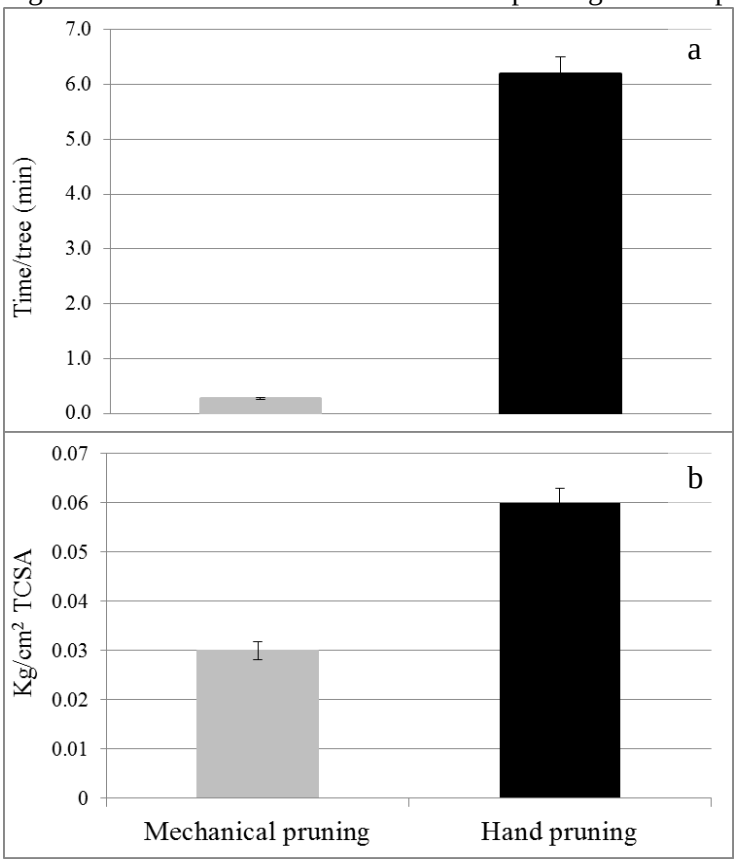

Figure 1. Pruning time per tree (min) for 'Tieton'/Gisela 5 trees pruned either mechanically or by hand. Figure 2. Kg of wood removed/ cm^2 of trunk cross sectional area (TCSA) removed by the pruning treatments. Data represent the mean +/- SE.

Hand pruning removed 2 times for wood than mechanical pruning (Figure 2) with 10 kg wood removed/tree approximately. Most of the cuts performed by hand were thinning cuts; differently, the machine made heading cuts. It is believed that hand pruning removed more wood than mechanical pruning because it is more selective. It was also observed that some of the branches were scrapped and/or peeled by the machine, which was identified as "dirty cuts" compared to cleaner cuts made by hand. (Figure 3).

Figure 3. a) "Dirty cuts" performed by the hedger compared to b) wood pruned by hand.

Recent observations were made in the trial block, evidencing that the wood has been healing without problems; nonetheless, further evaluations will be performed to corroborate the absence of diseases or insect impact. We are planning to investigate the effect to the combination of mechanical pruning and hand pruning this year.

Apple

Fuji/NIC29

Dormant mechanical pruning was 2 times faster that hand pruning with a tractor speed of 1.2 m/h (Figure 4), with an average of 9 sec/ pruned tree with the bar. We believe that the sickle bar was not as effective as expected because the machine was used for the first time with this trial. General observations of the performance of the machine allowed troubleshooting some issues and improving the performance of the hedger for the summer pruning. An initial tractor speed of 1.8 m/h was tested yielding negative results, at this speed the sickle bar hit the branches instead of cutting them, causing damages in the structure of the tree. In the summer, at 12-15 leaves stage and 20 leaves stage, the average time to prune a tree was approximately 4 seconds.

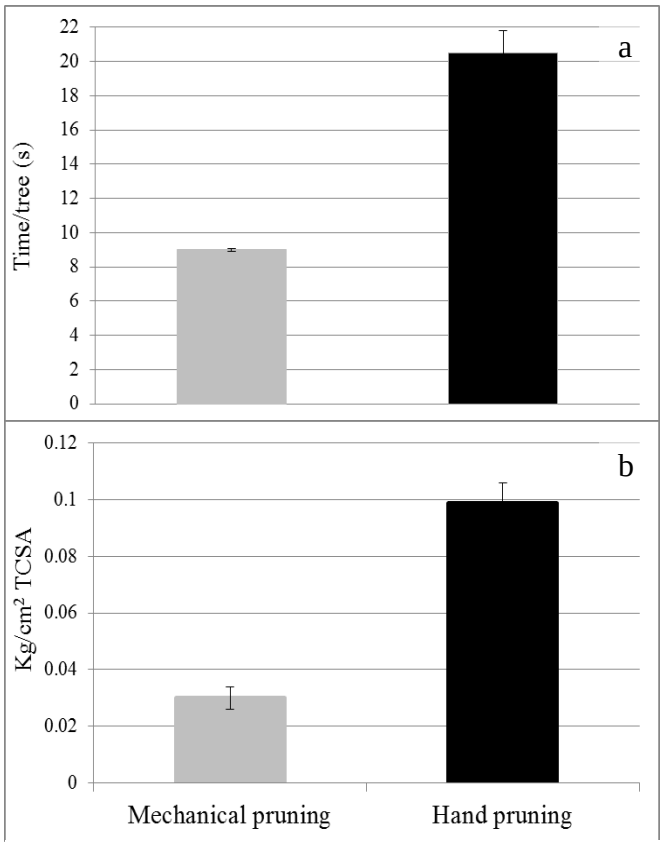

Figure 4. Time per tree (s) of 'Fuji'/Nic 29 dormant mechanical and hand pruning in 2014. Figure 5. Kg of wood removed/ cm^2 of trunk cross sectional area (TCSA) with dormant hand and mechanical pruning of 'Fuji'/Nic 29 in 2014

Dormant hand pruning removed 3 times more wood than dormant mechanical pruning with an average of 1.03 kg of wood removed/tree (Figure 5). Similarly to sweet cherry, we observed "dirty cuts' performed by the machine mainly due to the kind of equipment utilized, and the effect on wood will be evaluated next season. Of course hand pruning was more selective than the machine and most of the cuts were thinning cuts. In 2015 we will combine the hand pruning with mechanical.

After summer pruning, a maximum of 6.5% of damaged fruit/tree was observed before green thinning. We expect that a thinning crew could easily recognize these fruit and remove it during thinning.

A distribution graph was elaborated for the apple trial because of the variability that exists in the Fuji's block. Due to the site conditions and especially the type of soil, some trees are not uniform. We have identified that between 9-11 cm^2 of TCSA there is the least variability in the orchard, and we have chosen trees in this range to evaluate yield, fruit quality, return bloom and re-growth. Unfortunatelly, we lost the 2014 Fuji harvest and we could not evaluate yield and fruit quality. We will evaluate this parameters with next production (2015).

Pink Lady/M9-337

In summer, the mechanical pruning cut one tree in 1.78 sec, removing 0.32 kg of fresh material/tree (62.5% leaves and 37.5% wood) and 9 fruits/tree, as average.

Fruit size distribution assessment revealed that the mechanical pruning had a beneficial effect on the fruit size in the highest size classes (85 and >90 mm equal to 3.35" and >3.54") in comparison to control (Figure 6). Yield data showed a higher production for summer pruning and control than winter pruning. Winter pruned trees probably were more affected by this practice since they had less fruit/tree than the other two treatments (Table 1).

Summer pruning fruit showed a higher starch degradation, but lower SSC and acidity than the other fruit, while winter pruning fruit resulted redder with higher vividness (data not shown).

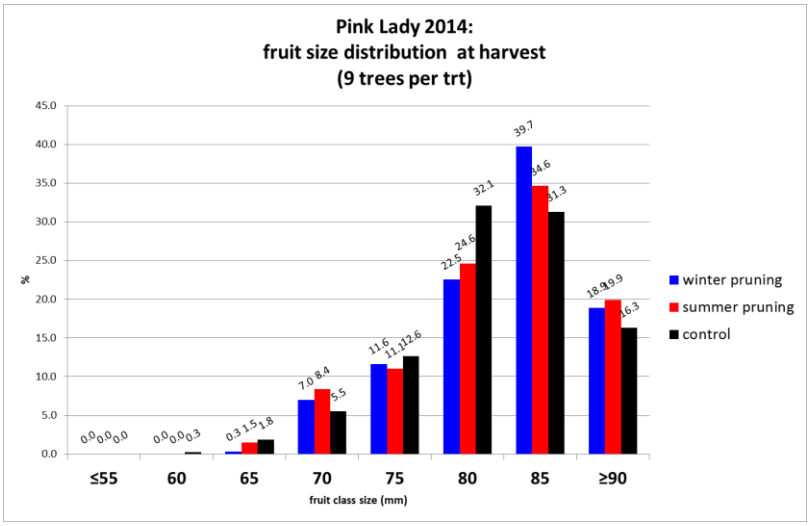

Figure 6: Pink Lady/M9-337 fruit size distribution: between winter, summer pruning and control.

Table 1. Pink Lady/M9-337 mechanical pruning trial: yield data (Mattawa, WA, Oct. 2, 2014).

Pruning treatments	number fruit/tree		net weight (kg) fruit /tree		TCSA (cm^2)		Avg. fruit weight (g)	Yield efficiency (kg/cm^2)	
Control	42	a	10.4	a	11.9	b	248	0.89	a
Summer pruning	44	a	10.9	a	13.7	a	251	0.82	ab
Winter pruning	34	b	9.0	b	12.4	b	261	0.74	b
Significance[1]	***		***		**		ns	**	

[1] $p<0.05$, *; $p<0.01$, **; $p<0.001$, ***; *ns*, not significant.

CONTINUING PROJECT REPORT **YEAR**: 1 of 3
WTFRC Project Number: TR 14-103

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

PI: Vincent P. Jones
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Co-PI (2): Ute Chambers
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Cooperators: Betsy Beers, WSU-TFREC.

Total Project Request: **Year 1:** $75,154 **Year 2: $78,160** **Year 3:** $81,285

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 plus an additional 1.0 FTE for a second programmer for one year.

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	45,567
Benefits[2]	14,983	15,582	16,205
Wages	12,480	12,979	13,498
Benefits[3]	262	273	283
Equipment	0	0	0
Supplies[4]	2,500	2,600	2,704
Travel[5]	2,800	2,912	3,028
Miscellaneous	0	0	0
Plot Fees	0	0	0
Total	75,154	**78,160**	81,285

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:

- The lower threshold of European red mites has been validated as 43.5ºF. More data is required for two-spotted spider mites diapause termination.
- The model for woolly apple aphid population growth is developed and requires field data for validation.
- The models for the two green lacewings have been completed and validated. Data analysis for the syrphid fly *Eupeodes fumipennis* and *Deraeocoris brevis* are ongoing.
- San Jose scale field observations matched the model predictions for 1st generation crawler emergence, while first male flight occurred earlier than predicted. More field data and in-depth analysis are needed to draw final conclusions about the quality of the current model.

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

Methods:
The models for European red mite (ERM) and two-spotted spider mite (TSSM) were developed from the literature data last year. Both of these data sets were extensive and may allow us to develop more comprehensive models such as those already developed for codling moth, obliquebanded leafroller, and the two lacewings *Chrysopa nigricornis* and *Chrysoperla carnea*. As stated in the grant proposal from last year, a big concern for both ERM and TSSM was the timing of overwintering egg hatch (ERM) and when adult females break diapause (TSSM).

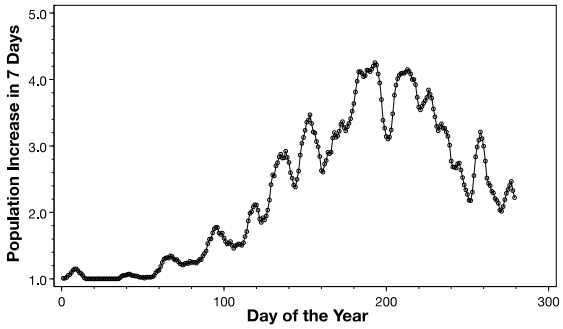
We obtained data for the egg hatch of ERM this past year by collecting overwintering eggs on twigs and bringing them to the lab, placing them into growth chambers and recording daily when egg hatch occurred. The ERM eggs were held at either 61ºF or 72ºF. In addition, we evaluated sticky tape as well as beat samples in three orchards 1-2 times a week for emergence of eggs (ERM) and the incidence of diapause coloration in the overwintering females (TSSM). Temperature data was collected for all sites using loggers installed in the orchards and/or records of the nearest AWN station.

Results & Discussion:
ERM: As mentioned last year, there were two conflicting times for emergence of ERM overwintering eggs. Our field data and the lab data agreed strongly with the records found in the literature from Yakima (this was data from 1922) and from four other studies. This means our lower temperature threshold is 43.5ºF. Average egg hatch of the incubated ERM was observed at 409 DD. However, in the orchard, average egg hatch occurred at 327 DD. We suspect that solar radiation, which can substantially increase the temperature of the tree bark where the ERM eggs overwinter, led to this accelerated development in terms of cumulative degree-days. We still need to synthesize our field data and evaluate how close the phenology is from our initial ERM model that was developed last year.

TSSM: Only one of the three monitored orchards had enough spider mites present for analysis (one orchard was sprayed for mites; the other had very low infestation). The beat and tape samples of the highly infested orchard showed that first post-diapause females, characterized by a greenish body

color as opposed to orange or red, were observed at 100 DD. At 550 DD and thereafter all females found in the samples had broken diapause. More in-depth analysis is necessary, but we hope to have it done by the research review.

Fig. 1. Population increase of WAA within 7 days over the year at WSU-TFREC in 2014.

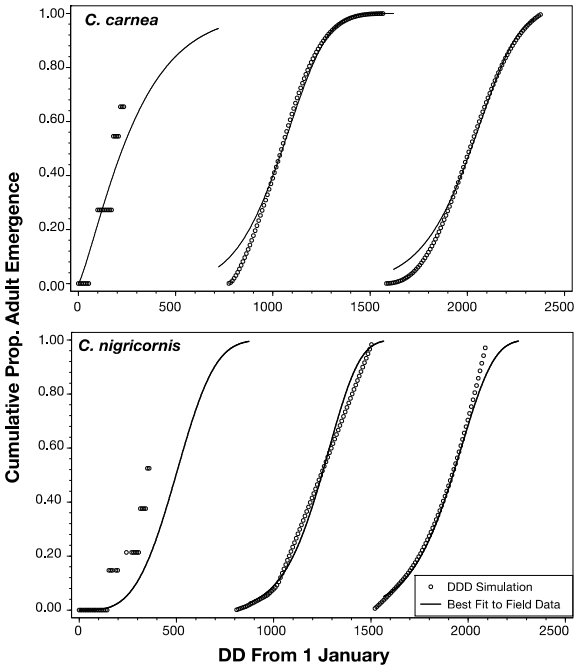


Woolly apple aphid: The literature data on WAA has been synthesized and a model that shows the population growth rate over the season has been developed. As with the ERM and TSSM, the literature review actually discovered more information than we thought and it is possible that we might be able to develop a more comprehensive model than we initially had planned. We now have the lower and upper thresholds (43.5° and 79.2°F), duration of the different stages (425 DD from egg-adult), longevity of the adults (mean = 688 DD), and the oviposition curve. We have also synthesized the population growth rates and can project population growth throughout the season (Fig 1). We also have some field data collected using sticky tapes, but more original data will be needed.

We obtained some field data from Betsy Beers (WSU-TFREC), but it appears that we will need to take more comprehensive data, including soil temperature data before we can be comfortable that the phenology is accurately predicted. Betsy's data clearly show that there are times where migration up and down the tree trunk occurs, but using the threshold data with air temperature did not give adequate predictability of the migration. At this point, we are comfortable with the growth rate projections, but not the phenology data.

Work this coming year: We will be taking more data on ERM, TSSM, and WAA this coming year, as well as data on green and rosy apple aphids. We will also begin the synthesis of the models for green and rosy apple aphids and the western orchard predatory mite (*Typhlodromus occidentalis*) and determine where holes in the data occur and begin collecting that information before the season starts.

Fig. 2. Comparison of the phenology from simulations and the best-fit equations to field data. Top: *Chrysoperla carnea*. Bottom: *Chrysopa nigricornis*.



Objective 2. Validate natural enemy models already developed in the SCRI biological control grant.

Methods:
This year, we collected additional data for the lacewing *Chrysoperla carnea*, the syrphid fly, *Eupeodes fummipennis*, and the predator *Deraeocoris brevis*. We used our natural enemy lures and beating samples to collect data from six orchards throughout the year.

Results & Discussion:
The models for the two lacewings *Chrysopa nigricornis* and *Chrysoperla carnea* are both

completed and validated and we have been able to develop the demographic models needed to evaluate pesticide effects on both species. These data were reported in the apple crop protection progress report. Both models track the phenology in the field almost exactly (Fig. 2) and we will begin to incorporate the results of the models into our management recommendations almost immediately. We expect that we should have the models themselves integrated into DAS within a year.

The data collected for *E. fummipennis* and *D. brevis* have not been fully analyzed. However, a quick review of the data showed that we still need more data, as both species are not found in high population levels in all six orchards. Three of the monitored orchards had sufficient numbers of the woolly apple aphid parasitoid *Aphelinus mali* for initial work on a phenology model.

Work this coming year: E. fummipennis and *D. brevis* may require two more years of data in more orchards than we were able to monitor this past year (because of the uncertainty of whether this proposal would be funded, we were restricted as to the number of orchards where we could get full season data from). We will increase the number of orchards we monitor the next two years and fully expect to complete these two models. For the two lacewing models, we will incorporate the results into our management recommendations this year and decide on the form in which to incorporate them and start the process on our other funding from WSU-Extension.

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

Methods:
To validate the San Jose scale (SJS) model, pheromone traps were place in three apple orchards that reportedly had SJS damage the previous year. Traps were placed in mid-April 2014 and checked twice a week. Due to low SJS male numbers caught, SJS crawler emergence was monitored in two experimental orchards using double-sided sticky tape from May through September, which was replaced 1-2 times a week and the number of crawlers determined under the microscope. This tape method also worked well, if not better, for monitoring adult male activity periods.

Results & Discussion:
Pheromone traps caught very low numbers of SJS males in two of the three monitored orchards during the first flight. Using 51ºF as the lower temperature threshold for SJS, first males were caught at 174 DD and 192 DD in the two locations. The local CM biofix (173 DD), currently used to start DD accumulations for SJS, occurred 4 and 3 days before the first males were caught in the pheromone traps, respectively. With the sticky tapes we observed two additional male flights.

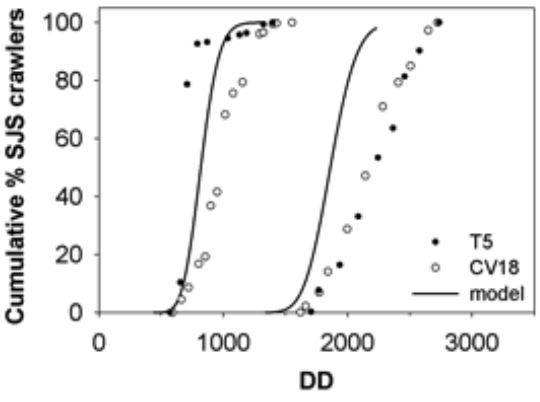


Fig. 3. Comparison of the current SJS model and field data for SJS crawlers in two experimental orchards.

Because SJS male flight is so short, the literature suggests that the first males caught in an orchard (biofix) correspond with 50% male flight in the PETE model. According to the SJS PETE model (Jorgensen et al. 1981), 50% male flight (=biofix) occurs at 275 DD since Jan 1st. The WSU Orchard Pest Management Online SJS development table uses 275 DD as the biofix for 20% male emergence. More field data of the first male flight is needed to clarify which degree-days correspond to the actual first and peak male catches in pheromone traps as well as on sticky tapes and

whether it is necessary to continue using the 275 DD as biofix.

The sticky tapes revealed two crawler generations in 2014. The first crawlers of the first generation were found at 663 DD and 655 DD in the two experimental orchards. The PETE model predicts 5% of crawlers to emerge by about 646 DD since Jan 1st (or 370 DD after the 275 DD biofix). The observed crawler emergence corresponds relatively well with the PETE model (Fig. 3). However, the second crawler emergence occurred later than the PETE model predicted. The developers of the PETE model were aware that their model only fits the first generation. However, we are hopeful that we can improve this model with additional field data to fit the entire seasonal phenology.

Work this coming year: Monitoring of SJS males and crawlers will be intensified over the remaining two years, using pheromone and sticky tapes in heavily infested orchards. We need sufficient numbers to compare observations and model predictions. The main focus will be on the crawler stage as that is the one used to time management tactics. With additional field data we feel confident to finally clear up the confusion regarding the biofix of SJS.

Reference:

Jorgensen, C.D., R.E. Rice, S.C. Hoyt and P.H. Westigard. 1981. Phenology of the San Jose scale (Homoptera: Diaspididae). *Can. Ent.* 113: 149-159.

CONTINUING PROJECT REPORT **YEAR**: 2 of 3
WTFRC Project Number: TR-13-107

Project Title: Efficient strategy to diagnose important virus diseases

PI: Ken Eastwell
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City/State/Zip: Prosser, WA 99350

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

Total Project Request: $109,256 **Year 1:** $35,000 **Year 2:** $36,400 **Year 3**: **$37, 951**

Percentage time per crop: Apple: 5% Pear: 0% Cherry: 90% Stone Fruit: 5%

Other funding sources
Agency Names: WTFRC Cherry, OSCC and CCB
Amt. awarded: $45,020 from WTFRC, but $10,000 from OSCC and CCB has not been confirmed.
Notes: Assuming full funding, the portion of the total funding to the virology program will be $16,071. The funding will help defray costs of testing grower samples and research samples from cooperators on the little cherry disease project.

Budget 1
Organization Name: Washington State University **Contract Administrator:** Carrie Johnston
Telephone: (509) 335-4563 **Email address:** carriej@wsu.edu

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[4]**

Footnotes:
1. A Post Doctoral Research Associate at 33% of full time and a Scientific Assistant at 10% of full time.
2. Benefits calculated at the state standard rate.
3. Purchase of enzymes and primers; high throughput sequencing of virus and phytoplasma isolates.
4. The requested amount is slightly higher than the $37,856 that was anticipated in the original proposal budget. The $95 increase is the consequence of higher than expected increases in staff salaries and benefits effective January 1, 2015.

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. Correct diagnosis of the underlying cause of disease is critical for implementation of effective management practices. Because of the recent escalation of little cherry disease in Washington, the agents associated with this disease are the highest priority. Reverse transcription recombinase polymerase amplification (RT-RPA) technology holds promise as a useful element in the virus diagnostic tool kit. Its application for little cherry disease and other diseases is being evaluated as appropriate. Although development of RT-RPA is emphasized, the development of sequence information and the evaluation of primer sequences is providing valuable information that can be applied in other molecular detection strategies if RPA fails to live up to expectations.

Specific objectives of this project for the next year:

1. Validate a re-designed the RT-RPA assay system for *Little cherry virus 2* (LChV2). As indicated below, the trial use of RT-RPA kits in 2014 revealed significant caveats to their use. The kit formulation and protocol is currently be modified based on our data. Once sample product is available, kit evaluation will be initiated on a limited basis on research plantings. However, as commercial fruit production approaches maturity, trees of interest (symptomatic and non-symptomatic) will be flagged. Once the fruit is harvested a side-by-side comparison of RT-RPA with other testing methods will be performed.
2. Design and evaluate a lateral flow RPA assay for Western X phytoplasma (WX) and *Little cherry virus 1* (LChV1). The evaluation of sequence is complete and primers and probes designed for the real time platform. Once the best configuration of primers and probes is verified, the reagents for the lateral flow platform of RPA will be synthesized for use on a trial basis during the late summer of 2015.
3. Develop a sequence specific diagnostic platform for detection of the agent causing apple green crinkle disease. Based on the sequence analysis derived by our project, prospective molecular assays are being designed to specially identify each of the two potential agents of apple green crinkle disease. These assays will be used to verify the association of one or both of these virus variants with the disease.

SIGNIFICANT FINDINGS:

- For the detection of LChV2, the RT-RPA assay system is much more accurate at or after fruit harvest.
- A genetic variant of LChV2 was detected in some symptomatic cherry trees.
- None of the trees identified by growers as symptomatic for little cherry disease were infected with LChV1 alone. In the absence of a second pathogen, it may be difficult to recognize trees in the orchard infected with only LChV1.
- Western X phytoplasma contributed significantly to the large number of symptomatic little cherry diseased trees. Two unique genes of the phytoplasma from Pacific Northwest isolates were identified and sequenced.

METHODS:

LChV2:

1. Data developed by our research team is provided to Agdia Inc. to develop kit components that will detect all isolates of LChV2. Options include a degenerate probe or a multiplex probe format (two probes of different sequences in a single reaction).

2. Once a kit is available with new components, the team at WSU will evaluate it with samples prepared from research samples.
3. Based on promising preliminary assessment of the redesigned kit, it will be applied to a larger number of orchard samples. Accuracy of the RT-RPA assay will be compared to RT-PCR, the most accurate system available for this virus.

LChV1 and WX:

1. Needed sequence information has been obtained to design RT-RPA or RPA for LChV1 and WX, respectively. A series of primers and probes have been designed for preliminary assessment.
2. Known infected samples will be tested with the real time RPA format to verify their utility. Not all of the parameters that affect RPA sensitivity are fully known, so real time evaluation is the most efficient method of verifying the robustness of proposed components.
3. The most successful RPA components determined in #2 above will be translated into the corresponding lateral flow RPA system and evaluated with orchard samples.

Other viruses:

1. Sequences from each of the virus sequence populations that are associated with apple green crinkle disease will be compared to those sequences that appear in viruses found in non-symptomatic trees. Unique regions of the virus genome will be used to design diagnostic primers for detection. The expression of apple green crinkle disease is very season-dependent. If no symptoms are expressed in our existing research block, research efforts will be deferred in favor of virus diseases of *Prunus* for this season.
2. Primers for RT-PCR for apricot ringpox-associated virus, cherry twisted leaf associated-virus, and cherry rusty mottle-associated virus have been designed and evaluated. The corresponding regions of the genome will be scanned for the design of RT-RPA primers and probes. The requirements for RT-RPA are slightly different to RT-PCR.

RESULTS AND DISCUSSION:

Validation of *Little cherry virus 2* (LChV2) field kits:

A diagnostic 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. and the preliminary assessment of this assay based on these new sequences is underway. It is anticipated that a re-designed RT-RPA assay kit will be available by mid-summer.

Development of an RPA test for LChV1 and WX phytoplasma:

The results from the 2014 validation experiments emphasized the need to have access to reliable diagnostic methods for all three pathogens associated with little cherry disease. Prior to this project, limited sequence information was available describing the genome of LChV1. Therefore, the development of an RT-RPA assay for LChV1 was initiated by identifying conserved regions from full to near full genome sequences from ten different LChV1 isolates. These sequences were obtained by

high throughput sequencing. 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 the Clean Plant Center Northwest. 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. Preliminary tests using the real time platform of RT-RPA with crude extracts from three LChV1 isolates yielded promising results. The lateral flow platform of RT-RPA suitable for use in field offices is being designed.

Current RT-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 RT-RPA assay. 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. To verify the potential of these regions for diagnostic purposes, sequence information of the imp and idpA regions of two WA isolates was obtained by high throughput sequencing. This sequence information along with the WX sequence from the public database enabled the design of four primers and corresponding probes targeting the imp and idpA regions. Selection of the best primers and probes for use in the RT-RPA assay is currently being pursued. At the very least, these newly identified regions will provide a much more specific RT-PCR assay.

Assays for other fruit tree viruses:

The original proposal included the development of the RT-RPA assay system for the rusty mottle group of cherry viruses. Several members of this complex group of viruses may move into cherry orchards from surrounding native vegetation. Similarly, an assay for the identification of apple stem pitting virus associated with apple green crinkle disease was also proposed. These remain critical objectives. However, the rapidly unfolding situation with little cherry disease in Washington State usurped much of the effort towards these remaining objectives. Nevertheless, significant progress has been made.

In addressing the cherry rusty mottle group of viruses, a study was completed that characterized the viruses associated with a group of diseases including cherry rusty mottle, cherry necrotic rusty mottle, cherry twisted leaf, and apricot ringpox. 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*), they form a distinct population with unique genomic organizations, genome sequences, and host range. Based on these criteria, a new genus of viruses 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 pathogens separately, as well as broad spectrum primers that will detect multiple components of this complex group. This baseline information is a necessary precursor to the development of an RT-RPA assay.

Genetic analysis of the virus(es) associated with apple green crinkle disease is nearing completion. There are two candidate variants of apple stem pitting virus that may be directly responsible for this troublesome disease of apple production. Analysis of symptomatic trees will continue in order to resolve if either or both of these variants are uniquely associated with apple green crinkle disease.

CONTINUING PROJECT REPORT **YEAR**: 1 of 2
WTFRC Project Number: TR-14-104

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

PI: Lee Kalcsits
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Cooperators: Stefano Musacchi, WSU TFREC, Sara Serra, WSU TFREC, Allen Brothers Fruit Company, Naches

Total Project Request: **Year 1:** 57,496 **Year 2: 19,144**

Percentage time per crop: Apple: 60% Pear: 30% Cherry: 10% Stone Fruit: 0%

Other funding sources: None

Organization Name: WSU TFREC **Contract Administrator:** Carrie Johnston/Joni Cartwright
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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	**$19,144**

Footnotes:
[1]25% research intern at $48,000 per year in the first year and at $49,920 in the second year.
[2]Benefit rate for the second year is 29.9%.
[3]Portable x-ray fluorometer. Any required accessories and the vacuum pump will be covered by Kalcsits' start-up funding.
[4]Cost of leaf and fruit elemental analysis.
[5]Two trips to Naches for fruit and leaf collection in addition to trips to commercial orchards for sample collection.

Objectives

The goal of this project is to test and apply a new, portable, non-destructive approach to measuring mineral content in plant tissue. To do this, we will:

1. Compare the measures of elemental composition using the portable x-ray fluorometer with traditional elemental analysis for leaves, developing fruit and mature fruit of 'Honeycrisp' (2014 and 2015).

2. Test whether there is a relationship between storability and calcium, magnesium and potassium concentrations along with Ca^{2+}/Mg^{2+} and Ca^{2+}/K^{+} ratios measured using the portable x-ray fluorometer for 'Honeycrisp' apples. (2014 and 2015).

3. Determine the absorption efficacy of foliar applications of calcium at different times during fruit development (2015).

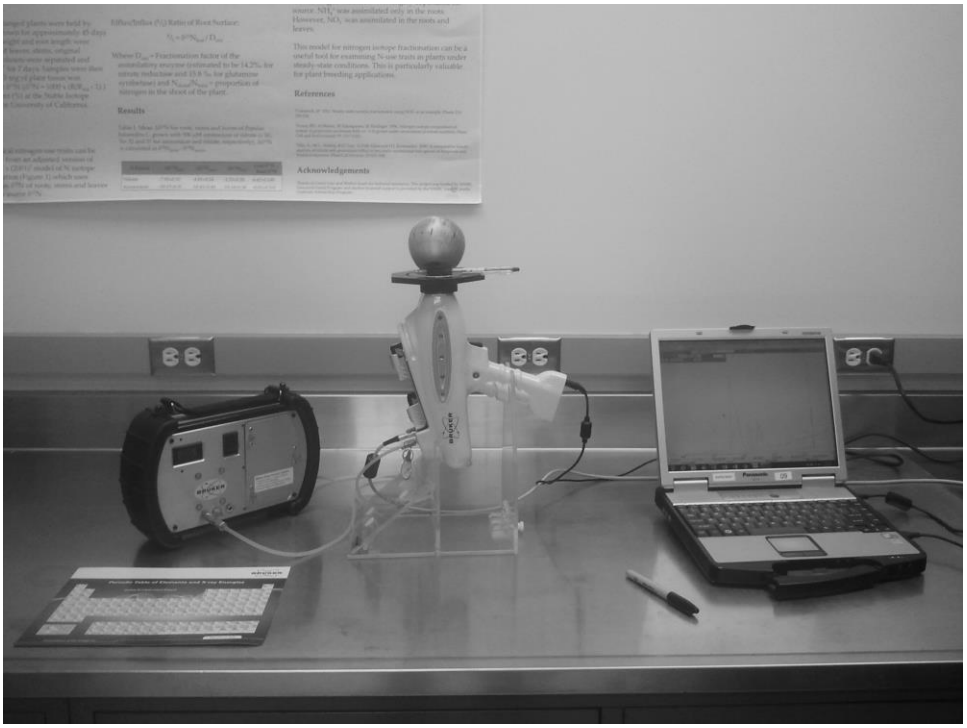

Figure 1. Instrument set up in lab in September, 2014.

Significant Findings

1. The instrument arrived at the end of September, 2014 and was set up in the lab. Bruker is working with us to provide technical support and will loan us an additional instrument for times when we need analysis done quickly.
2. Liquid standards indicate good accuracy and precision in measuring calcium in a liquid which can save time and cost for calcium analysis in acid digests.
3. The machine does not detect magnesium in small enough concentrations to measure it in fruit. I will verify this in 2015.
4. Previous literature indicates a strong relationship between calcium measured using the portable x-ray fluorometer (PXRF) in materials with a wide range in densities (liquid to rock) and measured using traditional destructive analysis (ICP-MS, ICP-OES, AAS). In 2015, this relationship will be verified in fruit, leaves and branches.
5. 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.
6. Presence of bitterpit is associated with high ratios of K/Ca measured using the PXRF.
7. There may be different thresholds among varieties that trigger the development of bitterpit. This idea will be developed in more detail in 2015.
8. There is a high variability in calcium within a tree and is likely related to location on the tree (high or low or outside or inside the canopy), fruit-shoot proximity and crop load. This will be explored in more detail in 2015.

Methods

First, we will compare the measures of elemental composition using the portable x-ray fluorometer with traditional elemental analysis for leaves, developing fruit and mature fruit of 'Honeycrisp' (2014 and 2015). Since this is a new technology for use in plants, it requires validation and testing in tree fruit. Previously published articles have highlighted the correlation between fruit measured using PXRF compared to traditional lab analysis, particularly for elements in high concentration such as potassium and calcium. We are in the process of digesting tissue samples with nitric acid to send to Pullman for calcium analysis. We have also added approximately 200 skin and flesh samples of pear for comparison to apple (collaboration with Musacchi and Serra). From the pear and apple samples, we expect that we will have approximately 400 samples to develop tissue specific calibrations to be able to non-destructively quantify calcium and potassium in skin and flesh of apples and pears. In 2015, this will also be tested in sweet cherry.

Next, we tested whether there is a relationship between storability and calcium, magnesium and potassium concentrations along with Ca^{2+}/Mg^{2+} and Ca^{2+}/K^{+} ratios measured using the portable x-ray fluorometer for 'Honeycrisp' apples. (2014 and 2015). In collaboration with Lav Khot and Sindhuja Sankaran, healthy and bitterpit apples from three varieties (Granny Smith, Golden Delicious and Honeycrisp) were analyzed using the PXRF. The surface of healthy and bitterpit apples were analyzed for calcium and potassium at three locations in the widest portion of the apples.

Using apples provided by Stemilt in October, 2014, we analyzed 72 apples from two locations using the PXRF and then placed the apples in regular atmosphere storage to monitor the development of bitterpit. We identified 23 apples from one field and 36 apples from another field that have high K/Ca. We are testing whether we can predict the development of bitterpit using this non-destructive analysis and then monitoring for bitterpit during storage.

After three months, the apples will be removed from storage for 7 days to accelerate the development of bitterpit and the apples will be assayed for bitterpit and compared to measured Ca and K values using the PXRF.

Lastly, we want to use this instrument in the field to determine the absorption efficacy of foliar applications of calcium at different times during fruit development (2015). To do this, we will conduct a calcium application trial with different frequencies of applications. At different points during the growing season, fruit will be measured for calcium and potassium concentrations and then fruit will be subsampled at harvest to screen for the development of bitterpit. Furthermore, repeated sampling of fruit within the canopy will provide information on the distribution of calcium and potassium in fruit within the canopy.

Results and Discussion

Objective 1.

To perform some initial tests using the instrument, we analyzed 'Honeycrisp' apples from a crop load trial (collaboration with Musacchi and Serra). We observed higher calcium concentrations when crop load is near optimum (50 fruit inch^{-2}) (Figure 2). Lower calcium in low crop loads are likely indicative of leaf-fruit competition for calcium and transpirational balance weighted to the leaves. Lower calcium and potassium in higher crop loads are likely a result of fruit-fruit competition for a limited resource pool being supplied by the roots.

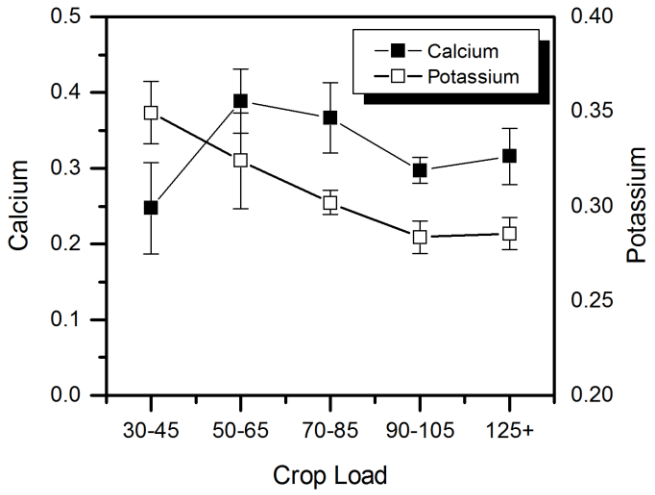

Figure 2. Semi-quantitative measurements of potassium and calcium.

We looked at the relationship between skin and flesh calcium in pear and there is a significant relationship (Figure 3). We will be doing similar measurements in apple this winter and will be able to present more complete results in the final report.

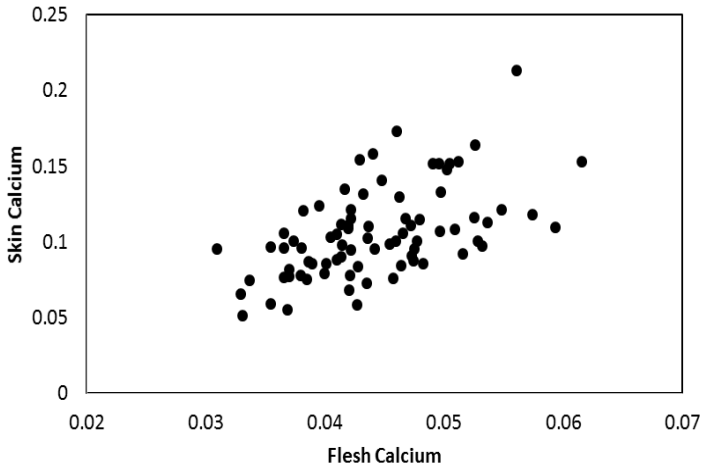

Figure 3. Relationship between skin and flesh calcium in pears measured using the PXRF.

Objective 2.

Initial testing indicates high variability in calcium and potassium on the surface of apples (Figure 4). There was not sufficient ability to measure magnesium in fruit. There is a gradient of calcium from the stem end to the calyx end and calcium can be highly variable at the surface near the stem. It appears that sampling around the middle of the apple is the most representative for sampling potassium and calcium. Initial results indicate that calcium levels were lower in Honeycrisp than the other two varieties and that calcium can vary between fields. When the potassium/calcium ratio was calculated, there was no difference in the ratios between fields for Honeycrisp (Figure 5). Healthy apples had a 50% lower potassium/calcium ratio than apples with bitterpit. This was consistent across all varieties and fields.

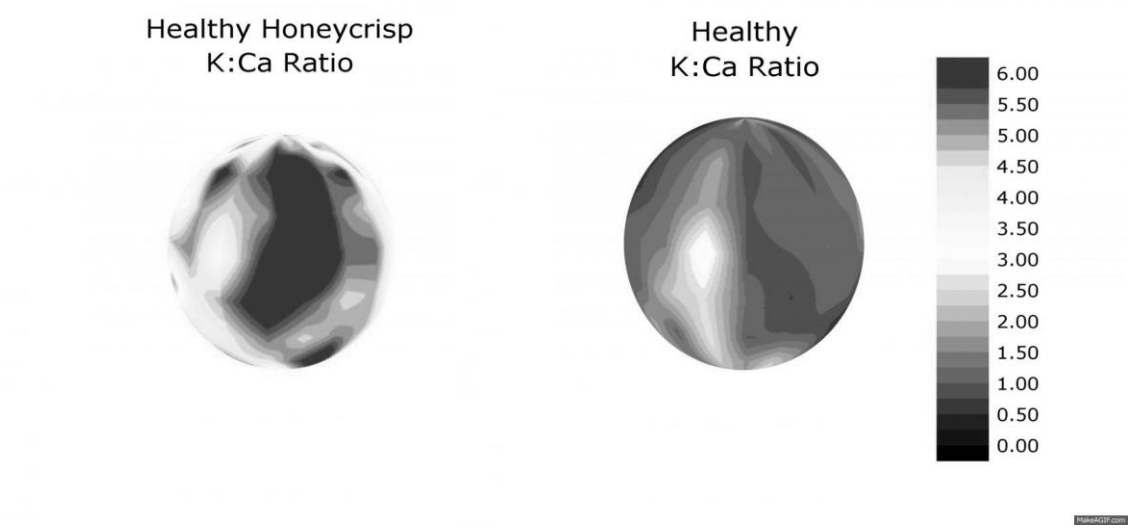

Figure 4. Surface map of the potassium:calcium ratio in a healthy Honeycrisp apple and a Honeycrisp apple with bitterpit showing high variability in calcium distribution on the fruit surface.

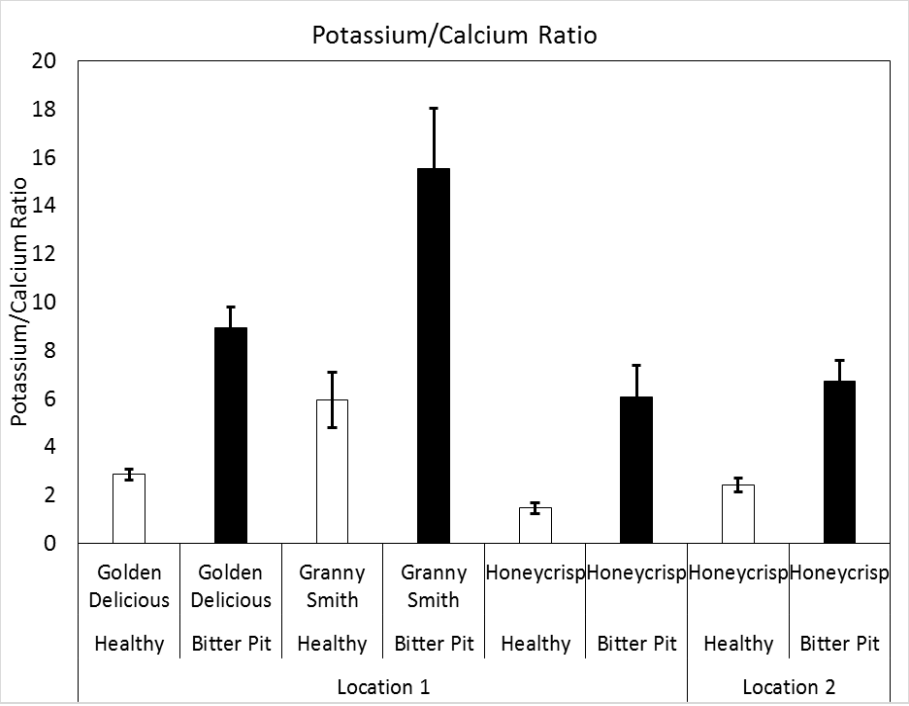

Figure 5. Mean potassium/calcium ratio of Golden Delicious, Granny Smith and Honeycrisp apples either showing bitterpit symptoms or not. Each mean is an average of 20 apples and error bars represent standard error.

Objective 3.

This objective will be completed in 2015. I am currently lining up a trial to look at the frequency of foliar calcium applications of equal rates (once per month, once per two weeks, once per week and twice per week). The efficacy of these applications will be tested using the PXRF to determine whether it has the precision to detect differences in frequencies of foliar calcium applications.

Other Progress

We are currently running a deficiency study in the greenhouse to look at whether we can detect low levels of nutrients (Ca, K, P, S, Zn, Mg, Cu, Fe and Mn). Plants are being fertilized with a balanced fertilizer minus a key element and being compared to a complete fertilizer control. We are analyzing leaves at time intervals to monitor the development of the deficiency and connect visual development of the deficiency with elemental levels within the leaf. This is something that is difficult to do with traditional analysis compared to a quick, non-destructive analysis such as PXRF.

Take home message

There is value to the Washington State tree fruit industry by using this instrument in research and consultation. By being able to non-destructively determine potassium and calcium in fruit and leaves, there is potential to advance the understanding of the role of calcium in bitterpit development and also to test horticultural approaches (summer pruning, growth regulators, foliar calcium applications, architecture, etc.) that may limit calcium deficiencies in fruit. This is the first time that this instrument has been used in tree fruit and from a physiology research standpoint, the applications of this type of instrument to improve nutritional balance in tree fruit are endless.