Northwest Cherry Research Review

Confluence Technology Center

Tuesday, 11/12/19

Time	Page	Presenter	Project Title	Yrs
8:00		Hanrahan	Welcome - WTFRC 50th anniversary video	
			Continuing/short project reports 8:15 - 10:30	
8:15	1	Schmidt	Cherry MRL[1,3], WTFRC technology projects - (see reports in Appendix)[2]	20
8:25	3	Akbari	Engineered transgenic D. suzukii for wild population suppression; *Videoconference*	19-21
8:35	10	Harper	How do Western X phytoplasma and LChV-2 cause little cherry disease?	18-20
8:45	16	Gibeaut	Development index model of sweet cherry: *Funded off cycle*	19-20
8:55	22	McCord	Supporting a robust PNW sweet cherry breeding and genetics program	19-21
			Equipping the re-launched PNW sweet cherry breeding program (update w/above)	19
9:05	30	Johnson	Durable genetic solutions to powdery mildew infection in sweet cherry	19-20
9:15	36	Grove	Fungicide resistance: a vital need to protect PNW cherries from mildew	19-20
9:25			Coffee break with scientists	
			Final project reports	
	42	Chiu	Electronic sensors to capture spatiotemporal population density of SWD: *Written report only*	18
9:45	53	Whiting	Advancing precision pollination systems for yield security	17-18
10:00	64	Iezzoni	MSU sweet cherry rootstocks: pre-commercialization	17-19
10:15	76	Swamy	ABC of sweet cherry powdery mildew: adaptation, behavior and control	16-18
10:30	88	Choi	Non-nutritive sugar-based control strategy for spotted wing drosophila	18-19
10:45	94	Choi	Non-toxic RNAi-based biopesticide to control SWD	17-19
11:00	102	Beers	Integrated pest management of spotted wing drosophila in sweet cherry	16-18
11:15	113	Harper	Native hosts of the Western X phytoplasma	18-19
11:30	122	Pscheidt	Mid-Columbia survey for sweet cherry viruses and vectors	18-19
11:45	132	Harper	Orchard management practices for *Little Cherry Virus 2*	17-19
12:00		Smith	Canine detection of little cherry disease: *Funded off cycle*	19
12:15 - 1:15			OSCC/WTFRC/CCB working lunch/continuing report discussions	
1:30		Rood	California Update	

CONTINUING REPORT **PROJECT LENGTH (CROP YEARS)**: 2018-2020

Project Title: Pesticide residues on WA cherries

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Cooperators: Gerardo Garcia, Sandy Stone, Pacific Agricultural Labs, Northwest Hort Council, Doug Stockwell, Doyle Smith, various ag chemical companies

Item	2018	2019	2020 (est.)
Salaries			
Benefits			
Wages[1]	1250	1350	1269
Benefits[1]	650	700	680
RCA Room Rental			
Shipping[2]	300	300	300
Supplies/Chemicals	250	275	300
Travel[3]	700	750	800
Plot Fees			
Analytical lab fees	3500	3750	4000
Total gross costs	6,650	7,125	7,349
Anticipated Income (contracts and gift grants)	0	0	0
Total net costs	6,650	7,125	7,349

Footnotes: Schmidt estimates 10% of his time is dedicated to this project on an annual basis
Most pesticides tested are donated by their registrants or an ag chemical supply company

1 Wages & benefits primarily for Garcia (spray applications), crew help for Garcia, and Stone (data entry & review)
2 Est. costs to ship cherries overnight to Sherwood, OR
3 Travel costs include hauling equipment to & from plots

2019 WTFRC CHERRY PESTICIDE RESIDUE STUDY

For the ninth consecutive year, the WA Tree Fruit Research Commission conducted a study of residues of commonly used pesticides on cherry fruit at harvest. Digital versions of this report and similar studies on apple and cherry including comprehensive summaries of multiple years' results are available at www.treefruitresearch.com. For current information on maximum residues levels (MRLs) and other regulatory issues, please consult the Northwest Horticultural Council at http://nwhort.org/export-manual.

Airblast application @ 400 gal/acre

TRIAL DETAILS

- Mature 'Bing'/Mazzard multiple leader open vase trees on 10' x 20' spacing near Orondo, WA
- 12 insecticides/acaricides & 5 fungicides applied at or near maximum rates and minimum pre-harvest and re-treatment intervals
- Ground applications made by Rears PakBlast PTO-driven airblast sprayer of the same rate of product per acre with 8 oz Regulaid surfactant/100 gal water in either 200 or 400 gal water/acre
- Approx. 1" of rain fell on trial block over several days in late May; residues of materials applied 35 days before harvest (buprofezin and tolfenpyrad) may be slightly diminished, but subsequent applications of those or other materials were not significantly affected by rain events
- Samples submitted overnight to Pacific Agricultural Labs (Sherwood, OR) for chemical analysis

RESULTS & DISCUSSION

This study generally simulates a *worst case scenario* for residues of legally applied pesticides using aggressive rates, timings, and spray intervals. Most materials were applied twice as allowed by product labels, whether or not typical commercial use patterns would do the same. With that approach, all residues complied with domestic tolerances but some **exceeded some foreign tolerances,** whether from published MRLs or national default values:

Insectides/acaricides: Bexar, Agri-Mek 0.15SEC, Mustang MAX, Closer, Danitol 2.4EC, Perm-Up 3.2EC, Carbaryl 4L, Onager

Fungicides: TopGuard, Gatten, Orbit, Topsin 4.5FL

MRLs are known to change frequently and cherry producers should routinely monitor the most current information (http://nwhort.org/export-manual) to facilitate compliance with shifting foreign standards. As with previous years' results, differences in residues between dilute (400 gal/acre) and concentrate (200 gal/acre) sprays were inconsistent and preclude any firm conclusions regarding the effects of sprayer carrier volume on pesticide residue levels. While fruit from this study were not rinsed prior to analysis, similar studies in 2011 and 2012 found no clear evidence of consistent residue reduction from commercial hydrocooler cycles.

Cherries dripping after 200 gal/acre application

Measured residue levels vs. MRLs for pesticides applied at uniform rates/acre on cherry fruit in either 200 gal or 400 gal water/acre. 'Bing'/Mazzard, Orondo, WA. WTFRC 2019.

Common name	Trade name	Application rate[1]	Application timing(s)	200 gal water/acre	400 gal water/acre	US tolerance[2]	Lowest export tolerance[3]
		per acre	*days before harvest*	*ppm*	*ppm*	*ppm*	*ppm*
buprofezin	Centaur	34.5 oz	28, 21	0.58	0.86	1.9	1 (Kor)
tolfenpyrad	Bexar	27 oz	28, 14	0.50	1.20	2	0.01 (Tai)
abamectin	Agri-Mek 0.15SEC	20 oz	21	0.019	0.011	0.09	0.01 (EU)
zeta-cypermethrin	Mustang MAX	4 oz	21, 14	0.14	0.14	1	0.1 (Can)
thiamethoxam	Actara	5.5 oz	21, 14	0.26	0.22	0.5	0.5 (many)
acetamiprid	Assail 70WP	3.4 oz	21, 7	0.47	0.45	1.2	1 (Tai)
beta-cyfluthrin	Baythroid XL	2.8 oz	21, 7	<0.05	<0.05	0.3	0.01 (Tai)
flutriafol	TopGuard	14 oz	14, 7	0.23	0.31	1.5	0.01 (Jap)
myclobutanil	Rally 40WSP	6 oz	14, 7	0.41	0.38	5	1 (Can, Tai)
sulfoxaflor	Closer	5.75 oz	14, 7	0.31	0.35	3	0.01 (Tai)
fenpropathrin	Danitol 2.4EC	21.3 oz	14, 3	1.00	0.74	5	0.01 (EU)
permethrin	Perm-Up 3.2EC	8 oz	14, 3	0.33	0.34	4	0.05 (EU)
carbaryl	Carbaryl 4L	96 oz	10, 3	2.8	2.1	10	0.01 (EU)
flutianil	Gatten	8 oz	10, 3	0.044	0.042	0.4	0.01 (EU, Jap)
propiconazole	Orbit	4 oz	10, 1	0.31	0.15	4	0.01 (EU)
thiophanate-methyl*	Topsin 4.5FL	30 oz	10, 1	1.05	0.89	20	0.3 (EU)
hexythiazox	Onager	24 oz	7	0.23	0.21	1	0.1 (Kor)

[1] All materials were applied by Rears PakBlast sprayer with 8 oz Regulaid/100 gal water

[2] 13 Aug 2019. http://nwhort.org/export-manual/comparisonmrls/cherry-mrls/

[3] Major export markets for Pacific Northwest cherries; 13 Aug 2019; tolerances may be based on published MRLs or default values. http://nwhort.org/export-manual/comparisonmrls/cherry-mrls/

* Reported thiophanate-methyl values reflect sum total of thiophanate-methyl and carbenzadim residue levels

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WASHINGTON TREE FRUIT RESEARCH COMMISSION

Results of this lone unreplicated trial are shared for informational purposes only and should not be construed as endorsements of any product, reflections of their efficacy against any arthropod or fungal pest, or a guarantee of similar results regarding residues for any user. Cherry growers should consult with extension team members, crop advisors, and warehouses to develop responsible pest control programs.

CONTINUING PROJECT REPORT **YEAR: 2020 Year 2**

Project Title: Engineered transgenic *D. suzukii* for wild population suppression

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Cooperators: None

Total Project Request: **Year 1:** $46,609 **Year 2: $50,946** **Year 3:** $52,445

Other funding sources

Agency Name: California Cherry Board
Amt. requested: approx. $75,000

Budget 1
Organization Name: UC San Diego **Contract Administrator:** Susan Pastell
Telephone: 858-534-4896 **Email address:** spastell@ucsd.edu

Item	2019	2020	2021
Salaries	$31,555	$35,221	$36,437
Benefits	$6,383	$7,104	$7,387
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$8,050	$8,000	$8,000
Travel			
Plot Fees			
Miscellaneous	$621	$621	$621
Total	$46,609	**$50,946**	$52,445

Objectives: Spotted wing Drosophila, *D. suzukii*, is a major worldwide crop pest of various soft-skinned fruits[1]. Unlike other Drosophilids that prefer to oviposit on overripe fruits, *D. suzukii* utilizes its serrated ovipositor to lay eggs inside ripening fruits, causing significant crop losses[1–3]. Found only in Japan prior to the 1930's[4], in the last several decades *D. suzukii* has spread invasively to every continent except Antarctica[1,5]. An alternative, highly promising approach to controlling *D. suzkuii* that could complement existing control methods is genetic pest management[6], which includes strategies such as gene drive[7,8] and transgenic-based precision-guided Sterile Insect Technique (pgSIT)[9,10] (Figure 1 left). SIT has been a successful technology for insect population suppression, which is achieved by introducing large number sterile males into a target population. In classic SIT, insects are irradiated with ionising radiation to induce male sterility. The sterile males are then released to mate with wild females resulting in non-viable progeny. Over time, repeated mass releases of sterile males suppresses and can even eliminate the target population (Figure 1 left) This approach was used to eradicate the screwworm fly, *Cochliomyia hominivorax*, [11], the Mexican fruit fly, *Anastrepha ludens*, and the Mediterranean fruit fly, *Ceratitis capitata,* from regions of North America *[12]*. While the classic irradiation-based SIT presents a environment-friendly method of a local population suppression, it is not technically feasible or scalable for the control of most insects. PgSIT, on the other hand, is a simplified way to generate sterile males and should be less expensive and labor intensive than irradiation-based SIT even at scale. SIT technologies have been determined to be very cost effective for some pest species, for example *Ceratitis capitata [13]* reducing the costs of this technology through genetic-based SIT methods, such as pgSIT, would make this approach to population suppression even more cost effective.

We also propose to engineered *D. suzukii* gene drive strains, which can be utilized to more rapidly spread desirable genes (e.g., susceptibility to a novel bio-friendly pesticide) throughout, or to entirely suppress/eradicate, wild *D. suzukii* populations. Such an approach is catalytic, with release of only modest numbers of engineered insects required to spread desirable genes or achieve population suppression. Additionally, since such a system relies on only a few releases of transgenic insects to do all of the work on an ongoing basis, it is affordable as compared to the use of insecticides, which need to be applied regularly. Finally, such an approach is environmentally friendly and entirely insect-specific and would have no effect on crops or on beneficial organisms.

Our objective is to therefore engineer *D. suzukii* gene drive strains that could be utilized as part of current integrated pest management programs to control wild *D. suzukii* populations. Specifically, out of the multiple types of gene drive systems that can be utilized in a genetic pest management program[8,14]. We aim to develop a pgSIT system in *D. suzukii* using the design principles we have optimized in *D. melanogaster[9]*. We also aim to develop synthetic *Medea* elements that can be used to suppress wild *D. suzukii* populations. Ultimately, our goal is to develop a product (a genetically modified *D. suzukii*) that can be mass-reared and deployed into the wild to catalytically suppress, and completely eliminate, the wild populations of this significant pest.

Objective A - Refinement of a *Medea* drive system for *D. suzukii* population suppression. We have developed a synthetic *Medea* gene drive system for population suppression [15]. *Medea* was first discovered in the flour beetle[16], and multiple versions were later reverse engineered from scratch and shown to act as robust gene drives in the laboratory fruit fly, *Drosophila melanogaster[17,18]*. Such engineered *Medea* systems rely on a *Medea* element consisting of a toxin-antidote combination. The toxin consists of a miRNA that is expressed during oogenesis in *Medea*-bearing females, disrupting an embryonic essential gene. A linked antidote is expressed early during embryogenesis and consists of a recoded version of the target gene that is resistant to the miRNA. This combination results in the survival of half of the embryos originating from a *Medea*-bearing heterozygous female, as those that do not inherit the *Medea* element perish. If a heterozygous *Medea* female has mated with a heterozygous *Medea* male, the antidote from the male will also take effect in the embryo, resulting in 3/4 of the embryos surviving (Figure 4). Therefore, *Medea* will rapidly spread through a population, carrying any linked genes with it.

We have already engineered a first-generation *Medea* system in *D. suzukii* [15], which is the first functional gene drive developed in this pest. We had rigorously tested it in laboratory cage populations, and had characterized it in different genetic backgrounds to determine effectiveness and fecundity (our results on this project were published in *PNAS* this year [15]). We found that this first-generation *Medea* system was capable of biasing Mendelian inheritance rates with up to 100% efficiency and could maintain itself at high frequencies in a wild population; however, drive resistance, resulting from naturally occurring genetic variation and associated fitness costs, was present and could hinder the spread of such a drive. Therefore, since mathematical modeling indicates that our *Medea* drive system could spread to fixation if resistance was reduced[15], we need to engineer a second-generation *Medea* system that should obviate the specific resistance that we observed. To safeguard, reduce risk, and mitigate the spread of the *D. suzukii Medea* system into wild populations, we also aim to develop a reversal *Medea* (RM) system that can be used to replace the original *Medea* in case a recall is necessary. Reversing the drive of a *Medea* system has been theorized; however, it has never been experimentally demonstrated. Finally, in order to use *Medea* to bring about population suppression, we need to link it to a cargo gene capable of killing *D. suzukii* under specific conditions to bring about a population crash. We have already identified several promising putative cargo genes and are testing them in *D. melanogaster*, a closely related species to *D. suzukii* that is easier to work with and provides a useful testing platform for transgenes. However, we will still need to build and test them in *D. suzukii*. Successful completion of the above objectives would lead to the development of a genetically modified *D. suzukii* strain (carrying a synthetic *Medea* element) that can be mass-reared and deployed into the wild to catalytically suppress, and completely eliminate, wild populations of *D. suzukii*.

Objective B: Precision guided sterile insect technique (pgSIT) for *D. suzukii* population suppression. The Sterile Insect Technique (SIT) is an alternative, proven pest management approach that could complement existing control methods[19–21]. SIT involves the mass-production and release of sterile males, and has historically been used to control, and eradicate, insect pest populations dating back to the mid-1930s[22–26]. Traditional SIT methodologies have relied on DNA-damaging agents for sterilization, substantially reducing overall fitness and mating competitiveness of released males. A next-generation highly-efficient technology that can be used for biocontrol of *D. suzukii* is precision guided SIT (pgSIT). PgSIT functions by exploiting the precision and accuracy of CRISPR to simultaneously disrupt genes essential for either female viability or male fertility. It utilizes a simple breeding scheme requiring two homozygous strains - one expressing Cas9 and the other expressing double guide RNAs (dgRNAs). A single mating between these strains mechanistically results in synchronous RNA-guided dominant biallelic knockouts of both target genes throughout development, resulting in the complete penetrance of desired phenotypes in all progeny. We have previously built pgSIT in *Drosophila melanogaster*, a model organism that is closely related to *D. suzukii*, and shown that it is extremely robust at genetically sexing and simultaneously sterilizing resulting progeny reproducibly with 100% efficiency, and that pgSIT sterile males are fit and can compete for mates[9]. We therefore aim to develop pgSIT technology in *D. suzukii* (Objective B). Successful development of this technology would produce a genetic-based sterile insect strain that can be mass-reared and released to reduce populations of *D. suzukii* in a straightforward manner with respect to regulations.

Significant Findings:

Objective A:

- We have developed a modified version of our original *Medea* system that is designed to reduce resistance to the drive. We are currently rigorously testing this second-generation *Medea* element and planning for longer term population cage studies.
- We have developed a second-generation "reversal" *Medea* system that should be more robust in the face of genetic diversity in general and could be used to replace the original *Medea* in case a recall is necessary. We are currently testing this system and planning for longer term population

cage studies.

- We have identified several promising putative cargo genes that could be spread with the *Medea* gene drive to cause population suppression. Multiple genes have been tested in *D. melanogaster* as proof of principle and are now being transitioned to *D. suzukii*.

Objective B:

- Designed and injected constructs that express gRNAs targeting the female viability genes and *beta tubulin* (***β-tub***), a male fertility gene. We are expanding these lines and will test them in crosses to multiple Cas9 expression lines to determine the most efficient gRNA and Cas9 line combinations to generate sterile male progeny.
- Established six transgenic gRNA lines targeting both *sxl* and ***β-tub*** simultaneously.

Methods:

Objective A - Refinement of a *Medea* drive system for *D. suzukii* population suppression. We have developed the first proof of concept *Medea* drive in *D. suzukii* [15]. Given our observations regarding resistance and its effect on *Medea* function, we now need to engineer improved *Medea* systems that could reduce the chances of resistance acting as an impediment to spread. So far, we have performed some sequencing-based characterization of naturally occurring genetic variation in various geographically distinct target populations to help guide selection of target sites that are well conserved across all populations in which the drive is intended to function. We then designed a modified version of the original *Medea* system that targeted different, conserved sequences (still in the 5'UTR of the *myd88* target gene), reasoning that such a *Medea* element should function very similarly to the original element but not be impeded by the resistance we previously observed. We are now obtaining transgenic lines for this improved *Medea* element, and preliminary data indicates that it works better than the original *Medea*, producing 100% inheritance bias. We need to continue to rigorously test this second-generation *Medea* element to characterize its function and ability to bias inheritance 100% in geographically distinct populations. We also will need to perform multiple long term multi-generational population cage experiments to determine whether this *Medea* can drive robust population replacement.

Additionally, we hypothesized that to reduce resistance, miRNA target site selection could be limited to the coding DNA sequence regions of a genome, which tend to be strongly conserved, as opposed to regions such as the 5'UTR, which canonically have higher tolerance for sequence variation. We have therefore also developed a second-generation "reversal" *Medea* system in *D. suzukii* that should be more robust in the face of genetic diversity in general (because it targets coding DNA regions as opposed to the 5'UTR) and could be used to replace the original *Medea* in case a recall is necessary. Specifically, to reduce risk and mitigate the spread of the *D. suzukii Medea* system into wild populations, it is important to develop a reversal Medea (RM) system and demonstrate that it can function as predicted. Reversing the drive of a *Medea* system has been theorized; however, it has never been experimentally demonstrated. Therefore, this should be of high impact and relevance when it comes to regulators assessing the risk associated with gene drives. We have finished designing and building a reversal *Medea* system capable of spreading on its own and of replacing the first *Medea* described above and are in the process of obtaining transgenic *D. suzukii* individuals containing this *Medea.* Once we have transgenic lines for this construct, we need to rigorously test them for their ability to bias inheritance in both wild type and original *Medea* backgrounds. We will then need to perform multiple long term multi-generational population cage experiments to determine whether this *Medea* can actually spread on its own (in a wild type population) and replace the original *Medea*.

Identification of Putative "Cargo" Genes: For *D. suzukii*, elimination of the pest populations is ultimately the goal. An engineered *Medea* system could achieve this by spreading a "cargo" gene proffering susceptibility to a particular pesticide, or a conditional lethal gene that would be activated by some substance or environmental cue such as high temperature or diapause. One promising type of candidate "cargo" gene is a thermally activated TRPA1 cation channel[27]. Specifically, TRPA1 is an ion channel located on the plasma membrane of many human and animal cells, and is finely tuned to

detect specific temperatures ranging from extreme cold to noxious heat[27]. Upon exposure to a critical "threshold" temperature, this cation channel can "open" and modulate Ca2+ and Mg2+ entry into the cell[28]; when TRPA1 is overexpressed in an exogenous tissue (such as the fly brain, for example), this "opening" can lead to total fly paralysis and death. We therefore have started to engineer *D. suzukii* to express a specific TRPA1 channel in the brain, so that exposure of the engineered individuals to a threshold temperature (determined by the specific TRPA1 channel used) would paralyze/kill the flies. In the next year, we can test whether these transgenic lines are able to spread this temperature-activated "cargo" gene through wild populations by using our *Medea* system at cooler temperatures and achieve population suppression when the TRPA1 gene is activated at warmer temperatures.

Developing a field-ready strain: Similar to the other suppression drives, when we build an optimized *Medea* drive, we will also need to conduct laboratory and caged field trials to determine mating competitiveness, longevity, and fitness of these strains. This data will be used and fed into mathematical models to predict the numbers of flies we will need to release to achieve suppression.

Objective B: Precision guided sterile insect technique (pgSIT) for *D. suzukii* population suppression. In order to construct a pgSIT system, we need functional Cas9 tools (including gRNA lines that target genes essential for female viability and male sterility and Cas9 expressing lines (Figure 2) in *D. suzukii*. We have now developed multiple transgenic lines that express Cas9 (*bicC-cas9, dhd-cas9, vasa-cas9, nanos-cas9, ubiq-cas9*). Also, essential to building a pgSIT system are guide RNA (gRNA) lines that target genes essential for female viability and male fertility. We have previously identified genes essential for female viability or male fertility in *D. melanogaster* and have shown that disrupting these genes via CRISPR/Cas9 produces the desired results (e.g., female death or conversion of females into sterile intersex individuals for the former group, male sterility for the latter [29]. Since *D. melanogaster* is closely related to *D. suzukii*, we reasoned that disruption of these same genes would have a similar effect in *D. suzukii* and are focusing our efforts on these validated target genes. Specifically, to disrupt female viability, we are targeting several sex-specifically alternatively spliced sex-determination genes including *sex lethal* (*sxl*), *transformer* (*tra*), and *doublesex* (*dsxF*), as well as *zero population growth* (*zpg*), a germline-specific gap junction gene. So far, we have identified *D. suzukii* homologues of all of these genes and have carefully selected two gRNA target sites in each gene that are highly conserved and thus unlikely to harbor sequence variation. We have generated multiple transgenic lines for each gRNA target and we are currently in the process of crossing each one separately to our five Cas9 strains to see whether the combinations of Cas9+gRNA will produce female lethality and male sterility. So far, we have six gRNA lines that generate the expected 100% sterile male phenotype in preliminary crosses. However, now we need to rigorously test these strains to ensure these results are reproducible over many replicates to ensure these lines can consistently kill females and sterilize males. We also plan to conduct male competition and fitness studies to ensure the sterile males are fit to compete in field conditions. We will also explore additional gRNA targets and combinations to ensure the final gRNA lines are optimized for the field.

Efficient sex sorting:In order to be easily implemented, the pgSIT approach also requires the ability to efficiently separate animals by sex to set up appropriate crosses (i.e., crossing Cas9 and gRNA parents together) for sterile male generation[9] . Therefore, we are also testing a sex-specific fluorescent reporter transgene that can facilitate automated sex sorting. Specifically, we have designed a transgene that contains a fluorescent marker (dsRed) under the control of a ubiquitous promoter. This transgene includes a female-specific intron from the Drosophila *transformer* (*tra*) gene that can be processed only in female flies. Linking this intron to a fluorescent marker should generate a transgene where successful splicing and expression of dsRed occurs exclusively in females, therefore generating a system where only females express a fluorescent marker for easy sorting. We have already obtained transgenic insects with the above transgene and demonstrated that only females express the red marker. We are now testing similar constructs with different fluorophores (yellow, green, blue) to create more options for efficient sex-specific sorting of flies of various genotypes.

Developing a field-ready strain: Once all of these components are individually validated, we

can proceed to assemble a single transgene that, coupled with a Cas9 strain, can be used to generate a pgSIT strain ready for use in the field for *D. suzukii* biocontrol. Laboratory and caged field trials will also be conducted on this strain to determine mating competitiveness, longevity, and fitness compared to wild flies. This data will be used and fed into mathematical models to predict the introduction frequencies we will need to use to achieve suppression. Gene drive experiments will be initiated at various introduction frequencies to characterize the population suppression dynamics. Modeling work will occur in collaboration with Dr. John Marshall (UC Berkeley), a mathematical biologist with whom we have worked on a number of modeling studies.

Since the ultimate goal here is to develop a product (a genetically modified *D. suzukii*) that can be mass-reared and deployed into the wild to suppress, and completely eliminate, the wild populations of *D. suzukii*, we will need regulatory bodies to permit such releases. In brief, we have requested a field cage study permit from USDA-APHIS BRS/PPQ. APHIS is responsible for issuing permits for the import, transit and release of regulated animals, animal products, veterinary biologics, plants, plant products, pests, organisms, soil, and genetically engineered organisms. We have also applied for a BRS 2000 (Application for Permit or Courtesy Permit for Movement or Release of Genetically Engineered Organisms). These permits have been successfully issued for the release of transgenic insects in the USA. For example, in 2009 the USDA approved the integration of genetically engineered pest insects (including pink bollworm moth (*P. gossypiella*), Mediterranean fruit fly (*Ceratitis capitata*), Mexican fruit fly (*Anastrepha ludens*), and oriental fruit fly (*Bactrocera dorsalis*)) into ongoing SIT programs[30]. Some key advantages of the pgSIT approach will be that only males will need to be released (so crops will not be damaged); that it is very species-specific, since the released males will be sterile and not capable of mating with wild *D. suzukii* or any other species; and that the approach is self-limiting, which makes it a safer alternative than self-sustaining approaches and thus more likely to win public and regulatory approval. Therefore, the key point here is that obtaining regulatory approval for releasing transgenic insects in the USA, that are engineered to reduce wild populations and prevent crop damage, has been achieved in the past, and therefore we do not envision it to be a limitation with our approach.

Results and Discussion:

Objective A: We have developed a modified version of our original *Medea* system that is designed to reduce resistance to the drive. Given our observations regarding resistance and its effect on Medea function, we set out to engineer improved Medea systems that could reduce the chances of resistance acting as an impediment to spread. Specifically, we performed some sequencing-based characterization of naturally occurring genetic variation in various geographically distinct target populations to help guide selection of target sites that are well conserved across all populations in which the drive is intended to function. We then designed a modified version of the original *Medea* system that targeted different, conserved sequences (still in the 5'UTR of the myd88 target gene), reasoning that such a *Medea* element should function very similarly to the original element but not be impeded by the resistance we previously observed. We have obtained transgenic lines for this improved Medea element, and preliminary data indicates that it works better than the original *Medea*, producing 100% inheritance bias. We are currently rigorously testing this second-generation *Medea* element and planning for longer term population cage studies.

We have developed a second-generation "reversal" *Medea* system that should be more robust in the face of genetic diversity in general and could be used to replace the original *Medea* in case a recall is necessary. We have finished designing and building a Reversal Medea system capable of spreading on its own and of replacing the first *Medea* described above and are in the process of obtaining transgenic *D. suzukii* individuals containing this *Medea* and of rigorously characterizing this system. We are currently testing this system and planning for longer term population cage studies.

We have identified several promising putative cargo genes that could be spread with the *Medea* gene drive to cause population suppression. To achieve this, we are working to leverage data from the Montell lab (UCSD), which is developing this technology for mosquito control. The Montell lab is currently testing several TRPA1 channels with different activation temperatures (including rattlesnake

TRPA1, python snake TRPA1, boa snake TRPA1 and fruit fly TRPA1) in *D. melanogaster* as a proof of principle, and has preliminary data indicating that at least some of the tested TRPA1 channels, when expressed in the fly brain, work as expected. Once we know which TRPA1 channel appears most promising, we will insert it into our best Medea element and begin testing this approach in *D. suzukii*. However, multiple genes have been tested in *D. melanogaster* as proof of principle and are now being transitioned to *D. suzukii*.

Objective B: We designed (>20) and injected (>10) constructs that express gRNAs targeting the female viability genes and *beta tubulin* (***β-tub***), a male fertility gene. We are expanding these lines and will test them in crosses to multiple Cas9 expression lines to determine the most efficient gRNA and Cas9 line combinations to generate sterile male progeny. We established six transgenic gRNA lines targeting both *sxl* and ***β-tub*** simultaneously; Exciting preliminary results indicate that there are **no female transheterozygote progeny** from gRNA and vasa-Cas9 lines crosses (Table 1), which indicates females that inherit these transgenes are killed as we expected!!!! Additionally, when the transheterzygote progeny were crossed with wildtype (WT) females, as expected they **produced no viable progeny** (Table 2). Taken together, these data strongly indicate that we may **have a functioning pgSIT system in *D. suzukii***, however we need to continue to assess these lines in more replicates and also expand and homozygose these lines and measure fitness and mating competitiveness.

Additional Items:

	1056H L.3 ♂ x vas-Cas9 ♀	1056J L.2 ♂ x vas-Cas9 ♀	1056J L.3 ♂ x vas-Cas9 ♀
WT M	36	16	34
WT F	-	-	-
Inherited 1056 M	3	12	12
Inherited 1056 F	-	-	-
Inherited Cas9 M	77	63	62
Inherited Cas9 F	-	-	-
Transhet Females	0	0	0
Intersex	2	0	3
Transhet Males	**23**	**42**	**9**
n=	141	133	120

Table 1. Preliminary results from vasa-Cas9 and *sxl* and *β-tub* gRNA line crosses demonstrate female killing. The table depicts the number of resulting progeny of crosses between three different gRNA lines that simultaneously target *sxl* and ***β-tub*** (genes required for female viability and male fertility, respectively) and a vasa-Cas9 line. The highlighted row shows that no transheterozygous (transhet) females were generated from these crosses demonstrating that females inheriting both transgenes are efficiently killed.

		Genetic Cross			
Female	*Male*	*# of WT Females*	*# of transhet Males*	*Embryo Count*	*Emerged*
WT	1056H L.3 ; vasa-Cas9	15	5	385	0
WT	1056J L.2 ; vasa-Cas9	15	5	364	0
WT	1056J L.3 ; vasa-Cas9	15	5	in progress	in progress
WT	1056J L.5 ; vasa-Cas9	15	5	in progress	in progress
WT	1056K L.1 ; vasa-Cas9	15	5	194	0
WT	1056K L.3 ; vasa-Cas9	15	5	279	0

Table 2. Preliminary results from crosses between male transheterozygote progeny and wildtype (WT) females demonstrate male sterility. The transheterozygote male progeny generated from crosses between the gRNA and Cas9 lines (e.g. Table 1) were crossed to WT females to assess male fertility. No males resulted from these crosses (highlighted column) indicating that the male progeny are sterile.

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CONTINUING REPORT **YEAR**: 2 of 3 Years
WTFRC Project Number: CH-18-101

Project Title: How do Western X phytoplasma and LChV-2 cause little cherry disease?

PI: Dr. Scott Harper
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Co-PI: Dr. Alice Wright
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Cooperators: None

Total Project Request: $115,291 **Year 1:** $41,058 **Year 2:** $38,058 **Year 3: $36,175**

Other funding sources

None.

Budget
Organization Name: Washington State University **Contract Administrator:** Katy Roberts
Telephone: 509-335-2885 **Email address:** arcgrants@wsu.edu

Item	2018	2019	**2020**
Salaries	$12,106	$20,145	**$21,789**
Benefits	$4,152	$7,313	**$7,186**
Wages			
Benefits			
Equipment			
Supplies	$24,800	$10,600	**$7,200**
Travel			
Miscellaneous			
Plot Fees			
Total	$41,058	$38,058	**$36,175**

Footnotes:

1. Salary & Benefits at 0.25 for the first year. From 2018 onwards, an increase to 0.4 FTE (due an increasing workload in sample collection, processing and analysis in years 2 and 3) thereafter for salary for Dr. Wright is requested to conduct this research. However, if the new 2020 proposal "Understanding little cherry disease pathogenicity" is funded, this current project will be absorbed into the new project and no further funding will be required.
2. Supplies include laboratory consumables and reagents, and sequencing/HPLC services.

OBJECTIVES

1. *Perform transcriptomic analysis to compare healthy cherry trees, asymptomatic Western X & LChV-2 infected trees, and symptomatic Western X and LChV-2 infected trees to identify genes that may lead to symptom development.*

Based on preliminary RNA-seq data from year 1, we chose to focus on fruit, fruit stem, and leaf tissues at shuckfall and within two weeks of harvest from Bing cherries. RNA-seq has been performed on these tissues for healthy and WX infected trees at shuckfall. RNA samples are being prepared for LChV-2 infected, WX infected, and healthy trees at harvest. These will be submitted for RNA-seq analysis. Samples were collected at harvest from healthy (or asymptomatic) and infected trees for additional cherry cultivars. Genes of interest from the RNA-seq analysis will be examined by qPCR in these other cultivars.

2. *Address differences in physiology of the trees in objective one by determining rate of photosynthesis and sugar content in phloem exudate in those trees.*

Preliminary analysis of sugar content in phloem exudate and cherry pulp from infected and healthy Bing cherries revealed no difference in phloem exudate but a significant decrease in sucrose in cherry pulp of LChV-2 infected cherries. Cherries have been collected from multiple cultivars with healthy, asymptomatic, and infected trees. Sucrose, glucose, fructose, and sorbitol content will be measured. In addition, total phenolic content, total flavonoid content, and total anthocyanin content will be measured by spectrophotometric assays. These data will provide a direction for a more targeted HPLC analysis. Photosynthesis was not measured. As the third year of this study is proposed to be continued in the new proposal, photosynthesis will be measured in the research block described in that proposal as all those trees will be in the same location, minimizing environmental variables.

3. *Determine relevant protein-protein interactions between Western X phytoplasma/LChV-2 protein products and protein products of selected genes from objective one using yeast two hybrid and bimolecular fluorescence complementation technologies.*

This objective is dependent upon the results from the transcriptomic studies in objective one. Based on the findings of the transcriptomic analysis, yeast two hybrid studies will begin in year three.

SIGNIFICANT FINDINGS

- **In the cherry pulp, sucrose was barely detected in LChV-2 infected trees, compared to Western X infected trees and healthy trees. Fructose was also reduced in LChV-2 infected trees. There was no significant difference for any of the three sugars when the phloem exudate was examined and for both phloem exudate and cherry pulp, there was no significant difference between Western X infected trees and healthy trees.**
- **Several genes associated with terpenoid production, sugar metabolism and transport, and plant development exhibited differential expression in fruit from WX infected trees at shuckfall.**

METHODS

1. *Perform transcriptomic analysis to compare healthy cherry trees, asymptomatic Western X & LChV-2 infected trees, and symptomatic Western X and LChV-2 infected trees to identify genes that may lead to symptom development.*

To determine which genes may be involved in development of little cherry symptoms, a transcriptomics study will be conducted. Healthy cherry trees and symptomatic trees harboring Western X and LChV-2 were selected for the study. The presence of Western X has been confirmed by PCR and LChV-2 by RT-PCR. In the first year, in June, fruit stem and leaf/midrib tissue was collected from each tree, total RNA was extracted from the tissue, and samples were submitted for library preparation and sequencing. The resulting data was analyzed to generate a transcriptome against which individual samples can be compared for differential gene expression analysis. In the second year, two time points were selected:

shuckfall and within two weeks of harvest (shuckfall was excluded for LChV-2 this year due to tree removal). Leaves, fruit stems, and fruit were processed. The shuckfall time point has been completed for WX and the data is in the process of being analyzed. RNA extractions are in process for the at harvest time points. Genes of interest will be selected from the RNA-seq data and gene expression will be examined in other cultivars that were sampled this summer.

2. *Address differences in physiology of the trees in objective one by determining rate of photosynthesis and sugar content in phloem exudate in those trees.*

Production of poor fruit in infected trees may be a result of physiological changes induced by the virus. To investigate this, in year one sugar content of phloem exudate and cherry pulp will be determined for the trees described in objective one. To determine sugar content, phloem exudate was collected using the centrifugation technique described by Hijaz and Killiny (2014). Cherry pulp was collected by quartering multiple cherries and homogenizing the tissue with a mortar and pestle. Both phloem exudate and cherry pulp were collected one week prior to harvest. As there was no significant difference in sugar concentration in phloem exudate, focus was shifted to cherry pulp. Cherries were collected from asymptomatic and symptomatic trees. Cherries were homogenized using a blender and the mixture was filtered twice through cheesecloth. Aliquots were stored at -80°C. Quantification of sucrose, fructose, glucose, and sorbital will be performed using enzymatic assays. Spectrophotometric assays will be used to determine total phenolic, total flavonoid, and total anthocyanin content in each sample. These data, combined with the transcriptomics data, will be used as a guide to determine which metabolites to focus on for HPLC analysis. These assays, due to their inexpensiveness and rapid generation of results, can be used to look at cherries from several different cultivars, not just the Bings used in objective one. The effect of disease state on rate of photosynthesis is also of interest. This will be investigated in the new proposal with which year three of this study will be combined. In the new proposal, a field block containing multiple varieties with replicates for healthy, LChV-2 infected, and WX infected trees will be established. Since these trees will be in the same block, they will be subjected to similar environmental conditions. This will remove or reduce much of the variability due to environmental conditions, making it likely that any observed differences between healthy and infected trees will be due to disease state.

3. *Determine relevant protein-protein interactions between Western X phytoplasma/LChV-2 protein products and protein products of selected genes from objective one using yeast two hybrid and bimolecular fluorescence complementation technologies.*

Symptom development for little cherry disease may be a result of protein-protein interactions between cherry proteins and pathogen proteins. To investigate this, relevant genes identified in objective one for both cherry and the pathogens will be selected for a yeast two hybrid screen. For Western X phytoplasma, which has a much larger genome than LChV-2 (~1200 compared to LChV-2's 10 genes), it will be necessary to use the differential gene expression data from objective one, as well as information regarding gene function, to select genes of interest. Yeast two hybrid analysis will be performed using the Clontech Matchmaker® gold yeast two hybrid system and will identify proteins that have the potential to interact. The yeast two hybrid system is a relatively quick means of identifying potential protein-protein interactions, however it occurs in artificial environment. To rule out any false positives, protein-protein interactions identified in the yeast two hybrid assay will be further investigated using bimolecular fluorescence complementation assays. These assays examine protein-protein interactions in plant cells, creating a more realistic environment than the yeast two hybrid assay.

RESULTS & DISCUSSION

1. *Perform transcriptomic analysis to compare healthy cherry trees, asymptomatic Western X & LChV-2 infected trees, and symptomatic Western X and LChV-2 infected trees to identify genes that may lead to symptom development.*

Symptomatic and healthy trees were selected for the study this year. Fruit, fruit stem, and leaf/midrib tissues were harvested from these trees for the transcriptomic study at shuckfall and close to harvest. Tissue was harvested only for WX infected and healthy trees at shuckfall; LChV2 was not included due to unexpected tree removal during the 2019 season. RNA-seq analysis has been performed for the shuckfall time point. While the data are still being analyzed, some genes of interested have been identified in the fruit tissue. These genes are associated with flavor (Table 1), development (Table 2), and starch and sugar synthesis (Table 3). A subset of genes associated with terpene and flavonoid synthesis were differentially expressed (Table 1) – changes in production of these metabolites may adversely affect flavor. The expression at shuckfall will need to be compared with those at harvest to identify any metabolites that are candidates for further study, such as HPLC analysis. Genes associated with development were among those differentially expressed (Table 2), which is not surprising as Western X cherries often appear as though they were halted in their development. Some of these genes were associated with the major phytohomones: abscisic acid, ethylene, auxin, gibberellins, and cytokinins. Interestingly a beta-D-glucosyl crocetin beta-1,6-glucosyltransferase was downregulated – the product of this gene is part of the pathway that generates crocin, a fruit pigment. Cytochrome P450 78A9 and HAIKU1 are important to fruit and seed development, as is hydroxyproline O-galactosyltransferase GALT6. These will need to be investigated further. Lastly, sugar and starch synthesis was examined (Table 3). Genes associated with glycolysis, sucrose synthesis, and starch synthesis were downregulated. A SWEET1 sugar transporter was downregulated while an ERD6-like transporter was upregulated. Sorbitol metabolism also seemed to be affected. Collectively, the genes in these three tables represent those that merit further study and will need to be compared with the findings for the tissues collected at harvest.

Tissue has been collect from fruit, fruit stem, and leaves for LChV-2 positive, WX positive, and healthy Bing cherries. RNA extractions are in progress for these tissues. RNA-seq analysis will be performed on these tissues and compared to the shuckfall data. In addition, leaf, fruit, and fruit stem tissues were collected from other cultivars at harvest. For genes of interest, qPCR analysis can be performed on these cultivars to determine if the gene expression patter is similar across multiple cherry cultivars. As no cultivar has thus far been proven to be tolerant to WX or LChV-2, a comparison across cultivars may help identify genes are pathways that are important to understanding this disease.

Table 1. Genes associated with flavor that are differentially expressed in WX infected fruit at shuckfall.

Gene	Change	Function
(-)-alpha-pinene synthase	Downregulated	Monoterpene synthesis
7-deoxyloganetin glucosyltransferase	Downregulated	Iridoid synthesis
Flavonoid 3'-monooxygenase	Downregulated	Flavonoid synthesis
Geraniol 8-hydroxylase	Upregulated	Terpenoid synthesis
Isoflavone 3'-hydroxylase	Upregulated	Isoflavonoid synthesis
S-linalool synthase	Upregulated	Monoterpene synthesis
Vinorine synthase	Upregulated	Alkaloid synthesis

Table 2. Genes associated with development that are differentially expressed in WX infected fruit at shuckfall.

Gene	Change	Function
1-aminocyclopropane-1-carboxylate synthase	Downregulated	Ethylene synthesis
Abscisic acid 8'-hydroxylase 1	Downregulated	Abscisic acid degradation
Abscisic acid 8'-hydroxylase 4	Upregulated	Abscisic acid degradation
Auxin-responsive protein SAUR50	Downregulated	Plant growth
Auxin-responsive protein SAUR71	Upregulated	Cell expansion regulation
Beta-D-glucosyl crocetin beta-1,6-glucosyltransferase	Downregulated	Fruit pigmentation
Cytochrome P450 714A1	Upregulated	Gibberellin synthesis
Cytochrome P450 78A9	Downregulated	Seed and fruit development
Cytokinin dehydrogenase 1	Upregulated	Cytokinin synthesis
Cytokinin riboside 5'-monophosphate phosphoribohydrolase LOG3	Downregulated	Cytokinin activation
Hydroxyproline O-galactosyltransferase GALT6	Downregulated	Reproductive plant growth
Protein BIG GRAIN 1-like E	Upregulated	Auxin transport
Protein HAIKU1	Downregulated	Regulates seed size
Xyloglucan endotransglucosylase/hydrolase 2	Downregulated	Tissue growth
Xyloglucan endotransglucosylase/hydrolase protein 23	Downregulated	Tissue growth

Table 3. Genes associated with starch and sugar synthesis that are differentially expressed in WX infected fruit at shuckfall.

Gene	Change	Function
ATP-dependent 6-phosphofructokinase 2	Downregulated	Glycolysis
Bidirectional sugar transporter SWEET1	Downregulated	Glucose transport, also fructose, mannose and galactose
Fructose-1,6-bisphosphatase, chloroplastic	Downregulated	Sucrose synthesis
Isoamylase 1, chloroplastic	Downregulated	Starch synthesis
Sorbitol dehydrogenase	Downregulated	Sorbitol metabolism
Sucrose synthase	Downregulated	Sucrose synthesis
Sugar transporter ERD6-like 7	Upregulated	Sugar transport

2. *Address differences in physiology of the trees in objective one by determining rate of photosynthesis and sugar content in phloem exudate in those trees.*

Like the first year, photosynthesis was not assessed this year as it was felt that the trees were in sufficiently different environments that environmental variables would overshadow any effects LChV-2 or Western X presence might have on the photosynthetic rate. Grafting of buds from infected trees onto rootstocks was planned as a way of addressing this. However, we are proposing carrying the third year of this study into the new proposal. In the new proposal we will have a field block with multiple cultivars, each cultivar having healthy, LChV-2 infected, and WX infected trees. Having these trees in the same location means they are exposed to the same environment, reducing the effects environment will have not only on photosynthetic rate, but also gene expression and metabolite production.

In year one, no difference was observed in sugar content for phloem exudate from infected and healthy trees. However, a significant decrease in sucrose was observed for LChV-2 infected trees (Figure 1). This year, fruit was collected not only from healthy and infected Bings, but other cultivars as well. In the first year, only sucrose, fructose, and glucose was measured. In addition to repeating those tests, the amount of sorbitol, total phenolics, total flavonoids, and total anthocyanins will be measured. These are simple, spectrophotometric tests that can be performed on a large number of samples. Based on the findings of these tests and the RNA-seq analysis, specific molecules will be targeted for HPLC analysis.

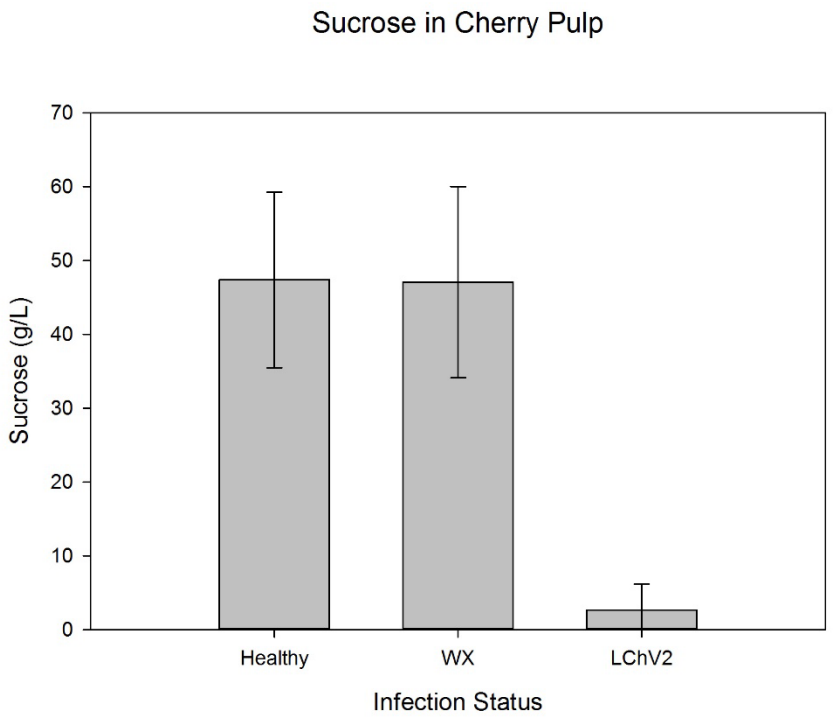

Figure 1. Sucrose measurements in cherry pulp from healthy, WX infected, and LChV-2 infected Bing trees.

3. *Determine relevant protein-protein interactions between Western X phytoplasma/LChV-2 protein products and protein products of selected genes from objective one using yeast two hybrid and bimolecular fluorescence complementation technologies.*

Candidate proteins for yeast two hybrid screening will be identified from the transcriptomics analysis. Work on this objective will begin during year three.

CONTINUING PROJECT REPORT **YEAR: 1 of 3**

Project Title: Development index model of sweet cherry

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Cooperators: Alan Reitz Mount, Adams Fruit; Garrett Bishop, GS Long; Mark Lapierre, Wilbur Ellis; Eric Shrum, Mike Omeg, Orchard View Farms

Total Project Request: Year 1: $131,909 **Year 2: $136,083**

Other funding sources: Year 1: Columbia Gorge Fruit Growers, $23,562; Washington Blueberry Commission, $5,250 **Year 2:** Columbia Gorge Fruit Growers, $23,562; Washington Blueberry Commission, $5,250

Budget 1
Organization Name: OSU-ARF **Contract Administrator:** Russ Karow
Telephone: 541-737-3228 **Email address:** Russell.Karow@oregonstate.edu

Item	2019	2020
Salaries[1]	$39,026	$40,197
Benefits	$23,696	$24,407
Wages[2]	$17,213	$17,729
Benefits	$13,657	$14,067
Equipment	-	-
Supplies[3]	$1,000	$1,000
Travel[4]	$2,000	$2,000
Plot Fees	-	-
Miscellaneous	-	-
Total	$96,592	**$99,400**

Footnotes:
[1]Postdoctoral Research Associate: 0.7 FTE with 3% increase factored into Year 2.
[2]Biological Science Tech: 0.5 FTE with 3% increase factored into Year 2.
[3]Miscellaneous supplies for sample collection and preparation.
[4]Travel to grower field for sample collection.

Budget 2

Organization Name: WSU **Contract Administrator:** Karen Kniep, Katy Roberts
Email address: **kmkniep@wsu.edu**, Katy.Roberts@wsu.edu

Item	**2019**	**2020**
Salaries	25,357	26371
Benefits	8,757	9,108
Wages		
Benefits		
Equipment		
Supplies		
Travel	1,203	1,204
Miscellaneous		
Plot Fees		
Total	35,317	**36,683**

Footnotes:
[1]**Systems Analyst:** 4.7 months at 1.0 FTE

JUSTIFICATION

Modelling is an extremely useful tool to assist scientists, growers and distributors with planning and execution of strategic objectives. This suite of models named AUTUMN WINTER SPRING (AWS), is aimed at achieving the following:

Objective 1. Predicting critical temperatures of **freeze tolerance** for flowers of sweet cherry (beginning in September and ending at bloom).

Activity 1.

- Assessed freeze tolerance during vernalization and dormancy using Differential Thermal Analysis (DTA) for cultivars at MCAREC: Attika, Benton, Bing, Chelan, Lapins, Rainier, Regina and Skeena.
- Assessed relative water content data of floral buds from MCAREC, and from other locations and cultivars provided by collaborators.
- Assessed bloom timing by flower counts for MCAREC, and for other locations and cultivars provided by collaborators.

Significant Finding 1. The relationship of floral bud phenology, as measured by relative water content, and 50% Lethal Temperature was updated (Table 1).

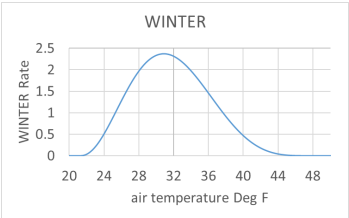
Table 1. Updated AWAKENING table for the estimation of lethal temperatures and phenology. These values were determined experimentally using Differential Thermal Analysis (DTA) and include data from 2018-19.

rwc	Lt50 Regina Deg F	Lt50 all other cultivars Deg F
0.56	-7.6	-4.2
0.58	-5.6	-2.2
0.6	4.8	8.6
0.62	10.9	14.6
0.64	15.3	18.1
0.66	18.7	20.6
0.68	22.1	22.4
0.7	23.5	23.5
0.72	24.4	24.4
0.74	25.2	25.2
0.76	25.9	25.9
0.78	26.4	26.4
0.8	27.0	27.0
0.82	27.3	27.3
0.84	27.5	27.5

Objective 2. Developmental model of sweet cherry (September - fruit maturation): location & cultivar dependent

Activity 2. Modelling of lethal temperature during autumn (double Beta-function) and winter (exponential rates of acclimation and deacclimation—*aka fteed*) has not changed. However, this approach did not well predict the date of Vernalization. Vernalization is the date at which warming temperature will induce developmental changes leading to flowering, therefore a good prediction is critical to improving AWS. A chilling –degree type model, WINTER (Fig. 1) was added to AWS.

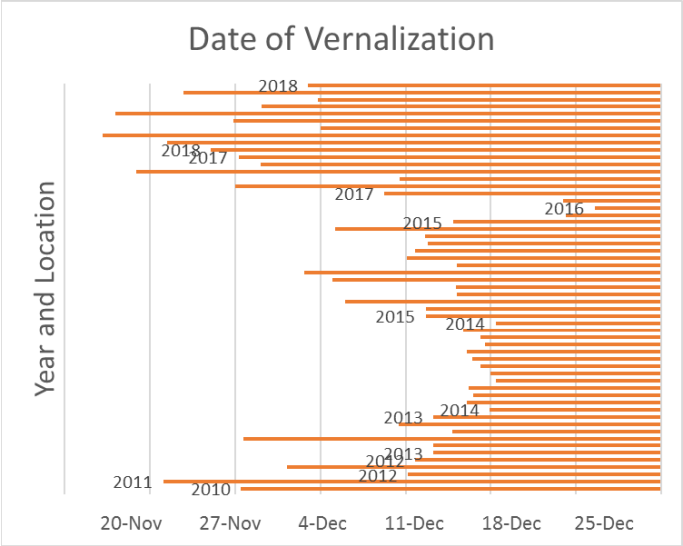
Fig 1. A chilling-degree model for WINTER versus temperature. This models accounts for freezing temperatures



Significant Finding 2.

- We now refer to this suite of models as: AUTUMN WINTER SPRING (AWS)
- WINTER accumulates to a Vernalization set point that estimates the date of Vernalization (Fig 2.). The set point was optimized by changing the variables of the Beta-function. When the Vernalization set point is met, the SPRING model begins to accumulate (Fig 3).

Fig 2. Dates for Vernalization from 57 instances, years 2010 to 2018, outside left of each bar.

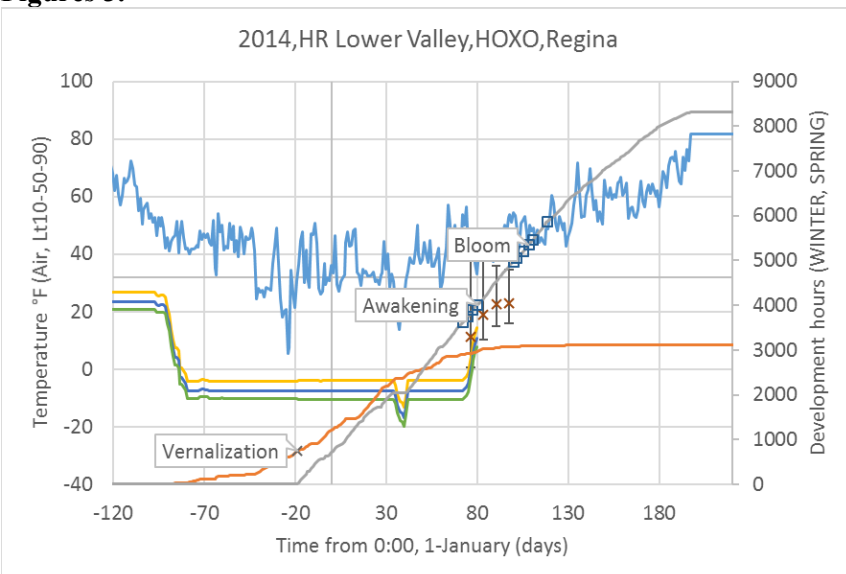


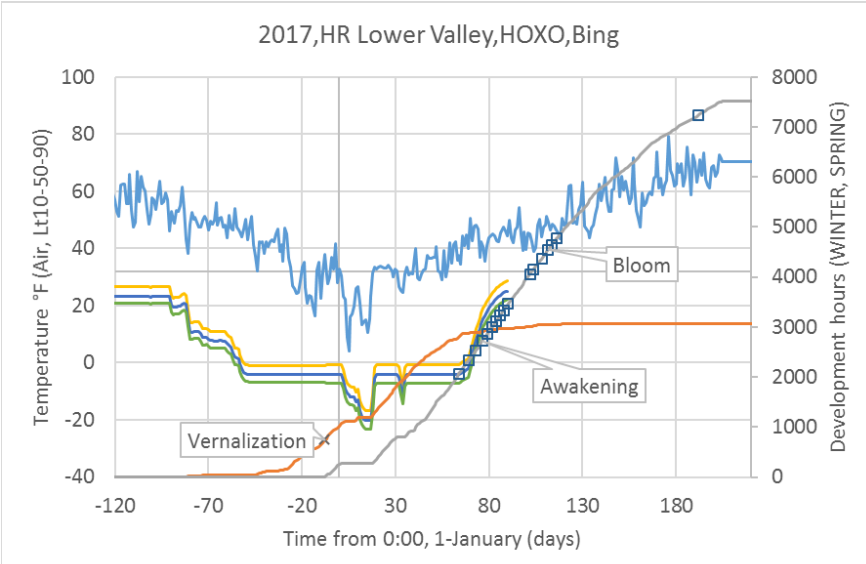
Objective 3. Presenting these models on AgWeatherNet for all to access.

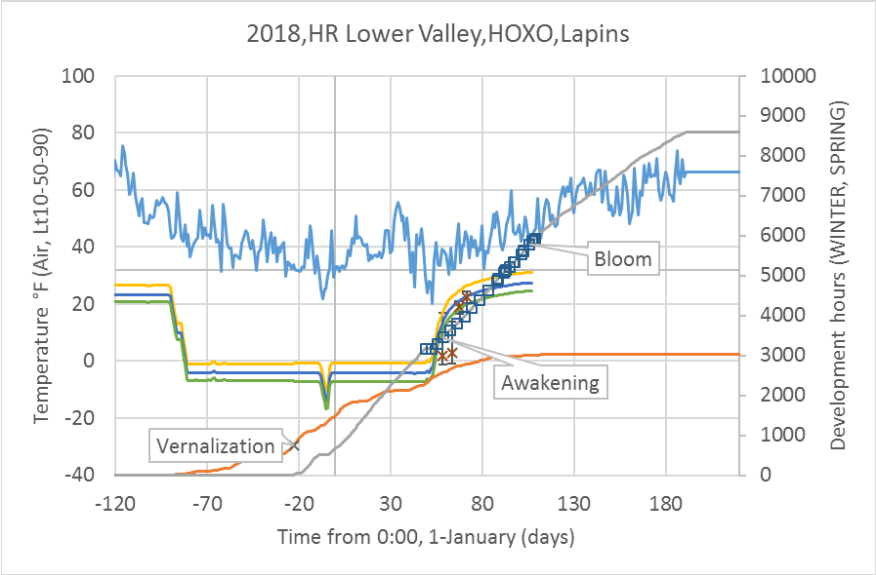
Activity 3. The freeze tolerance portions of AWS are coded and currently running but at present, only visible to beta-testers and researchers.

Significant Finding 3. Examples of AWS model outputs with some of the DTA (x's) and phenology (squares) data that were used to optimize the models. The upper lines are the instantaneous air temperature, the three parallel are Lt 10-50-90%, Vernalization is the date when the set point for the chilling-model, WINTER, was achieved. The growing-degree model, SPRING, begins at Vernalization.

Figures 3.






2018,HR Lower Valley,HOXO,Lapins
Temperature °F (Air, Lt10-50-90)
Development hours (WINTER, SPRING)
Time from 0:00, 1-January (days)
Bloom
Awakening
Vernalization
100
80
60
40
20
0
-20
-40
10000
9000
8000
7000
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5000
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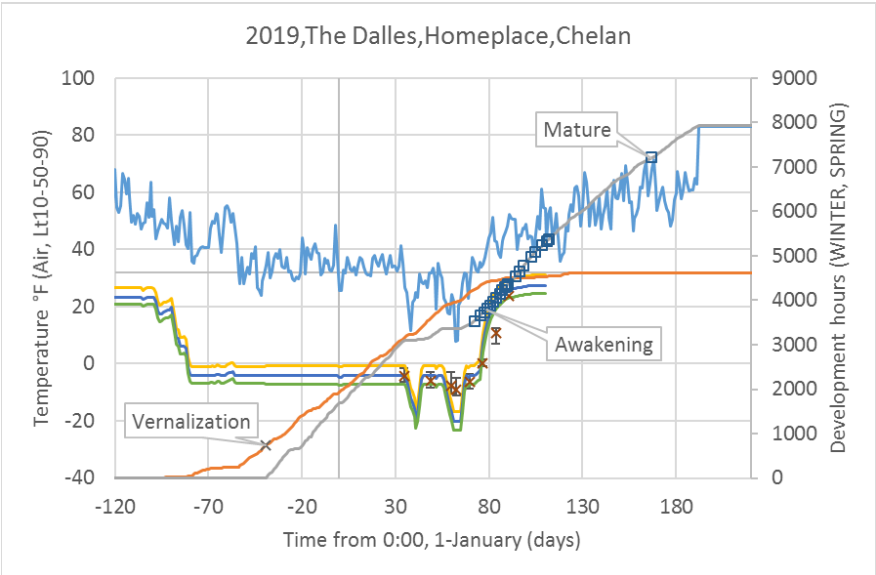
0
-120
-70
-20
30
80
130
180


2019,The Dalles,Homeplace,Chelan
Temperature °F (Air, Lt10-50-90)
Development hours (WINTER, SPRING)
Time from 0:00, 1-January (days)
Mature
Awakening
Vernalization
100
80
60
40
20
0
-20
-40
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-120
-70
-20
30
80
130
180

CONTINUING REPORT **YEAR**: 1 of 3 Years

Project Title: Supporting a robust PNW sweet cherry breeding and genetics program

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Cooperators: Allan Bros. Fruit, Orchardview Farms, Stemilt Growers, Breeding Program Advisory Committee (BPAC) members

Total Project Request: **Year 1:** $48,623 **Year 2: $174,559** **Year 3:** $183,584

Other funding sources

Agency Name: USDA NIFA – SCRI
Amt. awarded: $10 million (2014-2019)
Notes: RosBREED 2 project. PI: Amy Iezzoni. Co-PIs include Cameron Peace and Per McCord.

Agency Name: WSDA Specialty Crop Block Grant
Amt. awarded: $188,757 (2019-2022)
Notes: "Reducing Cold Damage in Tree Fruit". Co-PI: Matt Whiting.

Agency Name: WTFRC/OSCC
Amt. awarded: $79,000 (2019)
Notes: "Equipping the re-launched PNW cherry breeding program".

Agency Name: WTFRC/OSCC
Amt. awarded: $88,000 (2019-2020)
Notes: "Durable genetic solutions to powdery mildew infection in sweet cherry". PI: Cameron Peace. Co-PIs: Per McCord, Prashant Swamy.

Agency Name: WTFRC/OSCC
Amt. requested: $458,022 (2020-2022)
Notes: "Understanding little cherry disease pathogenicity". PI: Scott Harper. Co-PIs: Alice Wright, Per McCord.

Budget 1
Organization Name: Washington State University **Contract Administrator:** Katy Roberts
Telephone: (509) 335-2885 **Email address:** arcgrants@wsu.edu

Item	2019	**2020**	2021
Salaries[1]	$45,760	$37,440	$38,938
Benefits	$19,493	$16,230	$17,327
Wages[2]	$31,200	$32,450	$33,750
Benefits[3]	$10,564	$5,390	$5,606
Equipment			
Supplies[3]	$9,760	$33,325	$37,425
Travel	$4,000	$5,500	$6,100
Miscellaneous[4]	$40,000	$19,259	$17,438
Plot Fees	$4,275	$7,630	$8,800
Carryover from 2018 request	-$132,665		
Total	$32,387	**$157,224**	$165,384

Footnotes: [1]Includes Horticultural Support in 2019 (only), plus 1.0 FTE research technician. [2]Includes temporary labor for crossing, harvesting, seed extraction/transplanting, plus farm crew wages. [3]Reduction of benefit costs for 2020-21 reflects a more accurate estimate based on actual 2019 expenses. [4]Supplies for fruit evaluation, DNA extraction/genotyping, embryo rescue, propagation supplies/services, orchard maintenance. Amount is increased from original request as a result of more detailed expense information. [5]Irregular expenses. Amount is reduced from original request as a result of more detailed expense information.

Budget 2
Organization Name: OSU-MCAREC **Contract Administrator:** Russell Karow
Telephone: 541-737-3228 **Email address:** russell.karow@oregonstate.edu

Item	2019	**2020**	2021
Salaries[1]	$5,405	$6,005	$6,305
Benefits	$4,486	$4,985	$5,234
Wages[2]	$3,840	$3,840	$4,032
Benefits	$384	$384	$403
Equipment			
Supplies			
Travel			
Plot Fees			
Miscellaneous[3]	$2,121	$2,121	$2,226
Total	$16,236	**$17,335**	$18,200

Footnotes: [1] Estimated salary for technician to complete pruning, thinning and data collection. [2] Wages for one part-time employee ($16/hr) to assist with orchard activities. [3] Fees include per-acre research plot fees ($3104/acre), 2 months cold storage room fee ($1.24/square foot) and miscellaneous lab supplies.

OBJECTIVES

1. Build a well-trained support team to maintain and improve horticultural practices in the breeding orchard and maximize breeding efforts
2. Continue to rigorously evaluate existing selections in Phase 2 (P2) and seedlings in Phase 1 (P1). *Advance selections as warranted to Phase 3 (P3)*
3. Increase the number of targeted crosses made, seeds germinated, and seedlings transplanted
4. Enhance precocity and reduce external variation in the seedling blocks (*postponed to Year 2*)

SIGNIFICANT FINDINGS

- Hired Research Associate technician in January 2019. Another, WSU-funded technician also started in January 2019
- Trained new technicians, temporary employees in proper pruning techniques
- Fertilization of orchard blocks guided by soil and foliar analyses
- Followed up on 2018 bioassay virus screen (C53 block) by testing additional trees via real-time RT-PCR. Thirty-seven additional positive trees identified (to be removed this fall)
- Removal of trees in A36 block is nearly complete (will finish by the end of 2019)
- Screened A37 block (242 trees) for *Prune dwarf virus* using real-time RT-PCR. Over 140 trees tested positive and will be removed
- Evaluated 143 P1 and P1.5 selections
- Advanced one P1, one P1.5, and four of the Prosser-only P2 selections to full (multi-location) P2 trials
- Evaluated 14 P2 selections (7 multi-location, 7 at Prosser only)
- Advanced 3 mahogany P2 selections to P3 (R3, R19, R29)
- Established new P1 block at Prosser IAREC headquarters with 752 seedlings
    - Eliminated approximately 20 percent of seedlings prior to transplanting using DNA tests for self-fertility and powdery mildew resistance
- 61 bi-parental crosses were made in the 2019 crossing season, along with seed collected from 7 open-pollinated families, resulting in approximately 6,970 seed
- Tests of the ability of ReTain to boost seed set in emasculated, hand-pollinated crosses show some promise, but more testing is required
- Solitary bees (mason bees) used successfully in cages, producing nearly 2,700 seed from 9 crosses
- Successfully initiated embryo rescue as part of the CBP, with over 770 embryos. Testing of different growth media is underway

METHODS

1. *Support team and horticultural practices*

Ms. Corina Serban (funded by WTFRC/OSCC) and Dr. Michael Stein (funded by WSU) both began working for the breeding program in January 2019. Corina has primary responsibility for the orchards, while Michael's primary responsibilities include the greenhouse, laboratory, and data management. Both are being cross-trained so that they can support each other in all other aspects of the breeding program.

Horticultural practices were continued or implemented generally as follows:

- Nutrient management: standard practices for soil and foliage analysis to guide fertilizer applications. For the new seedling planting (described below), a double drip system (micro-sprinklers and drip tape) was installed to allow fertigation. We expect to use this system for future seedling blocks.

- Weed control was maintained satisfactorily through a combination of mowing, herbicide spraying, and manual weeding.
- In the absence of a dedicated orchard manager, spray recommendations were provided by a commercial crop consultant and implemented by the farm manager and his crew after consulting with Dr. McCord.
- Under the direction and guidance of Dr. Sallato, the Sagemoor and Hood River (MCAREC) blocks were pruned during the winter, as well as portions of the Roza (particularly weaker trees). The remainder of the Roza (particularly C53 and the seedlings) was pruned during the summer.
- Thinning to 30 fruit/foot was performed as needed on P2 selections and standard cultivars. A crop load rating before thinning was determined for reference.
- Virus monitoring and control: As a follow-up to the 2018 bioassay for *Prune dwarf virus* (PDV) and *Prunus necrotic ringspot virus* (PNRSV) conducted in block C53, additional trees (neighbors of the 2018 positives) were tested for PDV (which is more prevalent) via real-time RT-PCR. The entire A37 block (comprising mostly Tom Toyama's breeding selections) was also screened for PDV. These screens were done in-house by Dr. McCord and other CBP employees. Finally, 122 additional samples were screened by the Clean Plant Center NW for PDV, PNRSV, *Little cherry virus*-2 (LChV-2), Western X phytoplasma, *cherry leaf roll virus* (CLRV), *cherry rasp leaf virus* (CRLV), and *cherry virus A* (CVA). Note: not all samples sent to the CPCNW were screened for all viruses.

2. *P1 and P2 evaluations*

As in prior years, BPAC members were invited to inspect P1 seedlings during the fruiting season. Walkthroughs were conducted 1-2 times per week, with BPAC members visiting once per week. Selection criteria in the field was based on fruit size, firmness. Fruit from selected P1 seedlings, all current P2 selections, and standard cultivars were evaluated in the laboratory for defects (harvest and post-harvest), weight, diameter, firmness, stem pull force (P2 only), color, Brix, and titratable acidity.

In order to maximize efficiency, P1 selections that did not meet the thresholds of weight (minimum 9 grams) or firmness (minimum 270 g/mm) were not evaluated for downstream traits. We improved our storage conditions for post-harvest analysis by reducing the temperature in our walk-in cooler to approximately 35°F, and using modified-atmosphere packaging. We also increased our storage time from 2 to 4 weeks. An 'induced pitting' protocol was implemented by putting fruit in a bucket on an orbital shaker platform for 3 minutes at 200 rpm. When sufficient fruit was available, we also performed an induced cracking test based on a 4-hour soak in deionized water. This season, laboratory data collection was completely digital (using Android and cloud-based forms), and we are using a database (Breeding Information Management System, BIMS) to store data and query data.

Existing P2 trials suffer from a lack of proper experimental design. Moving forward, we will incorporate proper replication and randomization to improve data quality and allow statistical comparisons to be made between selections and standard cultivars. Specifically, we are increasing the number of trees per selection to 15, which will allow for replication of plot sizes with several trees. We are also in conversations with one of our cooperators to add an additional P2 site.

3. *Crossing and seedling production*

In order to make better progress in selecting superior new varieties, consistent, larger numbers of seedlings need to enter the selection pipeline. Our goal is to produce 10,000 seeds per year, with an average germination of 50%. For the 2019 crossing season, the majority of crosses were made using emasculated, hand-pollinated flowers. In an effort to improve fruit set (which is generally low using this method), we tested the effects of the ethylene inhibitor ReTain (aminoethoxyvinylglycine).

Retain (880 mg/L of a commercial preparation) was sprayed on flower buds either 1 or 2 days before pollination. For flowers being pollinated the following day, emasculation occurred the same day as the Retain treatment. For flowers being pollinated 2 days later, emasculation occurred the day after Retain application. A control group of flowers on each tree in the experiment was left unsprayed. Fruits were counted in May, once it could be reliably determined which flowers had set fruit.

For crosses made in cages using bees, a new approach was the use of mason bees (*Osmia ligaria*). Mason bees are said to be much more efficient than honeybees, and are less expensive when used for crossing purposes. A bundle of 5 reeds (with approximately 5 bees per reed) was secured to each mother tree with stretch tape, approximately 3 feet from the ground, in an east-west orientation. Male flowers were placed in a small garbage can, and the mother tree was caged in bee-proof netting. We intended to also use honeybees as a comparison, but due to very rapid bloom progression, only 1 bee-pollinated cross was made with honeybees, vs. 9 crosses with mason bees.

Embryo rescue was utilized in 2019 for the first time in the history of the CBP. Crosses targeting early maturity were the major focus of the effort, as well as crosses between sweet cherry and other *Prunus* species. Immature fruit were harvested and rinsed with water. Several sterilization methods were informally tested. The general method for early crosses was to first rinse the fruit with tap water for 20 seconds. After the stones were extracted from the fruit, they were soaked in a 10% solution of household bleach for 10 minutes, followed by 3 rinses with deionized water. The stones were then transferred to a laminar flow hood, where they were further sterilized by soaking in 70% ethanol (with a drop of dishwashing detergent as a surfactant) for 3 minutes. The ethanol was poured off, and a pair of anvil pruners (sprayed with 70% ethanol) was used to crack the seeds, which were then placed on nutrient medium. Two different media were tested; Woody Plant Medium and a medium developed by INRA at Bordeaux, France. Embryos were incubated in the dark at approximately ~5 °C. After 1.5 months, a subset were transferred to lighted conditions (~150 µmol/s-m) at ~22 °C. The remaining embryos are being moved to lighted conditions only after visible germination has occurred.

For the seedlings from 2018 crosses, leaf tissue was collected in 96-well strip tubes, desiccated using silica gel, and sent to Cameron Peace's lab in Pullman for DNA extraction and testing. DNA tests included self-fertility (2 sources), fruit size (for information only, not used for seedling elimination), and powdery mildew resistance. Surviving seedlings were transplanted to the field in two cohorts. The main group were planted in late May, and a smaller group was planted in early September.

4. *Enhancing precocity and reducing variation in seedling blocks*

On average, it takes from 3-5 years for a seedling cherry to begin fruiting. Increasing precocity in seedlings can shorten the breeding cycle and increase efficiency by more quickly allowing the breeder to identify desirable seedlings. Budding seedlings onto a precocious rootstock is expected to not only shorten the time to first fruiting, but also to enhance fruit production in succeeding years. In addition, budding has the advantage of eliminating the variation caused by trees growing on their own roots. To determine whether these expected advantages outweigh the labor and cost of budding seedlings, we will perform a pilot study with 100-200 seedlings from several families. Gisela-6 rootstocks were delivered in March 2019, but were too small for budding. In addition, the seedlings, though they had sufficient caliper, were not hardened off sufficiently for budding. The rootstocks were transplanted to the same field as the seedlings (described above), and will be budded in spring 2020. Additional Gisela-12 rootstocks were ordered in summer 2019. They are being maintained in the greenhouse in the hope that both they and the new crop of seedlings in the greenhouse will be large enough to be budded in the spring prior to transplanting.

RESULTS & DISCUSSION

1. *Support team and horticultural practices*

Since joining the CBP in January 2019, Corina and Michael have made significant contributions to the success of the program. Their input and dedication were critical to successful crossing and harvest seasons, both of which were much more extensive than the 2018 season (Dr. McCord's first year). The expansion of the breeding program, improvements to operations, and critical research activities, would not have been possible without their assistance.
As for 2018, fertilizer applications were guided by soil and foliar analyses. The new seedling planting was laid out with drip tape and microsprinklers. We intend to use this system for all future plantings, which should make fertilizer applications more flexible and precise. The spray program, gave acceptable levels of control of target pests and diseases, such as SWD, western cherry fruit fly, and powdery mildew.
We followed up on our virus bioassay screen of the C53 ('RosBREED') block by screening additional trees using real-time RT-PCR, which is more sensitive than the bioassay. We identified thirty-seven additional PDV-positive trees, which will be removed this fall. In most cases, the positive trees were replicates of the trees that tested positive last year, suggesting that the trees became infected through the use of infected budwood, and not from infected pollen. We also screened every tree in block A37 for PDV. The majority of the trees tested positive. All positive trees are slated for removal. We will propagate the PDV-negative trees prior to the removal of the entire block, and will further test the newly budded trees to ensure they are free of PDV (and PNRSV).
Some trees in the D53 (Pathology) block showed symptoms of Little Cherry Disease. In response, we surveyed C53 and B53 for both LChv-2 and X-disease phytoplasma. No positive trees were found in these blocks, although additional tests on these trees detected a small number infected with other viruses. We will continue to screen our parental blocks as well as selections advancing through the program.

2. *P1 and P2 evaluations*

A total of 143 P1 and P1.5 individuals passed field evaluations and were further tested in the lab. Two selections, CR01T078 (P1) and R35 (P1.5), showed strong performance over at least 4 years of observation, and are being advanced to Phase 2. Their characteristics are described in Table 1.

Table 1. Characteristics of selections advanced to Phase 2

ID	Timing	Fruit weight (g)	Row size	Firmness (g/mm)	Color	Notes
CR01T078	Bing +8 (2017-2019)	11.5	~9 (29.5mm)	354	Mahogany	Low cracking (2019)
R35	Bing +15 (2019)	12.7	9 (30.1mm)	324	Blush	

For Phase 2, seven selections were evaluated across multiple locations, and an additional seven were evaluated only at Prosser. All are mahogany. Of the first seven, three are of particular interest, and are being advanced to Phase 3 (pre-commercial) trials (Table 2). R19 is the earliest of these selections, ripening with Chelan. It is larger, firmer, and has much better flavor than Chelan (and is also self-fertile), but it may have problems with decay in post-harvest. R3 is slightly later than R19, but is larger, with good firmness and texture, and acceptable flavor when allowed to ripen fully. For mid-season, R29 has excellent size, is self-fertile, and appears to hold up very well in storage.
Table 2. Characteristics of seven Phase 2 selections planted in multiple locations (Prosser, Sagemoor (Pasco), and MCAREC (Hood River).

ID	Timing	Fruit weight (g)	Row size	Firmness (g/mm)	Notes	Status
Chelan		7.5	11 (24.1mm)	276		
R3	Chelan +4	10.9	9.5 (28.5mm)	338	Good size for timing	Advance
R19	Chelan +0	9.3	9.5-10 (27.8mm)	367	Very sweet, firm, self-fertile Storage issues	Advance
Bing		8.3	10.5 (25.7mm)	269		
R29	Bing +5	13.4	8.5-9 (30.8mm)	315	Very large, self-fertile, stores well	Advance
R1	Bing -1	11.1	9.5 (28.9mm)	317	Cracks, so-so size for timing	Drop
R6	Bing +6	10.7	9.5 (28.0mm)	260	Sour, uneven ripening	Drop?
R17	Bing +6	10.7	9.5 (27.9mm)	308	OK	Evaluate
R21	Bing +6	9.1	10 (26.3mm)	349	Small	Drop

Of the second group of Phase 2 selections (Table 3), three were dropped from further consideration, and the remaining five are being propagated for planting in a proper multi-location Phase 2 trial (including a new trial at Prosser). This trial will include the two selections from Phase 1/1.5.

Table 3. Characteristics of Phase 2 selections currently only at Prosser

ID	Timing	Fruit weight (g)	Row size	Firmness (g/mm)	Notes	Status
Bing		6.4	12 (21.2mm)	238		
R45	Bing +27[1]	7.8	9.5-10 (27.7mm)	273	Very light crop (small trees)	Advance
R46	Bing -5	9.8	10 (27mm)	278	Early mid-season	Advance
R47	Bing -5	9.9	10 (26.5mm)	372	Very firm	Advance
R48	Bing -5	8.9	10 (27.1mm)[2]	232	Soft, cracking issues	Drop
R49	Bing +17	9.6	9.5 (28.0mm)	329	Stem browning	Drop
R50	Bing +17	10.4	9.5 (28.5mm)	308	Decent size,	Advance
R51	Bing +17	8.9	10 (26.6mm)	296	Small for timing	Drop

[1]R45 was harvested when over-mature. [2]Diameter is from post-harvest samples.

3. *Crossing and seedling production*

A total of 61 bi-parental crosses and 7 open-pollinated families resulted in more than 6,900 seed (vs. 6,000 in 2018). We are therefore making good progress towards the CBP goal of 10,000 seeds annually. Expanded use of bees in cages, and techniques to increase fruit set in hand-pollinated crosses (see below) should enable us to reach this goal. Cross combinations were guided by DNA information and phenotypic performance. Major targets for crosses made included early/late maturity, fruit size, powdery mildew resistance, and self-fertility.

The Retain experiment involved more than 6,150 flowers across all treatments. Untreated flowers had an average of 10% fruit set, compared to 8% for flowers sprayed the day before pollination, and 27% for flowers sprayed 2 days before pollination. However, the variation within treatments was such that there was no statistically significant difference between treatments. This may have been due to the wide range of variation in fruit set from one cross to another (data not shown). We will test Retain again during the 2020 crossing season.

We were very pleased with the results from using mason bees in the crossing program. Nine crosses were made with these bees, resulting in an average of 296 seeds per cross (2667 total). In addition, the bees are easier to work with than honeybees; the reeds are kept in a refrigerator until shortly before use, and the bees are still inside the reeds when they are put into the cage. Finally, the cost per cross is less than half that of honeybees. We will expand the use of mason bees in the 2020 crossing season.

The embryo rescue experiment is still ongoing, but preliminary results are encouraging. Seeds have continued to develop in the nutrient medium, a good number have germinated, and we are just beginning to transfer young seedlings into pots. At this point, there does not seem to be a difference between the two nutrient media. Improvements/changes for the 2020 crossing season include increasing the number of embryos rescued, and harvesting the fruit slightly sooner.

Of the approximately 6,000 seeds generated in the 2018 crossing season, 1531 seedlings were sampled for DNA extraction and genotyping. Based on the results of the tests for self-fertility and powdery mildew resistance, 300 seedlings (19.6%) were discarded, and 752 of the surviving seedlings have currently been planted in the field. We expect a slightly higher percentage of seedlings will be culled from the 2019 seedlings, as more of the families will be segregating for the markers identified by the DNA tests. We are also expecting better germination from the use of fungicide, and we are seeing germination earlier in the season, most likely due to cracking of the seeds prior to stratification.

In summary, the Pacific Northwest sweet cherry breeding program (CBP) is making significant progress in its mission to develop superior cherry cultivars for the Oregon and Washington industries. The hiring of two dedicated technicians has been critical to the relaunch and expansion of the program. Horticultural practices put in place in recent years are being maintained in order to reduce viruses, and enhance tree health and fruit quality. Promising selections are being advanced to Phase 3 trials, and the pipeline is being fed by the advancement of seedlings into improved Phase 2 trials, and the planting of a new seedling block. The use of DNA information has led to better-informed crosses, and improved the quality of seedlings transplanted to the field. Improvements to crossing and seed-handling procedures are expected to increase the number of seedlings, including those targeting early maturity.

CONTINUING PROJECT REPORT **YEAR**: 1 of 2 Years

PROJECT TITLE: Durable genetic solutions to powdery mildew infection in sweet cherry

PI: Cameron Peace
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City/State/Zip: Prosser/WA/99350

Cooperators: Alexandra Johnson (WSU graduate student – Horticulture, Pullman); Gary Grove (WSU – IAREC Plant Pathology, Prosser)

TOTAL PROJECT REQUEST: Year 1: $44,000 **Year 2: $44,000**

Other funding sources:

Agency Name: USDA NIFA – SCRI
Amt. awarded: $10 million (Sep 2014 – Aug 2019)
Notes: RosBREED 2 project for expanding DNA-informed breeding strategies, tools, and knowledge for rosaceous crops. PI: Amy Iezzoni. Co-PIs include Cameron Peace and Per McCord.

Agency Name: WTFRC/OSCC
Amt. awarded: $539,661 (2019-2021)
Notes: "Supporting a robust PNW sweet cherry breeding and genetics program". PI: Per McCord. Co-PIs: Cameron Peace, Bernardita Sallato, Mateus Pasa.

Agency Name: Washington State Department of Agriculture
Amt. requested: ~$240,000
Notes: Application period recently opened (pre-proposals due 2 Nov 2019). Intended research to include evaluating inheritance of genetic resistance in germplasm often with unknown/unclear ancestry, and seeking heightened genetic resistance in offspring combining resistant from multiple sources.

Agency Name: National Science Foundation – Graduate Research Fellowship Program
Amt. requested: ~$140,000
Notes: Applications due 18 Oct 2019. Independent funding sought by the graduate student participating in the project (Alexandra Johnson).

BUDGET

Organization Name: W.S.U. **Contract Administrator:** Katy Roberts
Telephone: 509-335-2885 **Email address:** katy.roberts@wsu.edu

Item	2019	**2020**
Salaries[a]	$26,236	**$27,285**
Benefits	$2,443	**$2,541**
Wages	$5,330	**$5,543**
Benefits		
Equipment		
Supplies[b]	$3,516	**$2,156**
Travel[c]	$2,000	**$2,000**
Plot Fees	$4,475	**$4,475**
Miscellaneous		
Total	$44,000	**$44,000**

Footnotes

[a] Graduate student support for Alexandra Johnson
[b] Single use, disposable materials for sample collection and laboratory assays
[c] Pullman-Prosser return for approx. 4-5 multi-day trips during spring and summer each year

OBJECTIVES

1. Determine the long-term durability of Pmr1 for providing mildew resistance to the PNW industry
  a. Ascertain the pathogen's ability to overcome Pmr1 resistance
  b. Update knowledge about which selections and other PNWSCBP germplasm have Pmr1

2. Determine usability of alternative genetic sources for powdery mildew resistance
  a. Evaluate a diverse set of germplasm for degree of fruit powdery mildew resistance
  b. Identify other genetic factors capable of conferring mildew resistance in PNWSCBP germplasm
  c. Refine the DNA test for resistance to encompass new sources if they exist
  d. Identify which alternative genetic resistance factors are present in important germplasm individuals

SIGNIFICANT FINDINGS

- Four genetic factors at the Pmr1 genomic locus under examination for their influence on genetic resistance/susceptibility to fruit and foliar powdery mildew (PM) resistance:
  - Pmr1a ('Moreau'/'Chelan'/PMR-1)
  - Pmr1b ('Hedelfingen'/'Venus' and Mildew-Immune Mazzards)
  - Pmr1c ('Schneiders'/ 'Regina')
  - pmr1 – the common genetic factor associated with susceptibility
- Pmr1a: Confers <u>complete resistance</u> to PM infection, as concluded in previous years.
- Pmr1b: So far appears to also confer <u>complete resistance</u> to PM infection, as no infection was observed in detached leaf disk assay. Infection rates will be ramped up higher in 2020 to test limits of resistance.
- Pmr1c: <u>Not reliable</u> for resistance to PM, as infection was observed in detached leaf disk assay. DNA test will be further refined to distinguish this genetic factor from all others.
- The resistance-associated alleles Pmr1a and Pmr1b appear to both act in a dominant fashion in that only a single copy is required in a cherry cultivar/selection to confer PM resistance.

METHODS

Two-year plan: **2019** – Use previously optimized foliar infection protocols (field and in vitro) for collection of a first season of data. Begin genetic dissection of fruit resistance. **2020** – Continue use of foliar infection protocols (field and lab) to collect a second season of data to validate 2019 results. Develop a refined DNA test using data gathered over both years.

Germplasm use and tree management: Trees used for evaluation were growing at the Roza experimental orchard, part of Washington State University-Irrigated Agriculture Research and Extension Center (WSU-IAREC). Individuals selected for this study came from genetic stock trees in the RosBREED block (C53), breeding program mother block (B53), and the Toyama selection block (A37); all of which represent the diversity of the WSU sweet cherry breeding program (about 510 genetically distinct trees total). High-resolution, DNA-profiles of trees in the germplasm from the RosBREED project included those individuals thought to harbor PM-resistance factors.
Germplasm used in 2019 included several offspring from resistance sources (15 individuals). These descendants along with their parental sources (40 trees, 25 individuals) included trees expected to be PM-resistant and others expected to be susceptible based on the genotypic presence/absence of Pmr1 and Pmr1-like genetic factors. Pedigree-connected cultivars known to be susceptible were included as positive controls, including 'Bing,' 'Rainier,' and 'Sweetheart.'

Management of orchard trees was conducted in accordance with standard practices of the WSU breeding program with the exception of a misapplication of fungicides mid-season.

Foliar PM-resistance evaluation – orchard: To assess initial infection within the orchard, chosen trees were observed for signs of mildew infection beginning with leaf emergence. Infection assessment was halted upon discovery of fungicidal applications.

Foliar PM-resistance evaluation – lab: A lab-based detached leaf disk assay was performed on the chosen germplasm set. Briefly, this previously optimized assay began with collecting the first fully expanded leaf from a terminal shoot and transporting it to the lab for surface-disinfestation (10% bleach solution for 3 minutes followed by a quadruple rinse in sterile distilled water). From each leaf, a circular disk (12 mm in diameter) was excised and placed abaxial side up on a new well containing 500 µl water agar of a 24-well plate. Assays conducted for PM resistance/susceptibility consisted of two leaf disks from two independent leaves sampled from each germplasm individual. Conidial suspensions of *P. clandestina* were generated by gathering infected leaves from the mildew block of 'Bing' and 'Sweetheart' trees at the Roza, submerging them in a 0.01% TWEEN solution, and agitating the mixture until conidia were present in solution at sufficient numbers. A 10 µl conidial suspension of 20,000 conidia per mL (quantified through manual count using a hemocytometer) was administered to each leaf. Upon deposition on the leaf disks, conidia were allowed to settle for 5 minutes before residual moisture was wicked away using a sterile cotton swab. Settling time maximizes number of infectious propagules achieving contact with leaf surface, which in turn maximizes likelihood for infection establishment. Plates were subsequently sealed with parafilm to prevent contamination as well as moisture loss, and leaves with conidia were co-cultivated for 14 days in a plant growth chamber at 20°C and a 14 h light period. Plates were then viewed using a stereoscope and mildew presence/absence was assessed. A result was noted as positive if any signs of infection were observed, and negative only if zero mildew was found.

Foliar PM-resistance evaluation – durability under high pathogen pressure – lab: Testing of individuals harboring *Pmr1* and *Pmr1*-like alleles for resistance breakdown under high pathogen pressure was planned to involve the standard detached leaf disk assay with different conidial concentrations. Concentrations of conidia were to contain low (5 conidia per mL) to very high (500,000 conidia per mL) levels of pathogenic material to determine if Pmr1 could be relied upon to confer complete resistance to infection, regardless of pathogen pressure.

Preliminary genetic dissection of resistance/susceptibility: Comparison of preliminary genome scan information gathered previously from the RosBREED project was used to facilitate the discovery of a genetic difference among the alleles located at the Pmr1 locus.

RESULTS & DISCUSSION

Summary: Resistance versus susceptibility to PM infection was discernable for all individuals tested. While most of the 2019 season was rendered inconclusive by field mis-application of fungicidal spray, results gained prior to the spray clearly indicate a second genetic source for resistance to PM infection is present. Intriguingly, the degree of resistance appeared to be lineage-specific; resistance Pmr1 and Pmr1-like alleles inherited from 'Moreau' (Pmr1a), 'Hedelfingen' (Pmr1b), and the MIMs (Pmr1b) appeared to be complete, while PM infection resistance from the Pmr1-like allele from 'Schneiders' (Pmr1c) appeared not reliable. Evaluations during the 2020 season will elucidate if any useful resistance qualities are associated with Pmr1c.

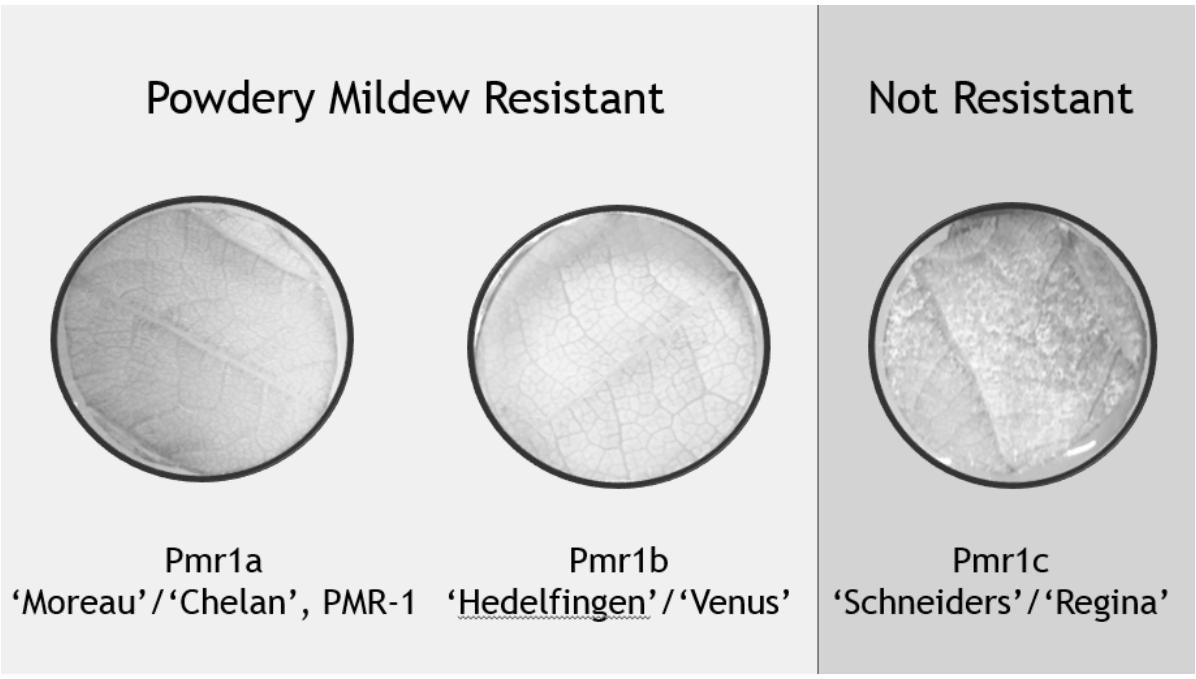

Figure 1. Grouping of mildew resistance determined by leaf disk assay results and DNA profiles

Foliar PM infection – in-orchard evaluation: Foliar infection was not observed in the field during the 2019 season on any trees, presumably because of the early season fungicidal spray applied.

Foliar PM infection – in-lab evaluation: Definitive results regarding resistance vs. susceptibility were obtained using the detached leaf disk assay. Of individuals containing one or two copies of Pmr1a ('Moreau' and its offspring), 100% of carriers were observed to have no forms of mildew infection. Additionally, individuals with one or two copies of the Pmr1b allele in the 'Hedelfingen' and MIMs lineages were also found to be completely free of PM infection, giving strong evidence for the presence of a second resistance factor. However, individuals within the 'Schneiders' family (including its offspring 'Regina') with one copy of Pmr1c (none were available with two copies) had PM infection. These results were based on a single detached leaf disk assay performed at the beginning of May and so they need to be validated during the 2020 season. Also, if we can identify individuals in the germplasm set that carry two copies of Pmr1c, it will be determined if having two copies is associated with no infection.

Durability of Pmr1 genetic resistance: Lab-standardized pathogen pressure (20,000 conidia per mL), which is higher than tends to be found in the orchard normally, was observed to be incapable of inducing infection in individuals harboring Pmr1a or Pmr1b but not those with only Pmr1c and/or pmr1. Moderate levels of mildew infection were observed in leaves of 'Schneiders' and 'Regina' with Pmr1c, and so Pmr1c was concluded to be incapable of resisting infection. Experiments in 2019 using the planned higher pathogen pressures were rendered ineffective by the orchard fungicide sprays (it was learned this was done after the high-pathogen leaf disk assays were conducted). The experiments will be conducted in 2020, ensuring that orchard fungicides are not applied. Based on 2019 results of the foliar infection in-lab evaluation (above), an additional focus of 2020 season will be to determine if presence of the Pmr1c allele in the 'Schneiders' lineage is associated with infection at any conidial load or if infection only occurs above a certain pathogen pressure.

Genetic dissection of newly discovered PM resistance: Discernibility was clear for the previously-identified Pmr1 resistance allele present in 'Moreau' (and offspring), here termed "Pmr1a". A second, detectable factor was found at the same locus, appearing in 'Hedelfingen', the MIMs, as well as 'Schneiders' (and their offspring). Due to the phenotypic differences observed in this group, the factor associated with resistance in 'Hedelfingen' and MIMs (as well as offspring) was termed "Pmr1b", and the non-resistant allele in 'Schneiders' (and offspring) was termed "Pmr1c". All other, common factors associated with susceptibility to PM infection are referred to as "pmr1". A DNA-based test for the Pmr1a resistance allele derived from the 'Moreau' source is already available. However, this DNA test elicits a positive response for PM resistance from presence of Pmr1a, Pmr1b, and Pmr1c. Given that 2019 results indicated lack of resistance associated with Pmr1c, that genetic factor needs to be distinguishable from the first two. DNA profile data from the RosBREED project indicate that the current DNA test can be refined.

Work is already underway to refine the current DNA-based PM-resistance test by fine-mapping of the *Pmr1* genomic region on chromosome 5 associated with infection resistance. Use of offspring in 2019 from certain resistant individuals derived from a genetic recombination at the *Pmr1* resistance locus were used to zoom in on the precise location of the underlying gene. Validation in 2020 of 2019 phenotypic results, along with closer dissection of the underlying genetics by assessing DNA-sequence data, should elucidate the precise location of the *Pmr1* locus, verify the identity of the gene, and provide sequence differences for discernment among the genetic factors.

Variety	Resistance Factor Group	2019 Observations	2020 Observations
'Moreau'	Pmr1a	Resistant	To be determined
PMR-1	Pmr1a	Resistant	
'Chelan'	Pmr1a	Resistant	
DD	Pmr1a	Resistant	
GG	Pmr1a	Resistant	
'Hedelfingen'	Pmr1b	Resistant	
'Venus'	Pmr1b	Resistant	
MIM 17	Pmr1b	Resistant	
MIM 23	Pmr1b	Resistant	
'Schneiders'	Pmr1c	Susceptible	
'Regina'	Pmr1c	Susceptible	
'Bing'	pmr1	Susceptible	
'Sweetheart'	pmr1	Susceptible	
'Rainier'	pmr1	Susceptible	

Table 1: Resistance status of chosen varieties by year of field and lab evaluation

CONTINUING PROJECT REPORT **YEAR**: 1 of 2 Years

Project Title: Fungicide resistance: A vital need to protect PNW cherries from mildew

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Cooperators: Tianna DuPont, Bernardita Sallato, Neusa Guerra

Total Project Request: Year 1: $60,175 **Year 2: $71,276**

Budget 1
Organization Name: WSU-IAREC **Contract Administrator:** Tim Palacios/Katy Roberts
Telephone: 509-786-9285/509-335-2885
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Item	2019	**2020**
Salaries[a]	$18,405	**$19,141**
Benefits	$8,958	**$9,316**
Wages[b]	$11,520	**$11,981**
Benefits	$1,152	**$1,198**
Equipment		
Supplies[c]	$18,250	**$27,750**
Travel[d]	$1,890	**$1,890**
Miscellaneous		
Plot Fees		
Total	$60,175	**$71,276**

Footnotes:
[a] 0.5 FTE for an associate in research
[b] Time slip field and laboratory workers
[c] DNA extraction kits, DNA sequencing, Next-Gen sequencing, laboratory chemicals and supplies. Additional $4,500 is requested to cover supplies, kits and chemicals for additional study (see below)
[d] Travel to various orchard site for mildew collections, spore collections and follow up trips in WA and OR

Explanation to additional funds request
Fungicide resistance mechanisms are complex, and pathogens adapt to several biochemically alternative pathways to overcome fungicides. It is also extremely important to test EC50 values for at least one fungicide from each of FRAC Group 3 (DMI), 7(SDHI) and 11 (QoI). We have experienced anomalies in the resistance bioassay and an estimate of the inhibitory concentration will be important to adjust volume/ concentration in the spray tank. The additional funds are requested to cover determining inhibitory concentrations, studying alternative resistance strategies, RNA extraction, qPCR, cloning and sequencing of succinate dehydrogenase (B, C, D), chitin synthase, CYP51 and cytb genes. The additional funds will also cover the investigation of another fungicide that failed in some bioassays (polyoxin-D), by high-throughput next-generation sequencing.

Recap of objectives

1. **Investigate the presence and extent of fungicide resistance in commercial orchards in the Pacific Northwest.** Significant progress was made on this objective in 2019. Overall, a total of 20 unique orchard sites representing different growing areas of Washington and Oregon were included in the collection. We will include at least 10 more sites in 2020 to get a uniform representation in both states and conduct molecular and leaf-disc bioassays for insensitivity studies. The activities in 2019 have been accomplished as planned and we anticipate completing remaining studies in 2020. Interestingly, there are some deviations forced by the data complexity (discussed in a later section).

2. **Identify and develop specific genetic markers for better identification of fungicide resistance.** We have been successful in designing, amplification and sequencing of fungicide target genes of FRAC Groups 11 and 3. The robustness of the assays was tested on several isolates and resistant colonies. We will initiate and accomplish designing and testing of FRAC Group 7 (SDHI) target gene(s) in 2020. The study was originally planned in year 2 (2020) of the project due to the complexity of different genes and the unavailability of sequence information in *P. clandestina*.

3. **Develop alternative programs for disease management, if significant fungicide resistance is documented in this study (Conditional).** As we discuss the results below, insensitivity to some FRAC groups is present in both states. As mentioned in objective 2, we will accomplish the additional FRAC Groups 3 and 7 testing and comprehensive data will determine our future focus of extension and outreach efforts. There is an urgent need for alternative strategies to be implemented for managing powdery mildew, as year 1 data suggests. The conclusion of this project will specifically address this objective in detail.

Significant findings

- We accomplished the collection of *P. clandestina* isolates in diverse locations of cherry growing areas in WA and OR.
- QoI target gene was tested in all cases and we found molecular evidence of fungicide resistance in 43% of isolates. There is no discernible pattern in geographic distribution of isolates resistant to FRAC Group 11.
- Leaf-disc bioassay confirmed our molecular data for FRAC Group 11 fungicides. Alternative mechanisms, other than genetic modification, may be responsible for the higher incidence of insensitive colonies in bioassay
- Several other FRAC Groups were also tested using the bioassay. In some cases, isolates were insensitive to fungicides from other FRAC Groups indicating that there is a good chance of fungicide resistance in FRAC Groups other than Group 11. We hope to complement the bioassay results with molecular confirmation.
- *P. clandestina* isolates from two organic orchards were sensitive to most fungicides.
- Myclobutanil (Rally, Group 3), trifloxystrobin (Gem; Group 11) and polyoxin-D (PH-D; Group 19) were the most problematic compounds.
- All isolates were susceptible to a premix of azoxystrobin and difenoconazole (Quadris Top).

Methods used (current and upcoming)

- The methods for handling of *P. clandestina* isolates were followed as proposed in the project. Additionally, we propose to specifically work with the pure cultures of the pathogen

immediately after they are brought to the lab because of 1) The pathogen is very dynamic and usually different mating-types coexist in populations and 2) Allows identifying genetic micro-variations of genes under investigation and possibly link it with fungicide resistance.

- We have found unexpectedly high DNA sequence variability in the cytochrome b (cytb) sequence, the molecular target gene of Group 11 (QoI) fungicides (but high similarity to deduced amino acid sequence). The approach to primer design for amplification of this gene from several samples is a challenge. During the upcoming year, we plan to clone and sequence a bigger fragment of cytb gene to design a single set of primers that can be used in fungicide resistance diagnostics.

- The CYP51 DNA sequences of myclobutanil and triflumizole- insensitive colonies (in the bioassay) were sequenced from several isolates. We did not find any known mutations that may correlate with bioassay. There is a possibility that *P. clandestina* uses alternative pathway to bypass the fungicide effects such as differential gene expression (Ma, Proffer et al. 2006). In 2020, plans are in place to extract RNA from FRAC Group 3 insensitive colonies (and individual isolates) and confirm by gene expression analysis. We have already designed and tested primers for gene expression.

- After the final project data analysis, we will consult with growers, WTFRC, pesticide companies and, extension specialists to prepare recommendations for resistance management. The resistance assays developed herein can be communicated to interested parties for the possible commercialization of the molecular diagnostic assays.

Table 1. List of *P. clandestina* isolates from sites in Eastern Washington and Eastern Oregon.

Isolate Code	Production Area	County	Variety	Conventional/ organic
DH	Columbia Basin	Franklin	Bing	Conventional
CS	Columbia Basin	Franklin	Rainier	Conventional
MH	Yakima Valley	Yakima	Bing	Conventional
HL	Columbia Basin	Grant	Rainier	Conventional
SC	Columbia Gorge	Wasco, OR	Rainier	Conventional
AR	Columbia Gorge	Hood River		Conventional
Roza	Yakima Valley	Benton	Bing	No fungicides
TP	Yakima Valley	Klickitat	Sweetheart	Conventional
RS	Wenatchee	Okanogan	Rainier	Organic
BR	Wenatchee	Okanogan	Sweetheart	Conventional
BO	Wenatchee	Chelan	Rainier	Conventional
HF	Wenatchee	Chelan	Rainier	Conventional
ST-1	Wenatchee	Chelan	Sweetheart	Organic
ST-2	Wenatchee	Chelan	Bing	Organic
BC	Yakima Valley	Benton	Bing	Conventional
BM	Columbia Basin	Grant	Bing	Conventional
HT	Yakima Valley	Yakima	Rainier	Conventional
SR	Willamette Valley*	Marion, OR	Unknown	Unknown

*Western Oregon

Results and discussion

Disease pressure in 2019 was relatively low in most collection areas. In the 2019 cherry growing season, several isolates of *P. clandestina* were collected from several susceptible cultivars (including organic orchards) in all cherry growing regions of Oregon and Washington (Table 1). All isolates were tested in mildew-susceptible leaf discs treated with candidate fungicides (Table 2) with an application rate equivalent to 200 gallons spray material per acre. At the end of 14 days incubation (post-inoculation), the apparently fungicide-insensitive mildew colonies were analyzed microscopically. Colonies appeared in FRAC Group 3 and 11 fungicide bioassays were then subjected to DNA extraction and sequencing of their respective target genes.

Table 2. List of fungicides used in the bioassay of *P. clandestina isolates*

Common name	Trade name	Class	FRAC Group	Resistance risk	Rate/ application (max)	Rate/ application*
penthiopyrad	Fontelis	Carboxyanilide (SDHI)	7	Medium to High	20 fl oz	781µl/l
triflumizole	Procure	DMI (triazole)	3	Medium	16 fl oz	624µl/l
myclobutanil	Rally	DMI (triazole)	3	Medium	6.0 oz	0.2252g/l
quinoxyfen	Quintec	Quinoline	13	Medium	7.0 fl oz	273µl/l
trifloxystrobin	Gem	Strobilurin (QoI)	11	High	3.8 fl oz	148µl/l
polyoxin-D	PH-D	Polyoxins	19	Medium	6.2 oz	0.2321 g/l
metrafone	Vivando	Aryl-phenylketone	U8	Medium	15.4 fl oz	601µl/l
pyraclostrobin/ boscalid	Pristine	Strobilurin(QoI)	7 and 11	Medium to High	14.5 oz	0.542g/l
		Carboxyanilide (SDHI)				
azoxystrobin/ difenoconazole	Quadris Top	Strobilurin (QoI)	11 and 3	High to Medium	14.0 fl oz	546µl/l
		DMI (triazole)				

*based on a spray volume of 200 g/ acre

The results of the bioassay indicated that most *P. clandestina* isolates were insensitive to polyoxin-D (PH-D) followed by trifloxystrobin (Gem) and myclobutanil (Rally; Table 3). Seven of 17 isolates insensitive to trifloxystrobin (Gem) were also insensitive to pyraclostrobin/ boscalid (Pristine) suggesting cross-insensitivities in FRAC Group 11. The other five isolates were insensitive to trifloxystrobin (Gem) but susceptible to a premix of pyraclostrobin and boscalid (Pristine), possibly due to susceptibility of those isolate to boscalid, a Group 7 (SDHI) fungicide. More than 50% of the isolates susceptible to FRAC Group 11 in bioassays contained the G143A mutation, hence confirmed fungicide resistance. The majority of remaining isolates were insensitive in fungicide bioassay but did not contain the mutation, possibly due to activation of an alternative oxidative pathway that causes a low level of resistance in response to QoI fungicides (Brent and Hollomon 2007). Myclobutanil, a moderate-risk FRAC Group 3 fungicide, was ineffective against 11 of 17 isolates but the molecular assay did not exhibit any amino acid substitutions as determined by sequencing of the target gene, CYP51/ ERG11. An alternate strategy may be responsible for these insensitive *P. clandestina* colonies as such mechanisms reported in the cherry leaf spot disease (Ma, Proffer et al. 2006). We will explore the possibility of overexpression of CYP51 gene in resistant colonies using qPCR methods in 2020.

Table 3. List of ineffective fungicides by collection site (as determined by bioassays) in different isolates of *P. clandestina* collected in 2019

Isolate	FRAC groups	Ineffective fungicides
DH	11, 13, 19, 11/7, U8	trifloxystrobin, quinoxyfen, polyoxin-D, pyraclostrobin/ boscalid, metrafone
CS	3, 11, 19, 11/7, U8	triflumizole, trifloxystrobin, polyoxin-D, pyraclostrobin/ boscalid, metrafone
MH	3, 7, 11, 13, 19, 11/7, U8	myclobutanil, triflumizole, penthiopyrad, trifloxystrobin, quinoxyfen, polyoxin-D, pyraclostrobin/ boscalid, metrafone
HL	11, 13, 19	trifloxystrobin, quinoxyfen, polyoxin-D
SC	3, 7, 11, 13, 19, 7/11	myclobutanil, penthiopyrad, trifloxystrobin, quinoxyfen, polyoxin-D, pyraclostrobin/ boscalid
AR	3, 7, 19	myclobutanil, penthiopyrad, polyoxin-D
Roza	3, 11, 19	myclobutanil, trifloxystrobin, polyoxin-D
TP	3	myclobutanil
RS	11	trifloxystrobin
BR	3	myclobutanil
BO	3, 7, 11, 13, 19, 11/7, U8	myclobutanil, penthiopyrad, trifloxystrobin, quinoxyfen, polyoxin-D, pyraclostrobin/ boscalid, metrafone
HF	3, 11, 19, 11/7	myclobutanil, trifloxystrobin, polyoxin-D, pyraclostrobin/ boscalid
ST-1	3, 7, 11, 19	myclobutanil, penthiopyrad, trifloxystrobin, polyoxin-D
ST-2	7, 19	penthiopyrad, polyoxin-D
BC	19, 11/7	polyoxin-D, pyraclostrobin/ boscalid
BM	3, 11, 11/7, U8	myclobutanil, trifloxystrobin, pyraclostrobin/ boscalid, metrafone
HT	11, 13, 19	trifloxystrobin, quinoxyfen, polyoxin-D

Table 4. Occurrence of apparent insensitive isolates of *P. clandestina* treated with various fungicides.

Fungicide common name	Occurrence (total isolates = 17)
Myclobutanil	11
Triflumizole	3
Penthiopyrad	6
Trifloxystrobin	11*
Quinoxyfen	6
polyoxin-D	14
azoxystrobin/ difenoconazole	0
pyraclostrobin/ boscalid	7*
Metrafone	6

*the results were confirmed by molecular data and confirmed to be fungicide resistant.

While testing other FRAC groups using the bioassay, we discovered that most of the isolates were also insensitive to polyoxin-D (PH-D) corresponding to FRAC Group 19, a group of fungicides that target chitin synthesis. If additional funds (explained above) are approved, we will investigate the chitin synthase gene for genetic mutations or alternative expression strategies, as applicable. Isolates insensitive to other FRAC groups such as 13 and U8 were discovered. If time permits, we will investigate the consequences of FRAC13 and U8 resistance by *P. clandestina* and perhaps modify the bioassay approach for these groups.

Overall, Group 11 fungicide resistance in PNW is widespread and many fungicides from several FRAC groups appear to be ineffective against some isolates. The results of the initial year of this study suggest that resistant management is critical at least for FRAC Group 11 fungicides, and further data is needed as to whether exclusion of these fungicide(s) would facilitate better management of cherry powdery mildew. In the upcoming season, we will confirm fungicide resistance of FRAC Group 3, 7, 11 and 19 (if time permits) only if gene expression and/ DNA sequencing data correlate with fungicide insensitivities in the bioassay experiments.

Literature cited

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Ma, Z., T. J. Proffer, J. L. Jacobs and G. W. Sundin (2006). "Overexpression of the 14α-Demethylase Target Gene (*CYP51*) Mediates Fungicide Resistance *Blumeriella jaapii*." Applied and Environmental Microbiology **72**(4): 2581.

FINAL REPORT

Project Title: Electronic sensors to capture spatiotemporal population density of SWD

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Cooperators: Eamonn Keogh (UC Riverside, Dept. of computer science and engineering)

Budget: **Year 1**: $31,384

Budget
Organization Name: UC Davis **Contract Administrator: Yang Yeh**
Telephone: (530) 752-3794 **Email address:** ypyeh@ucdavis.edu

Item	**2018**	**2019**
Salaries	$16,016	
Benefits	$8,168	
Wages	-	
Benefits	-	
Equipment	-	
Supplies	$5,200	
Travel	$2,000	
Miscellaneous		
Plot Fees	-	
Total	$31,384	**No-cost extension**

Footnotes:
Salaries and Benefits are for one SRAI (technician) for sensor testing and insect collection (33.3% time)
Supplies include funding to construct 20 sensors for testing ($4000) and for insect capture and maintenance ($1,200)
Travel funds ($2,000) are requested for SRAI to travel to Washington or Oregon to conduct field sensor testing

JUSTIFICATION

Sensor technologies and automated insect identification models were developed for the control of insects that spread human diseases. Our cooperator Dr. Keogh, a computer scientist at UC Riverside, has recently developed inexpensive pseudo-acoustic opto-electronic sensors and accompanying classification algorithm that can accurately classify multiple species of mosquitoes that vector pathogens such as Zika and West Nile virus (Chen et al. 2014) by using wing-beat frequencies, daily activity patterns, and geographical distribution. The ability to remotely capture real-time measurements and forecast insect density in a spatiotemporal manner allows for efficient and precise insect control response that could prevent public health crisis. ***The overall goal of this proposal was to adopt and translate this technology to optimize insect pest management programs and benefit agricultural stakeholders.*** We proposed to develop and ultimately deploy opto-electronic sensors that can accurately identify Spotted Wing Drosophila (SWD) and differentiate it from other insect inhabitants of cherry orchards.

SWD is a highly invasive pest species that cause up to $500 million in annual losses in the western United States because they oviposit into marketable, ripening fruit (Goodhue et al. 2011, Wiman et al. 2016). An insect sensor utilizing wing beat frequency for classification can theoretically be applied to identify any flying insect, but the substantial economic loss caused by SWD warrants the prioritization of optimizing this new technology for its control. It is important to stress that the electronic sensor technology we proposed to develop and optimize for SWD was not simply a modernized version of insect traps currently used for population monitoring. Besides supplanting conventional monitoring tools and greatly reducing the time necessary to process trap contents, we anticipated that the capability of the sensors to classify insects in real-time will revolutionize pest management research and lead to developments in precision agriculture. For example, current monitoring tools lack spatial and temporal resolution as conventional traps do not provide time-stamps for insect catches. Our sensors on the other hand can ultimately be connected to a central network and were capable of reporting real-time movement between crop and non-crop host plants, providing opportunities to target SWD for sprays at times when they are at maximum density in non-crop plants. This can reduce insecticide residues on crops, a major concern for export markets.

OBJECTIVES

Objective 1:

Measure wing beat frequency and circadian activity pattern of SWD to improve insect identification algorithm. Opto-electronic sensors will be installed in insect cages that house SWD to measure wing beat frequency and daily activity patterns simultaneously. Since biological parameters, e.g. sex, age, and seasonal morphology, may alter wing beat frequency and activity patterns, we plan to evaluate male and female SWD, different ages of SWD, and summer and winter forms of SWD. Various abiotic factors can also affect wing beat frequencies so we will evaluate recordings in a range of environmental conditions.

Objective 2:

Field recording to assess opto-electronic sensor and insect identification algorithm. We will deploy opto-electronic sensors housed in McPhail traps to assess the capability of the sensors to accurately identify SWD from other inhabitants of Cherry orchards.

METHODS

Objective 1: Refining insect identification algorithm using opto-electronic sensors

Overview: In order to automate the process of insect identification based on wing beat frequency, an algorithm was created and refined to take into account biotic and abiotic factors that may result in changes to insect wing beat frequency and activity pattern. Our cooperators have previously created an algorithm to accurately identify insects down to species and sex using wing beat frequency in controlled environments, which they have tested on mosquito species (Chen et al. 2014). To refine

this algorithm for SWD and use in the field, we recorded wing beat frequency of SWD and other insects commonly found in cherry orchards different environmental conditions (temperature, light cycle, humidity, etc.). The data acquired from these species in controlled environments were then incorporated in insect identification models to enable refinement of the algorithm.

Collection of data for insect identification algorithm refinement

Flies of a known species and sex (N=60) were placed into a modified McPhail trap outfitted with an opto-electronic sensor ring and connected to a recording device. This setup was then placed into a Digitherm incubator (Tritech Research) that allowed us to control the environmental conditions. Using this setup, we recorded wing beat frequency data in different temperatures, humidity, light-dark cycles with different photoperiods, etc. as well as wing beat frequency of different species and sexes. The data collected in these controlled environments were visualized using analysis programs using MATLAB (Mathworks). General trends were visualized using these analysis tools. Comparison between SWD and the closely related *Drosophila melanogaster* in controlled conditions showed distinct wing beat frequency patterns. Based on live capture in field in CA, we identified several closely related *Drosophila* species such as *D. simulans*, *D. biarmipes* and *D. tristis*. Recording using these different species and other relevant species present in cherry orchards were generated in order to refine the algorithm and improve identification accuracy.

Refinement of insect identification algorithm

Previously our cooperators have created an insect classification algorithm which they have used to accurately identify disease carrying mosquito species based on wing beat frequency alone (Chen et al. 2014). When more species were added or environmental conditions were changed the classification model was less accurate. Due to the large diversity of species present in the field and the heterogeneity of environmental conditions, it is important to have accurate classification established on a wide range of fluctuating parameters and species to mimic field scenarios. By creating a training dataset using the data we collected from *Drosophila* flies in various conditions, we were able to "train" the classification model to accurately identify insect pests in vastly different environments. We have already "trained" the insect classification model based on geographical and circadian rhythm data to increase the accuracy of the model in identifying mosquitos down to the species level. By "training" the insect classification model to correctly identify insects using a larger number of variables we were able to increase the accuracy of our identification process in the field. This was an iterative process of testing and refinement.

Objective 2: Assessment of insect identification algorithm and field deployment of sensors

Overview: With current monitoring methods, it is extremely time consuming to monitor insect pest species in the field because it requires the presence of a specialist to manually identify individuals. In addition, the time lapse between trapping and identification constitutes an important limitation to initiate a quick and appropriate response to slow down crop infestation. Our goal in refining the insect identification algorithm was to develop an automated identification process that is easier and faster to identify insect pests compared to current pest capture and identification processes. We assessed the ability of the sensors to correctly identify and monitor pest species both spatially and temporally in and around SWD habitats in CA.

Deployment of insect sensors in the field

Once the classification algorithm was found to be highly accurate (>99%), we deployed our system in SWD habitats. We used baited McPhail traps outfitted with sensors in the opening at the bottom to record the wing beat frequency and relevant environmental variables (temperature, humidity, time, etc.) of any insect that enters the trap and identifying them in real time. By deploying multiple trap/sensor setups, we were able to track the movements of SWD throughout the day, e.g. from crop to non-crop hosts. We envision this will allow for the development of more precise

strategies of pest management than are possible through conventional monitoring techniques using traps and manual identification. The automated process of insect identification also means that there will be far less processing time required to identify flies allowing growers and researchers to respond to the presence of pests as soon as they arrive and are detected in their fields.

RESULTS:

Objective 1:

Hardware optimization for insect sensors

We successfully went through several iterations of design and testing of the sensors. We have converged on a solution that we feel is robust, maintainable and cheap to produce in large numbers. Briefly, we use IR emitters and phototransistors working at a wavelength of 940nm, which is outside the visible light spectrum. Our emitters (OSRAM SFH 4043) and phototransistors (Everlight PT19-21C) use around ~20mA. There may be some other low power emitters and phototransistors out there that we can used to further improve our design in the future. We are currently using a cortex M4 MCU, which runs at 80Mhz. This chip has 32K of flash and 2K of RAM. It consumes around 5.5mA when running at 8Mhz but we can put it to sleep when there is no activity, in sleep mode it only consumes few micro amps. For transmission we are using Long Range Wide Area Network (LoRaWAN) technology. LoRaWAN is a wireless standard designed for long range communications at a low bit rate on a very low power budget. We use Semtech SX1272 LoRa module which has a range of 2 miles in non-line-of-sight environment and up to 15 miles in line-of-sight environment. It can achieve data rates up to 50 kbps. SX1272 consumes ~15mA while transmitting/receiving and a negligible power (1.5 uA) in idle state. We have started to install solar panel to the sensor unit, so that the sensors can be left unattended in the field for weeks at a time (Figures 1 to 3 and Figure 5).

Development of species ID algorithm

Since the completion of the activity and wing beat frequency recordings for 5 different *Drosophila* species (*D. simulans, D. tristis, D. suzukii, D. biarmipies, D. melanogaster*) at various temperature and photoperiod conditions (Figure 4), we have collected more than one million insect "encounters", in diverse conditions of light, temperature, humidity, life-stage, species, sex. Using this data, we have built the state-of-the-art classification model for insect classification, which is invariant to environmental conditions. For example, we can now train a model say in our dry hot California research station at ten meters above sea level and be confident that the model will generalize to the cooler humid conditions. Just a year ago, this environmental variability would cause our models to fail to generalize, drastically reducing our accuracy. Now the accuracy of the resulting species ID algorithm is easily over 90% accuracy and will continue to improve as we continue to feed the algorithm with data collected in the field.

Objective 2:

Field deployment and testing

For the purpose of testing the power and communication modules, we leveraged a field setup that was already in place to monitor Navel Orange Worm (NOW). We can then easily adopt the same setup for use with SWD monitors. We outfitted the sensors with solar panel and cellular data transmission for remote sensing. A first field trial was performed in an almonds orchard located in CA from May to September 2019 to trap (Figure 5). NOW can be trapped using sticky card loaded with species specific pheromone allowing us to collect and validate information for a given species before increasing complexity by adding more species to the system. During this test we were able to successfully validate:

1) the autonomy of the sensors.
2) the ability of the sensors to remotely send information to the database.

3) the accuracy of the system to match insect counts recorded by the sensor with the number of insects collected on sticky trap.

Indeed, the solar panel efficiently ensured full autonomy of the system as it provided enough power to sustain the sensor during the 4 months of the trial without any intervention required from us to recharge the battery. Data were send remotely using cellular signal to an online database where insect counts as well as environmental parameters associated (T°C, Humidity, Pressure, Light cycles...) were readily accessible. Finally we observed a strong correlation between information collected from the sensor and number of insects on the sticky card attesting of the efficacy of the sensor to detect pest pressure in real time (Figure 6).

Sensors to monitor for SWD (Figure 7) are currently deployed in the UC Davis orchard of Wolfskill. Traps are filled up with yeast-sugar solution, an attractive lure for *Drosophilds* allowing us to validate the capability of the sensor to accurately identify the presence of SWD among other species of fruit flies. Given the current percentage of accuracy provided by the algorithm we are confident that SWD recordings will result in high accuracy identification. Results from this trial are expected to set the stage for field application in the upcoming growing seasons. We hope to offer growers and PCA unprecedent tools to optimize insect pest management programs. Users interested in testing the smart sensor in their own crop are invited to request a demo by contacting the PI Joanna Chiu (jcchiu@ucdavis.edu) or by visiting the Farmsense website (www.farmsense.io).

LITERATURE REVIEW:

There have been some efforts in identifying insects based on recordings of their wing beat frequencies and these attempts date back to the advent of commercially available computers and audio recording devices (Reed et al. 1942, Foster and Robinson 1991, Moore and Miller 2002, Raman et al. 2007). These attempts have not been successful in creating an automated and accurate identification process based on recordings of wing beat frequencies. In most studies, wing beat frequency has been recorded using acoustic microphones, which are susceptible to noise from the wind as well as any ambient noise in the environment (Reed et al. 1942, Mankin et al. 2006, Raman et al. 2007, Villarreal et al. 2017). This made it very difficult to get quality recordings of insect wing beat frequency with acoustic recording devices. Because of this difficulty, wing beat frequency data is sparse, low quality, and typically recorded in unnatural conditions (Moore et al. 1986). Despite the sparseness and low quality of available insect wing beat frequency data, some researchers have attempted to create insect identification models with 300 or less recordings (Moore 1991). It is difficult to create models with such sparse data and this will cause the models to have very low accuracy in identifying insects (Banko and Brill 2001, Halevy et al. 2009). This is compounded by the fact that most attempts at classification of insects by recording wing beat frequency have used just one variable (wing beat frequency). Other environmental factors that cause wing beat frequency to change have also been ignored (Chen et al. 2014). By using pseudo-acoustic opto-electronic sensors, we will be able to record higher quality data. We will also be able to record larger volumes of data in more natural conditions than has been possible in the past, which will allow us to create a highly accurate insect classification model that can be used to identify SWD and differentiate it from other species in the field.

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FIGURES

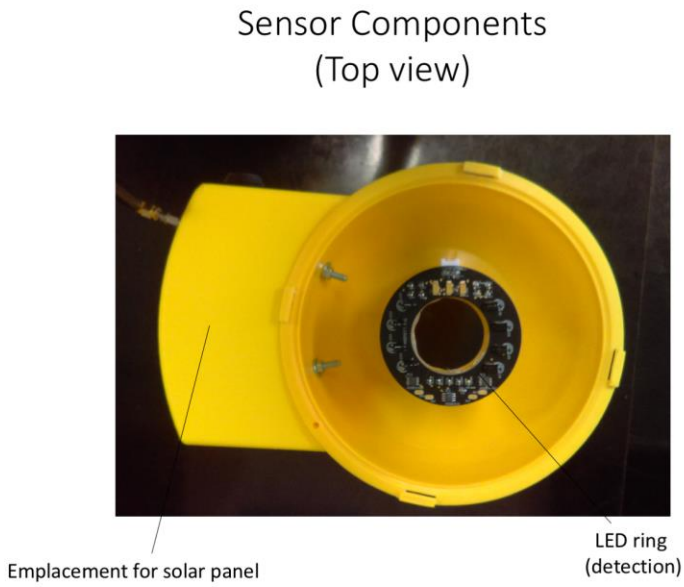

Figure 1: Top view of the modified Mcphail trap outfitted with the LED sensor ring, showing emplacement for solar panel.

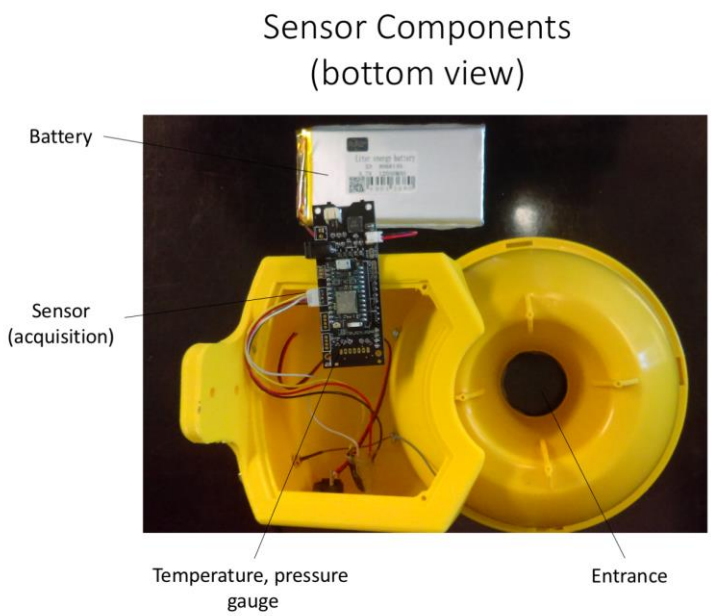

Figure 2: Bottom view of the modified Mcphail trap showing the battery unit, the data acquisition unit, the environmental measurement unit, and the entrance of the trap.

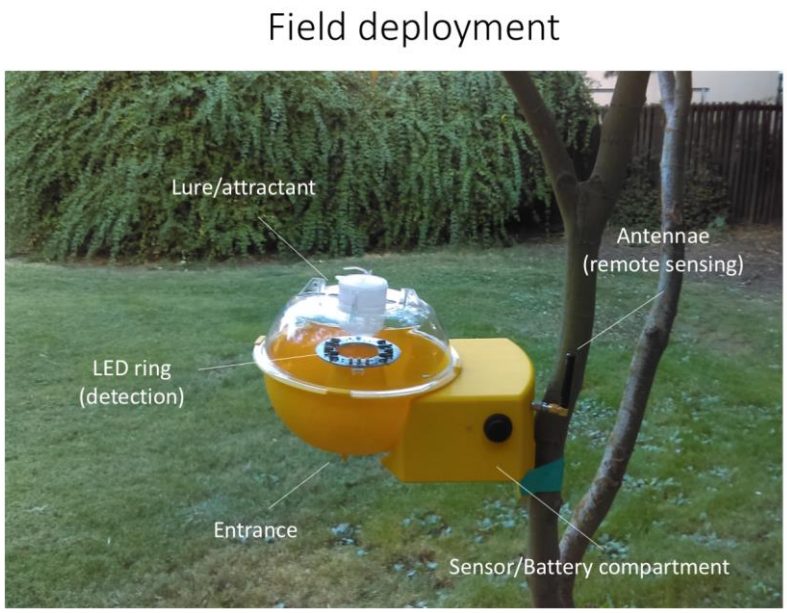

Figure 3: The modified Mcphail trap holding the sensor unit in a field setting. The antennae for transmitting remote sensing data is shown.

Temperature:		20C	25C	30C
D. sim	Male	x	x	x
	Female	x	x	x
D. tris	Male	x	x	x
	Female	x	x	x
D. suz	Male	x	x	x
	Female	x	x	x
D. biar	Male	x	x	x
	Female	x	x	x
D. mel	Male	x	x	x
	Female	x	x	x

Photoperiod		12:12
D. sim	Male	x
	Female	x
D. tris	Male	x
	Female	x
D. suz	Male	x
	Female	x
D. biar	Male	x
	Female	x
D. mel	Male	x
	Female	x

Figure 4: Temperature and photoperiod conditions for wing beat frequency recordings. Conditions marked with pink have been completed. *D. simulans (D. sim); D. tristis (D. tris); D. suzukii (D. suz); D. biarmipes (D. biar); D. melanogaster (D. mel).*

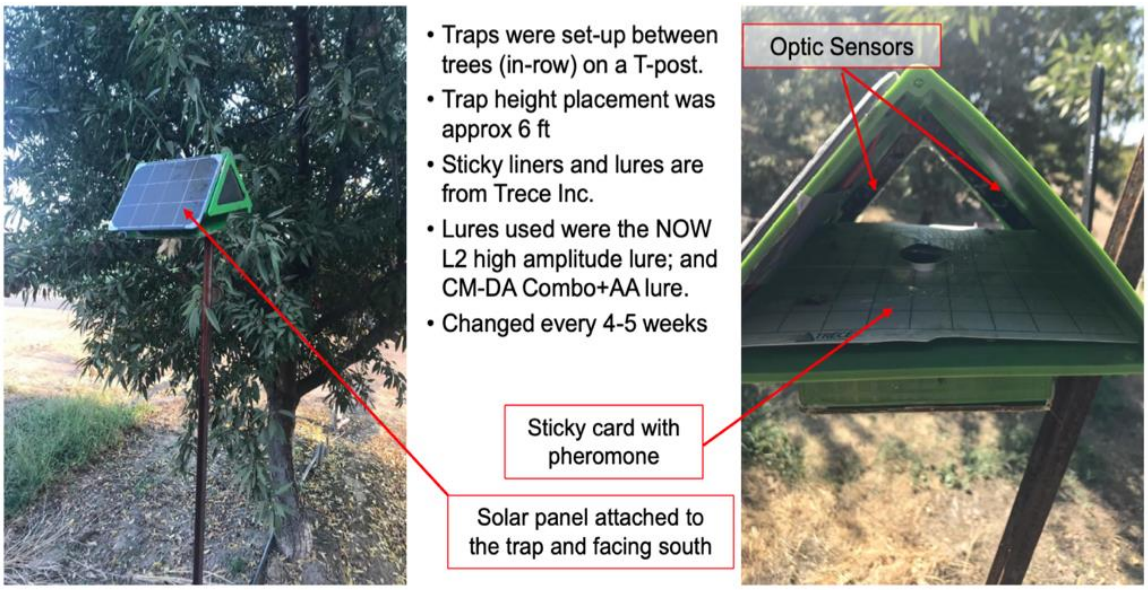

Figure 5: Experimental setup for field testing on NOW

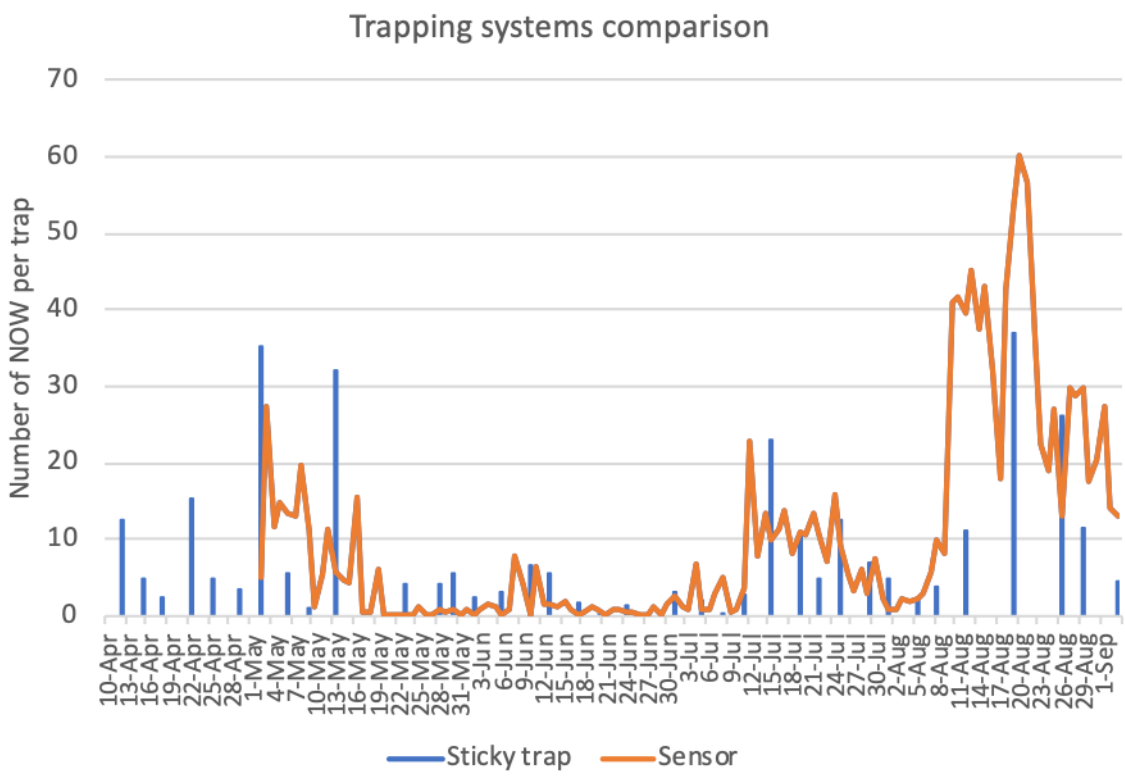

Figure 6: Insect count comparison between Sensor vs Sticky trap.

Figure 7: Field deployment of the sensors monitoring for SWD

EXECUTIVE SUMMARY

Project Title: Electronic sensors to capture spatiotemporal population density of SWD

Key Words: Spotted Wing Drosophila, *Drosophila suzukii*, remote sensing, insect identification

Spotted Wing Drosophila (SWD) is a highly invasive pest species that has now established itself as a keystone pest of U.S. fruit crops, including cherries. SWD oviposits into marketable, ripening fruits, leading to significant annual crop and economic losses. ***The overall goal of this proposal was to adopt electronic sensor technologies and develop automated insect identification models to enable remote and real-time SWD identification and monitoring to support management programs and IPM research efforts.*** Our cooperator Dr. Keogh, a computer scientist at UC Riverside, has previously developed inexpensive pseudo-acoustic opto-electronic sensors and accompanying classification algorithm that can accurately classify multiple species of mosquitoes that vector pathogens such as Zika and West Nile virus by using wing-beat frequencies, daily activity patterns, and geographical distribution. An insect sensor utilizing wing beat frequency for classification can theoretically be applied to identify any flying insect. We therefore proposed to optimize electronic sensor technologies for SWD identification. It is important to stress that the electronic sensor technology we proposed to develop and optimize for SWD is not simply a modernized version of insect traps currently used for population monitoring. Besides supplanting conventional monitoring tools and greatly reducing the time necessary to process trap contents, we anticipated that the capability of the sensors to classify insects in real-time will revolutionize pest management research and enable precision agriculture. For example, current monitoring tools lack spatial and temporal resolution as conventional traps do not provide time-stamps for insect catches. Our sensors on the other hand can ultimately be connected to a central network and were capable of reporting real-time movement between crop and non-crop host plants, providing opportunities to target SWD for sprays at times when they are at maximum density in non-crop plants. This can reduce insecticide residues on crops, a major concern for export markets.

Key products of project:

1. Software: We have collected more than one million insect “encounters” in diverse conditions of light, temperature, humidity, life-stage, species, sex, and completed the development of SWD species ID algorithm.
2. Hardware: We have successfully validated the solar power and remote communication modules of the sensor.
3. Field trials: We have initiated field testing of the SWD system and will continue repeated iterations of trial and optimization cycles. Measurements of field encounters will continue to improve the species ID algorithm. Demo units can now be requested.

FINAL PROJECT REPORT

Project Title: Advancing precision pollination systems to improve yield security

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Cooperators: Finley Cherries, Olsen Brothers, Hayden Farms, Russ LeSage, David Green, Chisa Fruit Co., Firman Pollen Co., On Target Spray Systems, Cameron Peace, Katie Taylor, Connor Dykes

Other funding sources
Firman Pollen company provided pollen for this project at no cost – estimated at $5,000; OnTarget Spray Systems is providing a technician (estimated 140 hrs/ year for this project) and a sprayer (retail value of $20,000) for this research

Total Project Funding: **Year 1:** 74,566 **Year 2:** 74,624

Budget History
Organization Name: Washington State University Contract Administrator: Katy Roberts
Telephone: 509.335.2885 Email address: katy.roberts@wsu.edu

Item	2017	2018	2019
Salaries	$40,856		$44,191
Benefits	$5,482		$6,053
Wages	$12,480		$13,500
Benefits	$1,248		$1,350
Equipment	$0		$0
Supplies	$8,000		$2,500
Travel	$6,500		$7,030
Plot Fees			
Miscellaneous			
Total	$74,566		$74,624

Objectives:
Our long term goal is to improve yield security and yield by developing and deploying a reliable precision pollination system that can 1) supplement current grower pollination practices or, 2) replace the current (ancient) system of planting pollinizers and renting pollinators. We will continue to work with grower collaborators, Firman Pollen Company, and On Target Spray Systems to be sure that research progress is easily translatable to commercial-scale solutions.

1. Refine pollen rate and application timing to improve efficiency of precision pollination systems
2. Optimize pollen suspension constituents to preserve pollen viability and improve solubility
3. Investigate pollen production systems
4. Use commission funded work to strengthen regional and national research proposals

Significant findings:

Overall:

- There is tremendous variability within and among orchards in fruit set
- Supplemental pollination treatment effects should be assessed on large-scale
- Supplemental pollination treatments can reduce variability in fruit set
- Supplemental pollination treatments, at 15 – 40 g/acre, can improve fruit set and yield
- Under favorable environmental conditions, pollination may be maximized with 4-5 hives per acre
- Pollen viability can be maintained in suspension media, in a commercial sprayer, for 100 minutes
- Pollen germinability is improved in the suspension media for up to 90 minutes
- There is high variability in pollen viability among genotypes, both within a year and among years

Finley 'Chelan'/Mazzard, mature block (2018 & 2019)

- Low fruit set overall, ca. 9% (2018) 25% (2019)
- High variability in branch-level fruit set (0% - 35%)
- No statistically significant effect on fruit set and yield (2018)
- 20% increase in fruit set with 20 g pollen per acre (2019)

Finley 'Chelan'/Mazzard, young block (2018 & 2019)

- High fruit set overall, ca. 47% in both years
- No statistically significant effect on fruit set and yield due to application timing being late (ca. 95% open flowers)
- 15% increase in fruit set from 20 g/acre treatment (2019)

Brewster 'Chelan'/'Gisela6' (2018)

- Moderate fruit set overall at ca. 20%
- High variability in branch-level fruit set (2% - 48%)
- No statistically significant effect on fruit set
- 10% increase in yield overall; 20% yield increase in top of tree

Angol, Chile 'Regina' (2018)

- Moderate fruit set overall at ca. 29%
- Range in branch-level fruit set of 15% - 40%
- Significant increase in fruit set from ca. 15 and 30 g pollen/ac

Pasco 'Benton' (2019)

- High fruit set overall, ca. 46% in control
- 7% and 28% increase in fruit set from 20 g and 40 g/acre, respectively

Pasco 'Skeena' (2019)

- 25% fruit set in control
- 46% increase in fruit set from 20 g/acre applied with electrostatic or airblast

Pollen viability

- Viability varies over time in suspension media and spray tank
- Viability improved or maintained for ca. 100 minutes
- Viability varies significantly among cultivars and between years

RESULTS & DISCUSSION

1. Refine pollen rate and application timing

Over two years, we conducted 10 trials, 8 across Washington, and 2 in Chile. Each supplemental pollination trial was conducted in a commercial orchard with grower established pollenizers and 4 to 5 hives per acre of pollinators. Our treatments examined the potential to improve fruit set and yield in these different orchards. Each orchard site was different in terms of pollenizer density and distribution (Table 1).

Table 1. Summary of orchard trial sites for 2018 and 2019.

Location	Brewster	Benton City	Finley	Finley	Pasco	Pasco
Year	2018	2018	2018 & 19	2018 & 19	2019	2019
Cultivar	'Chelan'	'Chelan'	'Chelan'	'Chelan'	'Benton'	'Skeena'
Rootstock	'Gisela 6'	Mazzard	Mazzard	Mazzard	'Gisela 6'	Mazzard
Pollinizer	'Santina'	'Index'	2017 'Coral'	'Coral Champagne' & 'Lapins'	'Cristalina'	none
Year planted	2008	2007	1996	2014	2014	2004
Architecture	steep leader	steep leader, 3 leaders/ tree	open center	steep leader, 2 leaders/ tree	steep leader, 2 leaders/ tree	steep leader, 3 leaders/ tree
Spacing	8' x 15'	10' x 15'	9' x 18'	6' x 18'	6' x 16'	9' x 18'
Treatment block size	2 rows x 35 trees	2 full rows	2 full rows	4 rows x 25 trees	3 rows x 42 trees	3 rows x 25 trees
# of trees/ block	70	N/A	200	100	126	75

Trial Site 1 (Finley, Old 'Chelan'/Mazzard): In 2018 we compared two pollen rates (15 and 30 g/acre) with untreated control. The grower collaborator used their electrostatic sprayer (100 gallon tank @ ca. 20 gallons/acre). Two applications were made (4 and 7 April) to 3 replicate blocks of two complete rows. Overall, fruit set was low in this block at about 9% across treatments (Table 2). Among the branches we evaluated for fruit set there was tremendous variability – a range from 0% - 35%. This variability was not associated with branch location (E vs. W sides of tree) nor the bloom density. There was no significant treatment effect on fruit set. Average fruit set was 8.9, 9.5, and 8.2% for the control, 15 g/acre, and 30 g/acre treatments, respectively. This lack of treatment effect may be related to the environmental conditions during application. It was cold during the first

application (51 F) and it rained following the second application. Low temperatures reduce pollen tube germination and growth (Zhang et al., 2018).

Fruit yield was similarly unaffected by pollen application. We weighed all fruit harvested from each two-row block on the day of commercial harvest. Tree yield was determined from total weight from each block divided by the number of trees per block. Yield varied slightly among treatments: 32, 33, and 27 lbs per tree for control, 15 g/acre, and 30 g/acre, respectively. These data translate to 4.3, 4.5, and 3.6 tons per acre.

In 2019 we setup a trial in the same orchard. Applications were made on April 12th and 13th using a 100-gallon On Target Systems electrostatic sprayer calibrated to ca. 20 gallons/acre. This year, fruit set in the control trees of the mature 'Chelan' orchard was 25%, nearly a 3-fold increase compared to the previous year, but again, significantly less than the adjacent, younger 'Chelan' orchard (see below). In this orchard two applications of 20 g pollen per acre had higher fruit set (29.6%) than the 40 g pollen per acre (24.6%) treatment and the control trees (25.3%). There was no similar effect on fruit yield however, with control, 20 g, and 40 g treatments all yielding about 80 lbs per tree, or roughly 10 tons/acre.

Table 2. Summary of fruit set (% available flowers) across all trials in 2018.

	Finley young 'Chelan'*	Finley old 'Chelan'	Brewster 'Chelan'	Benton City 'Chelan'	Angol 'Regina'
Control	48%	9%	19%	5%	21% b
Mix Only	55%				
15 G	48%	10%			34% a
30 G	45%	8%	20%	2%	32% a
30 G AB**				3%	
60 G	58%				24% b
Overall	48%	9%	20%	3%	29%

Fruit set = # fruit at harvest/ # flowers at full bloom. Finley young *n = 20, p = 0.19; Finley old n = 20, p =0 .71; Brewster n = 30, p= 0.36; Angol n = 25, p=0.02*. *second application only; **AB=airblast (all other pollen treatments applied with electrostatic sprayer).

Trial Site 2 (Finley, Young 'Chelan'/Mazzard): In 2018 at this site we compared 3 rates of pollen, the pollen suspension media alone, and a water-sprayed control. Pollen was applied on two days, the 7th and 11th of April. Unfortunately the pollen rates on the first application date were miscalculated and applied at ¼ of the intended rate (i.e., about 4, 8, and 15 g/acre instead of 15, 30, and 60 g/acre). The proper rates were applied on the second date, so our analyses are from those data only. We again recorded a tremendous variability in fruit set among branches. Irrespective of treatment, the range was about 23% - 83%. Overall, across treatments, fruit set was high in this trial – just under 48%. This is particularly interesting because the same genotype in an adjacent block (older Chelan block data above) exhibited much lower fruit set (about 9% overall). Bloom timing, tree age, pollinizer density, pollinator activity, etc., all will impact fruit set (Whiting et al., 2005; Sagredo et al., 2017). Climatic conditions influence ovule viability and longevity, pollen viability, pollen deposition, and stigma receptivity (Zhang et al., 2018). The effective pollination period, estimated at 3-6 days in sweet cherry (Sanzol and Herrero, 2001; Ughini and Roversi, 1996; Sagredo et al., 2017) was cool and windy, with some precipitation. Temperatures were between 50° - 55° F (Table 4). Rain occurred on the day of application in approximately half of our trials, and wind visibly influenced spray deposition in several of our trials. Further research is necessary to identify the key factors influencing fruit set across years and locations.

Yield per tree was variable in this block, ranging from 20 to 56 lbs per tree. The highest mean yield was for the 15 g/acre treatment at 43 lbs/tree. Other treatment yields were 42 lbs (60 g/acre), 37 lbs/tree (30 g/acre), 36 lbs/tree (control), and 34 lbs/tree (suspension media only) though differences among treatments were not statistically significant.

In 2019, the orchard was split into 12 treatment blocks of four rows by 25 trees separated by buffer sections of 15 – 20 trees and a buffer row between sections. Treatments were a no-spray control, 20 grams pollen/acre, or 40 grams pollen/acre assigned using a CRD. Each treatment was replicated four times. Applications were made on April 13th and 14th using a 100-gallon On Target Systems electrostatic sprayer at a rate of 15 gallons/acre. In 2019, untreated, control fruit set was nearly identical to the previous year at 45%. Both 20 g and 40 g per acre supplemental pollination treatments had higher fruit set – 52% and 49%, respectively ($P<0.295$). This apparently did not improve yield however, as all trees were similar at about 43 lbs/tree, or ca. 8.8 tons/acre.

Trial Site 3 (Benton City, 'Chelan'/Mazzard): In this trial we compared treatments of 30 g pollen/acre applied by electrostatic sprayer (ca. 15 gallons/acre) and airblast sprayer (ca. 85 gallons/acre) with untreated control. Fruit set was particularly low in this trial – an average of 3% across all treatments; ranging from a low of 0 to 17%. This is likely related to relatively poor environmental conditions during bloom and the lack of pollenizers. 'Index' trees were planted as every third tree in every third row as pollenizers and these trees were weakened with poor bloom density (one of the reasons we selected this block for a trial). Poor fruit set in treated blocks may be related to timing of application. During the first and second applications, the percent of bloom on labeled branches was 87% and 89%, respectively. There was a slight negative linear relationship between fruit set and the percent bloom during application. This block was left unharvested due to poor yield.

Trial Site 4 (Brewster, 'Chelan'/'Gisela6'): In this trial we compared a single application of 30 g pollen/acre vs. water-sprayed control. The application was made on 23 April using the 50 gallon electrostatic system at ca. 15 gallons/acre. Fruit set in this block was moderate overall at ca. 20%. This may be related to the relatively high pollinizer ratio of 33%. The block was setup with 6 rows of 'Chelan' and 2 rows of 'Santina'. Among the labeled branches, irrespective of treatment, we documented a large variability in fruit set, from 2 – 48%. This is consistent with the range observed in other blocks. There was no statistical difference in fruit set between treated (20%) and control (19%) branches (Table 2). This may be related to the timing of application – average percent open flowers on labeled branches was ca. 86%.

Yield data were collected by weighing bins of fruit during commercial harvest. Trees were harvested over two dates (20 and 23 June) with the first pick targeting ripe fruit in the upper portions of the canopies. From the treated blocks, about 65% of the fruit were harvested on the first date, from the tops of the trees. In control trees, about 58% was harvested on the first date. This difference may have been due to pollen application since our treatment would have been most effective in the upper regions of the trees – flowers in the lower halves of the trees were fully open and likely past optimum receptivity. Extrapolated to a per acre basis, there was about 680 lbs more fruit from the upper portions of treated trees compared to control though this was not statistically significant ($p = 0.35$). Again, the lack of a clear improvement in tree yield may be related to the effect of application timing, or, in this case, insufficient replication. We observed a negative linear relationship between the percent open flowers during pollen application and final fruit set – this suggests that applications made earlier may have been more effective.

Trial Site 5 (Angol, Chile, 'Regina'/Colt): This trial was setup with collaborators who used their own application equipment (On Target Systems electrostatic sprayer). A single application (made at 60-70% full bloom) of ca. 15, 30, or 60 g/acre of pollen was compared to the untreated control. Fruit set

overall was moderate at about 29% across treatments, with a range in set among individual branches of about 15 – 40%. Fruit set was improved significantly by treatments with both 15 and 30 g/acre of pollen, but interestingly, not with the highest pollen rate (Table 2). Compared to control fruit set of 21%, treatment with 15 or 30 g/acre led to fruit set of 34% and 32%, respectively. This represents an improvement of about 59% over the control. It is possible that the positive treatment effects in this trial were due to the timing of application. Again, our results suggest that applications made at later stages of flowering are less effective.

Table 3. Summary of fruit set (% of available flowers) from trial sites in 2019.

Site	Cultivar	Treatment			
		Control	20 g/ac	40 g/ac	*P*-value
Finley, WA	Young 'Chelan'	45%	52%	49%	0.295
	Mature 'Chelan'	25% ab	30% a	25% b	0.04[z]
Pasco, WA	'Benton'[y]	46%	49%	59%	0.114
		Control	Electrostatic	Airblast	
	'Skeena'	25%	34%	36%	0.168

[y] The 40g application was applied only once in this trial. All other applications were made on two separate days during the bloom time period.
[z] Statistical significance at $P \leq 0.05$; means separation was performed with a Tukey's honest significant difference test; means with no letter or the same letter are not different at $P \leq 0.05$.

Trial Site 6 (Pasco, 'Benton'/'Gisela 6'): Treatment blocks were three rows by approximately 42 trees with ~15-tree buffer sections between blocks and two buffer rows between treatment rows. There were 12 blocks with treatments assigned using a CRD. Treatments were a no-spray control, 20 grams pollen/acre applied twice, and 40 grams pollen/acre applied once. 20 gram and 40 gram rates were applied on April 18, and a second application to the 20 gram treatment blocks was on April 19. An On Target Spray Systems 100-gallon electrostatic sprayer was used at a rate of 15 gallons/acre. In this trial fruit set of untreated control was high, at ca. 46% (Table 3). Trees treated twice with supplemental pollination treatments at 20 g per acre had 49% fruit set, and those treated with 40 g per acre had 59% fruit set, a significant improvement compared to the control (P<0.11). The single application of 40 g/acre represents an increase of ca. 28% over the control. This translated into an increase in yield per tree of ca. 4 lbs, or about 1700 lbs per acre. Combined, these data also reveal a potential cause for low yield in 'Benton' – low flower density. This orchard had similar fruit set to the young 'Chelan' block in Finley (both nearly 46%) yet had a much lower yield – 9 tons/acre in the 'Chelan' orchard compared to 3.6 tons/acre in the 'Benton' orchard. These orchards were the same age, and a similar training system. In addition, the improvement in fruit set and yield with a single application in 'Benton' shows that supplemental pollination treatments can be effective in self-fertile orchards, despite there being an abundance of compatible pollen. Our previous data have shown greater fruit set from outcrossing 'Benton' compared to selfing (Whiting, unpublished).

Trial Site 7 (Pasco, 'Skeena'/Mazzard): In 2019 we also setup a trial comparing application technologies in a 'Skeena' orchard north of Pasco. There were no pollinizers in this block.

Treatment blocks were comprised of 3 rows by 25 trees with a 15-tree buffer section between blocks and two buffer rows between treatment rows. There were 16 treatment blocks and 3 treatments, each replicated 5 times. Treatments were a no-spray control, 40 grams pollen/acre applied with an airblast sprayer at a rate of ca. 100 gallons/acre, and 40 grams pollen/acre applied with an On Target Spray Systems electrostatic sprayer at a rate of ca. 15 gallons/acre. A single application was made on April 18, 2019. In this orchard, fruit set of the untreated control trees was moderate at 25% (Table 3). Both application technologies applying 40 g per acre had higher fruit set: 34% and 36% for electrostatic and airblast, respectively (P<0.17). This represents an increase of about 40% over the untreated control. We did not collect yield data in this block.

Interestingly, the greatest improvements in fruit set across both years came from single applications at the higher rate of 40 g/acre in self-fertile cultivars ('Skeena' and 'Benton'). Application timing will be important for single applications to be effective. From our first year we concluded that, in many cases, supplemental pollination treatments were applied too late (e.g., >80% open flowers). Depending on the environmental conditions, it is likely that a single application timed when there is a large population of recently-opened flowers may be sufficient to set a commercially acceptable crop. This is an area deserving of further study. In addition, we conclude that the lack of improvement in fruit set in some trials may simply be due to the high natural pollination in those blocks. Each trial site was stocked with 4-5 hives per acre, bloom overlap with pollenizers was generally quite good, and environmental conditions were favorable. In these conditions, it may be difficult to improve upon natural pollination with any technique. To be sure, supplemental pollination will only improve fruit set & yield when there is a limitation to pollination in the pollinator + pollenizer model. Our long-term goal of replacement pollination systems (i.e., in the absence of pollenizers and pollinators) will require a different approach – one in which blocks can be isolated from pollinators. This is the next step for this work.

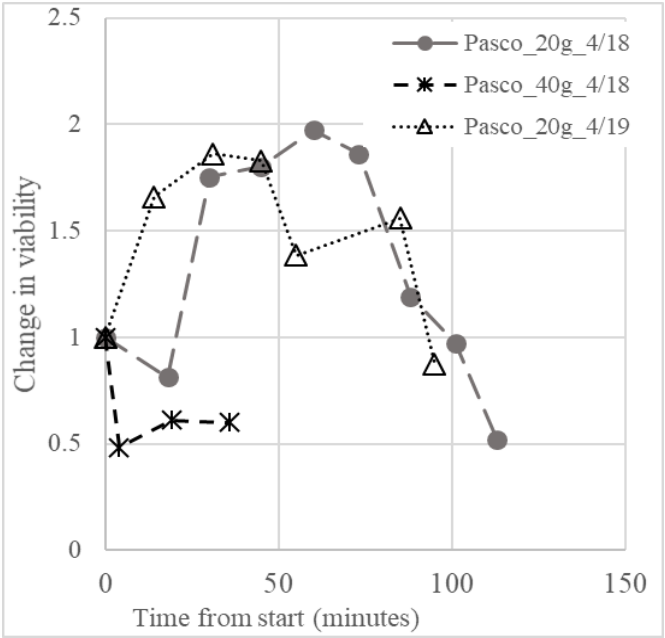

Figure 1. Relative change in pollen viability (time 0 = 1) by tree plate collection method in 3 separate electrostatic pollen applications in commercial sweet cherry orchards in eastern Washington during April, 2019. Mean sample size, *n,* for each point was 3.5 (Pasco_20g_4/19) and 2 for the other 2 trials . Starting viability, taken prior to liquid mixture, was 38% for Pasco_20g_4/ 18; 47% for Pasco_40g_4/18; and 41% for Pasco_20g_4/19.

2. *Optimize pollen suspension constituents*

The pollen suspension media used for all trials was developed in Whiting's lab, based on previous studies of viability over time in a laboratory setting. However, further development of the suspension media will occur privately, not through this project – in 2018 PI Whiting licensed the IP through WSU and is preparing it commercially (the budget request for 2019 was reduced to reflect this change).

To evaluate the impact of the application system on pollen viability during commercial application, samples were collected at various intervals during application. In 2019 pollen samples were collected via three means: 1) liquid suspension samples withdrawn from the sprayer tank, 2) pollen spray collected by holding petri dishes with

germination media in the spray cloud, and 3) hanging petri plates with germination media in the tree canopy during application. The latter was determined to be the preferred method because of high moisture in the other sampling techniques (data not shown). Compared to the germination rate of dry pollen on the germination media plates at the outset of the assessment, there was an increase in germination over the first twenty minutes and an arc that peaked at 40 – 60 minutes (Fig. 3). Pollen viability was maintained for 80 minutes and decreased to the level of the dust at approximately 95 minutes and continued to decline for the duration of the spray tank, 113 minutes (Fig. 3). Pollen is in a dehydrated state at time of anthesis and dispersal, which sustains viability until rehydration occurs on the stigma and triggers germination (Heslop-Harrison, 1979; Edlund et al., 2004; Radunić et al., 2017). Similarly, pollen is desiccated in storage conditions to preserve viability (Vaknin et al., 1999; Dafni & Firmage, 2000). Thus, a rehydration of the pollen grain is an important process expected to increase viability from the pollen dust. Zhang et al (2018) reported that rehydration levels on the stigmatic surface were optimum at anthesis and reduced when pollen was applied to stigmas open for 3 days or more. Vaknin et al. (1999) reported that controlled rehydration allows reorganization of the plasmalemma, increasing pollen germination rates. A period of rehydration could explain the increasing viability in the first thirty minutes of solution exposure.

3. Investigate pollen production systems

In 2017 and 2018 we worked with Firman Pollen Co. in establishing two new orchards for the purpose of pollen production. We are developing pruning and training strategies to promote high flower production and pollen yield. We anticipate the first significant harvest in 2020 from the 'Regina' and 'Black Gold' orchards planted in 2017 on 'Gisela5' rootstock. A block planted in 2018 is comprised of 'Regina' and 'Benton', both on 'Gisela5'.

Table 4. Percent of germinated pollen grains collected from sweet cherry trees in the WSU Roza Orchard in Prosser, WA in 2018 and 2019; analysis was by germination within 20 hours on an agar medium.

Cultivar	Germination (%)		*P* – value (between years)
	2018	2019	
'Chelan'	56 a[z]	68 a	0.024*
'Sweetheart'	59 a	60 ab	0.81
'Regina'		59 ab	
'Bing'	62 a	57 bc	0.32
'Lapins'	63 a	53 bc	0.022*
'Selah'	52 a	50 bc	0.64
'Van'	64 a	49 bcd	0.07
'Skeena'	56 a	48 cde	0.06
'Santina'	59 a	38 de	< 0.001*
'Benton'	53 a	37 e	0.001*
'Ulster'	18 b	12 f	0.006*

The bulk of our work in this area has been on determining the variability among genotypes in pollen viability. This information will be useful for future pollen production systems, and for new orchards being planted and decisions on the best pollenizer to use. Overall, average pollen germination rate across all genotypes was ca. 54% in 2019 and 47% in 2018. Across years and 11 genotypes, pollen germination exhibited significant variability (ca. 5-fold), ranging from 12% to 68%. Interestingly, the variability in germination was different in 2018 and 2019. In 2018, pollen viability was relatively consistent among the ten cultivars examined with a means separation test only yielding two groups: 'Van' (64% viable pollen), 'Lapins' (63%), 'Bing' (62%), 'Santina' (59%), 'Sweetheart' (59%), 'Chelan' (56%), 'Skeena' (56%), 'Benton' (53%), and 'Selah' (52%) were in the same statistical group for germination, with only 12% between the highest and lowest germinating cultivars

(Table 2). The overall mean for this group of cultivars was 58%. By contrast, 'Ulster' comprised the lower viability group, with mean germination of 18%, ca. 40% lower than the cultivars with higher germination (Table 2; $P < 0.0001$).

In 2019, pollen germination was considerably more variable among cultivars tested – means separation testing identified six distinct viability groups among the eleven cultivars tested. The range between the highest and lowest germination was 56%, compared to 46% in 2018. 'Chelan' (68%), 'Sweetheart' (60%), and 'Regina' (59%) had the highest viability. 'Bing' (57%), 'Lapins' (53%), and 'Selah' (50%) had lower viability than 'Chelan' and were equal to 'Van' (49%) and 'Skeena' (48%). 'Skeena' and 'Santina' (38%) had viability the same as 'Benton' (38%). And Ulster (12%) was again the cultivar with the least viable pollen in 2019, only about 17% the viability of 'Chelan' (Table 2). Five cultivars exhibited different germination rates between the two years. Of those 5, 'Chelan' was the only cultivar that exhibited higher viability in 2019 (68%) than 2018 (56%), a 21% increase (Table 2; $P = 0.024$). In 2019, germination rates for 'Ulster', 'Lapins', 'Benton', and 'Santina' were lower than in 2018 by ca. 6%, 10%, 15%, and 21%, respectively (Fig. 4; $P = 0.006$, $P = 0.022$, $P = 0.001$, $P < 0.001$). In contrast, five cultivars ('Bing', 'Selah', 'Skeena', 'Sweetheart', and Van') exhibited similar germination between the two years. Of these 5, all, except for Skeena ($S_1S_{4'}$), contained an S_3 allele. The only cultivar with higher germination in 2019 was 'Chelan' (S_3S_9), also containing the S_3 allele. Of the 4 cultivars exhibiting lower viability in 2019, 'Ulster' (S_3S_4) was the only one containing the S_3 allele and had the lowest germination rate in both years. Ulster and Bing are in the same incompatibility group, as are 'Lapins', 'Santina', and 'Skeena' ($S_1S_{4'}$), 'Sweetheart' and 'Selah' ($S_3S_{4'}$), and 'Regina' and 'Van' (S_1S_3) (Schuster, 2012). 'Benton' is $S_{4'}$ S_9 (Olmstead et al., 2011). It seems unlikely with these 11 cultivars that S-alleles had significant influence on germination rates. Environmental effects on pollen viability will be important to study in the future. If orchards continue to be established with a fixed quantity of pollenizers, variability in pollen viability may affect fruit set and yield. It would be advantageous to plant pollenizers with consistently high pollen viability to maximize pollination potential.

4. Use this work to strengthen larger proposals

We submitted a proposal to the Washington State Specialty Crop Block Grant program (request of ca. $250,000) to expand our research into precision pollination systems and the potential to improve yield security in tree fruit. The proposal was well-rated but not selected for funding.

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Executive Summary

Project Title: Advancing precision pollination systems to improve yield security

Keywords: fruit set, yield, pollen viability, pollination, germination

The project set out to 1) further investigate the potential to improve fruit set & yield security with supplemental pollination in commercial sweet cherry orchards, and 2) better understand the potential for pollen production systems. We documented improvements in fruit set and, in some cases, yield in several orchards. In 'Skeena', for example, we increased fruit set by 40% compared to untreated control trees with a single application of 40 g pollen/acre. We also documented an increase in yield of ca. 1700 lbs/acre in 'Benton' orchard treated with a single application of 40 g pollen/acre. In other trials, we did not record a significant effect on fruit set, nor yield. We draw two main conclusions for these results: 1) supplemental pollination treatments were made too late in bloom (ca. 90% open flowers), and 2) supplemental pollination treatments may not be effective when pollenizer bloom overlap is good, pollenizer density is high, pollinator activity is good, and pollinator density is high (5 hives/acre). Indeed, our approach of using supplemental pollination treatments to evaluate the potential for artificial pollination will be effective only if there is a pollination shortcoming under the growers' best management practices. Our treatments, in some cases a single pass alone, will only be effective in the population of flowers that are open, receptive, and, not already pollinated by the 'natural' process. Interestingly, the greatest improvements in fruit set across both years came from single applications at the higher rate of 40 g/acre in self-fertile cultivars ('Skeena' and 'Benton'). Application timing will be important for single applications to be effective. From our first year we concluded that, in many cases, supplemental pollination treatments were applied too late (e.g., >80% open flowers). Depending on the environmental conditions, it is likely that a single application timed when there is a large population of recently-opened flowers may be sufficient to set a commercially acceptable crop. This is an area deserving of further study. Our long-term goal of replacement pollination systems (i.e., in the absence of pollenizers and pollinators) will require a different approach – one in which blocks can be isolated from pollinators. This is the next step for this work.

Our investigations of variability in pollen viability across genotypes and years has revealed significant discrepancies for both factors. These data (Table 4) will be useful for pollen companies interested in planting orchards strictly (or predominantly) for pollen production, as well as growers considering pollenizer choices for new orchards. Clearly, genotypes that exhibit both high and consistent pollen viability will be preferred. 'Chelan' is one example of a cultivar having high pollen viability both years. We intend to continue these evaluations for an additional year and include various collection sites (e.g., low chill areas) to better understand the variability in pollen viability.

Our studies of pollen viability through the commercial application system showed that pollen remains viable for ca. 100 minutes from initial loading. Interestingly, pollen viability increases, nearly doubling, after 45 – 60 mins in suspension media (Fig. 1), then declines to initial levels by about 100 mins. We hypothesize that pollen in suspension media goes through the initial stages of germination (i.e., hydration) in the spray tank, improving its germinability. Importantly, commercial application of supplemental pollination treatments can be completed within about 90 minutes, before any significant loss of pollen viability.

With these results, we are optimistic for the potential to utilize full replacement pollination in the future – we have shown that pollen can be applied through commercial sprayers, and pollinate sweet cherry flowers, improving fruit set and yield. In addition, the application of supplemental pollination in existing orchards is useful for improving set and yield when the current pollinizer + pollinator model fails. Importantly, the entire process is now commercially available, with several thousand acres treated in 2019.

FINAL PROJECT REPORT

Project Title: MSU cherry rootstocks: pre-commercialization

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Cooperators: Tim Dahle, Scott McDougall, Dan Plath, Aran Urlacher, Dale Goldy, Denny Hayden, Ian Chandler (see Table 2)

Total Project Request: **Year 1:** $81,012 **Year 2:** $82,532 **Year 3:** $84,963

Other funding sources - None

Budget 1 – Amy Iezzoni
Organization Name: Michigan State University **Contract Administrator:** Greta McKinney
Telephone: (517) 353-0391 **Email address:** mckin134@anr.msu.edu

Item	**2017**	**2018**	**2019**
Salaries (technician)[a]	$ 5,500	$ 5,775	$ 6,064
Benefits[b]	2,335	2,492	2,635
Wages			
Benefits			
Equipment			
Supplies[c]	600	600	600
Travel[d]	4,500	4,500	4,500
Trees & shipping[e]	11,721		
Plot Fees			
Total	$ 24,656	$ 13,367	13,799

Footnotes:
[a]Technician will analyze and prepare summary tables and figures of the plot data and conduct the DNA diagnostics.
[b]Benefits calculated at 42.46%,43.15% and 43.46% for 2017, 2018 and 2019, respectively.
[c]Laboratory supplies for the DNA diagnostics.
[d]Travel for A. Iezzoni to visit the test plots, liner nurseries and finished tree nurseries.
[e] The cost of the trees and tree shipping for the 2017 plantings for Obj. 1 and 2.

Budget 2 – Lynn Long
Organization Name: Oregon State University **Contract Administrator:** Russel Karow
Telephone: **Email address:** Russel.Karow@oregonstate.edu

Item	2017	2018	2019
Salaries		$ 8,400	$ 8,400
Benefits		$ 2,772	$ 2,722
Wages	$ 5,100	$ 5,600	$ 6,400
Benefits	510	560	640
Equipment			
Supplies	200	200	200
Travel	40	40	40
Plot Fees	660	660	660
Miscellaneous			
Total	$ 6,510	$ 18,232	$ 19,112

Footnotes:
[1]Previous 2018 and 2019 totals were $7,060 and $ 7,940. The increase is due to the reduction in salary support by OSU and Wasco County due to Lynn Long's retirement.

Budget 3 – Bernardita Sallato[1]
Organization Name: Washington State Univ. **Contract Administrator:** Katy Roberts
Telephone: (509) 335-2885 **Email address:** katy.roberts@wsu.edu

Item	2017	2018	2019
Salaries	$ 2,990	$ 15,450	$ 16,068
Benefits	2,270	7,547	7,849
Wages	25,472	14,400	14,976
Benefits	17,489	10,498	10,918
Equipment			
Supplies			
Travel	1,625	3,038	2,241
Plot Fees			
Miscellaneous			
Total	$ 49,846	$ 50,933	$ 52,052

Footnotes:
[1]Budget for 2017 was to the WTFRC with Tom Auvil as Co-PI. Due to changes at the WTFRC, for 2018, and moving forward, this project was transferred to B. Sallato.

OBJECTIVES:

1. Compare the performance of the MSU cherry rootstocks (labeled as the Corette™ series) to currently available sweet cherry rootstocks using intensive cherry production systems.
2. Track the MSU rootstock performance in trials with PNW grower cooperators that are experimenting with a wider range of scions and orchard systems.
3. Collaborate with cooperating nurseries and the Clean Plant Center Northwest-Fruit Trees to ensure MSU cherry rootstocks are available as virus certified and genetically verified.

SIGNIFICANT FINDINGS

- For the three 2015 plantings, trees were generally largest (TCSA) on the Krymsk® rootstocks, followed by the Gisela® rootstocks and then the Corette™ rootstocks.
- Trees on K5 and K6 were too vigorous and not sufficiently precocious for the ultra-high density systems.
- The reduced size of trees on Clinton compared to Lake at The Dalles and Mattawa may be due to increased spur fruiting on Clinton and higher heat/stress tolerance on Lake.
- For 'Early Robin' in the 2015 plantings, there was not one rootstock whose projected yields per acre consistently outperformed the others, nor were there any consistent differences in fruit size. However, in E. Wenatchee in 2019, the highest 'Early Robin' yields were on Gi5 and Clinton.
- For 'Regina' at The Dalles 2015 planting, all the Corette™ rootstocks had equivalent of higher projected yields per acre compared to K6 and Gi5. Cass and Clare exhibited the highest projected tree yields without an associated reduction in fruit size.
- Across the 2015 plantings, 'Sweetheart' yields tended to be highest on Gi5, Gi6 and Clinton: however, the mean fruit size was small across all rootstocks.
- Trees with higher crop loads tended to have smaller fruit. However, there did not appear to be a genetic tendency for the small Corette™ rootstock trees to have smaller fruit independent of crop load.
- The SSA training system resulted in the trees on the Gisela® and Corette rootstocks coming into production earlier (third leaf) than trees trained to a KGB or steep leader.
- For the 2017 plantings, in general, the tree sizes (TCSA) for 'Regina' and 'Sweetheart' on Gi5, Clinton and Crawford were similar. Promalin-scoring techniques resulted in successful lateral shoot development.
- In one plot planted in 2017, 'Chelan' on all five Corette™ rootstocks had significantly more fruit and earlier ripening fruit than trees on the K6 control. In another plot, 'Coral' trained to an SSA, will have its first crop next year.
- The MSU rootstocks were trademarked under the name Corette™ to facilitate experimental plantings and are commercially available at several nurseries.
- The Iezzoni lab provided DNA diagnostic support as needed at no cost to the collaborating nurseries to assure rootstock trueness-to-type. The genetic test developed by the Iezzoni lab is now offered as a routine service provided by the Foundation Plant Services – U.C. Davis.

RESULTS AND DISCUSSION

Obj. 1. *Performance of the MSU rootstocks*

The performance of the Corette™ rootstocks was evaluated from plots planted in 2015 and 2017 in three locations, The Dalles, Mattawa, and E. Wenatchee. Summaries of the 2015 plantings are provided in Tables 1 and 2. The 2017 plantings, also in these three locations, included an additional MSU rootstock, Crawford, that was not in the prior plantings, and a subset of the scions and rootstocks planted in 2015.

Tree growth – 2015 plantings:

Results: For the 2015 plantings, in general across the three trials, the trees were largest (trunk cross-sectional area; TCSA) on the Krymsk® (K) rootstocks, followed by the Gisela® (Gi) rootstocks and then the Corette™ rootstocks (Table 3). When considering the three trials separately, trees planted in the two Super Slender Axe (SSA) trials in Wash. were significantly smaller than the trees in The Dalles trial that were trained as Kym Green Bush (KGB) and steep leader systems. The much greater shoot and branch growth of the KGB and steep leader systems compared to the SSA, contributed to the larger tree size. In addition, in the ultra-high density double row SSA systems used at Mattawa and E. Wenatchee, tree growth was influenced by inter-tree competition contributing to a reduction in tree size. Despite these limitations, K5 and K6 were found to be too vigorous for an SSA ultra-high density system.

Across all locations and scions, there were no significant differences for TCSA between the two Gisela controls (Gi 5 and Gi 6)(Table 3). This result was unexpected as Gi 5 is known to produce smaller trees than Gi 6. In contrast, when comparing the Gisela® and Corette™ rootstocks, the following differences in TCSA were observed (Table 3).

- At The Dalles, when each scion was considered separately, all the Corette™ rootstocks either had equivalent or smaller TCSA compared to the Gi controls.
- In Mattawa, the TCSA of 'Early Robin' on the Gisela® and Corette™ rootstocks were equivalent; however, 'Sweetheart' trees on Gi6 were significantly larger than trees on Cass and Clinton.
- In E. Wenatchee, for each scion, the TCSA of Gi5 and Clinton were not significantly different, and they were significantly larger than Clare ('Early Robin') and Cass and Clare ('Sweetheart'). The mean TCSA of Lake was similar to Gi5 for 'Sweetheart', but significantly smaller than Gi5 for 'Early Robin'.

Mattawa has sandier soil with less water holding capacity and can reach higher temperatures during the summer than the other sites. The differential growth of Clinton at Mattawa and E. Wenatchee (Table 3), suggests that of the Corette™ rootstocks, Clinton could be more susceptible to one or either of those conditions. In Michigan trials that are also on very sandy soils, Clinton consistently exhibited more symptoms of water/heat stress compared to the other MSU rootstocks. This trait was also observed in The Dalles. In contrast, in Mattawa, The Dalles and Michigan trials, Lake consistently appeared to have a higher water/heat stress tolerance than the other Corette™ and Gisela® rootstocks. The MSU rootstocks also differed in the presence or absence of suckers. Of the five Corette™ rootstocks, Lake, Cass and Clare produced suckers whereas Clinton and Crawford did not.

In addition to TCSA, another indicator for vigor is the ability to create new vegetative growth. In an SSA system, this branching ability is fundamental for the sustainability of the system. In an SSA system, fruiting is targeted to basal flower buds on one-year-old shoots as opposed to fruiting spurs on older wood. At Mattawa, 'Sweetheart' trees on Cass exhibited significantly less new shoot growth than trees on Gi6 and K5, but differences among the Corette™ rootstocks were not significant (data not presented). Anecdotally, under SSA, Gi 6 appeared to have more blind wood (not measured) compared to all other rootstocks.

Tree growth – 2017 plantings

The Corette™ rootstock Crawford was not included in the 2015 plantings as it was delayed being released from the CPCNW-FT. Therefore, in 2017, three smaller plots comparing Crawford with its most similar rootstocks, Clinton and Gi5 were planted in The Dalles, Mattawa, and E. Wenatchee. The two scions were 'Regina' and 'Sweetheart'. Trees in The Dalles were trained to a steep leader for 'Regina' and KGB for 'Sweetheart', while trees in the Wash. sites were trained to an SSA. For these 2017 plantings, tree sizes (measured as TCSA) at Mattawa and E. Wenatchee were not

significantly different for 'Regina' and 'Sweetheart' on Gi5, Clinton and Crawford (data not presented). However, at The Dalles, 'Regina' on Gi5 and Clinton were not significantly different but 'Regina' trees on Crawford were significantly smaller than those on Gi5 (data not presented). Promalin-scoring techniques resulted in successful lateral shoot development.

Discussion: The MSU rootstocks were previously compare to Gi5 and Gi6 in a six year trial planted in Prosser, Wash. in 2009 with 'Bing' scion. The trees were trained to a multiple leader system with eight feet between trees within the row, and fruit were hand-thinned when pea size to prevent over-cropping. In the sixth and final year of this trial, Gi5 and all the MSU rootstocks had significantly smaller TCSA than Gi6 (Long et al. 2019). The mean TCSA of trees on Clare was significantly smaller than for trees on Gi5, Clinton and Crawford. Tree sizes on Lake and Cass were intermediate between Gi5/Clinton/Crawford and Clare. In contrast to the 2009 planting, the TCSA of trees on Clinton in the 2015 plantings tended to be smaller than those of the other rootstocks at The Dalles and Mattawa, but not at E. Wenatchee. This suggests that Clinton is better suited for a cooler climate. It is also possible that in hotter and dryer sites, Cass, Clare and Lake may benefit from increased root volume. When trees in the 2009 WSU-Prosser planting were removed in 2014, three trees per rootstock were excavated to expose the root distribution and size. Visual observations suggested that the root masses differed with Gi6 having the most roots and Gi5, Clinton and Crawford having the least roots. Cass, Clare and Lake appeared intermediate.

<u>Yield and fruit size</u>:
Results:

The Dalles, Ore.: For 'Early Robin' trained to a KGB, there were some differences among rootstock tree yields over the three years (2015-17). However, as the different rootstocks would be planted at different spacings, the tree yields were used to calculate projected tons/acre using the following tree numbers/acre: 519 (6×14) for Krymsk and Gi6; 622 (5×14) for Gi5, Clinton and Lake; and 778 (4×14) for Clare and Cass. For the most part, the projected tons per acre among rootstocks were not significantly different (data not presented). However, in 2017, 2018, and 2019, the lowest projected yields (tons/ac) were for Gi6 (0.1), Lake (8.7) and Clinton (8.9), respectively, and the highest projected yields (tons/ac) were for Clinton (0.3), Gi6 (13.8) and Clare (16.4), respectively. In addition, in 2019 there were no significant differences in fruit size among the rootstocks. This similarity in fruit size and shifting yield rankings among years, illustrates the difficulty in identifying one rootstock that is best suited for 'Early Robin' trained to a KGB.

For 'Regina' trained to a steep leader, the comparison with Gi5 and Gi6 was only possible at The Dalles trial as all the 'Regina' on Gi5 and Gi6 at the Wash. trials turned out not to be 'Regina'. At The Dalles there were no significant rootstock effects on yield in 2017, and minimal differences in 2018 (Table 4). However, in 2019, Cass and Clare, followed by Clinton, resulted in significantly increased annual projected per acre yields when compared with K6 and Gi6. 'Regina' fruit size on Clinton was significantly smaller than that on Gi5 and Clare in 2019 and Clare and Lake in 2018 (Table 4).

For 'Sweetheart', also trained to a KGB, Gi5 tended to have the highest tree and per acre yields across the three years (data not presented). In 2019, the 'Sweetheart' trees at The Dalles overset. Trees on Gi5 were the most overset while K6 had a light crop and lowest yield efficiency, with Lake exhibiting the best balanced crop load rating (data not presented). As the fruit where hand thinned to equalized crop loads, there were no significant differences in fruit size. As 'Sweetheart' tends to over-crop when trained to systems that promote spur fruit production, there is little benefit to putting 'Sweetheart' on an extremely dwarfing rootstock.

Mattawa and E. Wenatchee, Wash.: In contrast to The Dalles, trees at Mattawa and E. Wenatchee were trained to a trellised SSA system at ultra-high density spacings (Table 2). In Mattawa, the 'Early Robin' crop was lost to frost in 2017; however, good yields were obtained in 2018 (Table 5). In

2018, 'Early Robin' on Gi5, Cass and Clare had significantly higher extrapolated yields per acre (as high as 10 tons/acre) compared to K6 (7.4 tons/acre), but these extrapolated yields were not significantly higher than on Gi6, Clinton or Lake (Table 5). 'Early Robin' data was also taken in 2019 but the yields were too low to provide a meaningful comparison. At E. Wenatchee, 'Early Robin' was not harvested in 2018 due to bird damage. Data was taken in 2017 and 2019, but the yields were extremely poor due to unfavorable weather conditions (Table 6); however, yields and yield efficiencies tended to be highest on Gi5 and Clinton.

'Regina' trained to an SSA, continually had low crops at E. Wenatchee and Mattawa. The highest per acre yield was obtained with Clare; extrapolated to be 4.7 tons/acre in 2017 in E. Wenatchee and 7.7 tons/acre in 2018 in Mattawa. These low yields of 'Regina' suggest that this cultivar may not result in increased per acre yields if trained to an SSA system.

'Sweetheart' rootstock performance at E. Wenatchee was similar to that in The Dalles in that the highest tree yields were obtained on Gi5/Gi6 and Clinton. However, in the E. Wenatchee trial, 'Sweetheart' exhibited high early yields in 2017 (third leaf) on all rootstocks except K6 compared to The Dalles KGB trees (Table 6). At E. Wenatchee, high extrapolated tons/acre were also obtained in 2018 and 2019 for all rootstocks except K5. However, the mean 'Sweetheart' fruit size was small across all rootstocks (generally ≥10 row) which is consistent with this productive cultivar having too high a crop load.

Unfortunately, in the Mattawa trial, poor weather in 2017 resulted in an extremely low 'Sweetheart' crop so data was not collected. In 2018, significantly lower yields were obtained on K5 which is consistent with this rootstock being less precocious compared to the Gisela® and Corette™ rootstocks (Table 6). Also in 2018, Gi5 and Clare had significantly higher extrapolated yields per acre compared to Gi6 and Clinton, while yields on Cass and Clare were intermediate. Among the rootstocks, fruit size tended to be smallest on Clinton and highest on Lake. 'Sweetheart' data was also taken in 2019 but the yields were too low to provide a meaningful comparison (Table 6).

Fruit quality: Across scions, locations and years, there were no consistent significant differences in fruit quality (soluble solids, brix, color) among the rootstocks. For example, 'Regina' fruit color was significantly lower on K5 compared to all the Corette™ rootstocks for E. Wenatchee and Mattawa in 2018 and Mattawa in 2019, but this difference was not significant in other years and locations (data not presented).

Discussion: Of the three 2015 plantings, tree yields and projected per acre yields were significantly higher at The Dalles in 2018 and 2019 where the trees were trained to a KGB or steep leader system compared to an SSA at the Wash. sites. However, the results from the E. Wenatchee trial highlight the potential of the SSA system to result in increased yields in the third leaf with precocious rootstocks. Yet, the results from Mattawa illustrated the challenges maintaining high production with an SSA system. For example, lower yields at Mattawa in 2019 compared to 2018 for 'Early Robin' and 'Sweetheart' were likely also due to difficulties in wood renewal with the SSA system. 'Early Robin' in particular exhibited more dead wood or blind wood than the other two cultivars. As 'Early Robin' and 'Regina' typically have lower fruit sets, the SSA system, with its limited number of flower buds may not be the best option for these cultivars. Cultivars with a relatively high number of flowers and good fertility of the basil portion of 1-year-old wood are more likely to benefit from an SSA system (Musacchi et al. 2015).

Previous results from the sixth leaf of the WSU-Prosser 'Bing' planting (2009-2014) illustrated the ability of the Corette™ rootstocks to maintain high tree yields without a reduction in fruit size despite their smaller tree sizes. However, key features of the WSU-Prosser plot included hand thinning of the fruit, lack of inter-tree competition and minimal heat stress compared to what is typically experienced at Mattawa. Results from the three 2015 trials illustrated the importance of matching the scion/rootstock/orchard environment and cultural practices as there was not one rootstock that performed best across all the scions and sites. At The Dalles, where more traditional

training systems were used and normal to heavy crops were obtained each year, the data suggests that there may be a benefit of experimenting with 'Regina' on Clare, Cass and Clinton. These rootstocks, along with Gi5, also have potential in the SSA system; however, the weather and bird related crop losses, inter-tree competition, lack of Gi5 and Gi6 controls for 'Regina' and the difficulty of maintaining 1-year-old fruiting wood, limited our ability to make recommendations among the rootstocks. Additional trails such as those described below in Obj. 2 will help determine what genetic/environment/cultural practices result in the most profitable plantings. These trials will also help determine if the perceived heat/drought tolerance of Lake would have value for sandy hot sites.

Obj. 2. *Track the MSU rootstock performance in trials with PNW grower cooperators that are experimenting with a wider range of scions and orchard systems*.

Project team members visited the plots planted in 2017 (Table 7) to monitor performance and provide cultural practice recommendations as requested. The goal of these plantings was to collectively provide critical comparisons of the new rootstocks with difference scions, high density orchard systems, and environments. The 'Coral', and 'Benton' plantings established in Wash. were managed to initiate an SSA system (Figure 1), while the 'Bing'/'Chelan' planting was managed to initiate a modified SSA/TSA system. All plantings are high density with trellis. In Oregon, the 'Coral' planting was trained to a KGB system and the 'Ebony Pearl'/ 'Burgundy Pearl' planting is being trained to a UFO.

In 2019, the 'Chelan' 2017 planting in Dallesport that includes all five Corette™ rootstocks and K6 control, fruited for the first time. Fruit was harvested from a subset of the trees to assess the early yields in this block (data provided by Ashley Thompson). There were no significant differences in tree yields or fruit quality measurements among the Corette™ rootstocks. However, there were virtually no fruit on the K6 control trees and the K6 fruit present on the day of harvest was just straw color. This illustrates the early yields and advance in maturity possible on dwarfing rootstocks that are precocious and have a reduced canopy size.

Figure 1. 2017 Coral Champagne trial at Denny Hayden orchard, Pasco, WA.

These 2017 plantings, along with the 2015 plantings also serve as demonstration plots. For example, In June, approximately 80 participants visited the Hayden Orchard 'Coral' rootstock plot in Pasco.

Obj 3. *Collaborate with cooperating nurseries and the Clean Plant Center Northwest-Fruit Trees to ensure MSU cherry rootstocks are available as virus certified and genetically verified.*

All five Corette™ cherry rootstocks have been virus certified by the CPCNW-FT; however, this virus certification had not included screening for Cherry Virus A (CVA). Therefore, all five Corette™ rootstocks were screened for CVA and found to be negative. To enable growers to have access to the

MSU rootstocks, they were trademarked under the name Corette™ and are available from several nurseries.

The Iezzoni lab provided DNA diagnostic support as needed at no cost to the collaborating nurseries to assure rootstock trueness-to-type at various stages of liner and finished tree production. In addition, a DNA diagnostic test to distinguish cherry rootstocks that was developed by the Iezzoni lab as part of RosBREED has been adopted by several nurseries and is a routine service provided by the Foundation Plant Services – U.C. Davis.

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Table 1. Summary of rootstock plantings made in 2015 with three scions at three locations: The Dalles, Ore., Mattawa & East Wenatchee, Wash.

	Scion		
Conditions	Regina	Early Robin	Sweetheart
Control Rootstocks	Gi5, Gi6, K6	Gi5, Gi6, K6	Gi5, Gi6, K5
Pollinizers	Sam	Chelan	
Training systems	Steep Leader[1], SSA[2]	KGB[1], SSA[2]	KGB[1], SSA[2]
Corette™ Rootstocks[3]	Clare, Cass, Lake, Clinton		

Training systems at [1]The Dalles; [2]Mattawa and Wenatchee.
[3]All locations and scions except 'Regina'/Cass was not included at East Wenatchee due to insufficient tree numbers.

Table 2. Summary of planting distances and number of trees/acre for rootstock plantings made in spring 2015 with three scions at three locations.

	Planting distance (ft) [trees/acre]		
Rootstock	The Dalles	Wenatchee[1]	Mattawa[1]
K5, K6, Gi5, Clinton	8 × 16 [340]	2 × 12 [1,815]	1.25 × 12 [2,904]
Gi6	8 × 16 [340]	2 × 12 [1,815]	1.5 × 12 [2,420]
Clare, Cass, Lake	8 × 16 [340]	1 × 12 [3,630]	1 × 12 [3,630]

[1]Orchard design consists of double rows, one on either side of a V-trellis.

Table 3. Mean comparison of trunk cross-sectional area (cm^2) in 2019 for three scions ('Early Robin', 'Regina', and 'Sweetheart') on four Corette™ rootstocks, K5, K6, Gi5, and Gi6 and planted in 2015 at three locations (The Dalles, OR; Mattawa and East Wenatchee, WA)[1].

	Trunk Cross-Sectional Area (cm^2)								
	The Dalles			Mattawa			East Wenatchee		
Rootstock	Early Robin	Regina	Sweet-heart	Early Robin	Regina	Sweet-heart	Early Robin	Regina	Sweet-heart
K5/6[2]	81 a[3]	101 a	111 a	24 a	31 a	33 a	29 a	31 a	35 a
Gi5	64 ac	102 a	76 b	20 ab	-[4]	15 bc	28 ab	-[4]	24 bc
Gi6	74 ab	76 ab	80 b	20 ab	-	18 b	27 a	-	27 b
CASS	57 ac	73 ab	48 cd	18 b	9 c	10 c	18 cd	-	17 d
CLARE	58 ac	52 b	47 cd	15 b	11 bc	11 bc	17 d	16 c	16 d
CLINTON	41 c	54 b	40 d	16 b	12 bc	11 c	23 bc	23 b	22 c
LAKE	55 bc	73 ab	64 bc	20 ab	15 b	14 bc	19 cd	16 c	21 cd

[1]KGB and steep leader systems at The Dalles produced much greater shoot and branch growth than SSA, contributing to the larger tree size.
[2]'Early Robin' and 'Regina' are on K6; 'Sweetheart' is on K5.
[3]Means that are significantly different ($P < 0.05$; Tukey) within column are denoted by different letters.
[4]Data for Gi5 and Gi6 from Mattawa and East Wenatchee were not available (-) as the trees unfortunately turned out not to be 'Regina'.**Table 4.** Individual tree yields, projected per acre yields and fruit size for 'Regina' on four Corette™ rootstocks, Gi5, Gi6 and K6 for trees planted in 2015 in The Dalles, OR.

The tree numbers/acre and spacings (ft) used for the projected yields were 519 (6×14) for K6 and Gi6; 622 (5×14) for Gi5, Clinton and Lake; and 778 (4×14) for Clare and Cass. Trees were trained to a steep leader. Data for three years are presented with yield efficiencies (YE) for 2019. In 2019, fruit were harvested on July 2.

Rootstock selection	Tree yield (lb)			Yield per acre (tons/ac)[1]			Row size		
	2017	2018	2019 / YE	2017	2018	2019	2017	2018	2019
Gi5	6.3 a[2]	23.8 ab	12.9 bc / 0.13 bc	2.0 a	7.4 ab	4.0 bc	9.7 a	9.8 ab	9.4 a
Gi6	5.2 a	30.1 a	16.6 abc / 0.23 ab	1.3 a	7.8 ab	4.3 c	9.8 a	10.1 ab	9.9 ab
K6	3.1 a	22.2 ab	6.5 c / 0.06 c	0.8 a	5.8 b	1.7 d	9.9 a	9.7 ab	9.8 ab
CASS	4.0 a	23.6 ab	27.5 a / 0.39 abc	1.5 a	9.2 a	10.7 a	9.9 a	9.8 ab	9.9 ab
CLARE	6.1 a	13.6 b	17.3 ab / 0.33 a	2.4 a	5.3 b	6.7 b	9.7 a	9.6 a	9.5 a
CLINTON	4.3 a	22.7 ab	19.4 ab / 0.39 a	1.3 a	7.0 ab	6.1 ab	9.8 a	10.2 b	10.1 b
LAKE	3.1 a	15.0 b	9.9 bc / 0.14 b	1.0 a	4.7 b	3.1 c	9.8 a	9.7 a	9.7 ab

[1]Ton=US short ton = 2000 lbs.
[2]Significantly different means (P < 0.05) are denoted by different letters.

Table 5. Individual tree yields, extrapolated yields per acre and fruit size for 'Early Robin' and 'Sweetheart' grown on four Corette™ rootstocks, Gi5, Gi6, K5 or K6 and trained to a Super Slender Axe (SSA) from trees planted in 2015 at Mattawa, WA. The tree numbers and spacings (ft) used for the extrapolated yields are in Table 2. Data are presented for two years with yield efficiencies (YE) presented for 2019. In 2019, fruit of 'Early Robin' and 'Sweetheart' were harvested June 14 and July 5, respectively. No fruit was harvested in 2017 due to a weather-related crop loss.

	Rootstock selection	Tree yield (lb)		Yield per acre (tons/ac)[1]		Row size	
		2018	2019 / YE	2018	2019	2018	2019
Early Robin	Gi5	6.7 ab[2]	1.0 ab / 0.05 b	9.7 a	1.5 ab	9.9 ab	9.7 ab
	Gi6	7.3 a	0.8 b / 0.04 b	8.8 ab	1.0 b	9.9 ab	9.8 ab
	K6	5.1 cd	2.0 a / 0.08 ab	7.4 b	2.8 a	9.4 a	10.0 b
	CASS	5.5 bcd	1.2 ab / 0.07 ab	10.0 a	2.1 ab	9.5 ab	9.5 a
	CLARE	5.5 bcd	1.7 ab / 0.11 a	10.0 a	3.1 a	9.6 ab	9.6 a
	CLINTON	6.0 abc	1.1 ab / 0.07 ab	8.7 ab	1.7 ab	10.7 b	9.7 ab
	LAKE	4.6 d	1.0 ab / 0.05 ab	8.3 ab	1.8 ab	9.9 ab	9.6 a
Sweetheart	Gi5	10.9 a	3.9 ab / 0.25 ab	15.8 a	5.6 ab	11.1 c	9.9 a
	Gi6	9.1 ab	5.6 a / 0.31 a	11.0 b	6.8 ab	10.4 ab	9.9 a
	K5	2.5 c	1.7 b / 0.06 b	3.6 c	2.4 b	10.5 b	10.0 a
	CASS	7.3 b	1.8 b / 0.18 ab	13.2 ab	3.2 ab	11.1 c	9.9 a
	CLARE	9.2 ab	4.1 ab / 0.38 a	16.7 a	7.5 a	10.5 b	9.7 a
	CLINTON	8.1 b	3.2 ab / 0.29 a	11.8 b	4.6 ab	11.5 d	10.0 a
	LAKE	7.7 b	2.6 ab / 0.18 ab	14.0 ab	4.7 ab	10.2 a	9.7 a

[1]Ton=US short ton = 2000 lbs.
[2]Significantly different means (P < 0.05) are denoted by different letters.

Table 6. Individual tree yields, extrapolated yields per acre and fruit size for 'Early Robin' and 'Sweetheart' grown on four Corette™ rootstocks, Gi5, Gi6 and K5 or K6 and trained using a Super Slender Axe (SSA) from trees planted in 2015 at East Wenatchee, WA. The tree numbers and spacings (ft) associated with the extrapolated yields per acre are in Table 2. Data are presented for three years with yield efficiencies (YE) presented for 2019. In 2019, 'Early Robin' was harvested on June 20 and 'Sweetheart' was harvested on July 14.

Rootstock selection	Tree yield (lb)			Yield per acre (tons/ac)[1]			Row size		
	2017	2018	2019 / YE	2017	2018	2019	2017	2018	2019
Early Robin									
Gi5	2.4a[2]	-[3]	6.6a / 0.24ab	2.2a	-	6.0a	9.6b	-	9.4a
Gi6	1.5bc	-	4.3ab / 0.16a	1.4ab	-	3.9bc	9.5ab	-	9.4a
K6	0.8d	-	2.5bc / 0.09bc	0.7b	-	2.3bc	10.1c	-	9.3a
CASS	1.0cd	-	0.9c / 0.05c	1.8a	-	1.6c	9.4a	-	9.1a
CLARE	1.2cd	-	0.7c / 0.05c	2.2a	-	1.3c	9.4a	-	9.1a
CLINTON	2.2ab	-	4.9ab / 0.22abc	2.0a	-	4.5ab	9.5ab	-	9.0a
LAKE	1.2cd	-	0.8c / 0.05c	2.2a	-	1.5c	10.0c	-	9.2a
Sweetheart									
Gi5	5.0a	11.7bc	12.0bc / 0.49bc	4.5a	10.6b	10.9bc	11.3b	10.4a	10.0ab
Gi6	3.1b	14.5ab	15.6ab / 0.56b	2.8b	13.2ab	14.2b	11.3b	10.4a	9.9a
K5	0.6d	1.1e	2.9d / 0.08d	0.5c	1.0c	2.6d	11.2ab	10.2a	10.1ab
CASS	2.0c	8.7cd	6.4d / 0.36c	3.6ab	15.8a	11.6c	11.1ab	10.0a	10.0ab
CLARE	1.9c	9.5cd	7.4cd / 0.45bc	3.4ab	17.2a	13.4c	11.0a	10.4a	10.1ab
CLINTON	4.0ab	15.6a	16.9a / 0.76a	3.6ab	14.2ab	15.4a	11.1a	10.4a	10.3b
LAKE	1.9c	6.3d	7.3cd / 0.35c	3.4ab	11.4b	13.3c	11.5c	10.4a	10.1ab

[1]Ton=US short ton = 2000 lbs.
[2]Significantly different means ($P < 0.05$) are denoted by different letters
[3]No fruit was harvested for 'Early Robin' in 2018 due to bird damage.

Table 7. PNW trials planted in 2017 testing the Corette™ cherry rootstocks.

Location	Scion(s)	No. of Corette™ Rootstocks (~ rep. size [1])	Producer
The Dalles, OR[2]	Ebony Pearl, Burgundy Pearl	All 5 (40)	Omeg Orchards
The Dalles, OR	Coral	All 5 (45)	Dahle Orchards
Dallesport, WA	Bing, Chelan	All 5 (30)	Orchard View
Mattawa, WA[3]	Benton	All 5 (200)	Zirkle Fruit
Mattawa, WA	Coral	All 5 (20)	Wash. Fruit & Produce
Pasco, WA	Coral	All 5 (20)	Hayden Farms

[1]The number of trees for each rootstock/scion combination.
[2]The rootstock liners were planted in the orchard in spring 2016 and budded fall 2016.
[3] The rootstock liners were budded in August 2016 and planted in place in spring 2017.
[4] Crawford was not included.

EXECUTIVE SUMMARY

Project Title: MSU Cherry Rootstocks: Pre-commercialization

Key Words: Rootstock, sweet cherry, Super Slender Axe

Five MSU sweet cherry rootstocks were previously identified that induced precocious abundant flowering and significantly reduced tree size compared to Gi6. This result was from a trial at WSU-Prosser with 'Bing' scion planted in 2009 and removed after the 2014 season. All five MSU rootstocks named after Michigan counties (Cass, Clare, Clinton, Crawford and Lake) and labeled as the Corette™ series, produced trees of similar size to Gi5 or smaller. In 2014, all five of the Corette™ rootstocks had yield efficiencies (kg fruit/cm^2) that were higher than that of Gi5 and Gi6, suggesting that they would be suitable for ultra-high density orchard systems. To test the Corette™ rootstocks in wider range of scions, systems and locations, plantings were established in 2015 at three locations (The Dalles, Mattawa, and East Wenatchee), with four Corette™ rootstocks (Cass, Clare, Clinton, and Lake) plus Gisela® and Krymsk® controls, and three scion cultivars ('Regina', 'Early Robin' and 'Sweetheart'). KGB and steep leader training systems were used for The Dalles trial, while ultra-high density double rows trained to a Slender Spindle Axe (SSA) were used for the Wash. trials. Crawford was not included in the 2015 plantings as it was delayed being released from the CPCNW-FT. Three smaller plots comparing Crawford with the most similar rootstocks, Clinton and Gi5, were planting in 2017, also in The Dalles, Mattawa, and E. Wenatchee.

For the 2015 plantings, in general across the three trials, the trees were largest (trunk cross-sectional area; TCSA) on the Krymsk® (K5/K6) rootstocks, followed by the Gisela® (Gi 5/6) rootstocks and the Corette™ rootstocks. Trees on the Krymsk® rootstocks were found to be too vigorous and of insufficient precocity for an SSA system. When comparing just the Gisela® and Corette™ rootstocks, the significant differences in TCSA varied slightly with cultivar/site/training system. The reduced size of trees on Clinton compared to Lake at The Dalles may be due to increased spur fruiting on Clinton and higher heat/stress tolerance on Lake.

For 'Early Robin' in the 2015 plantings, there was not one rootstock whose projected yields per acre consistently outperformed the others, nor were there any consistent differences in fruit size. However, in E. Wenatchee in 2019, the highest 'Early Robin' yields were on Gi5 and Clinton. For 'Regina' at The Dalles 2015 planting, all the Corette™ rootstocks had equivalent of higher projected yields per acre compared to K6 and Gi5. Cass and Clare exhibited the highest projected tree yields without a reduction in fruit size. Across the 2015 plantings, 'Sweetheart' yields tended to be highest on Gi5, Gi6 and Clinton but the mean fruit size was small across all rootstocks. However, there did not appear to be a genetic tendency for the small Corette™ rootstock trees to have smaller fruit independent of crop load. The SSA training system resulted in the trees on the Gisela® and Corette rootstocks coming into production earlier (third leaf) than trees trained to a KGB or steep leader. Yet, after the third leaf there were challenges renewing the wood necessary for the SSA system. For the 2017 plantings, in general, the tree sizes for 'Regina' and 'Sweetheart' on Gi5, Clinton and Crawford were similar. Promalin-scoring techniques resulted in successful lateral shoot development. Collectively these results illustrate the importance of matching the scion/rootstock/orchard environment and cultural practices, as there was not one rootstock that performed best across all scions and sites.

Project team members visited grower plots planted in 2017 that collectively encompass six scions and four training systems. These plantings not only provided critical comparisons of the new rootstocks, but they will also illustrate how these new rootstocks perform given different scions, orchard systems and environments. In one plot, 'Chelan' on all five Corette™ rootstocks had significantly more fruit and earlier ripening fruit than trees on the K6 control. In another plot, 'Coral' trained to an SSA, will have its first crop next year.

The Iezzoni lab provided DNA diagnostic support as needed at no cost to the collaborating nurseries to assure rootstock trueness-to-type. The genetic test developed by the Iezzoni lab is now offered as a routine service provided by the Foundation Plant Services – U.C. Davis.

FINAL PROJECT REPORT **YEAR**: 3 of 3 (No cost extension approved in 2018)
WTFRC Project Number: CH-16-104

Project Title: ABC of sweet cherry powdery mildew: adaption, behavior and control

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Total Project Request: **Year 1:** $81,321 **Year 2:** $82,187 **Year 3:** $84,435

Other funding sources: None

Budget 1
Organization Name: WSU-IAREC **Contract Administrator: Tim Palacios**
Telephone: 509-786-2226 **Email address: prosser.grants@wsu.edu**

Item	2016	2017	2018
Salaries[1]	36,504	37,964	39,483
Benefits[1]	17,522	18,223	18,952
Wages			
Benefits			
Equipment			
Supplies[2]	25,000	25,000	25,000
Travel[3]	1000	1000	1000
Miscellaneous[4]	1295*		
Plot Fees			
Total	81,321	82,187	84,435

Footnotes:
[1]Associate in Research
[2]Molecular supplies (DNA extraction, sequencing costs, PCR and qPCR related chemicals, primer development), Nitex mesh for *in vivo* studies, general supplies for greenhouse and laboratory (petri dishes, agar), fungicides
[3]Sampling trips through Washington and Oregon State
[4]Geneious Software license, international shipping of DNA samples

Recap of original objectives

1. **Adaptation:**
   a. Isolate and characterize cherry powdery mildew populations in commercial orchards in WA and OR
   b. DNA based identification of the causal agent of cherry powdery mildew and multigene phylogenetic reconstruction of the evolutionary relationships among global cherry mildew entities
2. **Behavior:**
   a. Compare virulence structures of identified clades/ subgroups
   b. Identify niche (host tissue) preferences
   c. Identify reproductive strategies and, if heterothallic, mating type frequencies
3. **Control:**
   a. Evaluate response of powdery mildew spores to fungicide sprays before fruit infection is established (visible) using viability qPCR
   b. Identify critical spray periods in which fungicidal protection is most needed to suppress onset or minimize severity of fruit infection

Significant Findings

ADAPTATION

- 93 l isolates of *P. clandestina* were collected in Washington State and Oregon in 2017**.** An Additional 55 isolates were collected throughout WA and OR state in 2018. The focus of 2018 collection effort was to include isolates from distinct geographic locations throughout WA and OR. In 2019, the next-generation and Sanger sequencing were performed to evaluate genetic similarity of these *P. clandestina* isolates. Overall, one hundred and forty-eight isolates of *P. clandestina* were genetically evaluated during the project period.

- Representative *P. clandestina* isolates collected in 2018 are presented in Table 1. Phylogenetic analysis of partial ITS nucleotide sequence showed that all *P. clandestina* isolates (except one from Mexico) clustered together in a separate clade confirming their high level of genetic relatedness. The result indicated that only *P. clandestina* is the causal agent of powdery mildew in cherry orchards of the Pacific Northwest (PNW).

- A few representative isolates were used in cloning and sequencing of the *cytochrome b* gene, a target gene of QoI class (FRAC Group 11) fungicides. This served two purposes: 1) An additional gene for isolate characterization and 2) Fungicide resistance based on molecular target identification.

BEHAVIOR

- Two distinct foliar mildew growth habits were discovered. Fruit mildew growth patterns were different than those found on leaves. The two distinct growth habits of *P. clandestina* isolates were found to be genetically identical and did not differ in fungicide sensitivities. In the leaf-disc assays, growth habits of A and B type are interchangeable.
- We found evidence of mildew infection on leaves (most common), fruit (often seen), buds (green tissue) and, pedicels (fruit stem) but not on flowers.
- Infections were most severe on sucker shoots and young leaves in the upper canopy.
- Chasmothecia (the overwintering propagule) were found at all locations. Mating type genes were identified. Opposite mating-type genes, alpha box domain (MAT1-1) and HMG

(MAT1-2) were found in all composite isolates collected in WA and OR. The presence of both mating-types in the orchard reiterates its significance in chasmothecia production.

CONTROL

- Foliar and fruit mildew disease pressure was monitored throughout the season. Two distinct peaks of increased conidia were identified in fruit infections. This information can be used to better time fungicide applications.
- Pilot-scale spray coverage was also studied to find out appropriate spray volumes using a water-soluble traceable dye. The current spray methods may not give adequate coverage on foliage higher in the canopies. The severe infestations in upper portions of the tree add significantly to the inoculum load, promote the spread of the pathogen, and provide a reservoir of chasmothecia to ensure available primary inoculum during the subsequent growing seasons.
- Although not a direct objective of this project, we used the opportunity to identify fungicide resistance using a pilot scale leaf-disc bioassay against different FRAC groups and molecular characterization of FRAC Group 11 (QoI) target gene, CytB. The results indicated presence of resistant isolates in Washington/ Oregon orchards. A separate project co-funded by Washington Tree Fruit Research Commission and Oregon Sweet Cherry Commission is currently investigating the nature, extent, and mitigation of fungicide resistance to different FRAC groups.

<u>Additional work accomplished:</u> Effects of postharvest treatments on harvested fruit in PNW

To aid in cherry export quarantine process to Australia and other countries requiring fumigation, we measured conidia viability on fruit after various applications of postharvest treatments. The results indicated the effectiveness of current PNW postharvest industry standards in removing all live and dead conidia. Specifically, hydrocooling removed more than 98% conidia and the remaining 2% were killed by fumigation and removed during packaging. The experiments were conducted in 2018 and results were published (Swamy, Probst, and Grove 2019). The final definitive results were further confirmed in 2019 using highly sensitive qPCR methods.

Methods, Results & Discussion

Adaptation and Behavior

Next-generation sequencing of P. clandestina isolates

High-throughput next generation sequencing of foliar powdery mildew isolates were performed in 2018. A minimum of 20 million reads were obtained from each sample indicating a greater depth in sequencing of *P. clandestina*. The reads were assembled in CLC Genomics Workbench platform (Qiagen, Redwood City, CA) and used in identification of full-length internal transcribed spacer (ITS) sequences. Additionally, the sequences were used to identify the full-length cytb gene, a target for QoI (FRAC group 11) fungicides. The sequence alignment and bioinformatics results indicated that most of the isolates used in the sequencing experiment harbored a mutation at position 143 of the cytb amino acid sequence, indicating a resistant isolate. The reads obtained in this study are also being used in 2019 co-funded project 'Fungicide Resistance: A Vital Need To Protect PNW Cherries From Mildew'

Phylogenetic analysis of P. clandestina isolates

Internal transcribed spacer (ITS) region analysis in 2017 and 2018 of all isolates exhibited high sequence identity confirming a single pathogen as the sole incitant of powdery mildew in Washington

and Oregon. ITS sequences were cloned and sequenced in 2018 for fuller coverage spanning the entire ITS region. Sequence analysis indicated that all isolates were nearly identical (>99% identity) confirmed genetic uniformity of the pathogen across the PNW. This was confirmed by phylogenetic analysis of partial ITS nucleotide sequences from representative *P. clandestina* isolates collected in 2018. All *P. clandestina* (except one from Mexico) clustered together in a separate clade confirming their high level of genetic relatedness (Figure 1).

In addition to previous powdery mildew isolate DNA collections, several new orchard sites were identified and *P. clandestina* isolates were collected from these locations. During morphological analysis of mildew isolates, two distinct foliar mildew growth habits were apparent. They were often present in mixed populations. Morphological observations such as conidiophore branching, length and width of each conidial type were investigated. A-type mildew was identified as being highly branched conidiophore arrangement. The conidia were larger in size compared to B-type (Figure 2). B-type was characterized as most widely present mildew with many conidia in linear chains. Each conidiophore contained only one conidial chain. Each isolate collected in 2018 was morphologically characterized (Table 1). The role of both growth habits in the epidemiology of powdery mildew requires further study.

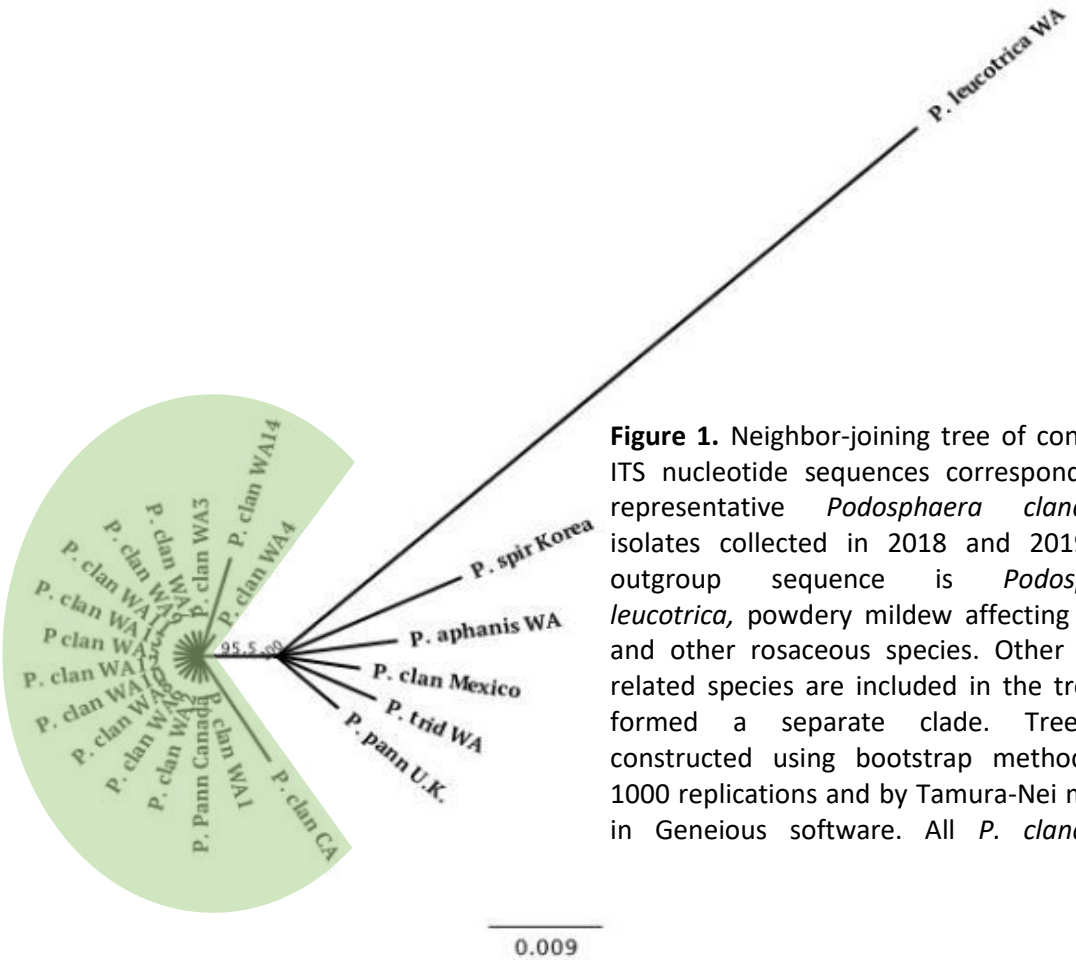

Figure 1. Neighbor-joining tree of consensus ITS nucleotide sequences corresponding to representative *Podosphaera clandestina* isolates collected in 2018 and 2019. The outgroup sequence is *Podosphaera leucotrica,* powdery mildew affecting apples and other rosaceous species. Other closely related species are included in the tree and formed a separate clade. Tree was constructed using bootstrap method with 1000 replications and by Tamura-Nei method in Geneious software. All *P. clandestina*

CPM-A *Globular* CPM-B *Filamentous*

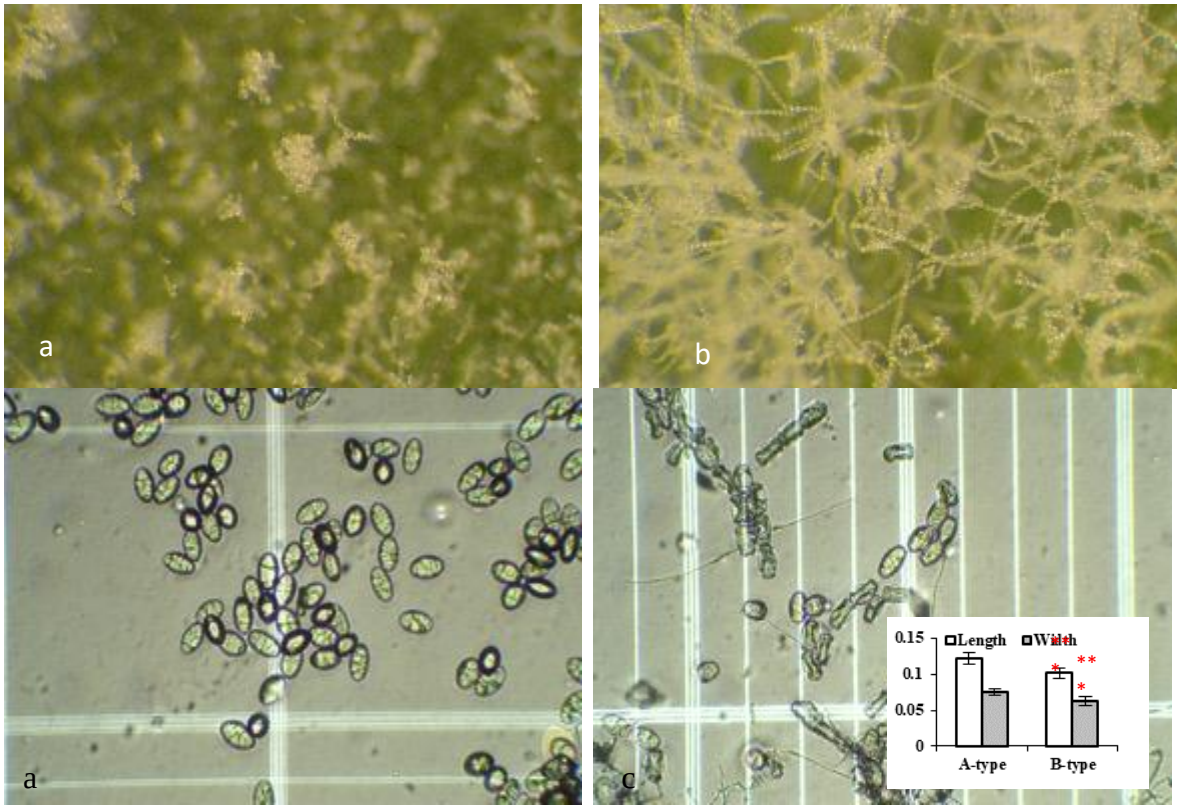

Figure 2. Note globular and highly branched CPM-A (a). Long linear chains observed in CPM-B (b). The sticky tape analysis exhibit slightly longer (and thicker) conidia of CPM-A (c) compared to CPM-B (d, e). Note the arrangement of conidia in both types. Significant differences (*asterisks) was determined using *t-test* ($p<0.001$).

Table 1. Cherry powdery mildew (*P. clandestina*) collections in 2018**.**

Region	Code	Host variety	Mildew type	ITS confirmation
Okanogan/ North Central Washington	BP-1	Lapins	A+B	Yes
	BP-2	Lapins	B	Yes
	OV-1	UN	B	No
	BR-1	UN	B	No
Chelan	MS-1	Bing	B	Yes
	MS-2	Bing	B	Yes
	MS-3	Bing	A+B	Yes
	MS-4	Bing	B	No
	MS-5	Bing	B	Yes
	MS-6	Skeena	B	Yes
	HF-1	UN	A+B	No
	MA-1	UN	B	No
Wenatchee	St1	Skeena	A+B	Yes
	St2	Skeena	A+B	Yes
IAREC Headquarters (Prosser, WA)	Roza-1	Bing	A	Yes
	Roza-2	Bing	B	No
	C-9	Bing	Fruit PM	No
Yakima Valley	MH-1	Lapins		Yes
	MH-2	Rainier	A+B	Yes
	MH-5	Bing	B	Yes
	MH-6	Bing	B	Yes
	MH-7	Lapins	B	No
	HT-1	Rainier	B	Yes
Columbia Basin	JP-1	Bing	A+B	Yes
	HL-1	Tieton	B	Yes
	HL-2	Santina	A+B	Yes
	HL-3	Santina	B	Yes
	HL-4	Bing	B	Yes
	HL-5	Rainier	B	Yes
	HL-6	Rainier	A+B	No
Lower Columbia Basin	DH-1	Bing	B	Yes
	DH-2	Bing	B	Yes
	DH-3	Bing	B	Yes
	DH-4	Bing	A+B	Yes
	DH-5	Bing	B	Yes
Dallesport area/ Oregon State	TP-1	Skeena	A+B	No
	SC-2	Skeena	B	Yes
	SC-4	Sweetheart	A+B	Yes
	SC-6	Rainier	A+B	Yes
	SC-7	Rainier	A+B	Yes
	AR-3	Bing	A+B	No

Mating-type and heterothallism

Chasmothecia, the overwintering propagules of *P. clandestina*, are an essential component in the epidemiology of powdery mildew (Grove and Boal 1991) in the PNW. Chasmothecia production depends on recognition of a mating type in the development of sexual structures, such as ascospores. This process is regulated by genes at mating-type loci in the fungal genome. In powdery mildews, the compatibility of mates is determined by mating-type locus, MAT-1 comprising of one of the two idiomorphs: MAT1-1 (alpha box domain) or MAT1-2 (HMG, high mobility group domain) but never both in each isolate. Identification of the reproductive strategy of *P. clandestina* is critical to the success of powdery mildew management in the PNW. At each location of isolate collection in 2018, late summer/ early winter visits were made to determine if the pathogen formed chasmothecia. We found chasmothecia in all sampling locations across PNW. These propagules serve as the source of primary inoculum for cherry mildew epidemics as reported earlier by Grove and Boal (1991). The presence of chasmothecia in each location implied that the pathogen is probably heterothallic and opposite mating types could be present throughout PNW. We used PCR-based approach to identify mating-types present in PNW to complement previous findings.

In order to identify the opposite mating-types, we used degenerate primers reported by (Brewer et al. 2011). The mating-type MAT1-1 (alpha box domain) was amplified as a 262 base pairs fragment, slightly longer than predicted. Other mating-type locus, MAT1-2 (HMG) was amplified as 282 base pairs fragment. The PCR amplicons were eluted from agarose gel and subsequently sequenced to design *P. clandestina* specific primers (Table 2) for analyzing the mating-type frequencies of isolates from Washington and Oregon cherry orchards. The multiplex PCR assay was performed on all single-colony or mixed cultures of *P. clandestina* isolates to measure the mating-type frequencies and to determine specificity of the primers that detect single or both mating-types in a single reaction. The results showed that all *P. clandestina* isolates (mixed cultures) amplified MAT1-1 and MAT1-2 in a single multiplex reaction (Figure 3). The populations of *P. clandestina* in the PNW contain both mating-types in equal frequencies.

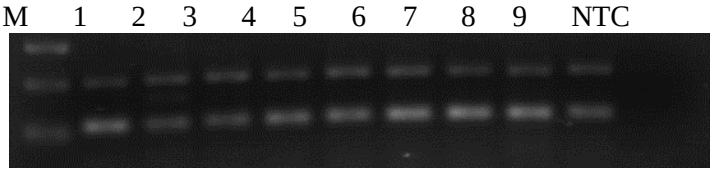

Figure 3. Amplification of MAT1-1 and MAT1-2 in a multiplex PCR reaction. Samples are *P. clandestina* composite isolates collected in Washington and Oregon orchards. M, marker; 1-9 DNA from *P. clandestina* isolates; NTC, no template control.

Table 2. *Podosphaera clandestina* specific PCR primers for identification of two mating-types in a multiplex reaction.

Primer type	Primer name	Primer sequence (5'→3')	Size of PCR product (bp)
P. clandestina specific primers	MAT1F	AGTCGGTGAATTCATGGATGGGA	108
	MAT109R	AGCGACACTGGGAAGACTAAAAA	
	HMG40F	TGAGGAAACTGTAGCCCGCA	207
	HMG247R	ACCAGGATTTTCAACAGCATGCT	

Control

Fungicide resistance

We investigated the occurrence of fungicide resistance of *P. clandestina* isolate collected at Washington State University's Roza farm in 2018. The isolate was subjected to leaf disc bioassays treated with fungicides from different FRAC groups, including a control that received no fungicide. These fungicides include myclobutanil (Rally, FRAC group 3), trifloxystrobin (Gem, FRAC group

11), penthiopyrad (Fontelis, FRAC group 7), quinoxyfen (Quintec, FRAC group 13), a combination of trifloxystrobin and fluopyram (Luna Sensation, FRAC group 11 and 7, respectively), a combination of fluopyram and tebuconazole (Luna Experience, FRAC group 7 and 3, respectively), a combination of azoxystrobin and difenoconazole (Quadris Top, FRAC group 11 and 3, respectively) and, a combination of pyraclostrobin and boscalid (Pristine, FRAC group 11 and 7, respectively). After 14 days incubation (following inoculation), the inoculated leaf discs were microscopically examined for the presence of infection. Our results indicate that the fungicides quinoxyfen (Quintec) and penthiopyrad (Fontelis) containing fungicides were most effective.

In our bioassays, *P. clandestina* grew on leaf discs (of some isolates) treated with myclobutanil (Rally), trifloxystrobin (Gem) and a combination of pyraclostrobin and boscalid (Pristine) (Table 3). Although the colonies were smaller than those on the untreated controls, they produced abundant conidia. To confirm the resistant colonies, the colonies were re-inoculated onto fungicide-treated leaf discs and observations were made after 14 days of incubation.

Table 3. Summary of leaf disc fungicide resistance assay of Roza *P. clandestina* isolate.

Common name	Trade name	FRAC group	Rate of application*	Total leaf discs	Resistant colonies
Penthiopyrad	Fontelis	7	20 fl oz.	80	0
Myclobutanil#	Rally	3	6.0 oz.	80	5
				100	14
Trifloxystrobin#	Gem	11	3.8 fl oz.	80	22
				100	23
Quinoxyfen	Quintec	13	7 fl. oz.	80	0
Fluopyram/ Tebuconazole	Luna Experience	7/3	8.6 fl. oz.	80	0
Azoxystrobin/ Difenoconazole	Quadris Top	11/3	14 fl. oz.	80	0
Trifloxystrobin/ Fluopyram	Luna sensation	11/7	7.6 fl. oz.	80	0
Pyraclostrobin/ Boscalid#	Pristine	11/7	14.5 oz.	80	17
				100	19

*Rate of application per acre assuming 400 g spray material per acre.
#These fungicides were screened in two independent experiments.

Monitoring foliar and fruit mildew inoculum

Foliar and fruit disease pressure was monitored throughout the growing season by measuring live and total conidia. Ten leaves and 50 fruits were processed using laboratory protocols. Conidial suspensions were split into two aliquots and one of them received PMA-treatment. After DNA extractions, quantitative PCR (qPCR) were performed using PCR primers specifically developed for short fragment of ITS gene (improvement to previous qPCR efforts). The results indicated that the live conidial density increased steadily in leaves while viable conidial incidence increased precipitously on fruit just one week prior to harvest (Figure 4) in cvs. 'Bing' and 'Sweetheart'.

Spray coverage

Preliminary studies on spray coverage on large trees were conducted in 2018. Pyranine dye was applied to mature trees at the WSU Roza orchard at 400 gallon per acre. Fruit samples were collected immediately after drying. The spray coverage was evaluated at different canopy levels (Figure 5). The results indicate that on large trees (traditional type with Mazzard rootstock) current industry application practices require further improvement. Spray coverage significantly decreased at higher canopy levels suggesting a need for improvement in current spray technologies and approaches. Results also explained the high number of infected fruits, the rapid increase of foliar powdery mildew following harvest, and number of in situ chasmothecia at higher levels in the canopy.

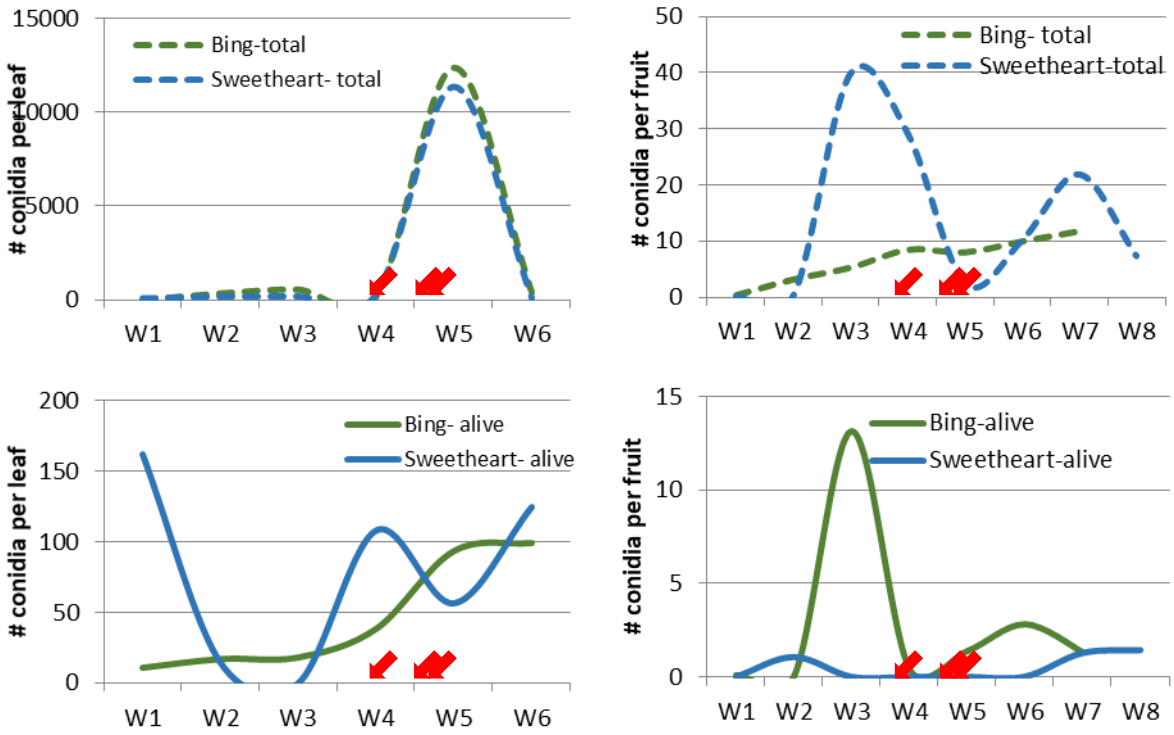

Figure 4. Conidial density of *Podosphaera clandestina* on cvs. Bing and Sweetheart trees. Leaves and fruits were analyzed separately from each tree. Red arrows indicate precipitation events. W, weeks after shuck fall.

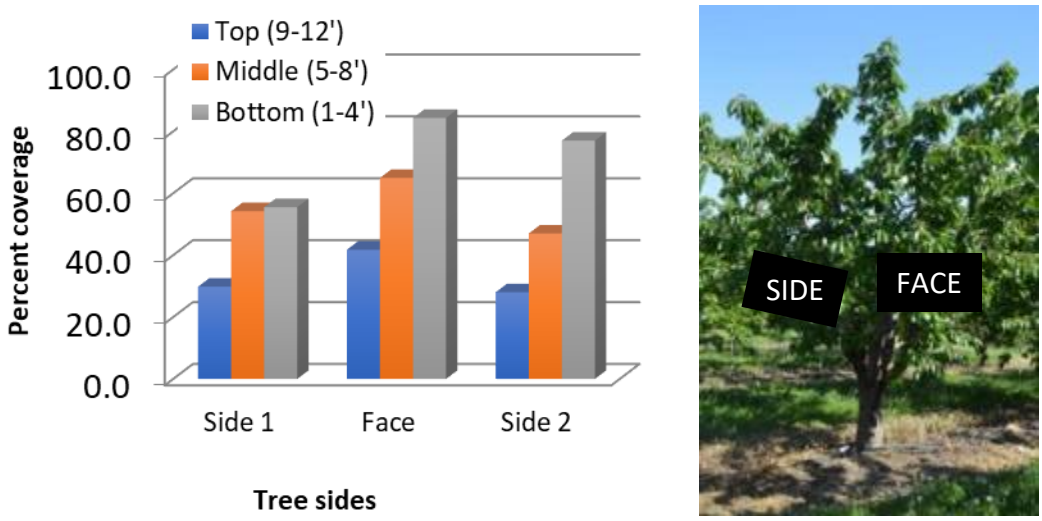

Figure 5. Spray coverage analysis at different canopy levels. Pyranine dye was quantified using images taken under UV-light and image processing was done using online tools.

EFFECTS OF POSTHARVEST TREATMENTS ON HARVESTED FRUIT

Mature Bing cherries were harvested in 2019 late June in commercial bins from the WSU's Roza experimental orchard. The cherry block was not treated with any fungicides in the past several years therefore powdery mildew infection on fruit was apparent and often exceeded 30% of the fruit surface. The cherries were immediately transported to Zirkle Fruit in Prosser for postharvest treatments. The fruit underwent hydrocooling (chilled chlorinated water) for about a minute and was later moved to a cold storage to achieve target fruit temperature of 48 °F for subsequent fumigation. Fumigation was carried out according to industry standards (for Australia as target market). The fruit on the following day received another round of hydrocooling on the packaging line and were packaged in 20 lbs. boxes. The boxes were then stored in cold storage to simulate shipping and arrival at the destination country. The samples (50 random fruit) were taken at each treatment stage in three replications for laboratory analysis. The fruit were processed according to established protocols (divided equally into two aliquots for further processing and PMA treatments) and DNA was extracted using a commercial kit. PMA was used to distinguish between membrane-compromised (dead) and total number of conidia (live and dead). A high sensitivity/specificity qPCR method was adopted to quantify conidia at each stage of postharvest treatment. The absolute number of conidia in each sample was deduced from a standard curve established on serial dilutions of known conidial concentrations. The fruit wash suspensions at each stage were used to inoculate foliage in the leaf-disc bioassay experiments. At the end of incubation period, none of the suspensions from fumigated or packaged cherries resulted in the establishment of mildew colonies on leaf discs. The results indicate that postharvest treatment of harvested fruit is extremely effective in removing all pathogenic conidia on the harvested fruit (Figure 6).

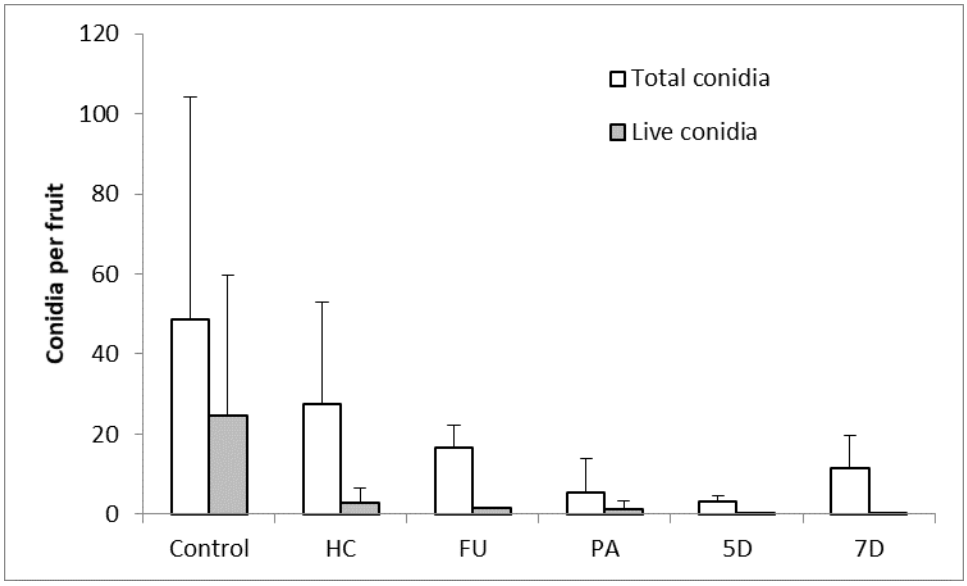

Figure 6. Number of total or pathogenic conidia at each stage of postharvest treatments of Bing cherries. Control: at harvest; HC: hydrocooling on receipt, FU: fumigation; PA: second hydrocooling at packaging; 5D: 5days post packaging; 7D: 7 days post packaging

Literature Cited

Brewer, Marin Talbot, Lance Cadle-Davidson, Paolo Cortesi, Pietro D. Spanu, and Michael G. Milgroom. 2011. 'Identification and structure of the mating-type locus and development of PCR-based markers for mating type in powdery mildew fungi', *Fungal Genetics and Biology*, 48: 704-13.

Grove, G. G., and R. J. Boal. 1991. 'Overwinter survival of *Podosphaera clandestina* in eastern Washington', *Phytopathology*, 81: 385-91.

Swamy, Prashant, Claudia Probst, and Gary G. Grove. 2019. 'Incidence of *Podosphaera clandestina* on sweet cherries (*Prunus avium*) and the influence of postharvest handling practices on the survival of conidia on harvested fruit', *Postharvest Biology and Technology*, 156: 110929.

EXECUTIVE SUMMARY

Project Title: ABC of sweet cherry powdery mildew: adaption, behavior and control
Keywords: *P. clandestina,* fungicide resistance, heterothallism, ITS, spray coverage

In the Pacific Northwest (PNW), powdery mildew of cherry foliage and fruit is caused by *Podosphaera clandestina*. Analysis of internal transcribed spacer (ITS) sequence on many isolates of *P. clandestina* collected across Washington and Oregon states indicated a very high degree of genetic relatedness and were confirmed as the same species. Next-generation sequencing of representative *P. clandestina* isolates was completed and the data was used in identifying various *P. clandestina* full-length genes for the first time and the data is currently been used in other related projects. The fungus was identified as heterothallic in PNW due to the presence of two compatible mating types in all orchard locations studied. This is critical in the production of overwintering chasmothecia, the means of pathogen overwintering and a source of primary inoculum. In the PNW, *P. clandestina* occurs as two distinct growth habits on foliage while drastic differences were seen on pathogen colonies on infected fruit. Although genetically identical, different growth habits did not exhibit any variations in infectivity or fungicide sensitivities suggesting that the two growth habits could be due to physiological differences of their microclimate at sites of collection or unknown host factors. In the orchard conditions, the increase in the concentration of viable conidia on fruit and leaves largely depends on the precipitation events followed by appropriate temperature. A large increase in the viable conidial concentration just before the physiological maturity of fruit may be the cause of fruit infections in cvs. Bing and Sweetheart, as seen in the viability assays. Effective management of a disease is the function of uniform spray coverage of effective pesticides. The consistent observation of increased disease pressure at a higher canopy prompted us to examine the spray coverage in an established cherry orchard. The results of the preliminary study indicated that the spray coverage was <40 % in the higher canopy probably due to an inappropriate sprayer, improper spray calibration or a large tree canopy. Even when the spray coverage is optimal, the likelihood of fungicide resistance encounter in the PNW in cherry orchards was suspected due to a high frequency of synthetic spray applications. We investigated one of the collection sites for the presence of fungicide resistance of the *P. clandestina* isolate against four different FRAC groups either as a component of a premix formulation or single as a single compound. The pilot-scale study suggested that the isolate had developed insensitivities to at least two different FRAC groups. The problem is being investigated in a separate project co-funded by Washington Tree Fruit Research Commission and Oregon Sweet Cherry Commission. Furthermore, this project was used as an opportunity to investigate the presence of powdery mildew conidia in the fruit destined for commercial export. We found that the PNW postharvest treatments effectively removed all viable conidia before packaging and contain no disease inoculum that could infect the host tissue in the country of its destination. Overall, we investigated the biology and genetics of *P. clandestina* infection, establishment, and perennation on a cherry host. Additionally, the project was critical in unraveling problems in current disease management and identified a potential PNW-wide presence of fungicide resistance.

FINAL PROJECT REPORT

Project Title: Non-nutritive sugar-based control strategy for spotted wing drosophila

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Cooperator: Dr. Ramesh Sagili, Associate professor, Oregon State University, Corvallis, OR

Other funding sources

Agency Name: USDA-ARS (Innovation-fund)
Amt. awarded: $25,000

Total project Funding: $82,720

Budget history

Item	2018	2019
[1]Salaries	$25,000	$25,750
[2]Benefits	$4,380	$4,510
[3]Wages	$2,880	$9,600
Equipment	$0	$0
[4]Supplies	$5,000	$4,000
[5]Travel	$800	$800
Plot Fees	$0	$0
Total	$38,060	$44,660

Footnotes: USDA ARS in-house fund supports for equipment, facilities and supplies for this project. [1]Salaries & [2]Benefit, 0.5 FTE Postdoc; [3]Wage, student assistant, $12/h x 10/w@4x6m; [4]Materialsand Supplies, non- & nutritive sugars; other supplies for bioassay, large potted blueberry plants, and Insect rearing materials and supplies; [5]Travel, PI and/or postdoc to attend and present results in the cherry commission or entomology meetings each year.

OBJECTIVES

Our approach for SWD control strategy is based on our current non-nutritive sugar study and previous research results. We recently discovered a variety of dosages of erythritol and other sugars in mixed or separate solutions had significantly reduced the survival of SWD adult flies, and suggested that erythritol alone or with sucrose had potential insecticidal activity. We have also found the impacts on fecundity and mortality from testing at a larger scale in greenhouse cages, and examined the nutritional pathway of ingested erythritol in the fly body. Undoubtedly, erythritol combined with sucrose reduced the survival and fecundity of SWD, which is caused by the physiological imbalance with the sugar osmolarity in the body. Based on those results, we proposed a possible mode of action of erythritol for insecticidal activity.

For practical applications, the erythritol formulation mixed with sucrose can be used as a potential insecticide or as a delivery agent combined with other biological insecticides such as RNAi (RNA interference) and microbial pathogen for SWD. To develop this new control method for cherry growers, we need to identify the mode of action of the erythritol formulation in SWD, evaluate the control efficacy from large scale tests, and investigate if negative impact(s) present on non-target insects. To achieve this goal, these specific objectives need to be accomplished in this project:

1. Test the efficacy of the erythritol formulation on SWD in a greenhouse
2. Test the efficacy of the erythritol formulation on SWD in a field
3. Evaluate the impact of the formulation applied on honeybees

SIGNIFICANT FINDINGS

- The erythritol formulation significantly reduced larval infestation and adult oviposition.
- The erythritol formulation reduced up to 90% larval infestation in the greenhouse trial.
- The erythritol formulation reduced infestations by SWD up to 96 % and overall by 49 % in the field.
- The erythritol formulation reduced up to 43% egg laying in the female fed the formulation.
- The erythritol formulation did not decrease survivorship of honeybees in a cage.

RESULTS & DISCUSSION

An erythritol formulation mixed with sucrose has reduced the lifespan and fecundity of SWD in the lab and greenhouse cages. The impacts of the formulation have been tested in the field or at a larger scale, and on honey bees. For these objectives, we conducted full arena greenhouse and field studies to evaluate the effectiveness of this non-toxic alternative. A part of the research results has been published in Journal of Economic Entomology, November 2018 entitled ‘Effect of Erythritol on *Drosophila suzukii* (Diptera: Drosophilidae) in the Presence of naturally-occurring sugar sources, and on the survival of *Apis mellifera* (Hymenoptera: Apidae) (doi: 10.1093/jee/toy362).

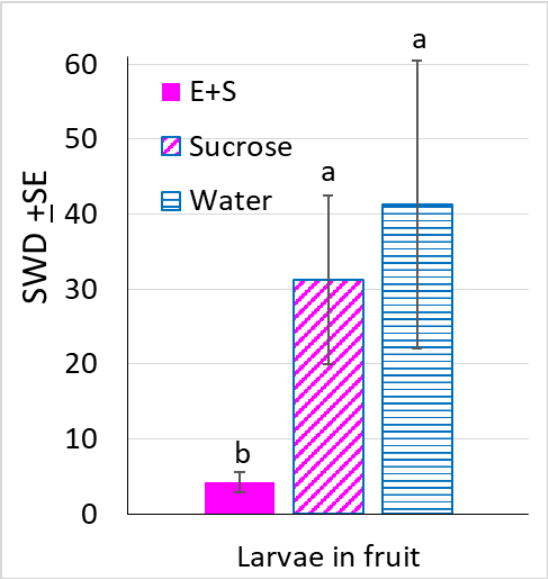

Figure 2. Numbers of SWD larvae infesting blueberries collected from bushes treated with E+S, sucrose or water.

1. Greenhouse trial

We evaluated the erythritol formulation to reduce infestation by SWD exposed among blueberry bushes in the greenhouse. We found 86-90% reduction of SWD larvae infestation on blueberries collected from bushes treated with E+S than the bushes treated with the water or sucrose controls (P = 0.0003) (Fig. 2). The sucrose control showed that a sticky sugar formulation alone does not reduce SWD infestation, but the presence of erythritol does.

We also found fewer adults were trapped post-fruit removal in the greenhouse when bushes were treated with E+S than water, but there were also fewer adults with sucrose than water. We know that sucrose feeding extends the longevity of SWD (Tochen et al. 2016), and that satiated SWD are less likely to be trapped with fermenting odors than hungry SWD (Wong et al. 2018). Therefore, the lowered trap counts following sprays with sucrose-only may reflect fed SWD not being as attracted to the trap to get trapped.

2. Field trial

The E+S formulation was sprayed on blueberry bushes in the field, and evaluated the efficacy for SWD infestation.

2-1. Bag trial for Reka

The field trial in 'Reka' blueberries in June and July occurred before SWD infestation was prevalent yielding minimal data. So, we conducted trials where SWD were confined in mesh bags over Reka clusters to assess whether SWD laid on field-treated blueberries. The erythritol formulation was evaluated for reducing oviposition by SWD exposed to field conditions. We found about 72-78% fewer eggs were laid in berries treated with erythritol (Fig. 3).

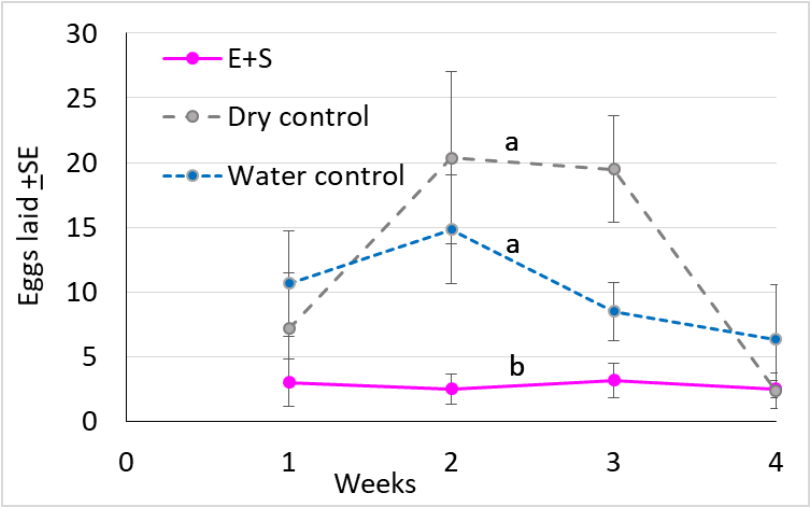

Figure 3. Numbers of eggs laid in blueberry fruits collected from plants treated with E+S, water control or dry control.

2-2. Field trial for Elliot

The E+S formulation was sprayed in 'Elliot' blueberry plants to reduce SWD infestation in the field. We found that SWD larvae was reduced by 49% overall among E+S plots. The difference was most pronounced at week 2 with a 96% reduction (Fig. 4).

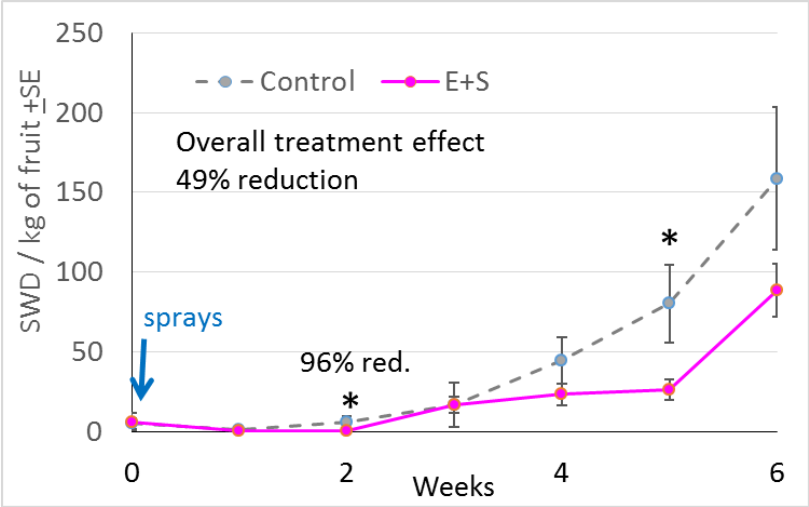

Figure 4. SWD infestation in blueberry fruits collected from plants treated with E+S or dry control.

3. Fly fecundity

To examine fecundity, flies were exposed to 5 blueberry fruits sprayed with E+S formulation, sucrose only or erythritol only for 48 h.

Eggs laid in the blueberries were counted under a microscope. When fed E+S or erythritol-only, females laid ~43% fewer eggs on blueberries than those fed sucrose in a 2 day period (Fig. 4A).

To clarify whether this reduction in fecundity was due to altered oviposition behavior or egg maturation, females were dissected to count ovarial eggs. Surprisingly, the number of ovarial eggs in E+S-fed flies was 37% or 45% more than sucrose- or erythritol-fed flies, with no difference between sucrose- and erythritol-fed flies (Fig. 4B). This suggests that females fed E+S have eggs available but exposure to erythritol reduces egg laying behavior.

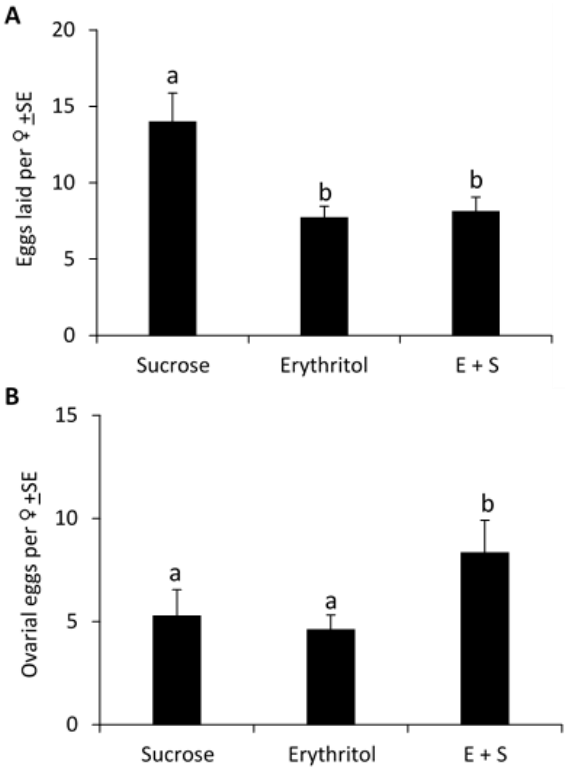

Figure 5. Fecundity (A) and ovarial eggs (B) of SWD females fed erythritol, E+S, or sucrose.

4. Impact on honeybee survivorship

The E+S formulation, erythritol, sucrose, or water were given to honeybees during foraging hours in a cage, and survival rates were similar among treatments at 74 – 85% (Fig. 5). If honeybees were exposed to erythritol for longer periods, differences may appear. But, the high exposure rate tested in the cage is not expected in the field. Honeybees are attracted to flowers via visual and odor cues. The attractiveness of the erythritol mixture is not known although sugars are odorless. Foraging workers can perceive sugar through the gustatory receptors. Future studies with erythritol sprays should monitor the presence of honeybees as well as other natural enemies to determine the extent of exposure, and test survivorship of other natural enemies with erythritol.

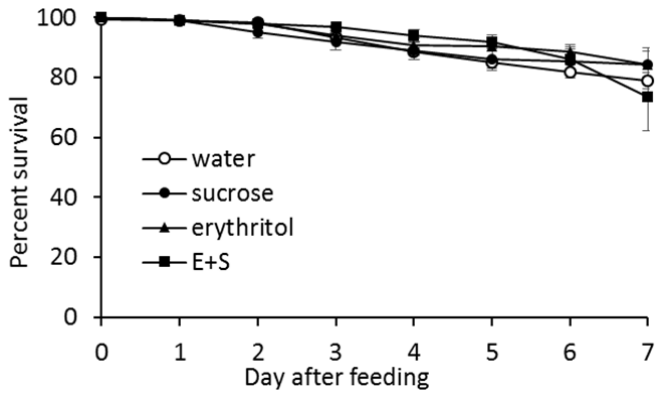

Figure 6. Survival rates of honeybees fed E+S, erythritol, sucrose, or water.

EXCUTIVE SUMMARY

Project Title: Non-nutritive sugar-based control strategy for spotted wing drosophila

Key Words: non-nutritive sugar, erythritol, non-toxic insecticide, *Drosophila suzukii*, *Aphis mellifera*

Spotted wing drosophila (SWD), *Drosophila suzukii*, is a destructive invasive pest, and attacks a wide range of ripening fruits including cherry crops. While numerous biological, cultural, mechanical, and chemical strategies are being developed for SWD control, currently the primary control methods rely on chemical pesticides despite human health and environmental risks. For insecticides to be part of a more sustainable program, efforts are underway to make insecticide applications more effective and reduce overall use, such as reduced spray programs and also to develop environmentally-friendly insecticides.

Sugars are used to stimulate pests to feed on insecticides, and thereby increase the effectiveness of the insecticide application. Sucrose as a feeding stimulant can be added to conventional or organic insecticides targeting the pest. Recently, the PIs investigated the effects of non-nutritive sugars and sugar alcohols on SWD by comparing the survivorship and fecundity of SWD. We found sucrose/erythritol formulations to have a potentially insecticidal effect on the fly. Our research results suggested that feeding caused mortality by the non-nutritive sugar increasing the osmotic pressure in the fly's blood system. The novel mode of action that we discovered represents a potentially useful strategy for a biological insecticide.

For practical applications, the erythritol formulation mixed with sucrose can be used as a potential insecticide or as a delivery agent combined with other biological insecticides. To develop this new control method for cherry growers, we proposed specific objectives: 1) Test the efficacy of the erythritol formulation on SWD in a greenhouse, 2) Test the efficacy of the erythritol formulation on SWD in a field, and 3) Evaluate the impact of the formulation applied on honeybees.

In this research, the significant outcomes are as follows. First, the erythritol formulation significantly reduced larval infestation and adult oviposition. Secondly, the erythritol formulation reduced up to 90% larval infestation in the greenhouse trial. Third, the erythritol formulation reduced infestation by up to 78 % and by 49 % overall in the fields. Fourth, the erythritol formulation reduced up to 43% egg laying in the female fed the formulation. Fifth, the erythritol formulation did not decrease survivorship of honeybees in a cage. In addition, we found the erythritol formulation reduced the longevity of SWD regardless of the presence of wounded blueberries as an alternative sugar source in a cup arena.

Besides causing death, erythritol ingestion appears to interfere with ovipositional behavior and/or physical process of egg laying. Since erythritol increases the osmotic pressure in the fly's hemolymph, this may impede physical movement needed for oviposition. It is also possible that erythritol could be an ovipositional deterrent, but this would need to be tested by applying erythritol to fruit and running no-choice and choice tests in future studies. The mixture did not decrease survivorship of honeybees within 7 days. Future studies should evaluate field applications, spray frequency and volume of erythritol mixture. While the present research focuses on *D. suzukii*, future research should investigate whether this tool can be applied to other Dipteran pests.

FINAL PROJECT REPORT

Project Title: Non-toxic RNAi-based biopesticide to control SWD

PI: Man-Yeon Choi
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Other funding sources

Agency Name: OBC, WBC, ORBC, WRRC, ARF
Amount awarded: $120,000

Total Project Funding: $140,400

Budget History:

Item	Year 1: 2017	Year 2: 2018	Year 3: 2019
Salaries[1]	$25,000	$25,750	$25,750
Benefits [2]	$4,380	$4,510	$4,510
Wages [3]	$6,000	$12,000	$12,000
Equipment	$0	$0	$0
Materials & Supplies[4]	$8,000	$5,500	$5,500
Travel[5]	$500	$500	$500
Plot Fees	$0	$0	$0
Total	$43,880	$48,260	$48,260

Footnotes: USDA ARS in-house fund supports for equipment, facilities and supplies for RNAi project. [1]Salaries & [2]Benefit, 0.5 FTE Postdoc; [3]Wage, student assistant, $12/h x 20/w@4x6m & OPE $480; [4]Materialsand Supplies Molecular biology materials & supplies, RNAi materials & supplies (relatively expensive), and Insect rearing materials and supplies; [5]Travel, PI and/or postdoc to attend a cherry commission & grower meetings each year.

OBJECTIVES

Objective of this research is the development of a novel environmentally-friendly control that is non-toxic insecticide and non-transgenic strategy to control SWD as well as other potential cherry pests. Although RNAi technology is a new insight and promising tool for insect pest management, there are still technical huddles remaining to successfully develop a next generation pesticide. RNAi approach to pest management consider three major challenges: 1) selection and identification of suitable target genes and/or physiological system with high level of gene silencing, 2) cost effective RNAi material production, and 3) development of a suitable delivery method into target pest. A large scale production of RNAi in vitro using kits is too expensive, and not a practical approach for growers (#2). Therefore, there is required a mass production system to synthesis dsRNA through a microbial-based process such as a bacterial-based dsRNA production provides more practical application. To solve this problem, we have established a mass production system using a microbial-induced dsRNA production to increase the feasibility of RNAi application for SWD control (see previous studies and preliminary data 2). To control SWD the strategy of our RNAi approach is non-planted incorporated delivery method such as spray and/or bait-station application (#3).

In the present proposal, therefore we focus on the screening and identification of suitable RNAi target(s) from SWD (#1). A feasible approach for RNAi target gene screening is to search previous targets or systems observed already from same or similar insect groups. Another cost-effective approach is to search RNAi targets from known functional gene analysis and RNA mechanism studies from model insects to increase likelihood of success from the initial stage. Therefore, the screening strategy to identify appropriate RNAi target genes is particularly important. A feasible approach for RNAi target gene screening is to search previous targets or systems observed already from same or similar insect groups.

Our approach for RNAi target gene screening is based on our current RNAi research and previous RNAi results. We recently started the screening of RNAi candidates from SWD, and currently evaluate their impacts through various bioassay. In this proposal we continue to screen more target genes selected from SWD, and evaluate and identify suitable RNAi targets. To achieve this goal the following specific objectives need to be accomplished in this project:

1. Cloning and identify potential RNAi target genes from SWD
2. Construct, design and biosynthesis dsRNAs for target genes
3. Screen for efficacy using bioassay to measure RNAi impacts on SWD
   3-1. Inject dsRNA into adult flies and monitor RNAi impacts
   3-2. Feed dsRNA to larvae and adults, and evaluate RNAi impacts

SIGNIFICANT FINDINGS

- Selected, identified 32 genes, and constructed double-stranded RNAs (dsRNAs) for SWD RNAi
- Screened 3 housekeeping and 3 receptor for potential RNAi targets
- Established a SWD specific nanoinjection system
- Evaluated RNAi impacts by injection and in vitro (cell lines)
- Found the activity of dsRNases in the SWD mid-gut

RESULTS & DISCUSSION

For optimal impact of dsRNA delivered to target cells through feeding, RNAi target genes should focus on systemic RNAi if dsRNA can be internalized into the target cells through feeding. We have selected 32 RNAi targets based on the previous studies for insect RNAi targets and biological functions. These target genes include essential housekeeping genes that are required for the maintenance of basic cellular functions, neuropeptide (NP) hormones and GPCRs for SWD life stages.

1. SWD RNAi target genes: The 32 SWD genes include 17 housekeeping genes, 10 neurohormone receptor genes, 3 neurohormone genes, and 2 chemosensory genes, for potential SWD RNAi targets (Table 1).

Table 1. SWD RNAi candidate genes, GFP, and nucleotide lengths of dsRNAs.

RNAi candidates	DNA template for RNAi synthesis	Gene family
SWD ID1	296 nucleotides	Neurohormone
SWD ID2	195 nucleotides	Neurohormone
SWD ID3	399 nucleotides	Hormone receptor
SWD ID4	244 nucleotides	Housekeeping
SWD ID5	253 nucleotides	Housekeeping
SWD ID6	255 nucleotides	Housekeeping
SWD ID7	253 nucleotides	Housekeeping
SWD ID8	250 nucleotides	Housekeeping
SWD ID9	251 nucleotides	Housekeeping
SWD ID10	254 nucleotides	Housekeeping
SWD ID11	254 nucleotides	Housekeeping
SWD ID12	250 nucleotides	Neurohormone
SWD ID13	299 nucleotides	Hormone receptor
SWD ID14	377 nucleotides	Housekeeping
SWD ID15	374 nucleotides	Housekeeping
SWD ID16	325 nucleotides	Hormone receptor
SWD ID17	299 nucleotides	Hormone receptor
SWD ID18	315 nucleotides	Hormone receptor
SWD ID19	325 nucleotides	Hormone receptor
SWD ID20	261 nucleotides	Hormone receptor
SWD ID21	362 nucleotides	Hormone receptor
SWD ID22	240 nucleotides	Hormone receptor
SWD ID23	360 nucleotides	Hormone receptor
SWD ID24	363 nucleotides	Housekeeping
SWD ID25	308 nucleotides	Housekeeping
SWD ID26	200 nucleotides	Housekeeping
SWD ID27	378 nucleotides	Housekeeping
SWD ID28	200 nucleotides	Housekeeping
SWD ID29	200 nucleotides	Housekeeping
SWD ID30	240 nucleotides	Housekeeping
SWD ID31	250 nucleotides	Chemosensory
SWD ID32	240 nucleotides	Chemosensory
GFP	350 nucleotides	Control, unrelated gene

Some of genes identified in this study were very different nucleotide sequences from the gene sequences published on the SWD genome data. Housekeeping genes as constitutive genes are expressed in all cell types at a level that does not fluctuate with the cell cycle. Functional examples of housekeeping genes for RNAi targets are related in the muscle physiology, detoxification, ATP metabolism, protein sorting and transporting, and cell membrane structure in cells.

For neurohormone receptor genes we particularly focused on G-protein coupled receptors (GPCRs), 7-transmembrane receptors, because the receptors are belonged in the largest superfamily of integral cell membrane proteins and activated for crucial roles of wide physiological processes including behavior, olfactory, hormones, reproduction and development through the life stages. Therefore, GPCRs have great potential for RNAi targets. Five SWD specific neurohormone and chemosensory genes were identified for RNAi candidates.

2. Designed, and constructed double-stranded RNA (dsRNA)

The dsRNAs for each SWD genes were designed and synthesized approximately 250 nucleotides from the parent genes (Fig. 1).

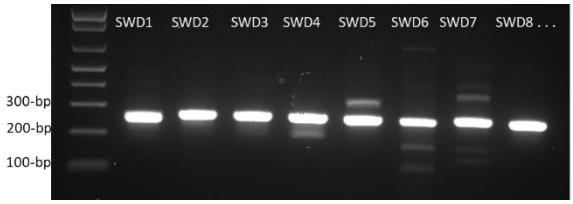


Figure 7. Examples of dsRNA synthesized for SWD RNAi test on injection and feeding.

3. Screen for efficacy using bioassay to measure RNAi impacts on SWD

3-1. Inject dsRNA into adult flies and monitor RNAi impacts: In this project, total 32 RNAi candidates plus two controls were injected over 4,000 flies for the project period (Fig. 2). This system is particularly important to inject a nano-liter (50nL = 0.05µL) of solution into small insects such as SWD with minimal or no physical damage to the fly. After injecting a sham or water control, we found that most SWD adults (> 90%) were not physically affected and survived at least for two weeks. Although individual RNAi injection into SWD is not a practical approach, it is the best and fastest method to screen RNAi targets from impacts on pheno-and genotypic effects. In this initial screening, dsRNA is injected into the hemocoel (= blood vessel). The injection system developed in this research is especially important for adult flies.

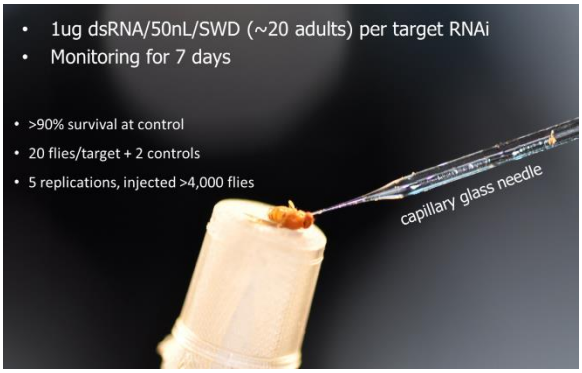


Figure 2. Photos of microinjection system equipped with micromanipulator and a vacuumed tubed plate, and SWD adult injected by a capillary glass needle.

Phenotypic impact of RNAi in SWD adults:

Phenotypic impacts after dsRNA injection into the flies, showed mainly mortality, and some of flies have recovered within few hours (Figs. 3 and 4). We found the maximum mortality up to about 60% on SWD flies injected with the specific dsRNA within 48h (Fig. 4).

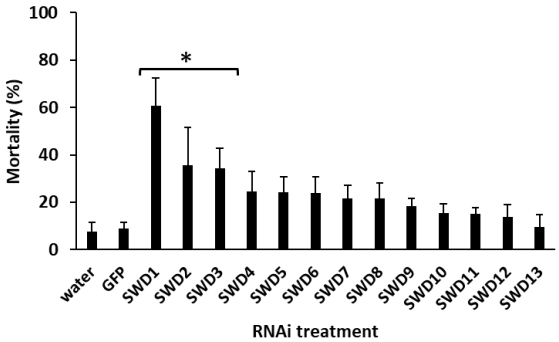
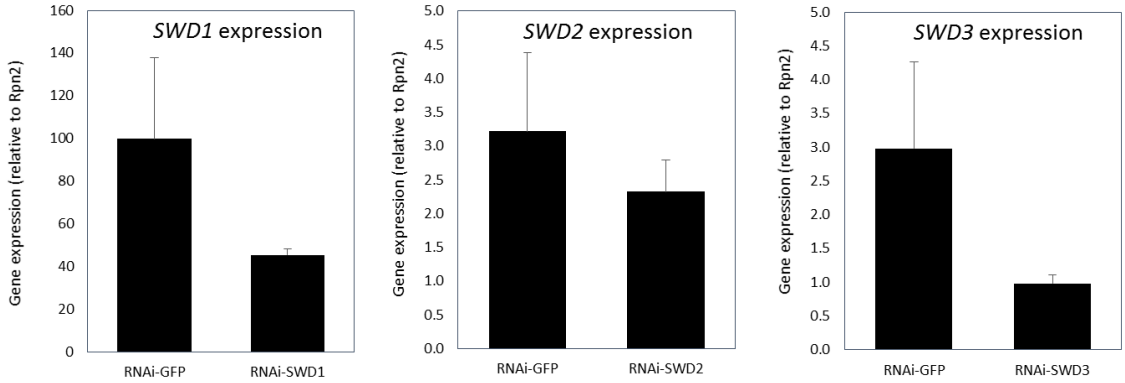


Figure 4. Photos of dead SWD flies after RNAi injected into adults within 48h.

Figure 3. SWD mortality after injected dsRNA (1ug/fly) for 48 hrs. Replicated five times at least.

Genotypic impact of RNAi in SWD adults: We narrowed down three housekeeping genes (SWD1, SWD2, SWD3), and further investigated the gene expression levels to find whether those genes are being suppressed or not after target RNAi (dsRNA) injected into SWD. Using a quantitative real-time PCR (qRT-PCR) three target genes were analyzed for the gene expressions. The result showed three RNAi target genes have been knock downed by dsRNA introduction to SWD (Fig. 5). The reduced

expressions of the genes were resulted from 33% to 67%, SWD3 was the highest genotypic impact, followed SWD1, and SWD2. The result is slightly different compare to the phenotypic impacts.



3-2.

Figure 5. Knock-down of housekeeping genes expression by RNAi. The mRNA expression levels of SWD1, SWD2, and SWD3 were compared between RNAi-GFP and RNAi-target in SWD 12h after dsRNA injection of SWD1, SWD2, SWD3, and GFP. Gene expression estimates are given per a copy of mRNA for the reference gene Rpn2

RNAi impact of the feeding by SWD adults: SWD1 dsRNA was mixed in the food, and introduced in 4-5-day old adults, then monitored the fly mortality for 7 days (Fig. 6). The result showed no significant between the treatment and control that flies fed sugar solution only. The feeding result was very different from the injection that dsRNA was directly introduced into the fly hemocoel. From two controversial results, we examined RNAi impact *in vitro* system which is using *Drosophila* cell line (S2 cell) because the *in vitro* cells could take dsRNA directly without the midgut membrane barrier (see the section 5).

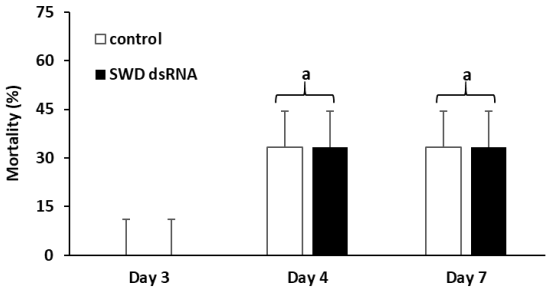


Figure 6. SWD mortality of SWD1 dsRNA-fed flies (1ug/fly) for 7 days. Replicated five times at least.

4. RNAi impact of a neurohormone receptor on SWD adult: Insect GPCR is a great potential target to develop biologically-based insecticide, because GPCRs are key elements to initiate almost all biological signals and physiological functions during insect life stages even the expression levels are very low.

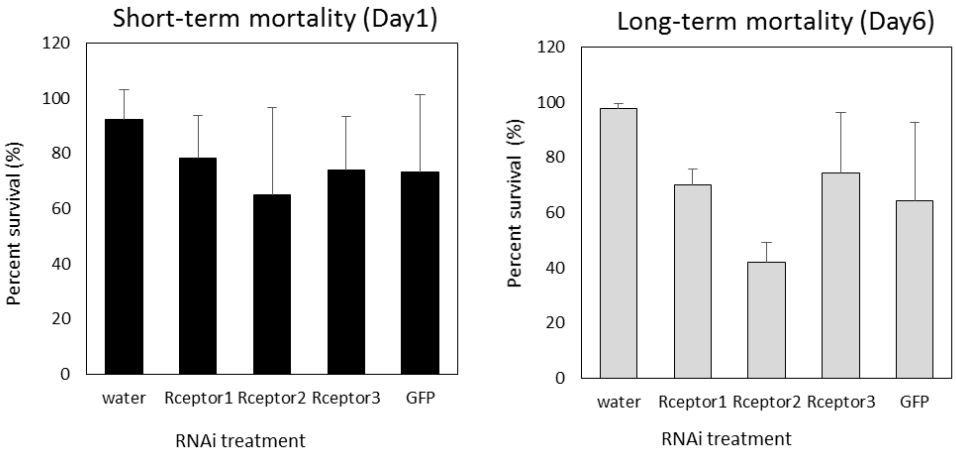


Figure 7. Knock-down of receptor genes expression by RNAi. The mRNA expression levels of Receptor 1, 2 &3 were compared between RNAi-GFP and RNAi-target for SWD 24h (left) and 6 days (right). Gene expression estimates are given per a copy of mRNA for the reference gene Rpn2

Therefore, we examined the gene expression levels of neurohormone receptors after target RNAi (dsRNA) applied to SWD. With qRT-PCR analysis, we found the receptor 2 was significantly suppressed at 48hr after the dsRNA introduced into SWD adults (Fig. 7). The receptor 2 RNAi is currently under more investigation with different bioassays.

5. Effect of RNAi in *Drosophila* cell line: S2 cells (*Drosophila* cells) were incubated, and transferred to a 96-well plate. Then, two amounts of the SWD dsRNAs (1ug – 5ug/well) were applied to the cells, and monitored the cell viability for 24h (Fig. 8). Surprisingly, many cells treated with the dsRNAs showed negative impacts such as cell death and/or decreased cell growth compare to the controls, water or GFP dsRNA treatment.

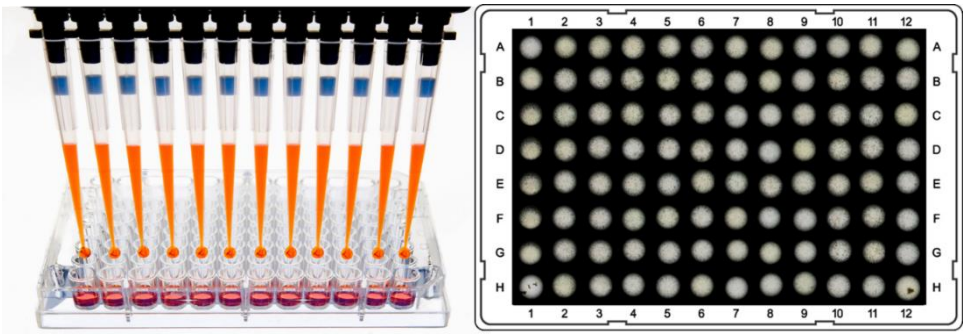


Figure 8. Photo of S2 cells treated with nine different dsRNAs (left) and cell viability after 24h incubation in a 96-well plate (right).

To identify the RNAi genotypic impacts the nine target genes were analyzed their gene expressions by the qRT-PCR. Eight of nine target genes were significantly suppressed with the gene expressions in the cells after the dsRNA treatments (Fig. 9). The phenotypic and genotypic results were consistent from the *in vitro* tests.

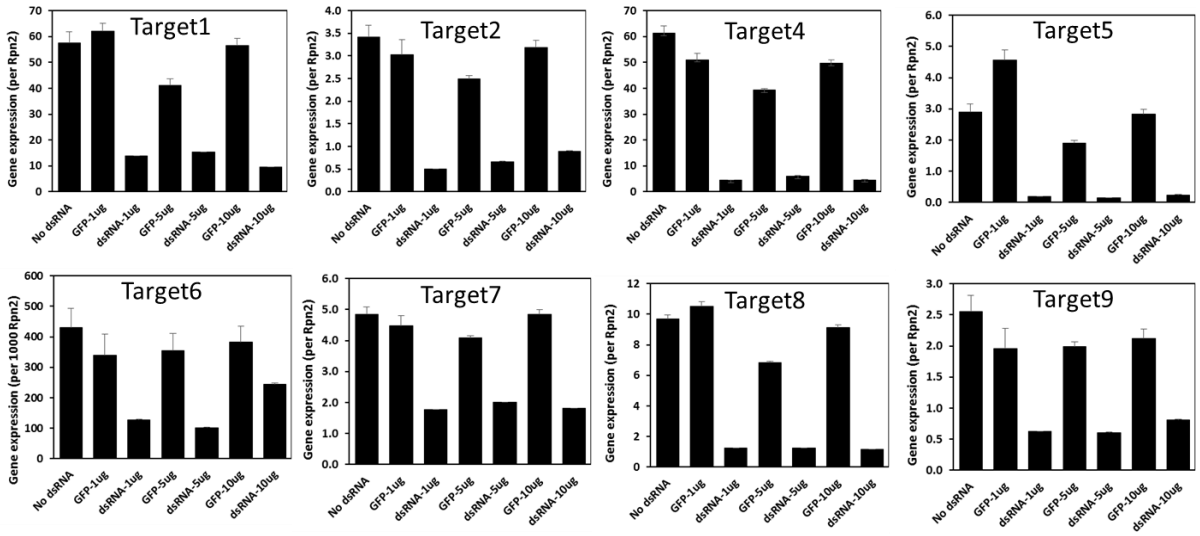


Figure 9. Knock-down of the genes expression by RNAi. The mRNA expression levels of eight genes were compared between RNAi-GFP after dsRNA introduced in the cells. Gene expression estimates are given per a copy of mRNA for the reference gene Rpn2.

From different outcomes from *in vivo* (= feeding) and *in vitro* (= injection and cell bioassays) experiments we particularly targeted the fly midgut because it is the most important component of the

digestive tract for absorbing nutrients from foods. RNAi activation material (= dsRNA) fed to insects should pass through the midgut barrier and then the dsRNA should be taken up by target cells.

6. Found RNase III type enzymes in the SWD mid-gut: We dissected the adult gut, separated the midgut and the other tissue. The tissues were homogenated, and incubated with the GFP dsRNA for 30 min. The amount of the dsRNA incubated with the midgut homogenate was significantly degraded as the RNase III incubation (Fig. 10), but the other gut tissues had no degradation activity (data not shown). The result indicates the midgut juice containing a dsRNA degradation enzyme. Currently, we characterize the enzyme genes, location, and activity in the midgut.

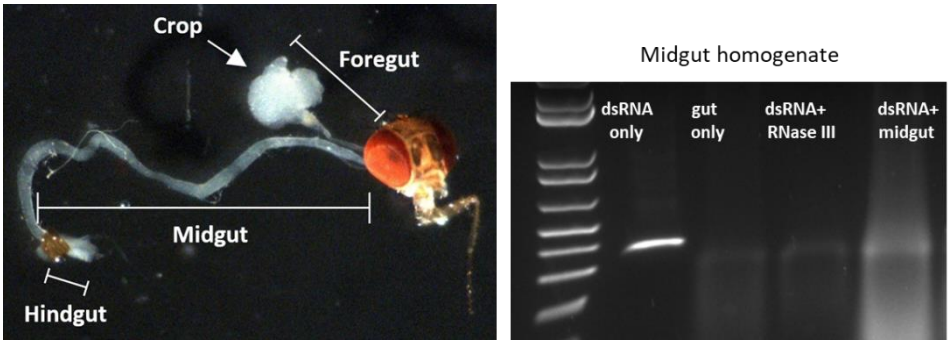


Figure 10. Photo of the adult fly digestive track (left), and gel photo of the GFP dsRNA incubated with RNase III or the midgut homogenate (right) for 30 min at the room temperature.

EXCUTUVE SUMMARY

Project Title: Non-toxic RNAi-based biopesticide to control SWD

Key words: Spotted wing drosophila, *Drosophila suzukii*, RNAi, Biopesticide

Spotted wing drosophila (SWD) is a destructive invasive pest introduced from Asia, and attacks a wide range of ripening fruits. The annual losses have been estimated at $800 million for the soft fruits and cherry industry in US. Recently, SWD management has been ranked a top research priority by small fruit growers. Currently the primary control methods rely on chemical pesticides despite environmental risks, and human health. Therefore, the heavy reliance on chemical insecticides should be replaced or at least complemented with environmentally friendly alternatives. To replace or reduce the use of chemical insecticides, currently alternative options are being developed, but there are still many critical gaps to be implemented against SWD in field.

RNAi for insect control represents a new direction for insect pest control. RNAi is the specific down-regulation or knockdown of gene expression that is a post-transcriptional gene-silencing mechanism insecticide. The mechanism of RNAi is introduced by the delivery of double-stranded RNA (dsRNA) into cells, resulting in interfere target messenger RNA (mRNA) molecules, and subsequently no specific protein produced. During the past decade the application of RNAi techniques has progressed rapidly, and it's becoming a promising pest control tool for chemical insecticide alternative because it poses little or no negative impact on the environment. To develop an RNAi-based biopesticide the major key step is to identify suitable RNAi target(s) from specific pest. Therefore, the selection of effective RNAi target(s) from multiple candidates is critical because RNAi impacts vary depending on insects and genes.

We focused on the identification of suitable RNAi target(s) for SWD. To screen RNAi targets our approach is based on current RNAi research and previous RNAi results. In this project, we proposed specific objectives: 1) cloning and identify potential RNAi target genes from SWD; 2) construct, design and biosynthesis dsRNAs for target genes; and 3) screen for efficacy using bioassay to measure RNAi impacts on SWD. F

In the research, the most significant outcomes are as follows. The first, we screened 32 RNAi candidates including 17 housekeeping genes, 10 G-protein coupled receptor (GPCR) genes, 3 neurohormone genes, and 2 chemosensory genes, from SWD. Second, we constructed all RNAi templates and dsRNAs. Third, we successfully established a specific nanoinjection system for SWD and other flies. Fourth, we screened 3 housekeeping & 3 receptor genes for potential RNAi targets through various bioassays. Unfortunately, the RNAi impacts from the feeding tests that the fly fed the diet or sugar solution mixed with dsRNA, or blueberry fruits sprayed with dsRNA, were not significant compare to the controls without dsRNA. Not like the *in vivo* tests (= feeding), however the *in vitro* test using *Drosophila* cells applied by various dsRNAs showed significant negative impacts such as cell death or decreased cell growth. In this test, eight of nine targets were significantly suppressed the mRNA expressions after dsRNA treatment.

Different RNAi outcomes between *in vivo* (= feeding) and *in vitro* (= injection and cells) results allowed us to focus the fly midgut, particularly, because it is the most important component of the digestive tract for absorbing nutrients from foods. Our RNAi delivery strategy is based on feeding/ingestion options including incorporation into baits for SWD. RNAi activation material (= dsRNA) fed to insects should pass through the midgut barrier and then the dsRNA should be taken up by target cells. We found a digestive activity of RNase III type enzyme (= dsRNA degradation enzyme) in the midgut. There are expected single or multiple enzymes to degrade the dsRNA delivered before pass through the midgut epithelium, so the RNAi impact would be significantly decreased or inactive.

Taken our results from this project suggest, SWD RNAi could be possible to develop a novel RNAi-based biopestide, since no or little Dipteran RNAi has been reported. Future studies should identify and characterize midgut enzymes for effective RNAi delivery through block the enzyme activity or avoid from the enzyme attacking.

FINAL PROJECT REPORT
WTFRC Project Number: CH-16-102

Project Title: Integrated pest management of spotted wing drosophila in sweet cherry

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Other funding sources

Agency Name: **WSDA SCBG**
Amt. awarded: $237,908 (September 30, 2013 - September 29, 2016)
Notes: Research Intern and time slip is paid from this grant through Sept. 29, 2016; hence the WA request for 2016 is limited to 6 months for these two budget items.
Previous WTFRC SWD project for Beers was used as match for SCBG.

Total Project Funding: $203,420

WTFRC Collaborative Expenses: None

Budget 1 History (Beers):
Organization Name: WSU-TFREC **Contract Administrator:** Katy Roberts/Shelli Tompkins
Telephone: 509-335-2885/509-293-8803 **Email:** arcgrants@wsu.edu/shelli.tompkins@wsu.edu

Item	2016	2017	2018	2019
Salaries[1]	16,042	32,085	33,368	0
Benefits[2]	6,192	12,385	12,880	0
Wages[3]	4,922	8,364	8,699	0
Benefits[4]	118	448	467	0
Equipment	0	0	0	0
Supplies[5]	5,000	1,000	1,000	0
Travel[6]	1,150	1,150	1,150	0
Miscellaneous	0	0	0	0
Plot Fees[7]	2,500	2,500	2,500	0
Total	$35,924	$57,932	$60,064	No Cost Extension

Footnotes: [1]Salaries 0.60 FTE Research Intern, [2]Benefits, Research Intern 38.6%; [3]Wages, student (summer) @$20.51/hr, 20 hrs/week x 12 weeks; Research Assistant $12/hr x 20 hr/week x 13 weeks; [4] Benefits student 2.4%, Research Assistant 10%; [5] Five whole tree cages @$800/cage (2016 only), SWD rearing supplies, traps and lures; [6]Travel to plots, $0.54/mile x 100 miles/year; [7]Plot fees. $1,000/acre x 2.5 acres for Sunrise 4 and Columbia View 14.

Budget 2 History (Shearer):

Item	2016	2017	2018	2019
Salaries[1]	0	0	0	0
Benefits[2]	0	0	0	0
Wages	31,320	0	0	0
Benefits	10,187	0	0	0
Equipment	0	0	0	0
Supplies[3]	3,411	0	0	0
Travel[4]	1,582	0	0	0
Plot Fees	3,000	0	0	0
Miscellaneous	0	0	0	0
Total	$49,500	0	0	0

Footnotes:

[1]Two Temp BSRT1, 6 mo ea, $15/hr, 3% raise each year
[2]Benefits: $850/mo
[3]Traps, lures, sampling equipment, insect rearing
[4]In state travel to research sites, $0.575/mile

Objectives:

1. *Test chemical control products to determine their ability to prevent infestation.* There is an ongoing need to determine efficacy of insecticides on SWD. Establishing the length of residual control, both for mortality and prevention of ovipositions, will aid in optimal timing and sequencing of products. New products must be screened to expand the selection of modes of action available for resistance management.
2. *Test chemical control products to determine ability to kill early stages of SWD in fruit.* If preventive sprays are not applied early enough, fruit infestation may occur under high pressure situations. Killing eggs or larvae in fruit will prevent the development of a complete generation of SWD that can attack nearby vulnerable crops (e.g., blueberry) or later maturing cultivars of cherry.
3. *Test provisional spray thresholds to determine initial and subsequent spray timing in commercial orchards.* In order for IPM to be implemented, action thresholds are necessary. Developing a sensitive and reliable monitoring tool is an essential first step in this process. Historic trap catch data coupled with records of infestation inform the need for insecticidal controls.

Significant Findings:

- Entrust and Delegate consistently provide long-term (up to 21 days) mortality of SWD
- GF-120 is still the most attractive/lethal attracticide tested, although new formulations may provide higher levels control in the field
- Some organic materials may provide overall control either by topical toxicity or oviposition deterrence, but lab studies need to be followed by field tests including phytotoxicity
- The spinosyns (Delegate and Entrust) have consistently provided the longest residual control of SWD, but the pyrethroids and diamides can play a role in resistance management
- The Scentry lure is the most attractive lure, but also has the most by-catch if used in a jar trap. Using this lure in combination with a species- and sex-specific trap (yellow sticky trap) may improve user-friendliness and ultimate adoption
- High pressure years for SWD can be predicted by the use of traps and help guide decisions on the intensity of control measures

Methods – *Field trial.* A field trial was performed in a research orchard to assess insecticide efficacy against SWD. Five insecticides were compared to an untreated check in a randomized complete block design, using single tree plots and rows as replicates. There were buffer rows and trees between the treated plots. Insecticides were applied a single time 2 weeks before harvest using an airblast sprayer calibrated to deliver 100 gpa. Four hundred fruit/replicate were collected and placed in plastic containers, and held at 72 °F for 10 days. The number of adult SWD that emerged from the fruit were recorded.

Results – *Field trial.* Fruit were infested to some degree prior to treatment, thus this was a test of both the insecticide's ability to kill pre-imaginal stages in fruit and prevent further infestation. The presence of buffers ensured a high level of pressure in this test. Entrust, Delegate, Exirel, and Warrior all provided similar levels of control. Admire Pro was not significantly different than the check (Fig. 1). This field test confirms

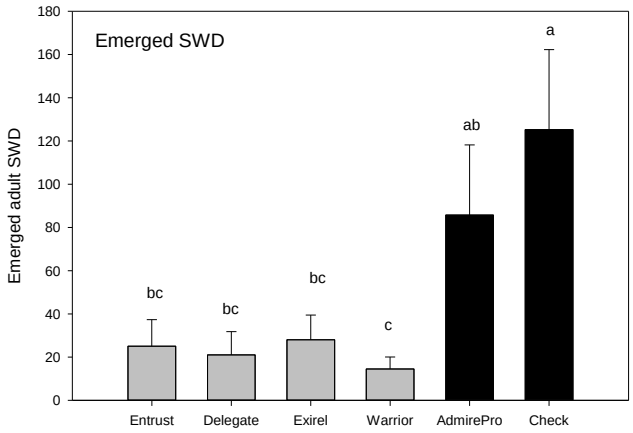

***Fig. 1**. Number of flies emerging from field-sprayed fruit.*

observations in previous work that imidacloprid is a weak material against SWD, and the spinosyns, Entrust and Delegate, provide high levels of control. This is the first field trial of a diamide, and it performed better than expected based on field-aged residue bioassays. The Warrior treatment had the lowest number of SWD adults in this trial, confirming the high levels of activity of this pyrethroid.

Methods - *Field-aged residue bioassays.* A series of experiments was performed using similar methods to test the longevity and efficacy of insecticide residues. Insecticides were applied to a 1-acre block of 'Sweetheart'/Mazzard cherries at the WSU Sunrise orchard near Rock Island, WA. The trees were planted in 2007 at a 10 x 14 ft spacing. Replicates consisted of 2-3 trees per treatment, with sufficient fruit on the trees for the bioassay samples. Pesticide treatments were applied with an airblast sprayer calibrated to deliver 100 gpa.

Five replicates of undamaged fruit and leaves for the bioassays were collected 1, 3, 7, 14 and 21 DAT. The fruit and leaves were placed in paper bags and kept cool during transport to the laboratory. SWD used in the bioassay were from a laboratory colony originally collected from a cherry orchard in the fall of 2017. Flies were reared in 30-ml polystyrene vials with commercial *Drosophila* medium. The colony was reared in a controlled temperature room at 22 °C with a photoperiod of 16:8 L:D.

The bioassay arena consisted of a 32-oz plastic container lined with treated cherry leaves held in place with staples, and a 1-oz plastic portion cup was glued to the bottom of the arena. Five cherries were suspended from the lid of the container by inserting the stem through slits in the top and securing them with hot-melt glue (Fig. 2); allowed females access to all surfaces or the fruit for resting or oviposition. A 10-mm diameter hole was cut in the lid and covered with surgical tape for ventilation. Ten mated female flies were anesthetized with CO_2 and introduced into the arenas. After 16 h, the lid with the suspended cherries was removed and replaced with a standard lid (with honey agar and ventilation). At that time, a second 1-oz cup containing *Drosophila* medium was placed inside the glued cup to provide a food and moisture source in the absence of cherries. After the cherries were removed, the oviposition punctures were counted and recorded. After 48 h, mortality of females was evaluated, after which the females and the original bioassay container with leaves was discarded. The lids with fruit were incubated at 22 °C for 16 d, and the number of emerged adults was recorded at this time. Mortality, oviposition and emergence data were analyzed using a mixed-model ANOVA and the Tukey-Kramer mean separation test.

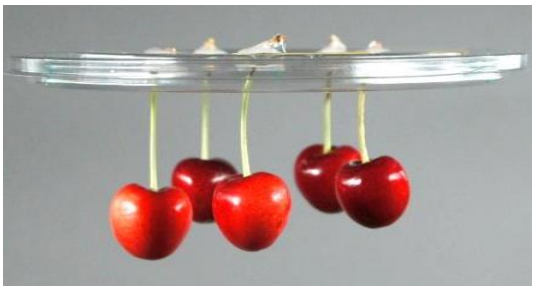

Fig. 8. *Sweetheart cherries suspended from lid of bioassay arena.*

Results - *Field-aged residue bioassay Trial 1.* Entrust caused 100% mortality through 14 DAT, and remained high at 21 DAT (Fig. 3). Fruit damage (ovipositions) were significantly lower than the check on 1, 3, and 14 DAT, and numerically lower on other dates. The total numbers of emerged flies (data not shown) was reduced commensurately with mortality and ovipositions, but the numbers of adults successfully emerging from the ovipositions was only occasionally different than the checks (3 and 21 DAT). The two rates of Cormoran behaved similarly, although the lower rate (21 fl oz) was not infrequently better than the higher rate (28 fl

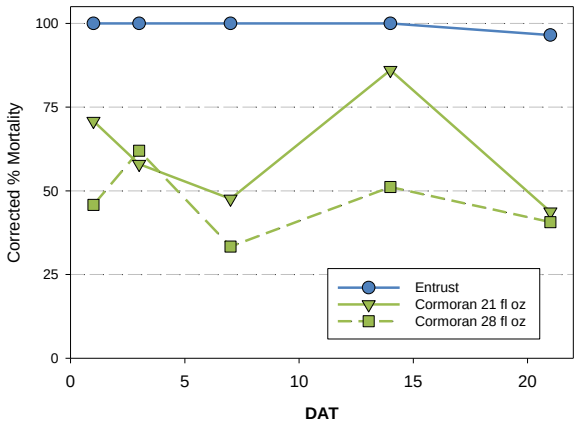

Fig. 9. *SWD mortality from 1 to 21 days, Trial 1.*

oz). Mortality was moderate on 1 DAT, and while significantly higher than the check, it was also significantly lower than Entrust. Mortality in the Cormoran treatments continued higher than the check on 3 and 14 DAT, generally staying between 40 and 90%. Surviving females were able to oviposit normally, and ovipositions resulted in adult development, indicating no effects on egg hatch or larval development.

Results - *Field-aged residue bioassay Trial 2.* Delegate and Entrust caused high levels of mortality (90-100%) through 21 DAT (Fig. 4). Exirel (cyantraniliprole) and Minecto Pro (a mixture of cyantraniliprole and abamectin) caused high levels of mortality initially (3 DAT), which decreased (50-70%) at 7 and 14 DAT, and increased (>80%) again at 21 DAT. V-10433 (a formulation of sabadilla) at 8 fl oz caused ≈70% mortality at 3 DAT, but was low thereafter. This initial high level appears doubtful give that the higher rate (22 fl oz) was low throughout the test. Azera did not cause any mortality of SWD at any point in time. Ovipositions (a measure of fruit damage) were highest throughout the test period in the check V-10433 and Azera treatments, and lower in the Delegate, Entrust, Exirel, and Minecto Pro treatments (Fig. 5).

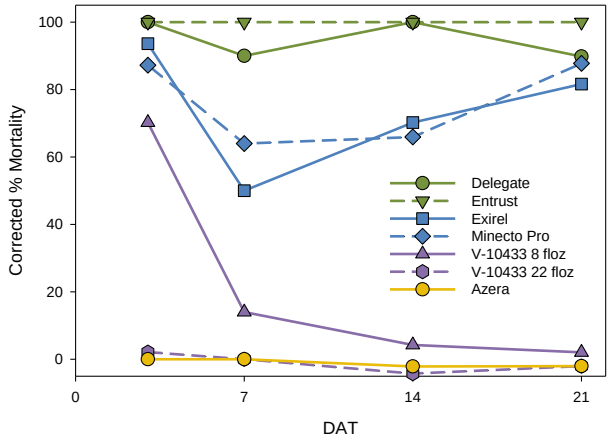

Fig. 4. *SWD mortality from 3 to 21 days, Trial 2.*

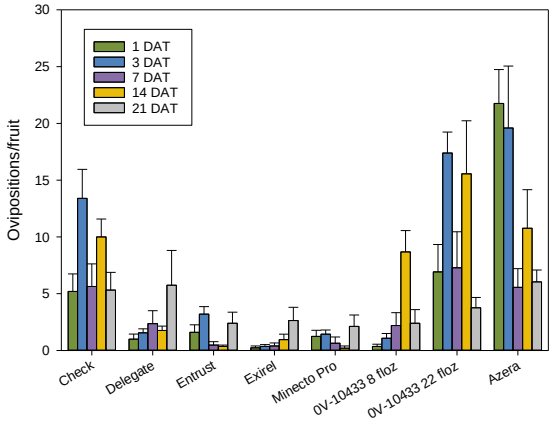

Fig. 5. *Ovipositions per fruit, 1 to 21 DAT, Trial 2*

Methods - *Laboratory bioassays.* Multiple experiments were conducted to determine if products either deterred female SWD from ovipositing, or killed eggs and larvae in fruit, thus preventing adult emergence. Five of these experiments tested the effects of two IGRs, Rimon and Dimilin, on SWD adults using different routes of exposure (topical, residual, ingestion). In these experiments, untreated leaves and fruit were collected from a research orchard, and treated with the two insecticides in the laboratory. The bioassay arenas were the same as described for the field-aged residue bioassays. For topical exposure, adults were anesthetized and transferred to a 14.7 ml plastic cup. Each group of SWD was sprayed with the specified rate of insecticide in a laboratory sprayer using 2 ml solution at 6.5 psi. Flies were transferred to prepared arenas with untreated leaves and fruit. For residual exposure, previously untreated cherry leaves were sprayed on a metal tray, and then stapled to arenas in groups of 3 leaves. Fruit suspended from arena lids were sprayed in a bucket sprayer with a laboratory mister, turning the lid ¼ turn after each spray in order to cover all fruit surfaces. Fruit and leaves were allowed to dry, and untreated adults transferred to the arena. For the ingestion treatments, the specified rates of the two insecticides were mixed in a 1:1 ratio with corn syrup. Twenty 25-µl droplets were applied to the leaves in the arena, and untreated adults transferred into it. Data collection and analysis of mortality, fruit damage, and adult emergence was the same as for the field-aged residue bioassays (above). In the first experiment, flies were 7 days old when treated; in the second experiment, they were 1 day old; and in the third experiment, they were 7 days old at the time of treatment, but a reduced rate of Rimon was used. In the fourth experiment, Rimon or Dimilin was incorporated into a semi-transparent agar medium where the females oviposited, allowing the

eggs and larvae to be dissected out to determine stage of mortality. In the final experiment in this series, the two compounds were sprayed on the surface of the agar medium (versus incorporation).

Results - *Rimon/Dimilin results.* *Bioassay #1***.** Adult female mortality was low regardless of route of exposure, which is typical for IGRs. Oviposition level was quite variable, but lowest in the Rimon/Topical treatment. The most interesting result is the complete shut-down of adult emergence in the Dimilin/Residual treatment, despite the high levels of oviposition and only 16 hours of exposure. The exact mechanism for this shutdown (activity on eggs, larvae, or pupae) cannot be determined from this bioassay, but it represents the potential for an additional tool to suppress SWD populations and prevent fruit cullage. *Bioassay #2.* Both Rimon and Dimilin in the residual and ingestion treatments suppressed oviposition and completely shut down adult emergence when the flies were exposed at a young age. This experiment confirms the results of the first bioassay, which used the same treatments and methods except for the age of the flies at time of exposure (Fig. 6). The impact appears to be greater on flies exposed immediately after eclosion than those that are 7 days old. *Bioassay #3*. Fly mortality, ovipositions/fruit and emerged adults were not significantly different than the check in this test. The Dimilin/residual treatment had the lowest adult emergence, but due to variability, a statistical difference was not detected. This may be an indication that while the higher (Dimilin 16 fl oz/acre, Rimon 40 fl oz/acre) rates were effective, the lower rate of Rimon (24 fl oz) was not; in theory, Dimilin at the same rate should have performed similarly to the first bioassay. The results of the diet incorporation study were striking; egg hatch was greatly suppressed by both materials, and in the Rimon treatment, all of the resulting larvae died. The trend was similar where only the surface of the medium was sprayed, but less pronounced. Egg hatch was reduced by both materials, but only to 60-88%; however, larval mortality was high for both materials (80-90), with a net result of very few surviving larvae. This series of tests indicates that these two IGR may greatly reduce the ability of SWD to successfully reproduce in sweet cherry.

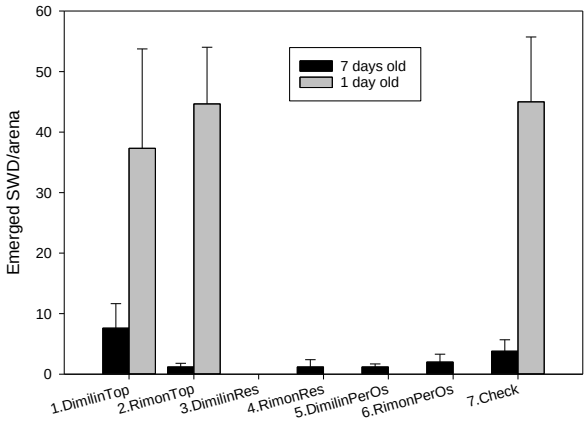

Fig. 6. *1 and 7 day old SWD emerging from fruit treated with Rimon or Dimilin (3 routes of exposure).*

Methods - *Bioassays of organic insecticides.* Additional bioassays were performed to determine if other pesticides (especially those registered for organic production) were effective against any stage of SWD. Contact bioassays were performed on 7-10 day old females in plastic Petri dishes, and sprayed with 2 ml of the solution at 15 psi. Residual control and oviposition deterrence bioassays were performed using the same bioassay arena as in previous experiments with untreated leaves, but with fruit sprayed with the candidate insecticides using a laboratory sprayer. The sprayed fruit were suspended from the lid of the bioassay arena, and 10 female SWD introduced. Mortality and ovipositions were assessed after 24 h, and adult emergence after 15 days. The ability of materials to kill eggs inside the fruit was tested by allowing females to oviposit and then spraying the fruit. A series of bioassays was performed with erythritol, a sugar alcohol known to be toxic to SWD. The first was a contact+residue bioassay (see above), where a food source (drosophila medium) was also contaminated; the second was incorporation in the larval diet (drosophila medium); the third type of bioassay incorporated erythritol into bait droplets, and measure adult mortality.

Results - *Bioassays of organic insecticides.* Cinnerate at all rates tested (25-40 fl oz/100 gal) does not appear to have any contact toxicity to SWD, but the contact-only bioassay is a severe test. When tested for oviposition deterrency, the 50 fl oz rate of Cinnerate had fewer ovipositions than the check,

but only the standard (1.5% petroleum oil) was significantly lower. Similarly, Cinnerate did not kill eggs after they had been oviposited, but 1.5% petroleum oil reduced the number of adults emerging by over one-half (Fig. 7). In further tests of oviposition deterrence of organic materials, only Ecotrol (0.5%) and methyl benzoate (1%) reduced the numbers of ovipositions in relation to the check; petroleum oils had generally lower numbers of ovipositions, but differences were not statistically significant. Lavender oil residues caused high levels of mortality at 1-5% concentration; the 0.5% level caused a moderate amount of mortality. Conversely, topical applications of sabadilla caused little contact mortality to adult female SWD.

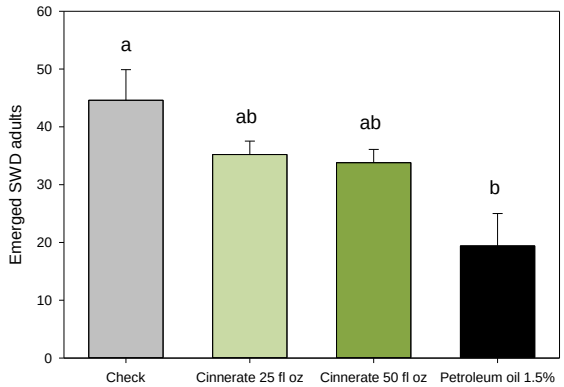

Fig. 7. *:Larvicidal activity of Cinnerate and oil for SWD.*

Erythritol sprayed on the adults (including exposure to residues in the arena and contaminated food) did not cause any mortality. Conversely, when erythritol was incorporated into the larval diet, there were very little survival at the two higher rates of erythritol (Fig. 8. Erythritol incorporated into bait droplets (Fig. 9) caused moderate amount of mortality at 1 DAT, but high levels at 4 DAT; however, there was correspondingly high levels of damage in the check, likely due to starvation. The sucrose standard treatments had the lowest levels of mortality (indicating an adequate nutrient source), with the erythritol+molasses treatments intermediate.

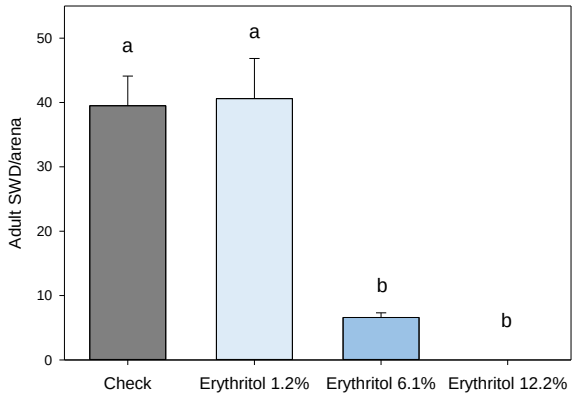

Fig. 8. *Survival of SWD larvae in drosophila medium with various concentrations of erythritol added.*

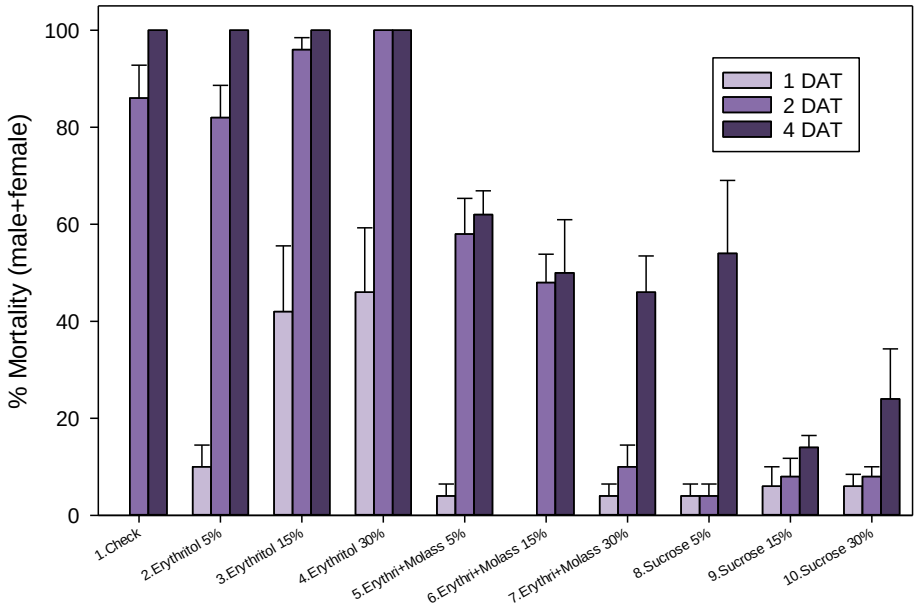

Fig. 9. *Mortality of SWD adults exposed to droplets of erythritol or sucrose.*

Methods - *Attracticides.* One category of insecticidal control, attracticides, was pursued in hopes of reducing the non-target impacts of full canopy sprays. A candidate material made by Scentry consisted of an attractant only, to which different insecticides might be added for resistance management schemes. Initial tests were done in laboratory assays, examining fly mortality when exposed to bait droplets in 1-liter arenas. Further tests were conducted in a field-aged residue bioassay where bait+insecticide droplets applied to leaves in a research cherry orchard, then collected at intervals to determine attractiveness and lethality. In all cases, the toxicant added to the Scentry attractant was spinosad (Entrust), and the comparison material used was GF-120 (which also has spinosad as the toxicant). In addition, control with attracticides was tested in whole-tree field cages, where known numbers of SWD adults could be added to control the amount of insect pressure. This trial compared an airblast application of Entrust to GF-120 bait spray.

Results – *Attracticides*. *Laboratory bioassays.* Neither of the attracticides caused a significant amount of mortality after 6 h, but levels increased at 24 and 48 h. After 24 h, the GF-120 treatment had significantly more mortality than the Scentry attracticide treatment, but they were not different at 48 h (Fig. 10). When field-aged residues were tested, the Scentry attracticide had lower mortality initially than GF-120, and decreased rapidly thereafter (Fig. 11). Overall, the goal of developing an attracticide that is more effective than GF-120 has not been realized. *Field cage trials*. Damage was low overall, and while not significantly different from the check, fruit damage was numerically lower in both the Entrust and GF-120 treatments (Fig. 12).

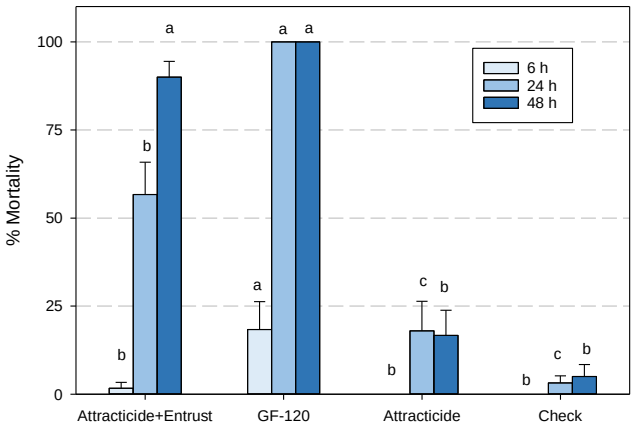

Fig. 10. *Percent mortality of female SWD following exposure to attracticides.*

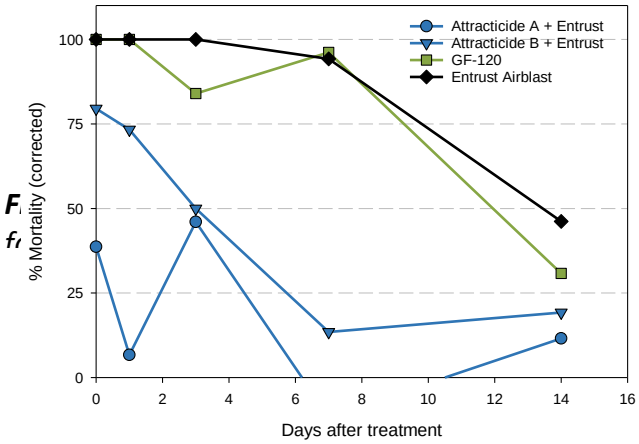

Fig. 11. *SWD mortality from 1 to 14 days, following exposure to attracticide.*

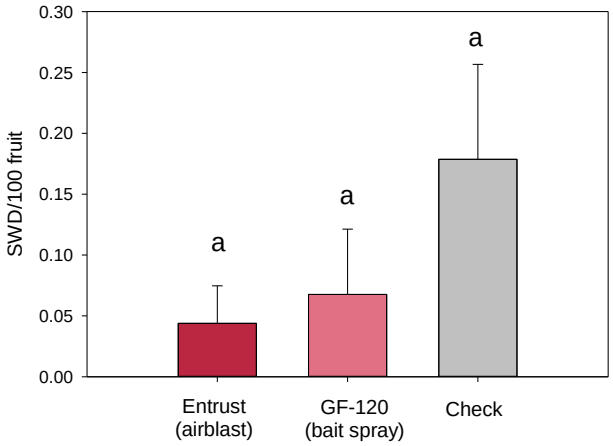

Fig. 12. *Fruit infestation by SWD following exposure to Entrust or GF-120.*

Monitoring. The efficacy of traps and lures were tested over a 4-year period, and provide insight into the most sensitive monitoring tool for SWD. Early trap and bait systems were adequate to detect range expansion, but monitoring for action thresholds will require a trap that is sensitive and (if possible) user friendly. The Scentry lure has consistently provided the highest trap captures of SWD, whether during low density periods (cherry maturation period) or higher densities (fall post-harvest period). This lure also has the highest levels of by-catch, which is problematic with drosophilids, which are small and similar in appearance. Only the spots on the wings of the males distinguishes SWD from non-pest species, but the females (the damaging stage) are difficult to differentiate. The liquid-based traps overall provide the highest level of catch, but the poorest level of user-friendliness. Specifically, the in-house fabricated PBJ trap (Fig. 13) consistently captures the most SWD; the Scentry trap (of the commercial traps) has also performed well. Sticky traps offer an easy-to-use alternative to the liquid traps, but their primary limitation is that while males are relatively easy to identify, the females are more problematic in field counts. Of the sticky traps, the yellow card has a slight edge over other colors. An additional unresolved problem is the preponderance of females in the population in the early season, when spray decisions for cherries are usually made. Whether male capture on these traps is sufficiently sensitive to be used as an action threshold remains to be determined.

Fig. 13. *Clockwise from left: PBJ trap, yellow sticky card, and male SWD on a sticky trap with wing spots clearly visible.*

Regional trends. SWD traps were deployed in orchards in North Central Washington from 2012 to 2019 in the same locations each year to track year-to-year variation in trap capture (Fig. 14). Starting in 2013, we coordinated sample collection and rearing with the WSDA cherry packinghouse inspection program to rear larval *Drosophila* species and determine if they were SWD (Fig. 15). The addition of PCR diagnostic tools in 2015 and 2016 allowed us to identify most of the specimens that died during rearing, and failed to produce an identifiable adult. The packinghouse sample information provides valuable insights into the variation in insect pressure from year to year, and the relevance of cherry season SWD trapping to regional reports of damage. While there many factors (including weather interfering with insecticide applications) that influence the efficacy of control

measures, it is clear that high trap numbers in the summer of 2015 corresponded with high numbers of SWD finds in the packinghouse. The packinghouse finds were similar from 2016 to 2018, with an uptick in 2019; this was the second worst year for SWD infestations since 2015. In general, high pressure can be predicted as early as May in order to intensify control measures; however, the lure shortage in 2019 meant no early season data were available to predict the higher risk of damage in 2019.

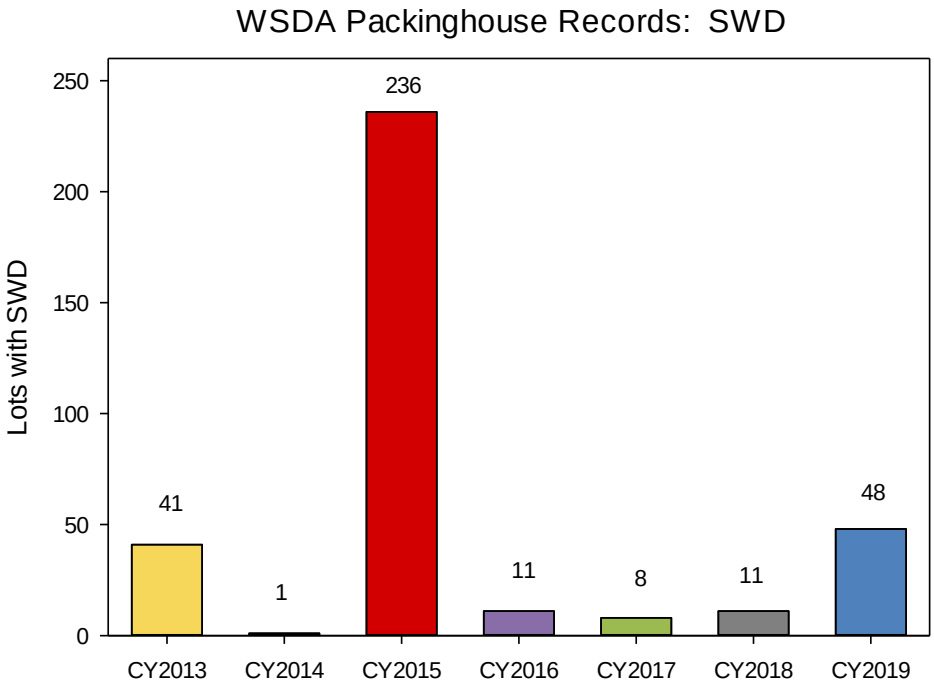

Fig. 14. WSDA larval SWD detections in packinghouses, 2013-2019.

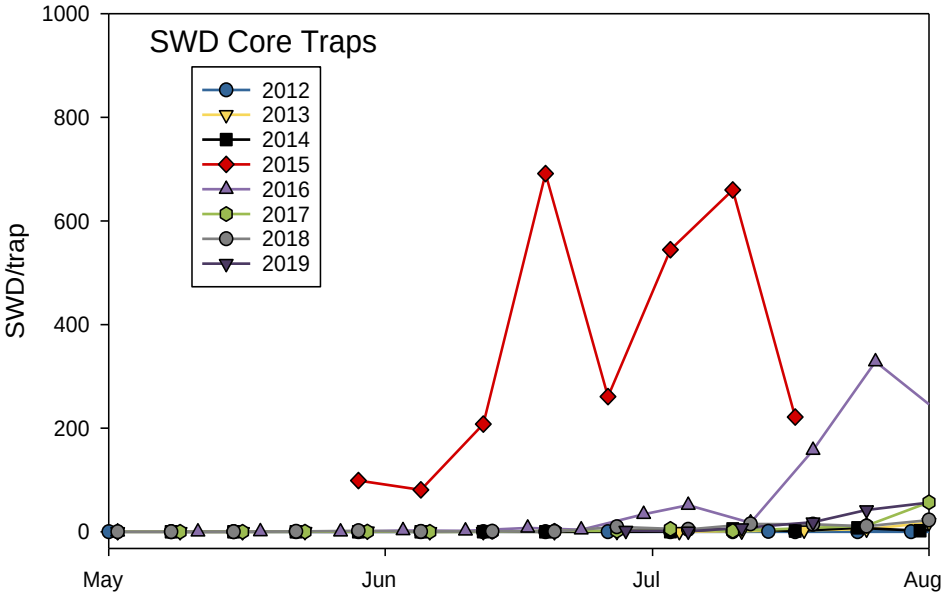

Fig. 15. SWD trap captures during cherry maturation and harvest, 2012-2019.

Executive Summary

Project Title: Integrated pest management of spotted wing drosophila in sweet cherry

Keywords: spotted wing drosophila, *Drosophila suzukii*, attracticide, oviposition deterrent

The first detection of spotted wing drosophila in eastern Washington caused a sudden shift in the insect management programs of sweet cherry growers in the region. While populations and damage levels have varied from year to year, this pest continues to be a challenge to cherry growers. Early research focused on assessment of chemical control options, and the cherry industry has picked 2-3 materials that give consistent results. Unfortunately, several are in the same chemical class, spinosyns, and warning were issued about the possibility of resistance. A previous project set baselines for some of the primary active ingredients, and can be used to track incipient resistance in the future. In the meantime, new techniques and approaches are needed to address the single-tactic approach to control.

On the research side, a continuing challenge is the unpredictability of this pest's appearance in prospective plots, and the reluctance of producers to risk part of a valuable cherry crop. WSU's two research orchards have had a testable population in only one year since 2010, viz., the high-pressure year of 2015. While laboratory or field-lab residue tests have help fill in the details of the characteristics of the candidate insecticides, field-level testing is necessary in order to validate these findings.

This project focused on alternatives to the standard adulticidal insecticides. The impetus for this arose from the high level of fruit infestation in 2015: if eggs or larvae are already present, how can I kill them? While less desirable than preventing infestation, adverse weather conditions or unusually high overwintering survival may result in some level of infestation. Many of the same materials that are used as adulticides also kill larvae developing the fruit. Laboratory tests have demonstrated there are two IGRs (Rimon and Dimilin) which cause little adult mortality, but cause a high level of egg and larval mortality. These may provide rotational materials for the currently used adulticides.

Another approach that was explore was the use oviposition deterrents. In theory, because the compound does not kill the female spotted wing drosophila, there should be less selection of physiological resistance. Many of the compounds screened are essential oil products (lavender, rosemary, cinnamon, garlic) and thus suitable for use in organic production. Erythritol shows promise as a toxicant for use in a bait spray, and should be tested further. The need for rotational materials in organic sweet cherries is acute, give the dominance of spinosad use in these orchards. The need is even more urgent because the same active ingredient is used in both organic and conventional orchards, or effectively industry-wide. Resistance to spinosad has already been documented from California, and may spread to other growing regions.

Finally, we have explored the possibility of a replacement for GF-120 that is engineered especially for spotted wing drosophila versus western cherry fruit fly, the original target. To date the candidate compounds have been less attractive than GF-120 in laboratory test, which only provided about 50% suppression in the field. Further research in collaboration with private companies may yield progress in the future.

FINAL REPORT **YEAR**: 2

Project Title: Native hosts of the X-disease phytoplasma

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Cooperators: Washington cherry growers

Total Project Request: $75,999 **Year 1:** $38,107 **Year 2:** $38,336

Other funding sources: None

Budget
Organization Name: Washington State University **Contract Administrator:** Katy Roberts
Telephone: 509-335-2885 **Email address:** arcgrants@wsu.edu

Item	2018	2019
Salaries[1]	$12,106	$20,145
Benefits	$4,152	$6,910
Wages[2]	$9,840	$0
Benefits[3]	$932	0
Equipment	0	0
Supplies[4]	$9,577	$10,281
Travel[5]	$1,000	$1,000
Miscellaneous	0	0
Plot Fees	0	0
Total	$38,107	$38,336

Footnotes:
1. 0.25 FTE Salary for Dr. Wright the first year, and 0.4 FTE for the second.
2. Wages for a field worker for sample collection.
3. Benefits calculated at standard WSU rates.
4. Supplies include: Field sampling supplies and laboratory consumables and reagents
5. Travel covers mileage to reach field sampling sites.

OBJECTIVES

1. *Survey plants both within and in the vicinity of cherry orchards for X-disease phytoplasma.*
Plants were collected around central and eastern Washington, both within cherry orchards afflicted with X-disease phytoplasma and from locations distant from orchards. Plants were photographed for later identification. Tissue was harvested for DNA extractions and screened for the presence of phytoplasma using a generic phytoplasma qPCR assay. Positive plants were further screened for the presence of X-disease using a qPCR assay. A total of 923 plants, including trees, shrubs, broadleaf weeds, and grasses, were tested for the presence of X-disease. Only 10 tested positive for phytoplasmas and of those 4 were positive for X-disease.

2. *Identify potential reservoirs and sources of spread for X-disease phytoplasma in Washington.*
This objective was initially proposed to examine leafhopper spread of X-disease from herbaceous weeds. However, the low incidence of X-disease found in weeds during the survey indicates that weeds alone cannot explain the incidence and severity of X-disease in cherry orchards. Given that weeds are unlikely to be the main source of this pathogen, studying leafhopper spread from weeds would not be informative. Therefore, this objective was refocused on three alternative reservoirs: 1) Cherry planting stock, for symptoms are only visible on fruit and nursery stock is rarely, if ever, tested for the presence of this phytoplasma, 2) Other *Prunus* species, for we have recently detected X-disease in peach and nectarine orchards in Washington, and 3) Pome fruit orchards in the vicinity of cherry orchards, for X-disease has been reported to infect apples in other states.

SIGNIFICANT FINDINGS

1. Less than two percent of plants tested for the presence of phytoplasma were positive and only a subset of those contained X-disease phytoplasma, suggesting that non-crop plants in and around orchards may not be the source of X-disease phytoplasma.

2. Samples from three large nurseries were screened for the presence of X-disease phytoplasma. From the first nursery, one of the 66 samples screened was positive for X-disease. In the second, one of 107 samples was positive. In the third, none of the 25 samples were positive. The presence of the pathogen in two of the three nurseries demonstrates that X-disease is present in nurseries at low numbers and has the potential to be amplified and spread.

3. X-disease phytoplasma was detected in peaches and nectarines in both Wapato and Pasco. X-disease was also detected in plums. The phytoplasma was present in these trees in very high numbers indicating that they may serve as a reservoir for spread of the pathogen. Apricots have not yet tested positive for this specific phytoplasma.

4. While X-disease phytoplasma has been reported to infect apple in both Pennsylvania and the Czech Republic, survey results in Washington apple orchards were negative.

RESULTS AND DISCUSSION

This project was developed to identify reservoirs for the X-disease phytoplasma as this pathogen has severely increased in incidence in eastern and central Washington. The intention was that identifying the environmental reservoirs would allow for another control strategy in that the reservoirs, once known, could be removed and thereby reduce spread of the disease. In the first year of the project a survey was conducted of weeds and non-crop perennials in and around orchards to identify plants that may serve as a reservoir for the X-disease phytoplasma. Plants were collected

March through September and multiple individuals for several species were sampled, including plants from the sagebrush, non-crop trees and shrubs, and annual weeds (Tables 1-3). DNA was extracted from each plant and screened for the presence of phytoplasma using a generic phytoplasma qPCR assay. Plants that were positive were subsequently screened by the more specific X-disease phytoplasma qPCR assay.

Table 1. Abbreviated list of annual weeds screened for phytoplasma.

Plant	Number Sampled	Locations	Phytoplasma Positive
Tumble mustard	45	17	2
Common mallow	24	11	0
Kochia	22	11	0
Puncturevine	22	9	1
Prickly lettuce	21	12	0
Redroot pigweed	20	9	0
Common lambsquarters	19	6	0
Perrenial pepperweed	17	9	0
Western yarrow	17	9	0
Common mullein	16	9	2
Field bindweed	16	10	0
Russian thistle	16	10	1
Scurf-pea	15	8	0
Canada thistle	13	7	0
Dandelion	13	7	0
Blue mustard	12	5	0
Flixweed	12	4	2
Salsify	12	6	0
Showy milkweed	12	7	0
White clover	12	5	0
Fiddleneck	11	11	0
Poison hemlock	11	5	0
Prostrate knotweed	11	6	0
Scotch thistle	11	6	0
Bushy wallflower	9	6	0
Common purselane	9	4	1
Shepherd's purse	4	2	1

Table 2. List of sagebrush plants screened for phytoplasma.

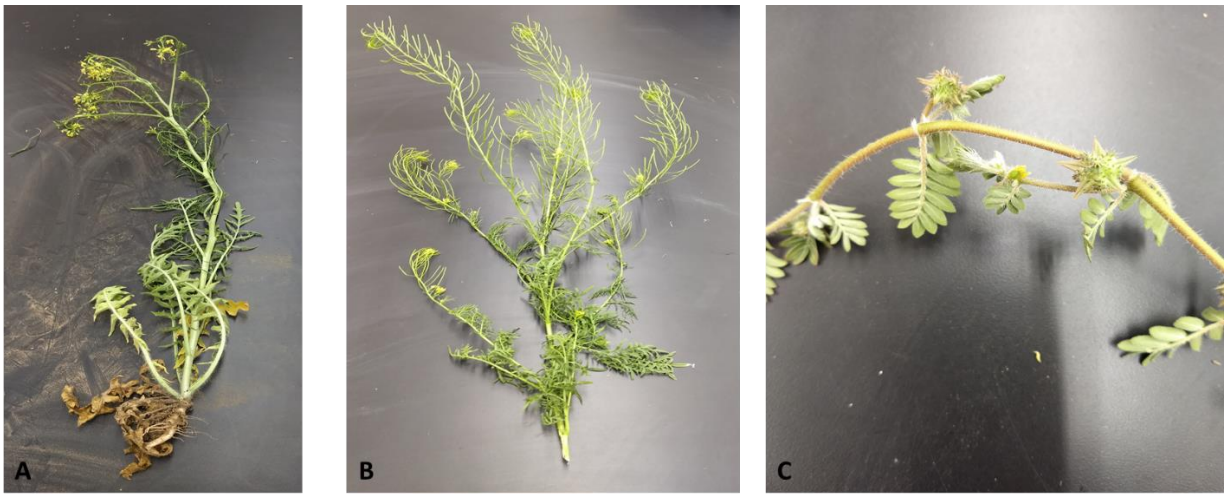
Plant	Number Sampled	Locations	Phytoplasma Positive
Sand sagebrush	12	7	0
Big sagebrush	6	6	0
Hopsage	3	3	0
Greasewood	2	1	0
Antelope bitterbrush	1	1	0
Douglas rabbitbrush	1	1	0
Gray rabbitbrush	1	1	0
Green rabbitbrush	1	1	0

Table 3. Abbreviated list of perennial species screened for phytoplasma.

Plant	Number Sampled	Locations	Phytoplasma Positive
Russian olive	11	7	0
Narrowleaf willow	10	7	0
English walnut	8	3	0
Wood's rose	8	6	0
Eastern cottonwood	7	6	0
Himalayan blackberry	6	4	0
Siberian pea tree	6	2	0
Elderberry	5	4	0
Apple	3	1	0
Netleaf hackberry	3	1	0
unknown perrenial	3	3	0
White alder	3	3	0
Golden currant	2	2	0
Oregon ash	2	1	0
Red birch	2	1	0
Nootka Rose	2	2	0

A total of 923 plants representing at least 173 species were screened for phytoplasmas (Tables 1, 2, and 3). Only ten plants were positive for phytoplasma and these were all annuals. The phytoplasma positive plants included flixweed, puncturevine, mullein, tumble mustard, shepherd's purse, Russian thistle, and common purselane. These ten were screened further using an X-disease specific qPCR assay. Only four, one flixweed, one puncturevine, and two tumble mustards, were positive for X-disease (Figure 1). This is less than 0.5% of the plants screened. The four positive plants had only low levels of X-disease present (Figure 1D). Of the weeds that were positive, these were among the most sampled species. Forty-five tumble mustards from 17 locations, 22 puncture

vines from 9 locations, and 12 flixweeds from 4 locations were tested. This would suggest a low incidence of phytoplasma infection among these species. Furthermore, it is not known if the insect vector(s) transmitting X-disease feed on these plants and if, particularly at such low concentrations, they are likely to acquire the pathogen. Also of note, no plants native to the sagebrush were positive for the pathogen. If X-disease is present in the sagebrush, it is likely to be at such low numbers that it is not a significant source of the pathogen. Collectively, these data indicate that the main source of X-disease lies elsewhere.



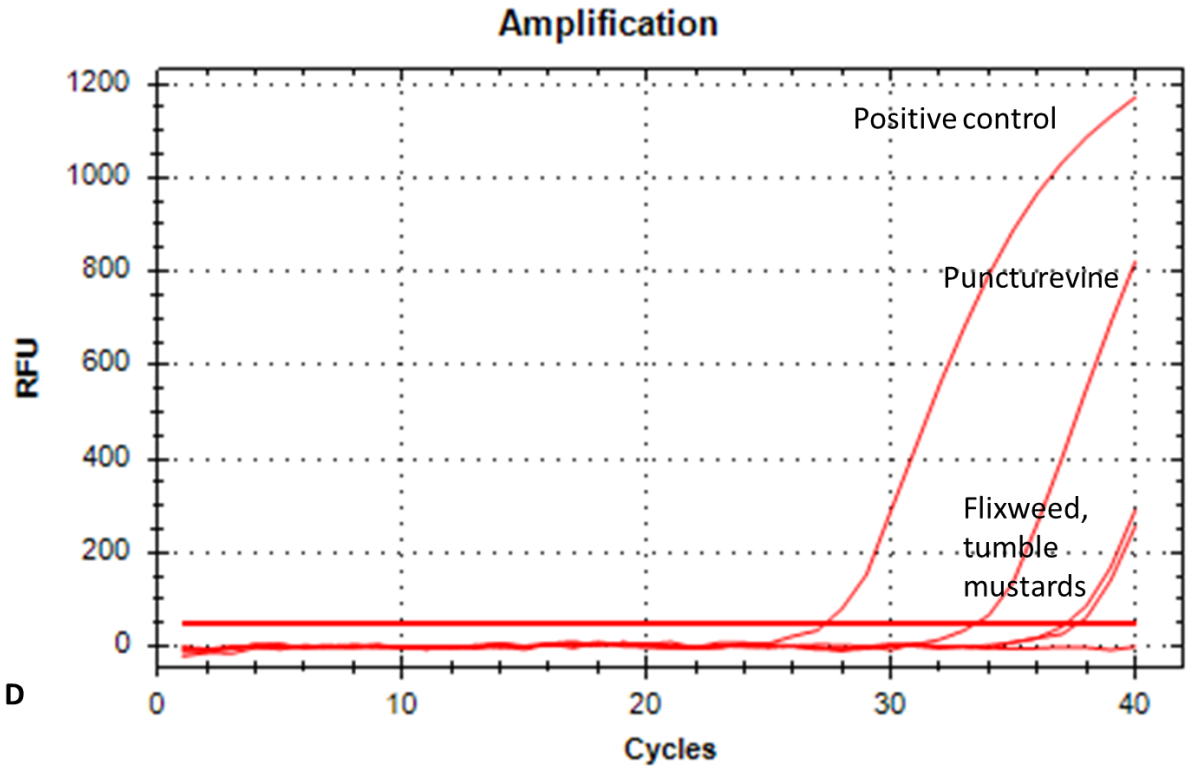


Figure 1. Representatives of plant species that tested positive for X-disease phytoplasma: A) tumble mustard, B) flixweed, and C) puncturevine. D) Quantitative PCR results for the X-disease phytoplasma assay. The red lines show amplification for, from left to right, the positive control, puncturevine, and the flixweed and tumble mustards.

While the survey does not rule out non-crop plants in and near orchards as a potential source of X-disease phytoplasma, it does indicate that the greatest reservoir for this pathogen may be located elsewhere. For the second year of our project, we chose to rewrite objective two to look for other sources of this pathogen. That included surveying nurseries and examining other crops, specifically other stone fruit and pome fruit.

Samples were collected from three large nurseries. In the first 66 samples were collected. In the second, 107 samples were collected. In a third, 25 samples were screened. For the first two, one sample each was positive for the X-disease phytoplasma while in the third, none were positive. While this may seem to be a small number of positives, given the number of trees produced by nurseries, this is a concern. If one of those positives served as a source of budwood, that would contribute to the spread of the disease.

Given that other stone fruit are susceptible to X-disease, we suspected that they may be serving as a reservoir to spread this disease. We found that peaches, nectarines, and plums in Washington are infected with this phytoplasma (Figure 2). The phytoplasma is at far higher concentrations in these trees than what was observed in the four weeds that were positive in the first year (Figures 1 and 3). Given the amount of pathogen present in these stone fruit, it is unlikely that they are a dead end host for this pathogen. Even if the insect vector has a poor acquisition rate from these reservoirs, the number of insects out there paired with the concentration of pathogen in the tree and how widespread it is throughout the Columbia River and Yakima Valley region make peaches, plums, and nectarines likely contributors to the spread of this pathogen.

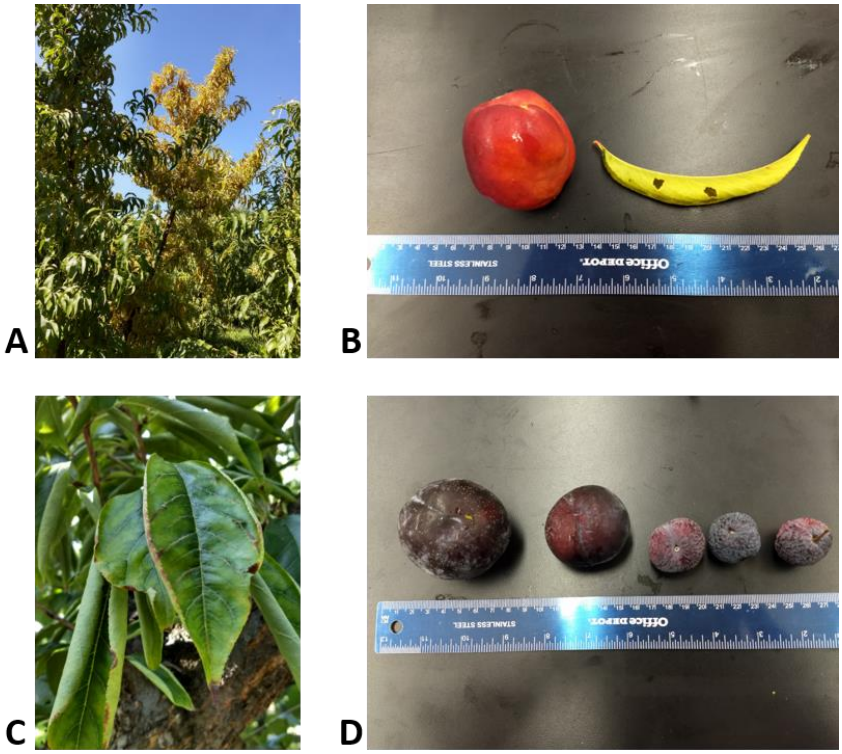


Figure 2. Trees infected with both X-disease and pear decline. A) Nectarine tree exhibiting yellowing symptoms. B) Misshapen fruit and a leaf showing shot-holing, characteristic of X-disease infection in nectarines. C) Plum tree infected with both X-disease and pear decline. D) Misshapen fruit from infected plum tree.

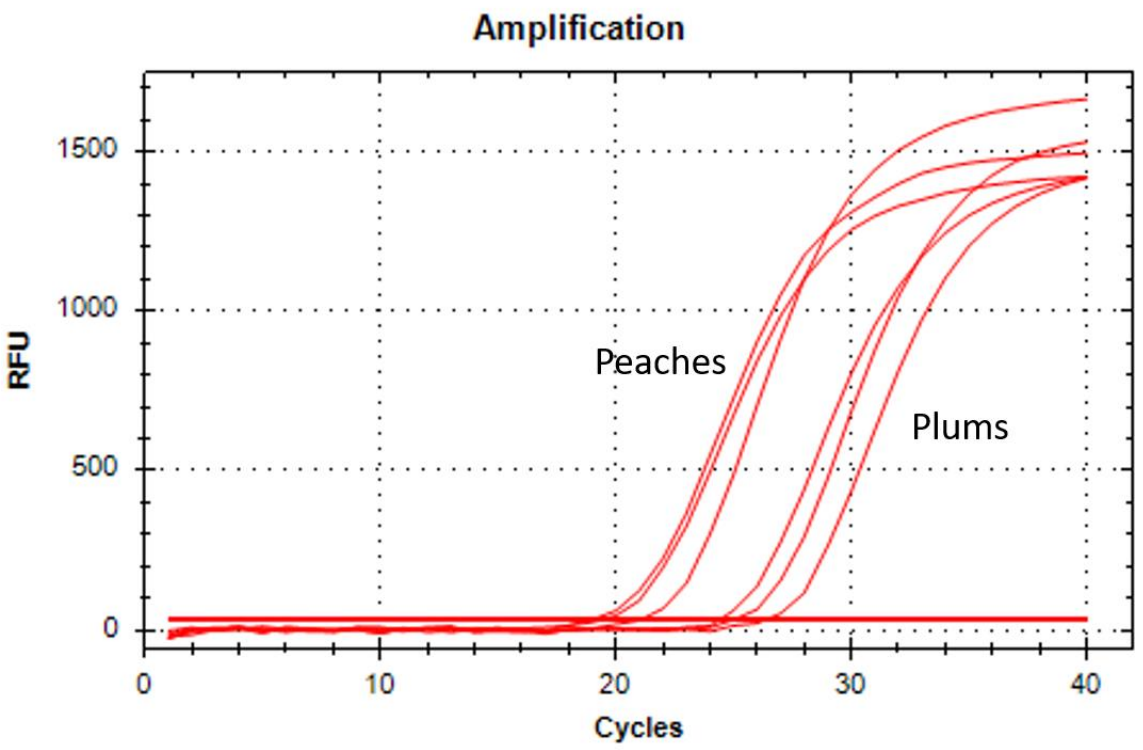


Figure 3. Detection of X-disease phytoplasma by qPCR.

Lastly, pome fruit were investigated. In tree fruit production, apples are often grown next to or near cherries. While it has been demonstrated that X-disease phytoplasma can infect apples in both Pennsylvania and the Czech Republic, we did not detected it in a survey of 69 apples samples in Washington State. This is encouraging as it suggests that apples are an unlikely reservoir for this pathogen.

In screening nearly a thousand non-crop plants it was found that less than 0.5% were positive for X-disease phytoplasmas. While this does not rule out weeds and other non-crop plants as a reservoir, it makes it very unlikely that these plants are the primary reservoir. Infection rates of X-disease among cherries are extremely high meaning that within orchard pathogen load is probably sufficient for tree to tree spread either by an insect vector or root grafting. As nurseries are often located near orchards, it is not surprising to see even a low incidence of X-disease presence in nurseries through random insect 'strikes' transmitting the pathogen. Furthermore, peaches, nectarines, and plums, which are also experiencing high rates of X-disease infection, are often grown next to or near cherries, raising the possibility that there is movement of the pathogen between these crops and cherries.

Moving forward, we recommend that nurseries be pro-active with regards to X-disease phytoplasma. Any mother trees that produce fruit should be inspected for little cherry disease symptoms. At harvest and through August would be ideal times for testing suspect trees or random testing to check for X-disease presence. Growers should also be aware that this pathogen infects other stone fruit and should be on the lookout for symptoms in peach, nectarine, and plum blocks. Most importantly, any infected trees should be promptly removed to reduce spread of this pathogen. In the long term, there is a need for testing or inspection of all Prunus material moving in to the state, for the X-disease phytoplasma is found throughout the country, and preventing its re-introduction will be critical to management.

EXECUTIVE SUMMARY

Project Title: Native hosts of the X-disease phytoplasma

Keywords: Little cherry disease, X-disease phytoplasma, nursery, stone fruit

Objectives

1. Survey plants both within and in the vicinity of cherry orchards for X-disease phytoplasma.
2. Identify potential reservoirs and sources of spread for X-disease phytoplasma in Washington.

Significant Outcomes

1. In a survey of weeds and non-crop perennials in and around orchards, less than two percent of plants tested for the presence of phytoplasma were positive and only a subset of those contained X-disease phytoplasma, suggesting that non-crop plants in and around orchards may not be the source of X-disease phytoplasma.

2. Samples from three large nurseries were screened for the presence of X-disease phytoplasma. From the first nursery, one of the 66 samples screened was positive for X-disease. In the second, one of 107 samples was positive. From the third, none of the 25 samples were positive. These data demonstrate that X-disease is present in nurseries at low numbers and has the potential to spread from nurseries.

3. X-disease phytoplasma was detected in peaches and nectarines in both Wapato and Pasco. X-disease was also detected in plums. In all three crops, the phytoplasma was present in very high numbers, indicating that these trees may serve as a reservoir for spread of the pathogen.

4. While X-disease phytoplasma has been reported to infect apple in both Pennsylvania and the Czech Republic, survey results in Washington apple orchards were negative.

Future Directions

Weeds in and around orchards are unlikely to serve as a major reservoir for X-disease. X-disease positive plants were not detected in the sagebrush – if any sagebrush species can harbor this pathogen, it is likely not at an incidence high enough to significantly impact spread of the pathogen. Instead, our work has shown that X-disease is most commonly found in stone fruit, specifically cherries, peaches, nectarines, and plums and is present at a high concentration in these trees. Infected stone fruit trees are likely serving as a reservoir, allowing for transmission of the pathogen to healthy trees via an insect vector or root grafting. Nurseries are not immune to X-disease. A short survey of three nurseries revealed the presence of X-disease at low numbers in two of the three nurseries. Given the propagation potential of a single nursery tree, nurseries should be on their guard against X-disease. Random testing and monitoring for symptom development in trees old enough to produce fruit is advised. Growers should watch for symptoms in cherries and other stone fruit and remove infected trees. As insect control is difficult to achieve, removal of reservoirs such as infected trees is essential to reducing the spread of the disease.

FINAL PROJECT REPORT

Project Title: Mid-Columbia survey for sweet cherry viruses and vectors

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Cooperators: Lauri Lutes (OSU), Steve Castagnoli (OSU), Ashley Thompson (OSU), Drew Hubbard (GS Long), Jeff Heater (Columbia Gorge Fruit Growers Association), Inga Zasada (USDA-ARS)

Other funding sources

Agency Name: OSU Extension Service
Amount Awarded: $4,000
Notes: Annual discretionary statewide travel funds used to get to sampling sites.

Agency Name: USDA-ARS-HCRL
Amount Awarded: $4,000
Notes: Use of consumable supplies budget leveraged from USDA virus project.

Total Project Funding: $109,306

WTFRC Funding: None **OSCC Funding:** $109,306

Budget History

Item	**(2017-2018)**	**(2018-2019)**
Salaries (GRA Stipend**)**	23,776	24,242
Benefits (Health Insurance**)**	6,720	6,855
Wages		
Benefits (OPE**)**	100	102
Equipment		
Supplies		
Travel		
Miscellaneous (OSU fees**)**	18,520	21,000
Plot Fees		
Total	$49,106	$52,200

OBJECTIVES

Objective 1: Determine areas with sweet cherry trees expressing symptoms associated with Little Cherry Disease (caused by the pathogens *Little cherry viruses 1 & 2* and/or X-Disease phytoplasma) in Oregon's Mid-Columbia region.

Objective 2: Conduct regional Mid-Columbia survey for *Cherry leaf roll virus* and the *Cherry leaf roll virus* complex with *Prune dwarf virus* and *Prunus necrotic ringspot virus* on sweet cherry.

Objective 3: Survey Mid-Columbia sweet cherry orchards for nematode-transmitted viruses (such as *Cherry rasp leaf virus, Tobacco ringspot virus,* and *Tomato ringspot virus*) and their vectors.

Objective 4: Investigate spread and diversity of *Tomato ringspot virus* isolates from cherry in known-infected areas.

SIGNIFICANT FINDINGS

- X-Disease found associated with little cherry symptoms in The Dalles, Dallesport (WA), and Mosier, but not Hood River valley.
- Several known *Cherry leaf roll virus*-infected trees removed in Oregon.
- Yet another orchard in The Dalles area was found with *Tomato ringspot virus* (ToRSV) infected trees.
- Nematode virus vector, *Xiphinema americanum*, was found in two commercial orchards in association with *Tomato ringspot virus* (ToRSV) infected trees. Visual symptoms were found reliably associated with ToRSV infected trees but a low percentage of samples without obvious symptoms also tested positive for ToRSV.

RESULTS & DISCUSSION

Objective 1: Determine areas with sweet cherry trees expressing symptoms associated with Little Cherry Disease (caused by the pathogens *Little cherry viruses 1 & 2* and/or X-Disease phytoplasma) in Oregon's Mid-Columbia region.

In 2018 and 2019, 55 symptomatic and 6 asymptomatic sweet cherry leaf samples were collected for diagnostic testing associated with Little Cherry Disease and X-Disease with the help of local cooperators. Four symptomatic samples were collected from the Willamette Valley, while the rest were from the Mid-Columbia region. All samples from the Willamette Valley tested negative for the X-Disease phytoplasma. All but 3 of the other 52 symptomatic samples expressing little, unripened fruit tested positive for the X-Disease phytoplasma, *Candidatus* phytoplasma pruni, using a general phytoplasma real-time PCR assay (Table 1). One native *Prunus* sp. and six asymptomatic samples tested negative for the X-Disease phytoplasma. All samples tested negative for little cherry virus 2.

Table 1: 2018-2019 Little Cherry/X-Disease Survey Results

			Number of positive samples	
Region	**Year**	**# symptomatic /total**	**LChV2 qRT-PCR**	**Phytoplasma qPCR**
Hood River Valley, OR	2018	0/1	0	0
	2019	1/1	0	0
Mosier, OR	2018	5/5	0	5
	2019	2/2	0	2
The Dalles, OR	2018	20/24	0	20
3 Mile		11/11	0	11
15 Mile		0/1	0	0
Dufur		0/1	0	0
Mill Creek		9/12	0	9
The Dalles, OR	2019	23/23	0	23
3 Mile		10/10	0	10
15 Mile		0/0	0	0
Dry Hollow		6/6	0	6
Dufur		1/1	0	1
Mill Creek		4/4	0	4
Unknown		2/2	0	0
Dallesport, WA	2018	2/2	0	2
Willamette Valley, OR	2019	4/4	0	0
TOTAL		**57/62**		

These results indicate that the X-Disease phytoplasma is present throughout cherry production regions in The Dalles and Mosier, OR, as well as across the river in Dallesport, WA, but not in the Hood River Valley or the Willamette Valley, OR. Awareness of this disease and removal of infected trees should help control spread throughout the region.

Objective 2: Conduct regional Mid-Columbia survey for cherry leaf roll virus and the cherry leaf roll virus complex with prune dwarf virus and prunus necrotic ringspot virus on sweet cherry.

Past surveys revealed the presence of cherry leaf roll virus (CLRV) in The Dalles. One of the CLRV-infected orchards was removed and replanted in 2017. Another CLRV-infected orchard was removed in 2018. No new CLRV symptoms were reported in 2018 or 2019. Growers with the last few remaining trees known to have CLRV were encouraged to remove them.

Objective 3: Survey Mid-Columbia sweet cherry orchards for nematode-transmitted viruses (such as cherry rasp leaf virus, tobacco ringspot virus, and tomato ringspot virus) and their vectors.

Historically, the nematode-transmitted viruses, *Tobacco ringspot* and *Tomato ringspot*, have been known to occur on sweet cherry in Oregon. There is also historical evidence of another nematode-transmitted virus, *Cherry rasp leaf*, the causal agent of flat apple in Oregon's prominent production region, Hood River. In a statewide survey for sweet cherry viruses, *Tomato ringspot virus*, was found in Hood River and the Grande Ronde Valley. Due to this, a follow-up survey was conducting after bringing awareness of these diseases to the sweet cherry industry. No enation symptoms were

submitted for testing in the Mid-Columbia region in 2018. In 2019, a sample was submitted from an orchard in The Dalles and tested positive for *Tomato ringspot virus*.

Objective 4: Investigate spread and diversity of *Tomato ringspot virus* isolates from cherry in known-infected areas.

Understanding the distribution of viral symptoms, the virus itself and its nematode vector will help us determine how to manage this disease. Two infected orchards were selected for a more in-depth study of disease and pathogen distribution.

Commercial Orchard #1 in The Dalles

On May 21, 2018, a commercial cherry orchard in The Dalles was surveyed for symptoms associated with tomato ringspot virus (ToRSV). A total of 1,952 trees were surveyed for enations, dwarfed leaves, rosetting and dieback and rated on a 0-4 scale to indicate severity (0 = no ToRSV symptoms (rosetting, enations, 1 = Minimal ToRSV symptoms present, 2 = Moderate ToRSV symptoms present, 3 = Obvious ToRSV symptoms present, 4 = Tree stump with ToRSV symptoms on leaves). Tree locations were marked with an "X" if the tree had been removed partially (stump) or with an "M" if it was missing entirely. The orchard was primarily cv. 'Bing' on Mazzard rootstock with Van and Rainier pollinizers. Pollinizers were painted with different colors and used as reference points. Pollinator locations were indicated with a "Y" for yellow indicating cv. 'Van' and "R" for red indicating cv. 'Rainier'. A "+" symbol was used to note if suspected virus symptoms were observed that were not expected to be associated with ToRSV. Leaf samples were collected from a subsection of 400 trees (large rectangle, Figure 1) for testing by ToRSV ELISA. Due to missing trees, a total of 379 samples were tested. Of the samples tested, 92.9% of the samples expressing symptoms associated ToRSV tested positive for ToRSV, 6.6% tested positive when no visual symptoms were observed, and 0.5% tested negative when symptoms were observed, Figure 2.

Soil samples were collected on June 5, 2018, from a smaller subsection of 50 trees (5 rows by 10 trees in each row), as well as two small sections in areas where symptoms were not observed (medium and small rectangles in Figure 1) for identification of nematodes. Dagger nematodes were identified in 77.6% of the sites in the subsection of 50 trees, and in 25% of the samples from the regions where symptoms are not present on the outer edge of the orchard., Figure 3A. All dagger nematodes present in June were juveniles.

Soil samples were collected again on October 17, 2018 from the same regions. Dagger nematodes were identified in 88% of the subsection of 50 trees, and in 75% of the samples from the regions where symptoms were not present on the outer edges of the orchard, Figure 3B. The number of nematodes present in October was greater than in June. In June, there were an average of 11.16 (range 0-117) nematodes/250 g of soil, while there were 66.35 (range 0-313.5) nematodes/250 g of soil in October. Adult female dagger nematodes were found in the October survey and were archived for future studies relating to virus detection and nematode characterization.

Figure 1: Disease severity rating in commercial sweet cherry cv. 'Bing' orchard in The Dalles, OR; 0 = No 1 = ToRSV Symptoms (rosetting, enations), 2 = Minimal ToRSV symptoms present, 3 = Obvious ToRSV symptoms present, 4 = Tree stump with ToRSV symptoms on leaves, X = Dead stump (no leaves), M = Tree removed, Y = yellow marked pollinator, R = red marked pollinator, + = Suspected virus symptoms not associated with ToRSV, hashlines = *Xiphinema* sp. nematodes

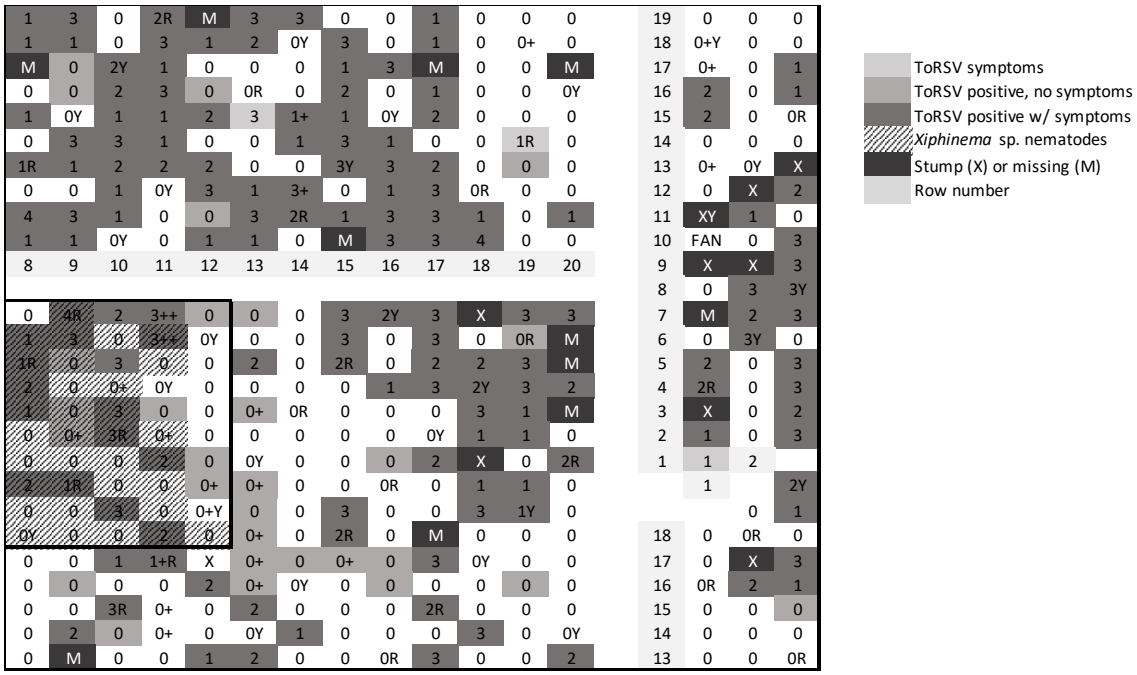

Figure 2 (a subsection of Figure 1 above): Correlation of symptom presence with diagnostic result for *Tomato ringspot virus* in commercial sweet cherry cv. 'Bing' orchard in The Dalles, OR

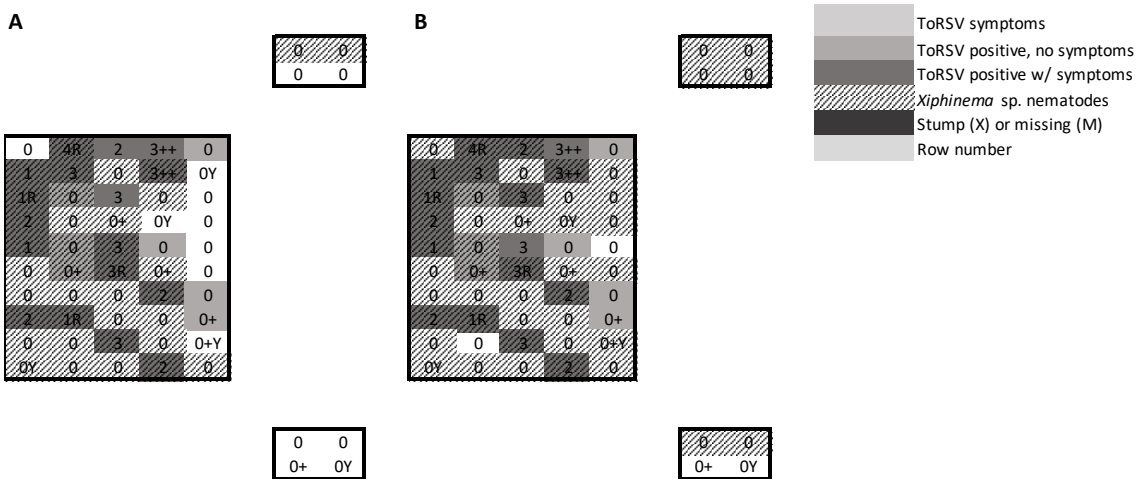

Figure 3 (a subsection of Figure 2 above): Dagger nematode presence in commercial sweet cherry cv. 'Bing' orchard in The Dalles, OR

Commercial Orchard #2 in The Dalles

On May 30, 2019, another commercial cherry orchard in The Dalles was surveyed for symptoms associated with tomato ringspot virus (ToRSV). A block of 455 trees (366 living) were surveyed for enations, dwarfed leaves, rosetting and dieback and rated on a 0-4 scale to indicate severity (0 = no ToRSV symptoms (rosetting, enations, 1 = Minimal ToRSV symptoms present, 2 = Moderate ToRSV symptoms present, 3 = Obvious ToRSV symptoms present, 4 = Tree stump with ToRSV symptoms on leaves). Tree locations were marked with an "X" if they had been removed partially (stump) or with an "M" if they were missing entirely. A "+" symbol was used to note if suspected virus symptoms were observed that were not expected to be associated with ToRSV, Figure 4. Due to missing trees, a total of 366 leaf samples were tested. Of the samples tested, 94.3% of the samples expressing symptoms associated ToRSV tested positive for ToRSV, 1.4% tested positive when no visual symptoms were observed, and 4.4% tested negative when symptoms were observed.

Soil samples were collected on June 6, 2019, from subsection of 45 trees for identification of nematodes in a section of the orchard that had symptomatic and asymptomatic trees. Four samples were also collected from two areas with asymptomatic plants. Dagger nematodes were identified in 33.3% of the sites in the subsection of 45 trees, and in 50% of the samples from the regions where symptoms are not present on the outer edge of the orchard. The average number of dagger nematodes found was 7.07 (range 0-71.5) nematodes/ 250 g of soil.

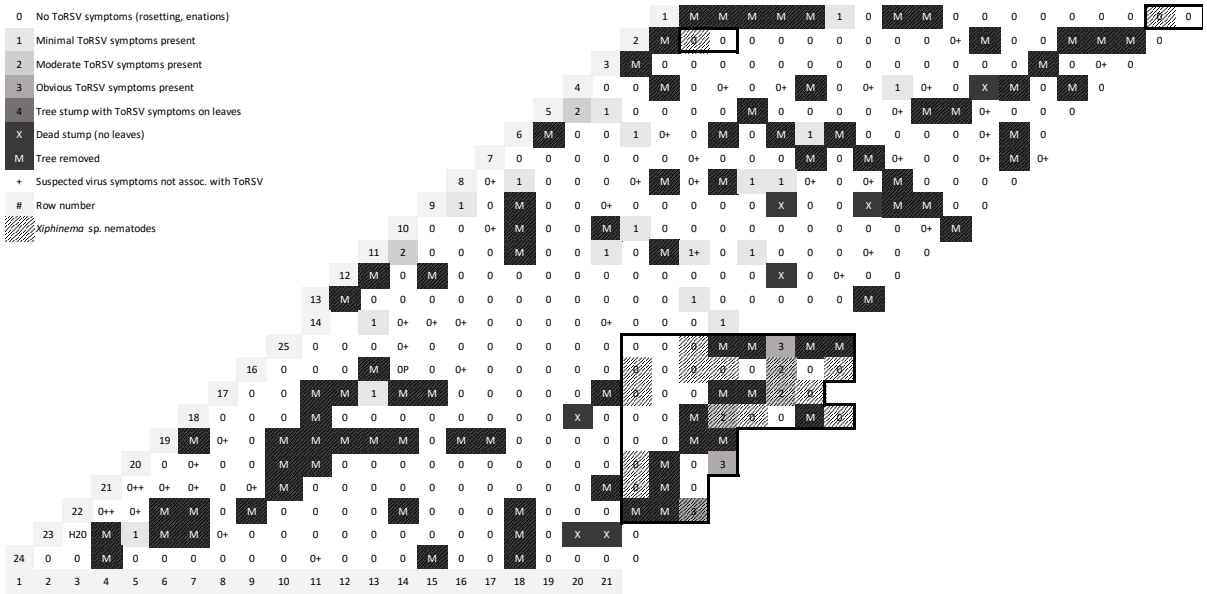

Figure 4: Disease severity rating in commercial sweet cherry orchard in The Dalles, OR; 0 = No 1 = ToRSV Symptoms (rosetting, enations), 2 = Minimal ToRSV symptoms present, 3 = Obvious ToRSV symptoms present, 4 = Tree stump with ToRSV symptoms on leaves, X = Dead stump (no leaves), M = Tree removed, + = Suspected virus symptoms not associated with ToRSV, hashlines = *Xiphinema* sp. nematodes

The two orchards surveyed for this study had tested positive for ToRSV; however, the distribution of the virus in the orchard and the correlation with the dagger nematode vector was unknown. The results suggest that there is no correlation between disease and nematode presence, as nematodes

were found near trees with and without disease and did not seem to be aggregated in one area. From this, we suggest to growers that if a nematode-transmitted disease is present in an orchard, it cannot be assumed that trees without symptoms are free of virus. If the virus and vector are present, the disease may be widespread in the orchard.

Mid-Columbia Research and Extension Center

Soil samples were collected on March 20, 2018, at a known *Tomato ringspot virus* (ToRSV)-infected research block at the Mid-Columbia Research and Extension Center. Samples were processed for the extraction and identification of dagger nematodes (*Xiphenema americanum*), the known vector of ToRSV. Matching the presence of nematodes in the orchard with locations of known ToRSV positive trees, allowed for the determination of eight areas where the virus and vector were present to test for the natural transmission of ToRSV to a variety of hosts. The following hosts were planted on April 13, 2018: apple (Scarlett Spur on MM106 rootstock), blueberry (Legacy), cherry (Lapins on Krymsk 6 rootstock), cherry (G6 rootstock), grape (self-rooted Chardonnay), peach (Loring on seedling rootstock) and raspberry (Meeker).

Bud, leaf, and/or root tissues were sampled from all hosts at planting and tested for the presence of ToRSV using ToRSV-specific ELISA. All samples tested negative for ToRSV (Table 2). Each tissue type (apple, blueberry, cherry, grape, peach and raspberry) was spiked with ToRSV-positive material in a 1:10, 1:100, 1:1000 serial dilution to account for possible inhibition of the test. None of the tissue types indicated the presence of inhibitors. Eight out of ten grape plants did not survive and were re-planted on June 4, 2018. Testing via ToRSV ELISA was performed again on root and leaf tissue to ensure plants were ToRSV negative (Table 2). Leaf samples were collected in September 2018 to test if transmission had occurred. None of the hosts tested positive, indicating no natural transmission had occurred.

On September 14, 2019, three cucumber seedlings at the cotyledon stage, as well as three cucumber seeds were planted in each plot positioned 8-10 inches around a sprinkler. Leaf and root samples were collected from each tree and small fruit host, cucumber, and weeds (plantains and dandelions) on October 4, 2019. Soil samples were collected from each of the eight plots to confirm presence of dagger nematodes. Results are pending. All foliar samples collected from the tree and small fruit hosts, cucumbers, and weeds tested negative for ToRSV (Table 2). Testing of root tissue is still in progress.

Table 2: Testing results for samples collected at Mid-Columbia Research and Extension Center

		4/13/18 (Pre-plant)		6/4/2018 (Re-plant)		7/19/18		9/17/18		10/4/19	
Host	**Tissue Type**	**n**	**# ToRSV + ELISA**	**n**	**# ToRSV + ELISA**	**n**	**# ToRSV + ELISA**	**n**	**# ToRSV + ELISA**	**n**	**# ToRSV + ELISA**
Apple (Scarlett Spur	bud	9	0	-	-	-	-	-	-	-	-
on MM106)	leaf	-	-	-	-	-	-	9	0	8	0
	root	9	0	-	-	-	-	-	-	8	TBD[1]
Blueberry (Legacy)	leaf	10	0	-	-	-	-	10	0	10	0
	root	10	0	-	-	-	-	-	-	10	TBD
Cherry (G6)	bud	10	0	-	-	-	-	-	-	-	-
	leaf	-	-	-	-	-	-	10	0	9	0
	root	-	-	-	-	-	-	-	-	9	TBD
Cherry (Lapins)	bud	6	0	-	-	-	-	-	-	-	-
	leaf	-	-	-	-	-	-	6	0	5	0
	root	6	0	-	-	-	-	-	-	5	TBD
Grape (Chardonnary,	leaf	-	-	8	0	-	-	10	0	10	0
self-rooted)	root	10	0	8	0	-	-	-	-	10	TBD
Peach (Loring	bud	9	0	-	-	-	-	-	-	-	-
on seeding)	leaf	-	-	-	-	-	-	9	0	8	0
	root	9	0	-	-	-	-	-	-	8	TBD
Raspberry (Meeker)	leaf	10	0	-	-	-	-	9	0	9	0
	root	10	0	-	-	-	-	-	-	9	TBD
Cucumber (seedlings)	leaf	-	-	-	-	-	-	-	-	7	0
	root	-	-	-	-	-	-	-	-	7	TBD
Cucumber (seeds)	leaf	-	-	-	-	-	-	-	-	3	0
	root	-	-	-	-	-	-	-	-	3	TBD
Weeds (dandelion,	leaf	-	-	-	-	1	0	-	-	8	0
plantain)	root	-	-	-	-	1	0	-	-	8	TBD

[1]TBD = To be determined

Economic Importance

We hope that this survey has been a wakeup call for the Oregon sweet cherry industry to take action on important diseases that threaten cherry production. These diseases kill trees and/or yields and efforts should be made to find and remove infected trees. Many of the infected trees are from older orchards that may have already been scheduled for renovation. Removal of infected trees and treatment for soilborne nematode vectors will help protect newer orchards that keep the area vital and productive.

REFERENCES

Lutes, L. A. and Pscheidt, J. W. 2019. Spread and host-specificity of *Tomato ringspot virus* on sweet cherry (*Prunus avium*) in Oregon. Phytopathology. (*Abstract accepted*)

Ito, T., and Suzaki, K. 2017. Universal detection of phytoplasmas and Xylella spp. by TaqMan singleplex and multiplex real-time PCR with dual priming oligonucleotides ed. Ruslan Kalendar. PLoS ONE. 12:e0185427.

EXECUTIVE SUMMARY

Project Title: Mid-Columbia survey for sweet cherry viruses and vectors

Key Words: sweet cherry, virus, Little Cherry Disease, X-Disease, Tomato ringspot virus

A statewide diagnostic survey in 2016-2017 revealed the presence of important virus and phytoplasma induced diseases that threaten the Oregon sweet cherry industry. A focused survey for these diseases in the Mid-Columbia, the most prominent sweet cherry production in the state, as well as, the adjacent region in Washington, was conducted.

Little Cherry/X-Disease symptoms were found in this survey in The Dalles, Dallesport, and Mosier, but not in the Hood River Valley. Samples from these orchards tested positive for the X-Disease phytoplasma but negative for *Little cherry virus 2*. A few symptomatic samples were collected from the Willamette Valley, but each tested negative.

Cherry leaf roll virus (CLRV) was found in the previous survey, but no new reports of CLRV were made in 2018-2019; however, several known CLRV-infected orchards were removed. During this survey another orchard in The Dalles area was found with *Tomato ringspot virus* (ToRSV) infected trees. Despite an increased awareness of nematode-transmitted disease among the Oregon sweet cherry industry, only one new positive result was found.

The distribution of *Tomato ringspot virus* (ToRSV) was studied at two commercial orchards in The Dalles. The nematode vector was found in association with symptoms at both orchards. Visual symptoms of enations (gall-like formation on the underside of the leaf), as well as rosetting (bunching of leaves due to shortened internodes) were found reliably associated with ToRSV infected trees. A low percentage (1-6%) of samples without obvious symptoms also tested positive for ToRSV, highlighting the importance of virus testing.

An experiment to test if ToRSV could be transmitted to other hosts was conducted at the Mid-Columbia Research and Extension Center in Hood River by interplanting several tree and small fruit hosts in a known-infected orchard in areas where the dagger nematode vector was present. After an 18-month period, ToRSV did not naturally infect any of the interplanted hosts, including cherry trees used as controls.

We hope these surveys have been a wakeup call for the Oregon sweet cherry industry to take action on important diseases that threaten cherry production. These diseases kill trees and/or yields and efforts should be made to find and remove infected trees.

FINAL PROJECT REPORT **YEAR**: 3

Project Title: Orchard management practices for *little cherry virus 2*

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Cooperators: None

Total Project Request: $171,172 Year 1: $57, 512 Year 2: $55,716 Year 3: $57,944

Other funding sources: None

Budget
Organization Name: Washington State University **Contract Administrator:** Katy Roberts
Telephone: (509) 335-2885 **Email address:** katy.roberts@wsu.edu

Item	2017	2018	2019
Salaries[1]	$7,361	$7,655	$24,213
Benefits[2]	$2,157	$2,243	$8,329
Wages	$3,120	$3,245	0
Benefits	$324	$337	0
Equipment	0	0	0
Supplies	$44,550	$42,236	$25,000
Travel	0	0	$0
Plot Fees	0	0	$0
Miscellaneous	0	0	$0
Total	$57,512	$55,716	$57,542

Footnotes:
1. 0.15 FTE for year 1 and 2, and 0.5 FTE for year 3.
2. Benefits calculated at standard Washington State rates.

OBJECTIVES

1) *Examine the effect of rootstocks on the concentration of LChV2.*

2) *Quantify the accumulation of LChV2 in different host tissues throughout the growing season.*

3) *Determine the population structure of LChV2 within Washington cherry production regions.*

SIGNIFICANT FINDINGS

- In established infections, LChV2 can be detected in all tissue of the plant year round. However, the virus was most abundant in the fruit stem and woody tissue.
- The X-disease phytoplasma cannot be detected year round. Concentrations of the phytoplasma reach detectable levels at harvest and can be detected into the fall, however distribution is not uniform throughout the tree. Leaves are unreliable. Fruit stem and wood tissue appear to be the best tissues to test for the X-disease phytoplasma.
- LChV2 infects multiple rootstocks. Early in infection movement of the virus is basipetal, with highest concentrations in the roots and below the graft.
- Single-strand conformation polymorphism analysis confirmed that there are multiple isolates of LChV2 in Washington state.

RESULTS AND DISCUSSION

LChV2 is one of the pathogens responsible for Little cherry disease (LCD), which produces small, misshapen fruit with poor taste. This project was undertaken to gain a better understanding of LChV2 biology with the goal of improving disease management. X-disease phytoplasma, which also causes little cherry disease, was included in the third year of the study as it has greatly increased in incidence in Washington along the Columbia River, and in the Wenatchee area.

To determine how LChV2 moves in different rootstocks, three rootstock varieties (Gisela 6, Gisela 12, and Krymsk 5) were chip grafted with wood from a tree infected with LChV2. At four, eight, twelve, and sixteen weeks, a subset of trees for each rootstock was dissected. Material was collected from fibrous roots, the primary root, wood scraping below the graft, wood scraping above the graft, pooled wood scrapings from branches, and a pooled leaf sample. This material was collected from four to five trees per rootstock at each time point. Quantitative PCR was performed to determine the concentration of LChV2 in each of these tissues. It was found the LChV2 moved basipetally, or downward towards the roots following initial infection, and was most concentrated below the graft and in the roots (Figure 1). Gisela 12 showed the most pronounced difference in LChV2 concentration in tissues below and above the graft. However, for all three rootstocks the virus did make it into the leaf tissue of the rootstock. Plans are in place to repeat this in Mazzard rootstocks.

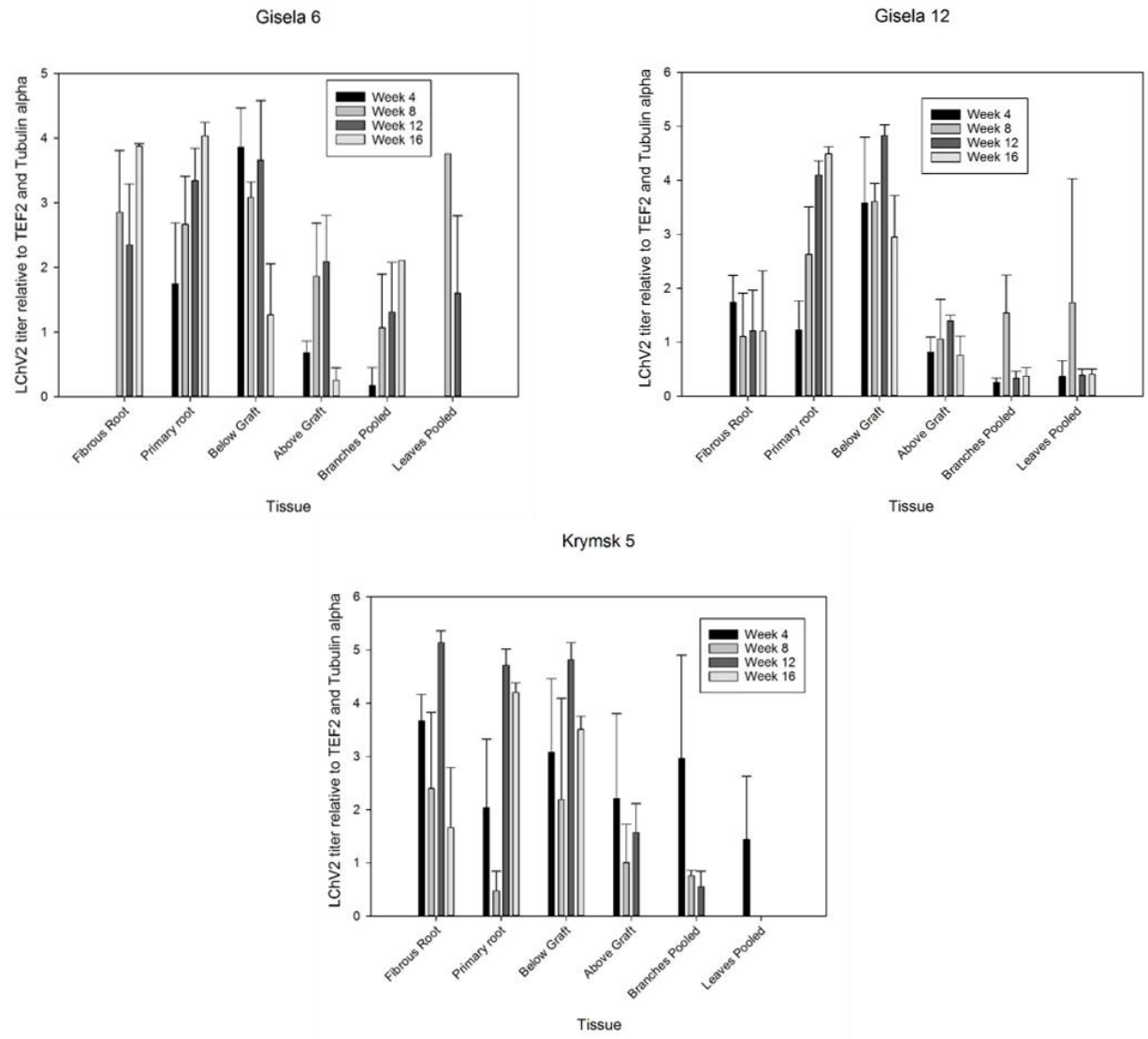

Figure 1. LChV2 titer across time and tissue type for Gisela 6, Gisela 12, and Krymsk 5 rootstocks.

A two year study was conducted examining titer and distribution of LChV2 in a Lambert cherry which had been infected for several years. Several tissue types were examined throughout the year (Table 1). RNA was extracted and qPCR analysis was performed to determine LChV2 titer. TEF2 and tubulin alpha were used as reference genes. LChV2 was distributed throughout the tree and was detected in all tissues throughout the year (Figure 2). However, LChV2 titer was greatest in the fruit stem and wood scraping. Although there were some differences between the two years, the overall pattern is similar. In both years, titer in leaf tissue was at its lowest in early May. Petiole and midrib concentrations were similar at each time point except in August of both years when midrib titer was higher. In the flower/fruit stem titer increased from April to May for both years. Titer in fruit increased closer to harvest although this increase was greater in the first year. Although in established infections LChV2 is distributed throughout the plant, it does appear that to improve the likelihood of detecting the virus when present, fruit stems and wood scrapings are the best material for analysis.

Table 1. Time points and tissues for titer and distribution of LChV2 (Tissues with highest concentration at that time point are in bold.

Time	Tissue
December - January	Buds, **Wood Scraping**, Roots
March - Mid April	Buds, **Wood Scraping**, Roots
Mid to Late April	**Wood Scraping**, Roots, Flower Stem
Late April - Early May	Midrib, Petiole, **Wood Scraping**, Fruit, Fruit Stem
Late May - Early June	Midrib, Petiole, Wood Scraping, Roots, Fruit, **Fruit Stem**
Mid June - Early July	Buds, Midrib, Petiole, Wood Scraping, Roots, Fruit, **Fruit Stem**
Mid August	Buds, Midrib, Petiole, **Wood Scraping**
Mid October	Buds, Midrib, Petiole, **Wood Scraping**

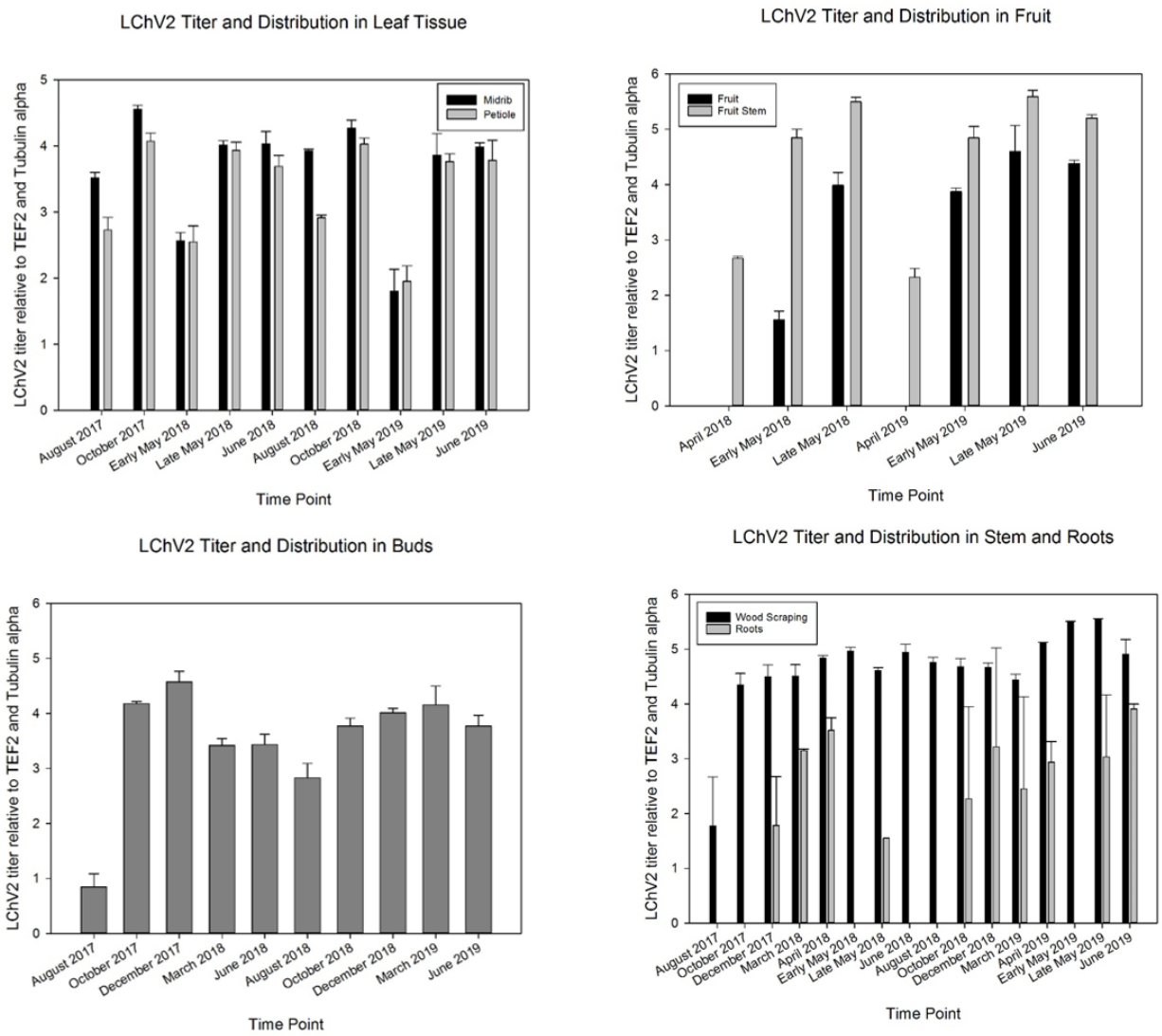

Figure 2. Titer and distribution of LChV2 in leaf, fruit, buds, stem and roots for year one and two.

The behavior of X-disease phytoplasma in cherry trees proved to be very different from LChV2. Samples were collected from trees that had tested positive in previous years. DNA was extracted from the samples and qPCR was performed using ITS as a reference gene. Sampling for titer and distribution was begun in the spring, however it was not until shuck fall in May that the X-disease phytoplasma could be detected (Figure 3). Even then it could not be detected in leaves or fruit, only fruit stem and wood scrapings. Two weeks before harvest the pathogen could be detected in all four tissues, however detection in leaves was not consistent. Concentration in the fruit stem did increase between May and June. After harvest, buds were included in testing and these were positive in July and early September. Leaves, the tissue most commonly screened for the X-disease phytoplasma, are not reliable and were not positive until close to harvest. It is unknown what role, if any the winter weather had in delaying detection of the X-disease phytoplasma. Another year of data collection is planned, however this will be included in a new proposal. In addition, leaf, fruit stem, and fruit tissue was collected from several infected cultivars at harvest. Titer and distribution will also be examined in these samples.

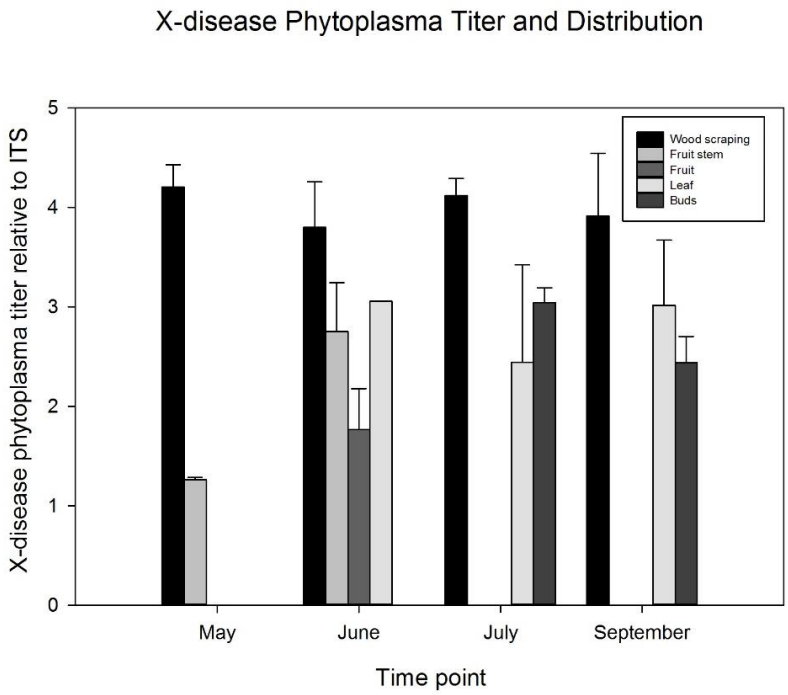

Figure 3. Titer and distribution of the X-disease phytoplasma.

Lastly, an examination of LChV2 samples from Washington State was conducted to identify unique isolates of the virus. This was done with the goal of increasing the amount of sequence data available for this virus so a more accurate qPCR assay can be developed. Positive samples from across the state were subjected to single strand conformation polymorphism analysis in which different sequences generate different banding patterns (Figure 4). While it was intended that the different variants identified be sequenced, the rapid escalation of the LCD epidemic in 2019 delayed this process into winter. Furthermore sampling during the 2019 season identified plants that were displaying classic LCD symptoms yet were negative for both LChV2 and X-disease phytoplasma. While it is unlikely that these are infected by an LChV2 isolate missed by extant tests and is more probably the X-disease phytoplasma, these samples will be sequenced over the winter to determine the causal agent. Together, this data will allow for a better assessment of the existing assays and development of an improved assays if necessary.

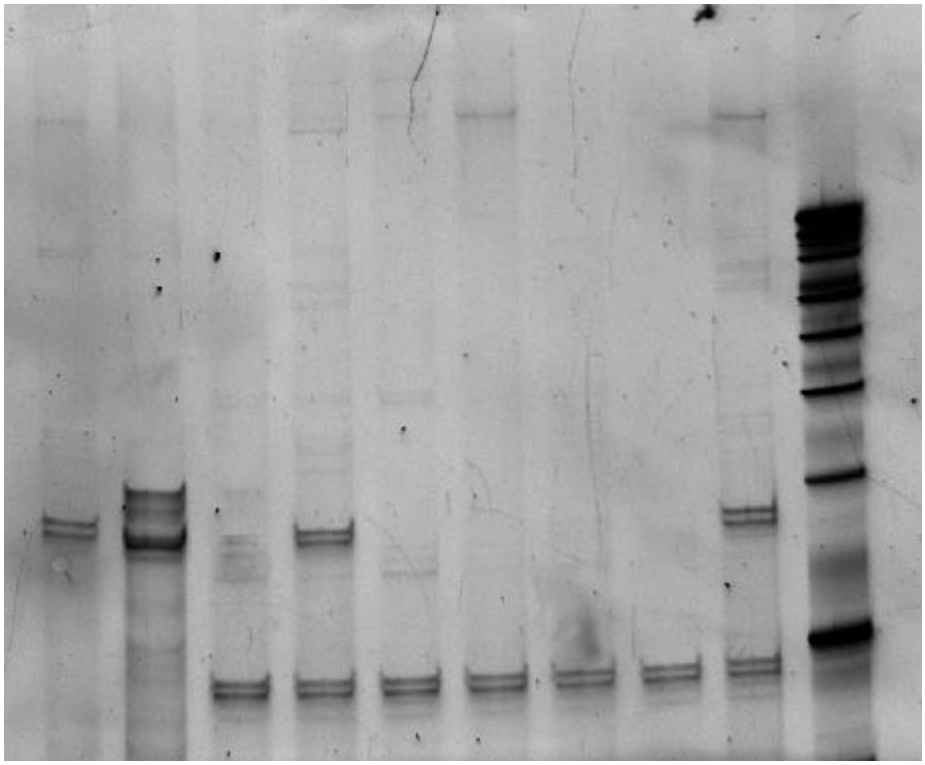

Figure 4. SSCP analysis on LChV2 samples. For each sample, the same region of LChV2 was amplified, however the banding patterns indicate that some of these samples have different sequences.

Collectively these data will lead to improved orchard management for control of LChV2. The rootstock analysis shows that, for these three rootstocks at least, once LChV2 is introduced, it can move throughout the plant even though it favors basipetal, or downward movement, towards the roots. The data for Mazzards will need to be included, but it is unlikely that rootstock choice affects susceptibility to LChV2. The titer and distribution study revealed that, in established infections, the virus is distributed throughout the tree and can be detected year round. SSCP analysis demonstrated that different isolates of LChV2 are present in the state and sequencing will determine how much these isolates differ. These data will inform decisions regarding LChV2 detection and should lead to improved detection of the virus. Early detection is an important part in managing this disease.

While growers should remain vigilant with regards to LChV2, the X-disease phytoplasma has in the last couple of years become the predominant cause of little cherry disease in Washington State. Testing of grower samples by the Clean Plant Center Northwest showed a 14% incidence of LChV2 and a 24% incidence of the X-disease phytoplasma. This year those numbers changed to 1% and 63%, respectively. LChV2 has declined while the X-disease phytoplasma has sharply increased. Additionally, the X-disease phytoplasma has been detected as far north as Wenatchee where LChV2 has traditionally been the predominant of the two pathogens. To address this, in the last year we examined titer and distribution of the X-disease phytoplasma. The phytoplasma is far more difficult to detect than the virus. Prior to harvest it is very difficult to detect in any tissue. Tissue distribution is not even and leaves in particular, which are the tissue most often used in testing, are unreliable. The lack of sequence data for this pathogen also makes it difficult to know if any assay will detect all strains. Future work will focus more heavily on the X-disease phytoplasma than LChV2 as the X-disease phytoplasma is the predominant pathogen in the state.

EXECUTIVE SUMMARY

Project Title: Orchard management practices for *little cherry virus 2*
Keywords: Little cherry disease, Little cherry virus 2 (LChV2), X-disease phytoplasma

OBJECTIVES

1) *Examine the effect of rootstocks on the concentration of LChV2.*
2) *Quantify the accumulation of LChV2 in different host tissues throughout the growing season.*
3) *Determine the population structure of LChV2 within Washington cherry production regions.*

SIGNIFICANT FINDINGS

- In established infections, LChV2 can be detected in all tissue of the plant year round. However, the virus was most abundant in the fruit stem and woody tissue.
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- LChV2 infects multiple rootstocks. Early in infection movement of the virus is basipetal, with highest concentrations in the roots and below the graft.
- Single-strand conformation polymorphism analysis confirmed that there are multiple isolates of LChV2 in Washington state.

FUTURE DIRECTIONS

The data presented here will result in improved detection and management of LChV2. Knowing how the virus is distributed allows for better sampling. Having sequence data from multiple isolates in the state will allow for the development of an improved qPCR assay if needed. Identification of trees with the virus, either through symptoms on the fruit or detection of the virus by qPCR, and removal of those infected trees is key to controlling and reducing the spread of LChV2.

While growers should remain vigilant with regards to LChV2, this pathogen is no longer the primary agent of little cherry disease. X-disease phytoplasma increased significantly in the state in the summer of 2019 and has spread north, being detected as far north as Wenatchee. Based on samples brought into the Clean Plant Center Northwest in 2018 and 2019, LChV2 incidence has dropped from 14% to 1% and X-disease phytoplasma incidence has increased from 24% to 63%. This one year increase is alarming. To address X-disease phytoplasa in terms of management, we began to look at titer and distribution of the phytoplasma. Unlike LChV2, which can be detected year round, the X-disease phytoplasma cannot be reliably detected in infected trees until summer. At harvest is the best time to begin sampling and sampling should be complete by the end of September. Leaves, the tissue most frequently sampled, were not reliable. Future projects will shift their focus mostly to the X-disease phytoplasma as this is the most problematic of the two pathogens. Currently, knowledge about this pathogen is very limited. Existing control options are not slowing down the spread. It is necessary to not only improve detection, but to understand the biology of this pathogen so either tolerant cultivars can be developed or improved control strategies can be employed.