APPLE CROP PROTECTION RESEARCH REVIEW
Thursday, January 24, 2019

Time	Page	Presenter	Title	Yrs
			Final Reports	
2	1	Hanrahan	Food safety update	*Internal*
	6	Irwin	Cold tolerance, diapause, and survival of Brown Marmorated Stink Bugs: ***written report only***	16-17
	16	Knight	Kairomones for monitoring and control of native and invasive moths; ***written report only***	15-17
	22	Norelli	Fire blight resistance and fruit quality in new Washington cultivars: ***writen report only***	15-17
Group			**Continuing Projects: 2:15 - 3:45**	
1	29	Schmidt	Apple pesticide residue studies	*Internal*
1	33	Khot	Data to model apple airblast spraying drift exposure levels	18-19
1	41	Amiri	Rapid lab and field detection of two major apple quarantine pathogens	17-19
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2	116	Beers	Brown marmorated stink bug control in Washington: ***No-cost extension***	16-18
2	92	Beers	Optimizing sterile insect release of codling moth in Washington	18-20
	99	Garczynski	Can we get codling moth females to stop laying eggs on apple?	
	106	Knight	Improved monitoring & lure & kill for cm management: ***No-cost extension; written report only***	18

CONTINUING REPORT **Year: 2018**

Project Title: WTFRC internal program – food safety efforts

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Cooperators: Jacqui Gordon (WSTFA), Faith Critzer & Girish Ganjyal (WSU), Manoella Mendoza and Mackenzie Perrault (WTFRC), Rob Atwill, Missy Partyka, and Ronny Bond (UC Davis), Bonnie Fernandez-Fenaroli (CPS)

Other funding sources

Agency Name: WA SCBGP
Amt. requested/awarded: $ 216,682 Title: Enhanced food safety education and training for tree fruit producers (awarded to WSTFA with Jacqui Gordon as PI)
Agency Name: FDA
Amt. requested/awarded: $243,651 for FY18 awarded to WCFSS (Atwill et al.) Title: Facilitating implementation of FSMA regulations for agricultural water quality
Agency Name: CPS
Amt. requested/awarded: $290,000 to Zhu and Suslow; Title: Control of Listeria monocytogenes on apple through spray manifold-applied antimicrobial intervention

WTFRC internal program budget:

Item	2018	2019
Salaries[1]	2,975	3,100
Benefits	1,220	1,325
Wages[2]	6,000	7,500
Benefits	3,180	3,230
RCA Room Rental		
Shipping		
Supplies[3]	200	350
Travel[4]	3,500	3,100
Plot Fees		
Miscellaneous		
Total	**17,075**	**18,605**

Footnotes:
[1]Salaries: 5% of Mendoza (with 41% benefits), not included in salaries: 15% of Hanrahan time, 1% Schmidt
[2]Wages: 53% benefit rate
[3]Supplies include 1 poster for ASHS and misc.
[4]Travel includes: CPS annual meeting, 3 trips to WSU in Pullman, in state day travel to attend trainings, Annual NW Food Safety and Sanitation Conference in Portland

OBJECTIVES

1. Enhance collaboration in all areas of food safety (research, policy, FSMA implementation)
    a. Participate in development of training for industry
    b. Develop an effective food safety outreach program

SIGNIFICANT ACCOMPLISHMENTS IN 2018

Food safety remains one of the highest priority items within the industry. As some compliance dates of FSMA have been effective, it is of utmost importance to continue to provide the Washington growers with timely assistance. Further, in order to attract microbiologists to work on problems related to food safety for tree fruit, a strong collaboration from scientists with a horticulture background is of great advantage to ensure that project goals and outcomes reflect immediately actionable items. Lastly, translating research into layman's terms and providing a bridge between science, politics and farming is another important goal of this project.

Research:

We participated in a number of on-going and new collaborative projects, funded by WTFRC, CPS, SCBG and FDA (see Table 1). Notably, WTFRC is increasingly sought as collaborator in national grant applications to NIFA or SCRI.

The WTFRC, under leadership of Ines Hanrahan, continued to serve as a partner in research for the Center for Produce Safety (CPS) and she attended the annual meeting in Charlotte, NC. Tree fruit specific research priorities were developed and integrated into the annual CPS call for proposals, with the help of the NHC Food Safety Committee. During the proposal process Dr. Hanrahan frequently serves as specialist to answer questions asked by scientists preparing to propose new research projects. Currently one project involving local scientists has been funded by CPS: 'Control of Listeria monocytogenes on apple through spray manifold-applied antimicrobial intervention' (Zhu/Suslow; $290,000). WTFRC has developed and executed a packing line survey for this project in 2017 to determine the current industry practices related to spray manifold interventions and is currently assisting Dr. Zhu's team to set-up packingline validation studies with industry collaborators. The team is also sourcing fruit for the experiments. In January of 2018 Hanrahan has also participated in a SCRI industry relevance review of 11 proposals submitted to USDA NIFA in the area of food safety.

Table 1: Summary of WTFRC collaborations* in food safety research in 2018 and pending research for 2019

Keyword	PI's	Affiliation(s)	Funding Source	Amount
Continuing/finishing/new in 2018				
Listeria storage*	Zhu/Amiri/Hanrahan	WSU, WTFRC	WTFRC	195,414
Imp. Dryer	Ganjyal/Zhu	WSU	WTFRC	57,000
Water sampling	Partyka/Bond	UC Davis, WTFRC	FDA	243,651
Food Safety Training	Gordon	WSTFA	SCBG	216,682
List. Cleaning*	Zhu/Hanrahan	WSU, WTFRC	WTFRC	306,285
FMSA PCHF	Ganjyal	WSU, WTFRC	WTFRC	98,971
Brush bed sanitation*	Critzer et al.	WSU, WTFRC	WTFRC	51,967
Listeria monitoring	Kovacevic et al.	OSU	ODA SCBG	174,540
Packing sanitation*	Critzer et al.	WSU, WTFRC	WTFRC	203,000
Rapid detection tools*	Critzer	WSU, WTFRC	WTFRC	112,000

Ozone in storage*	Zhu	WSU, WTFRC	WTFRC	300,000
E.Faecium as surrogate	Zhu et al.	WSU	WA-SCBG	250,000
Water treatment	Critzer	WSU	WA-SCBG	194,000
Pending for 2019				
Apple slices*	Zhu	WSU	WTFRC	TBD
Microbiome*	Zhu	WSU	WTFRC	TBD
PSR cost effective mgt.*	Danyluk et al.	U. Florida	SCRI-CAP	TBD
Antimicrobial coatings	Wang et al.	UC-Davis	USDA-NIFA	TBD
Lm survival/biocontrol	Amalaradjou et al.	U.Conn.	USDA-NIFA	TBD

*collaborations involve a separate WTFRC internal budget

FSMA implementation: In order to best serve the needs for timely information distribution related to new laws pertaining to food safety, WTFRC staff (Hanrahan) leads efforts to coordinate all outreach activities by the various industry organizations. Specifically, NHC (policy), WSTFA (education) and WTFRC (research) efforts have continued to be combined and talking points were coordinated to prevent further confusion, when learning how to implement the already complicated laws. Further, the WSTFA continued to host numerous PSA training sessions in 2018, and Hanrahan served as a trainer for two modules in April. WTFRC staff assisted in meeting logistics and Hanrahan frequently serves as expert to help field questions. In February, Hanrahan attended a two-day listening session related to the ag water rule and in March she was invited to present at the annual Western Regional Center to Enhance Food Safety Meeting at UC Davis. In May, Hanrahan attended an OFRR (on farm readiness review) training in Oregon and in November she participated in an FDA listening session on the draft guidance for the PSR by participating in a panel discussion.

Development of industry training modules: In collaboration with WSTFA, NHC, and WSU, we repeated the existing workshop module "Putting Cleaning and Sanitation Programs into Practice (in Spanish)" and "Verification of cleaning and sanitation programs for tree fruit packinghouses: a hands-on environmental monitoring workshop". These workshops provide a combination of classroom and hands-on activities and take place in collaborating packing facilities (Table 2). Dr. Hanrahan's contributions to these workshops include: leading of general curriculum development, being a trainer, delivering talks, and helping with logistical support (including staff). Secondly, the same group, in collaboration with UC Davis will hold a workshop named: FSMA water quality testing (moved to 2019). This module will also be a repeat of a curriculum originally developed in 2016. It is the first of its kind in the nation to address practical considerations for water testing under FSMA. Workshops were designed to give participants theoretical background in combination with outdoor activities geared towards learning based on examples coupled with hands on training (Table 2).
In addition, WTFRC is collaborating with the WSFTA to develop a series of food safety videos. In 2017 we finished and the WSTFA distributed two videos: Hand Washing Training, and Cross Contamination vs. Cross Contact. These videos are available in both English and Spanish upon request from Jacqui Gordon (jacqui@wstfa.org). For a 2018 release, we have finished a video on Good Agricultural Practices in the orchard and another video in the packinghouse (the four zones). WTFRC personnel contributes to content development, video shooting, voice over, and development of a training module to teach growers how to best use these materials when training their crews.

Table 2: WTFRC staff involvement in WSTFA sponsored food safety trainings in 2018

Name of Workshop/Training	**Date**
FSMA Water Quality Testing Workshop Wenatchee	In 2019
FSMA Water Quality Testing Workshop Yakima	TBD
Putting cleaning and sanitation programs into practice – Yakima (Spanish)	April
Environmental monitoring	July

Food Safety outreach:

Based on industry feedback, Dr. Hanrahan developed a Q&A series in collaboration with the Good Fruit Grower to answer frequently asked questions related to food safety. In 2017, a total of 5 pieces were published, and for 2018 another article has been published in the February 15th issue. More pieces will be written as needed.

In addition, Ines Hanrahan is serving as an adjunct faculty member for the WSU School of Food Science. She is currently participating as a committee member on two Ph.D. and two MSc. committees in the Food Science Department. All students will work in the general area of food safety on very relevant tree fruit industry topics and are interested in a career in tree fruit upon graduation.

Further, Dr. Hanrahan is serving on the stirring committee of the PNW Food Safety and Sanitation Conference, a regional conference with 450 attendees annually. Other outreach activities may include but are not limited to: posters at national/international meetings, invited talks, lectures for WSU classes.

FINAL PROJECT REPORT **YEAR**: 2 of 2
WTFRC Project Number:

Project Title: Cold tolerance, diapause, and survival of brown marmorated stink bugs (*Halyomorpha halys*)

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Cooperators: Dr. Lisa Neven, Yakima Agricultural Research Laboratory

Total Project Request: **Year 1:** $34,800 **Year 2:**$33,200
Other funding sources: None **WTFRC Collaborative expenses:** None

Item	2016-2017 July1 - June 30	2017-2018 July 1 – June 30
Salaries		
Benefits		
Wages	28560	28800
Benefits	1940	1950
RCA Room Rental		
Shipping		
Supplies	2800	950
Travel	1500	1500
Plot Fees		
Miscellaneous		
Total	34800	33200

Footnotes: Benefit rate is 9% for CWU academic year, 3% for summer.

Budget Explanation:

The salary requested will support a graduate student during the summer and academic year as she performs the activities outlined in this proposal. Irwin's laboratory already has a functioning respirometry system so funds are requested only for scrubbing chemicals (i.e., Ascarite and Drierite) and occasional small parts such as fittings and tubing. The laboratory is fully equipped for cold tolerance measurements so no significant costs will be incurred. Other than some inexpensive chemicals, Dr. Neven is not requesting any funds. Other expenses include a lumite outdoor insect cage (#1412C, BioQuip Products) and "Bug dorms" (#1462C, BioQuip Products). Both are requested to raise BMSB in captivity and within an outdoor field enclosure. Mileage is requested to defray the costs of travel to locate sites for collection and monitoring of BMSB.

OBJECTIVES

1) Describe characteristics of diapause in this species, including the seasonal timing of metabolic suppression and arrested development, and the cues for diapause induction (e.g., critical photoperiod).
2) Measure seasonal changes in cold tolerance for overwintering adults (e.g., determination of supercooling points and chill-intolerance survivorship).
3) Identify any potential links between diapause timing and seasonal changes in cold tolerance.
4) Describe overwintering site preferences, including microclimate, and measure winter survival under field conditions (including selected natural sites and an enclosure study).

SIGNIFICANT FINDINGS

Objective 1: We were able to successfully characterize the seasonal timing of diapause for BMSB in Eastern Washington, and provide the first verified measurement of critical photoperiod (e.g. photoperiodic threshold) for this species.

- Metabolic rates of field-maintained BMSB fell as they went into diapause (the insect version of hibernation) in late-October through November.
- Both metabolic rate and reproductive status of laboratory-maintained BMSB indicate that the critical photoperiod (that is, day length) of 13.5h induces diapause in this species.
- The critical photoperiod of 13.5h is necessary but not sufficient (that is, a required prerequisite) for diapause induction to occur naturally in BMSB.

Objective 2: Seasonal changes in the cold tolerance of overwintering adult BMSB were successfully quantified within Eastern Washington populations (from Walla Walla and Yakima).

- The supercooling point (SCP, the temperature at which ice forms in the body) of adult BMSB fell as Fall moved into Winter.
- Males supercooled better than females, with a mean SCP of 5.79 ± 0.2°F (mean ± standard error) in males and 9.26 ± 0.2°F in females.
- Supercooling points for BMSB were not below the minimum freezing temperatures experienced in Eastern Washington during our study and, thus, full mortality of the outdoor population was observed during both sampling seasons.
- BMSB in Eastern Washington are chill-intolerant (that is, individuals may die even before ice forms in their bodies), as has been observed in other parts of the United States.
- Temperatures of 14 to 5°F cause 50% mortality of naturally cold-acclimated BMSB, which are much higher than normal winter temperatures in this area.

Objective 3: There were no strong links between diapause and cold tolerance indicated in adult BMSB.

- Diapause induction proved to be necessary but not a sufficient stand-alone factor to induce high levels of supercooling ability in BMSB. A period of cold-acclimation is also necessary to achieve maximum supercooling ability.

Objective 4: A naturally selected overwintering site in Walla Walla, WA and our outdoor enclosure in Yakima, WA were successfully monitored for temperature throughout the fall and winter.

- BMSB sought refuge beneath the cedar-shingle siding of a residential house in Walla Walla, WA, within a few blocks of Franklin Park, a known BMSB 'hot spot'.
- Temperature probes placed internal and external the shingle siding recorded minimum air temperatures of 3.9°F and 1.3°F respectively, below the observed minimum SCP values, and

within the range where probability of survival is significantly decreased due to chill intolerance.

- Temperature probes at the Yakima enclosure experienced lower minimum temperatures than the Walla Walla site, with a recorded minimum of -0.6°F.

RESULTS & DISCUSSION

In our temperate climate, winter conditions affect the population dynamics of BMSB. In other stink bugs, photoperiod plays a key role in regulating the seasonal timing of diapause and plays a major role in limiting northward range expansion (Musolin & Numata 2003). Similarly, the northward expansion of BMSB may be limited in regions where cold weather arrives before diapause is induced via shorter photoperiods. Metabolic rates of BMSB from a naturally-acclimated outdoor population underwent significant metabolic suppression (an indicator of diapause) throughout the sampling period. When measured at three measurement temperatures, all treatments significantly differed across months in 59°F ($F_{(7)} = 6.4$, $P < 0.001$), 50°F ($F_{(6)} = 3.9$, $P < 0.05$), and 49°F ($F_{(7)} = 2.8$, $P < 0.05$). Metabolic rates showed no significant difference between sexes over time for 59°F ($F_{(1)} = 0.29$, $P = 0.59$), 50°F ($F_{(1)} = 1.75$, $P = 0.19$), and 49°F ($F_{(1)} = 3.87$, $P = 0.056$) (Fig. 1). The interaction term between 'date' and 'sex' was evaluated, and did not significantly improve the model. These results demonstrate that adult BMSB in eastern WA are able to fully transition into a diapause state that is prepared to overwinter.

A population of BMSB that enter diapause later than induced by day-length alone, could lack adequate cold tolerance to survive the onset of winter temperatures. Diapause has been found to enhance cold tolerance in other insects (Denlinger 1991), and the timing of diapause plays a key role. Entrance into diapause too early cuts short the growing season, whereas entrance too late can leave the insects susceptible to being killed by cold weather early in the fall. In some cases (for example, the Pitcher Plant Mosquito (*Wyeomyia smithii*)) the timing of seasonal development presents the most immediate impediment to range expansion in the temperate zone (Bradshaw et al. 2000, 2001). In the Southern Green Stink Bug (*Nezara viridula*) in Japan, the critical photoperiod for diapause induction proved to be maladaptive, preventing the species from moving northward. In colder regions, individuals were unable to enter diapause before seasonal cold temperatures inflicted high rates of mortality (Musolin 2007). Furthermore, diapause has been found as a prerequisite to maximum cold tolerance in the southern green stink bug (*Nezara* viridula) (Slachta et al. 2002). Determining the relationship between diapause and cold tolerance in BMSB has remained a knowledge gap in understanding how this pest will biologically function and survive within a region.

Prior research has assumed that a critical photoperiod (day length) of 13.5h is adequate to induce diapause in BMSB, but our study is the first to experimentally verify the accuracy of this value (Watanabe 1979; Nielsen et al 2016). Throughout the determination of the critical photoperiod, temperature was held constant at 68°F as the available light hours gradually decreased from 16L:8D to 12L:12D at a rate matching the natural seasonal changes of day-length in Yakima, WA. Metabolic rates of stink bugs from the artificially-acclimated laboratory population, measured at the incubation temperature of 59°F differed significantly across light hour availability ($F_{(7)} = 4.35$, $p < 0.001$). Metabolic rates did not differ significantly between sex ($F_{(1)} = 3.78$, $P > 0.05$) (Fig. 2), and the interaction between 'light hour' and 'sex' was also evaluated and did not significantly improve the model. BMSB are known to go into reproductive diapause prior to overwintering (Niva & Takeda 2003; Nielsen & Hamilton 2009), and the probability of female reproductivity observed from the same regime of decreasing photoperiod, also differed significantly across light hour availability ($X^2_{(8)} = 15.53$, P-value = 0.049) (Fig. 3). As the days grew shorter, BMSB showed a state of both metabolic and reproductive suppression (absence of mature oocytes during dissections) (Fig. 4), within the same light hour range of 14h to 13h (Fig. 2 & 3). Under controlled conditions (i.e., constant temperature with a step-wise decrease in photoperiod), our results strongly support that a critical photoperiod of 13.5h can induce suppression of metabolism and reproduction in BMSB.

Prior research has used the 13.5h day-length as the critical photoperiod to model population phenology and dynamics across various geographic regions (Nielsen et al. 2016), but our data suggest that this is too simplistic. These models assume that diapause is driven by photoperiod alone, independent of temperature in natural settings (Watanabe 1979; Yanagi & Hagihara 1980; Nielsen et al. 2016). Our data suggest that a critical photoperiod may be necessary, but is not sufficient to induce diapause in BMSB. Other seasonal factors such as food availability and fluctuating temperatures interacting with declining day-length, could potentially extend the point of diapause induction for BMSB, as observed in our outdoor population. This interaction of multiple environmental factors potentially influences the point at which BMSB begin to induce diapause. Metabolic suppression occurred naturally later in the season (Oct – Nov) (Fig. 1), where seasonal photoperiods were shorter than the laboratory determined critical photoperiod of 13.5h.

Mathematical models, based on climatic variables, predicting potential range expansion of BMSB demonstrated that minimum monthly temperature plays a significant role in determining range (Zhu et al. 2012). Supercooling ability is found in many insects, a process where no ice forms in the body even though it may be at temperatures well below freezing. The supercooling point (temperature at which ice forms in the body) serves as the theoretical temperature minimum at which insects can survive because once ice forms, the insect will die. Recent research by Cira et al. (2016), observed supercooling points (SCP) of BMSB in the eastern United States (Virginia & Minnesota), and found that the region of cold-acclimation, rather than geographical origin, strongly determined SCP ability. Our research performed in Washington State, showed similar supercooling points during the fall and winter to those found in Virginia and West Virginia. Our research also compared the SCP values of individuals from a naturally-acclimated outdoor population and an artificially-acclimated laboratory population. These comparisons allowed for a better understanding of how photoperiod and temperature influence cold tolerance in BMSB.

Supercooling points from the naturally-acclimated outdoor population showed significant difference between seasons (Fall = Sept – Nov, Winter = Dec – Feb) ($F_{(2)} = 3.32$, $P = 0.041$), while SCP from the artificially-acclimated laboratory population differed significantly between Light/Dark Hour (LD) regimes (LD = 16L:8D, SD = 12L:12D) ($F_{(1)} = 5.74$, $P = 0.023$). An analysis of both populations combined showed SCP values with significant differences across LD regime ($F_{(3)} = 4.55$, $P = 0.0048$), sex ($F_{(1)} = 9.49$, $P = 0.0026$), and mass ($F_{(2)} = 4.62$, $P = 0.033$) (Fig. 5). The addition of body mass as a covariate did not reduce the significance between sexes in the model. Supercooling points measured within the 'fall' group, had a mean SCP of 9.3 ± 0.7°F in females and 5.8 ± 0.7°F in males, while the 'short-day' group had a mean SCP of 4.9 ± 1.0°F) in females and 1.4 ± 1.2°F in males (Fig. 5). Our results for supercooling ability show that neither males or females supercool well enough to survive natural minimum temperatures recorded in eastern Washington during the sampling period (Fig. 7 & 8). The 'short-day' group were observed to have supercooling points significantly lower than field individuals measured during the coldest recorded instances in winter. This data suggests a relationship between cold-tolerance and diapause like those found in the Italian striped bug (*Graphosoma lineatum) (*Šlachta et al. 2002), in which diapause is necessary for developing cold tolerance, but is not the only driving factor. A subsequent process of cold acclimation is also necessary to achieve the maximum levels of cold tolerance in this species. The 'short-day' group illustrates this relationship, in which an acute and rapid cold-acclimation regime resulted in SCP values significantly lower than even those observed in the field population. The long-term and fluctuating rate of cold acclimation experienced by the field populations only produced SCP values similar to the non-diapausing 'long-day' lab population. No prior research has successfully provided quantifiable support to the relationship between diapause and cold tolerance in this species.

Supercooling is a known mechanism to survive adverse winter conditions, and no present research has found any true bug (Heteroptera) to survive freezing (Saulich & Musolin 2012). Though BMSB can supercool to very low temperatures, recent research by Cira et al. (2016) determined that the cold-tolerance strategy of BMSB is chill intolerance, in which adults die at significantly higher temperatures than they freeze. Our research produced comparable results. Survival rates began to

decline when temperature was reduced to 23°F, and showed a significantly reduced probability of survival between 14 and 5 °F. These temperatures at which survival declines are much higher than reported for supercooling ability. Naturally cold-acclimated adult BMSB in eastern Washington, exposed to minimum cooling temperatures of 32, 23, 14 and 5°F, showed significant differences in the probability of survival across temperature ($X^2_{(3)} = 9.02$, P-value = 0.028), sex ($X^2_{(1)} = 6.22$, P-value = 0.012), and mass ($X^2_{(1)} = 5.09$, P-value = 0.024). BMSB survival dropped significantly below a 50% threshold between the minimum temperatures of 14 and 5°F (Fig. 5).

To relate our experimental findings to BMSB populations overwintering in nature, we monitored ambient air temperatures at our field-maintained outdoor enclosure in Yakima, WA (Fig. 7), and a naturally-selected overwintering site in Walla Walla, WA (Fig. 8). At the latter site, we monitored air temperatures on the interior and exterior of cedar-shingle siding on a residential home. The observed minimum interior and exterior temperatures were 3.9°F and 1.3°F with an average temperature difference between the two of 2.5°F throughout the sampling period. While we were not able to directly monitor the mortality of overwintering BMSB at this site, observations of both the homeowners and a licensed pest manger (Dr. Albert Grable), detected negligible populations of BMSB emerging from the structure in the spring, which supports out assumptions of high mortality due to chill intolerance and minimum SCP (Fig. 7). BMSB are known to select for cool, dry, enclosed spaces as overwintering sites as a way to avoid contact with wet conditions, and man-made structures provide these exact specifications (Lee et al. 2014).

Our research emphasizes the importance of human structures in successful overwintering of BMSB in eastern and central Washington. BMSB in our area can complete their life cycle, enter diapause, and become cold tolerant. However, normal winter temperatures will kill BMSB – only with an artificially heated site could they survive. Growers may be able to minimize BMSB populations by carefully inspecting buildings and other thermally-buffered structures where BMSB might successfully overwinter.

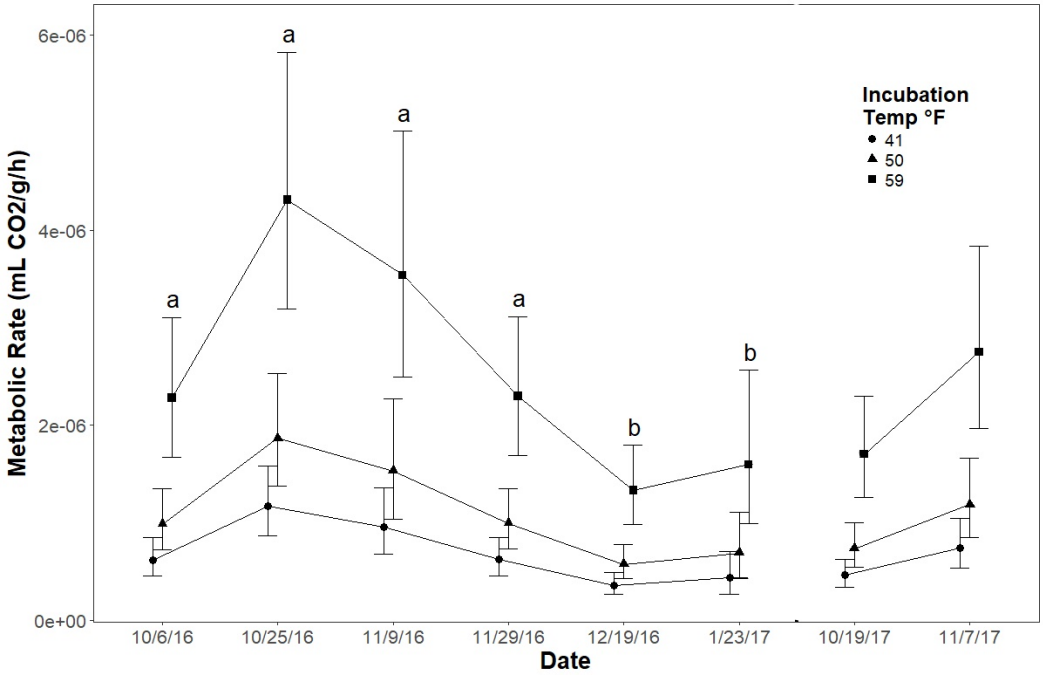

Figure 1. Mean metabolic rates including upper and lower confidence intervals, measured at 41, 50, and 59°F of overwintering adult brown-marmorated stink bugs (*Halyomorpha halys*). Metabolic rates of stink bugs, from the naturally-acclimated outdoor population, significantly differed across months in 59°F ($F_{(7)} = 6.4$, P <0.001), 50°F ($F_{(6)} = 3.9$, P <0.05), and 41°F ($F_{(7)} = 2.8$, P <0.05). When

comparing two sample points, those assigned different letters (i.e. a – b) are significantly different from each other.

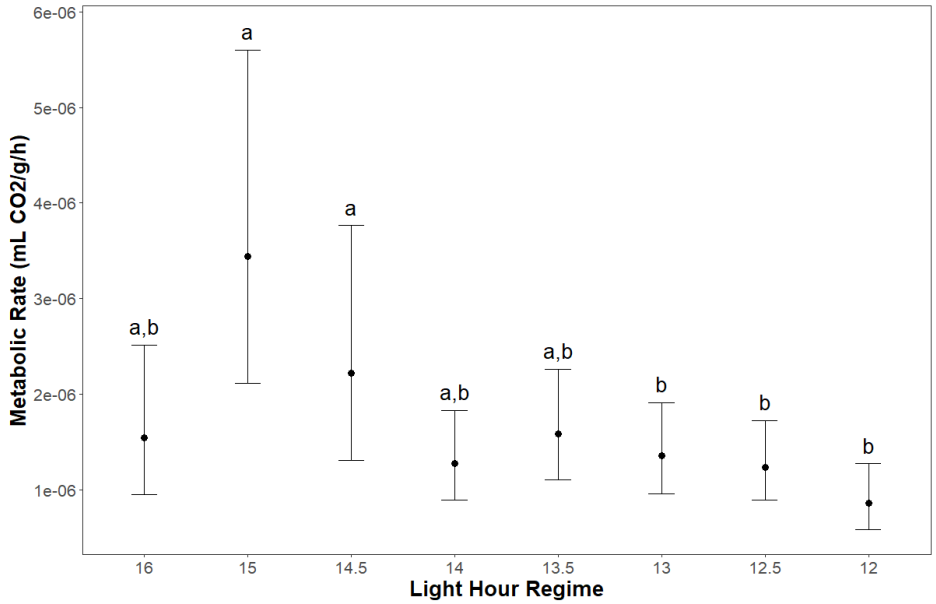

Figure 2. Mean metabolic rate including upper and lower confidence intervals, measured at 59°F of adult brown-marmorated stink bugs (*Halyomorpha halys*). Metabolic rates differed significantly across light hour availability ($F_{(7)} = 4.35$, $P < 0.001$). Metabolic rate response to incubation temperature showed an initial increase in metabolic rate during the photoperiod of 15 h, followed by a decreasing trend throughout the photoperiod transition to 12 h. When comparing two sample points, those assigned different letters (i.e. a – b) are significantly different from each other.

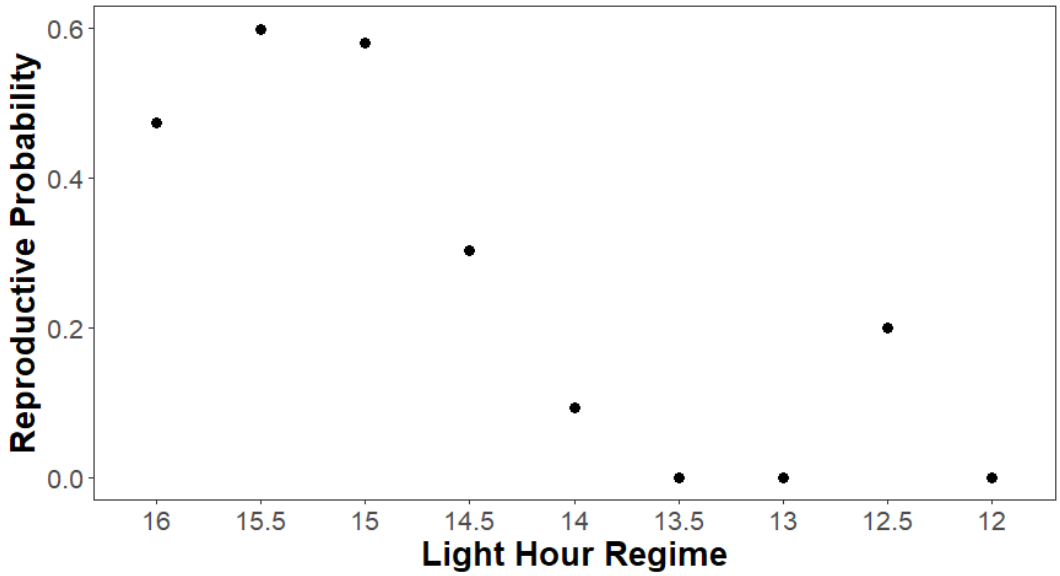

Figure 3. Probability of female Brown-marmorated stink bugs (*Halyommorpha halys*) being in a reproductive state at different day-lengths. The probability of reproductive BMSB differed across LD regime ($X^2_{(8)} = 15.53$, P-value = 0.049).

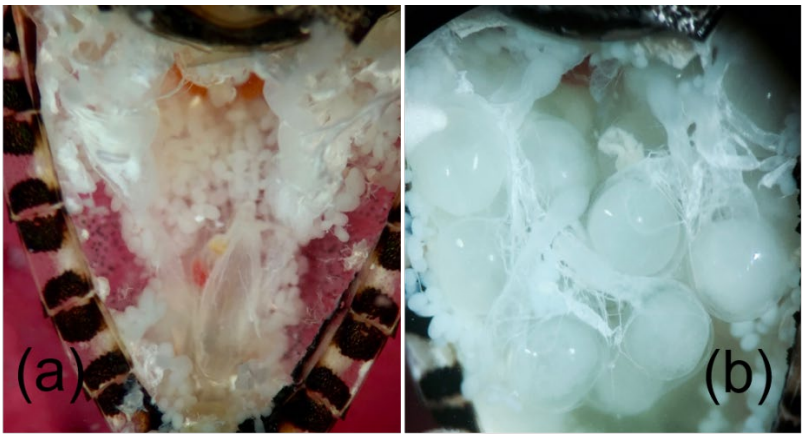

Figure 4. Female BMSB dissections demonstrating (a) lack of mature oocytes indicating a non-reproductive female (reproductive suppression during diapause) and (b) presence of mature oocytes indicating a reproductive female.

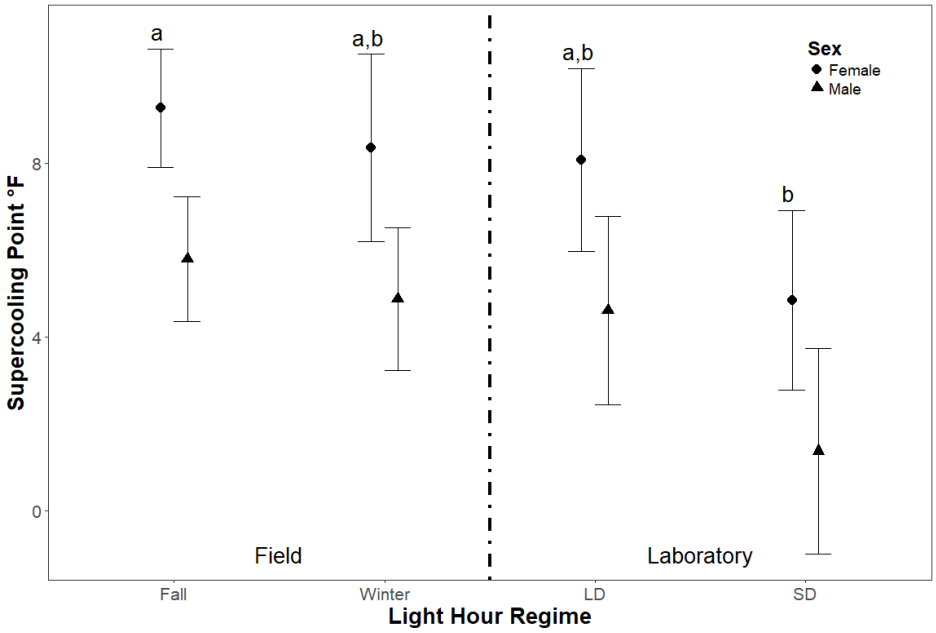

Figure 5. Mean supercooling points of adult brown-marmorated stink bugs (*Halyomorpha halys*) including upper and lower confidence intervals, that differed significantly across day length (LD = long day, SD = short day), sex and mass ($F_{(3)} = 4.55$, $P = 0.0048$; $F_{(1)} = 9.49$, $P = 0.0026$; $F_{(2)} = 4.62$, $P = 0.033$). When comparing two sample points, those assigned different letters are significantly different from each other for both males & females.

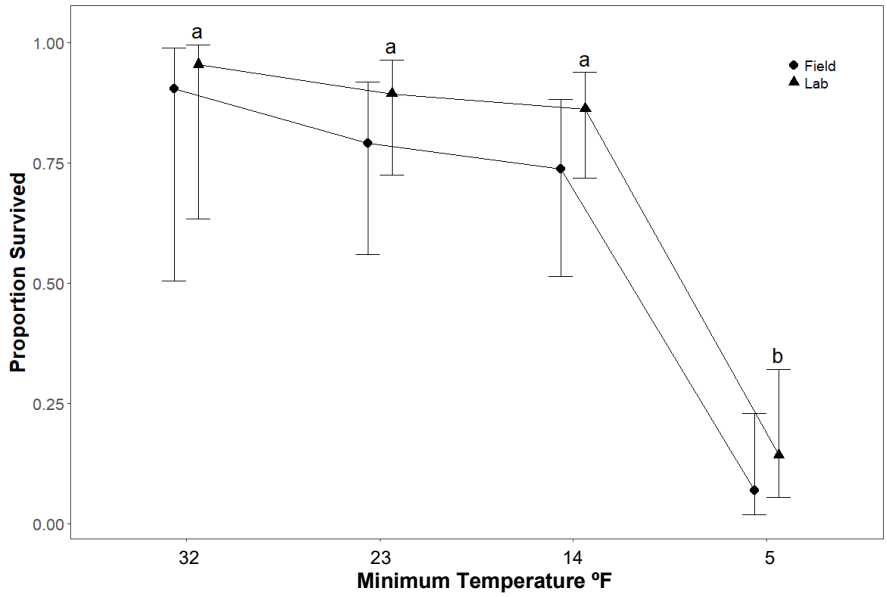

Figure 6. Survival of adult Brown-marmorated stink bugs (*Halyomorpha halys*) exposed to a series of minimum temperatures, show significant differences in the proportion survived across temperature treatments ($X^2_{(3)} = 51.598$, $p < 0.01$). Survival dropped significantly with exposure to 5ºF.

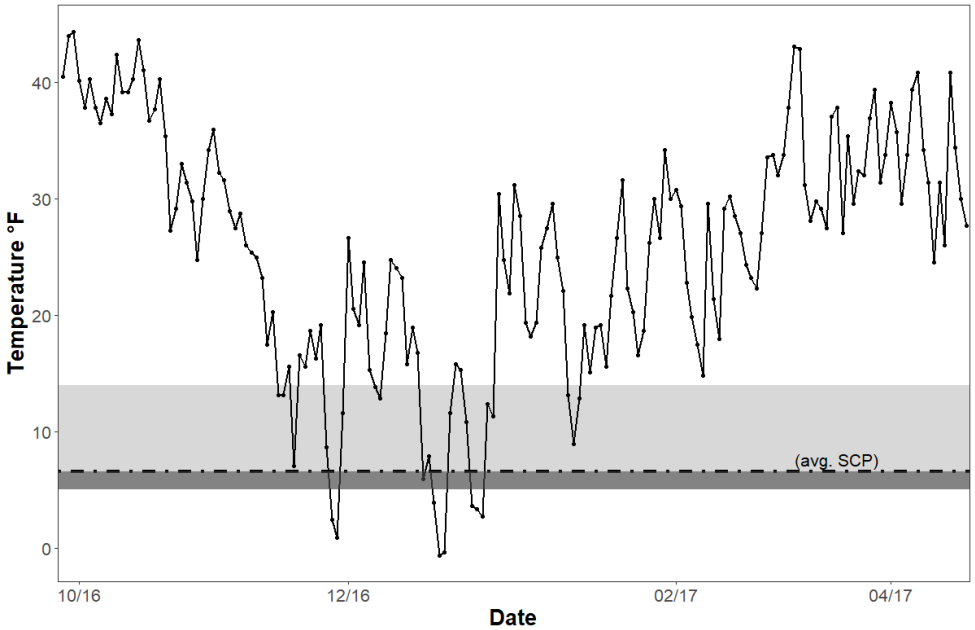

Figure 7. Daily minimum temperature (°F) recorded at an outdoor field enclosure in Yakima, WA from 10/28/16 to 4/15/17, with an observed minimum temperature of -0.65 °F on 1/6/17. The dashed line indicates the observed mean supercooling point of 6.6°F for BMSB, and the shaded area indicates the temperature range at which the probability of survival via chill intolerance is significantly reduced, with the shaded region below avg. SCP resulting in near full mortality.

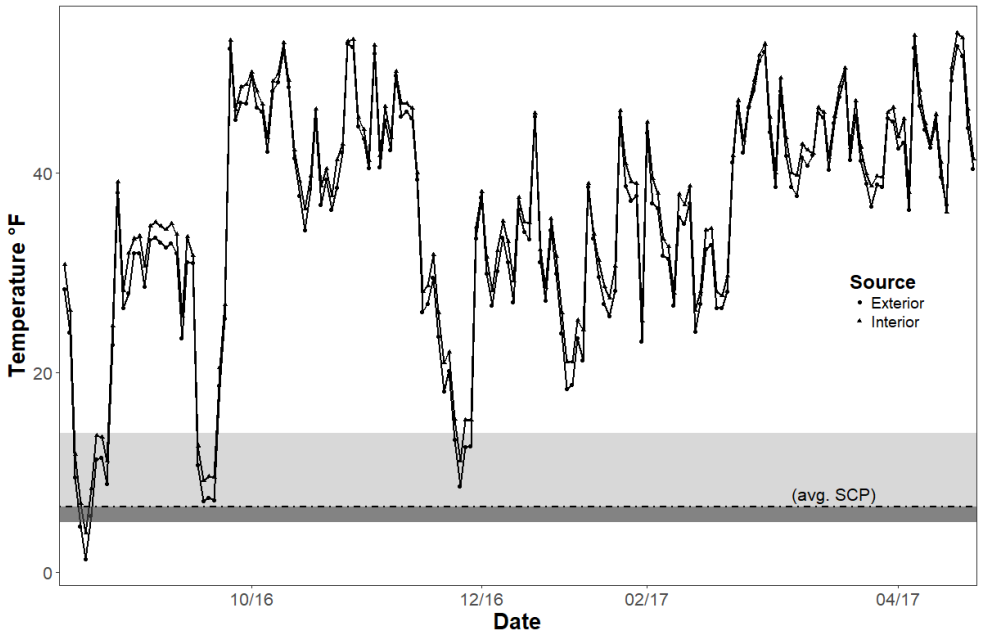

Figure 8. Daily minimum temperature observed in Walla Walla, WA, at a naturally-selected overwintering site of Brown-marmorated stink bug (*Halyomorpha halys*), from 10/27/16 to 4/15/17. BMSB selected the tight spaces beneath cedar-shingle siding as refuge, with observed minimum temperatures from the interior and exterior of 3.9°F and 1.3°F. The dashed line indicates the observed mean supercooling point of 6.6°F for BMSB, and the shaded area indicates the temperature range at which the probability of survival via chill intolerance is significantly reduced, with the shaded region below avg. SCP resulting in near full mortality.

EXECUTIVE SUMMARY

Our investigations into BMSB achieved all our objectives. We sought to describe both diapause regulation and cold tolerance in BMSB in eastern and central Washington State, both factors which have major impacts on the success of BMSB as it continues to expand northward in the Pacific Northwest. Metabolic suppression and cessation of egg development (indicators of diapause) occurred during the fall and winter, with deep suppression occurring late-October into November. Both metabolic rate and reproductive status of laboratory-maintained BMSB demonstrated that diapause is initiated by a day-length of 13.5h. This is similar to the critical day-length for diapause estimated by other studies, but this is the first systematic measurement of this value.

A comparison of laboratory and enclosure populations suggests that the 13.5h photoperiod is indeed necessary for diapause induction, but it is not sufficient on its own to induce diapause. Other seasonal factors (for example, food availability and fluctuating temperatures) interacting with declining day-length can delay diapause induction for BMSB, as we observed in our enclosure population. This is important, because a population of BMSB that experience postponed seasonal diapause induction, could risk obtaining insufficient levels of cold tolerance to survive the onset of cold temperatures, leaving them at higher risk of mortality.

The cold tolerance of BMSB in eastern WA is comparable to those in other parts of the USA. Supercooling ability changed little from fall into winter, and although males supercooled better than females (5.7°F vs. 9.2°F), an average minimum SCP value of 6.6°F for adult BMSB is not enough to survive the minimum freezing temperatures experienced in the region. BMSB also succumbed to chilling injury (death caused at temperatures before ice forms in the body) at temperatures between 14°F and the average minimum supercooling point of 6.6°F. Given their sensitivity to chill injury and their high supercooling points, BMSB are unlikely to survive the winter in central and eastern Washington State. We measured minimum air temperatures of -0.6°F and 1.3°F at our field-maintained outdoor enclosure in Yakima, WA and at our natural overwintering site in Walla Walla, WA, respectively. These were lower than the average SCP of 6.6°F measured from field-maintained individuals throughout the sampling period. For the population we maintained outdoors in Yakima, the mortality rate was 100%. While we were not able to directly monitor the mortality of overwintering BMSB at the Walla Walla site, observations of both the home owners and a licensed pest manger (Dr. Al Grable), detected no BMSB emerging from the structure in the spring, which supports our assumptions of high mortality due to chill intolerance and minimum SCP. BMSB often select cool, dry, enclosed spaces as overwintering sites, and these are often human-made structures.

Implications for control of BMSB

Our research further emphasizes the importance of human structures, especially buildings, to protect BMSB from the coldest winter temperatures. The cold winters of central and eastern Washington will significantly reduce or even eliminate BMSB overwintering in unheated sites. However, populations will persist where they can find thermally buffered or heated overwintering sites. Unfortunately, we expect that the mild winters of western Washington will not cause significant winter mortality so BMSB will likely become established unless other factors limit the species distribution.

Given that BMSB cluster during the winter in large aggregations, management of overwintering sites could provide an important area of management for the species. Eliminating populations overwintering in nearby buildings (especially heated buildings), would reduce the populations size in the subsequent growing season throughout the state.

FINAL PROJECT REPORT

Project Title: Kairomones for monitoring and control of native and invasive moths

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Other funding sources

Agency Name: Agriculture and Agri-Food Canada**,** Science and Technology Branch, Annual Call for Research, Development and Technology Transfer Proposals
Amt. requested: $90,000 USD total over three years (2016-2019)
Notes:

Total Project Request: **Year 1**: $55,000 **Year 2:** $59,000 **Year 3:** $65,000 **Year 4**: $0

Budget 1
Organization Name: ARS, USDA
Contract Administrator: Chuck Myers
Telephone: (510) 559-5769
Email address: chuck.myers@ars.usda.gov

Item	2015	2016	2017	2018
Salaries			38,000	0
Wages	17,500	20,000	22,000	0
Benefits	1,500	1,500	2,000	0
Supplies	6,000	7,500	8,000	0
Travel (local to research plots)	1,000	3,000	3,000	0
Miscellaneous			3,000	0
Plot Fees	3,000	3,000	3,000	0
Total	29,000	35,000	79,000	0

Footnotes: [a] $20,000 that was approved for 2016 ($59,000) was used in 2017 to support a visiting Italian scientist ($38,000 total for salaries).

Budget 2
Organization Name: Agriculture & Agri-Food Canada
Contract Administrator: Karen St. Martin, and Goewin Demmon
Telephone: 250-494-7711
Email address: KSM stmartink@agr.gc.ca, GD demmong@agr.gc.ca

Item	2015	2016	2017	2018[a]
Wages[1]	18,000	0	0	0
Benefits	2,500	0	0	0
Supplies	4,000	4,000	6,000	0
Travel[2]	1,000	0	0	0
Miscellaneous	500	0	0	0
Total	26,000	4,000	6,000	0

OBJECTIVES:

1. Optimization of one or more new kairomone attractants for North American tortricid pests, the work will include chemical analysis, electrophysiology work, and field testing of dosages and chemically-related compounds in lures and various traps.
2. Evaluate the attractiveness of new kairomones in apple and pear orchards situated within the major fruit growing districts of Washington State and British Columbia. This will include studying active space, trap design, trapping grid optimization, and development of long-lasting lures
3. Establish the correlation for each species of moth catches (both males and females) using new kairomones with larval densities and fruit injuries in the spring and summer.
4. Conduct small plot studies examining the potential of using new kairomones as a female moth removal tactic to manage these pests in apple and pear orchards.

SIGNIFICANT FINDINGS

- Volatile analyses of leafroller and budmoth infested apple shoots identified six specific compounds released only following herbivore damage.
- 2-phenylethanol (PET) and phenylacetonitrile (PAN) when used with acetic acid (AA) co-lures were identified from as being the most attractive to a large number of tortricid pests in North America, Europe, and New Zealand.
- A ternary blend and several binary ratios of three of these compounds were found not to significantly increase moth catches compared with the single most attractive compounds.
- Traps baited with the respective kairomone lures were highly effective in monitoring pest species in orchards but caught < 50% as many moths as sex pheromone-baited traps.

- Effective lures (10 mg red septa and long-lasting proprietary closed membrane cups) loaded with either PET + AA or PAN + AA were developed in collaboration with Trécé Inc (Adair, OK). All lures were found to be highly effective.
- PET+AA is more attractive for leafrollers and PAN+AA is more attractive to eye-spotted bud moth (ESBM). PET/PAN+AA was effective for both moth groups.
- Volatile captures by Valentino Giacomuzzi (Free University of Bolzen, Italy) of apple volatiles from foliage untreated or subjected to OBLR larval feeding were analyzed by Jim Mattheis (ARS, Wenatchee, WA) and provided a complete description of the array of important volatiles to be considered as attractants.
- No further discoveries of new attractive plant volatiles were made in extensive field trials, however, several compounds were found to reduce moth catch.
- Orchards treated with sex pheromone dispensers for OBLR were effectively monitored all season with PET+AA-baited traps.
- We showed that the new LR lures can be used in the same trap with lures for codling moth to monitor both pests with a single trap.
- Bucket traps using a solution of propylene glycol to retain moths were found to be the most effective low-maintenance trap for use in mass trapping.

- The project-developed a new longer-lasting AA lure that can be used over the entire season.
- A new combination host-plant volatile (HPV) lure (TRE1379) was developed that contains equal amounts of PET and PAN and has a broader activity for the various tortricid leafroller and budmoth pests that can exist in Washington and around the world. We demonstrated that this combination lure does not lose any efficacy for any of the tortricid pest species studied when compared to either of the two components used alone.
- We learned that combining the sex pheromone and either plant volatile in a trap is antagonistic and reduces captures of male leafrollers. However, including the acetic acid co-

lure cancels out this negative effect. Nevertheless, this suggests that the HPV+AA lure should be used alone instead of in combination with sex pheromone to monitor leafrollers, unlike what we previously found with pear ester and codlemone for codling moth.

- We refuted published reports that apple seedlings infested with leafroller larvae are more attractive than clean trees to adult leafrollers. Conversely, we demonstrated that several volatiles co-released with the attractants by infested apple seedlings are repellant to adult leafrollers.
- We showed that female moths captured on liners can lure males into traps and that with the oblique-banded leafroller this effect is more pronounced on hot melt pressure-sensitive adhesives compared with the standard Tangletrap adhesive. With codling moth this effect also occurs but the two adhesives do not differ.
- We demonstrated that adult feeding (water or honey water) by leafrollers is important for moths to allow full mating and egg laying to occur if the moths are unable to mate for 2 or more nights. Relative humidity is also an important environmental factor affecting moth longevity and realized fecundity.
- Using a proboscis-extension bioassay we have shown that the new attractants elicit a feeding response by both sexes of leafroller adults. This suggests that the volatiles may signal the presence of a food source for the moths.
- Characterization of apple volatiles and testing of their potential for leafroller attraction led to the discovery of a new attractant for codling moth
- Mass trapping studies were conducted in seven apple plots during 2017 for leafrollers and codling moth. Bucket traps baited with bisexual lures for both pests were used at a density of 24 per acre. Population densities of both pests were significantly reduced between generations, except in 1-acre plots surrounded by unmanaged and infested orchards.
- Studies with this range of tortricids demonstrated that the use of pear ester can be added to allow traps to monitor both codling moth and leafrollers in single traps.

- The combination PET+PAN kairomone lure was evaluated for light brown apple moth with help from Dr. Lucia Varela with UC Extension. Unlike in published reports the PET+AA outperformed PAN+AA for this species. This promotes the eventual commercial development of a single kairomone lure for world-wide leafroller monitoring.

RESULTS AND DISCUSSION

This project has been highly successful as eight peer-reviewed scientific articles have been published and several more are in preparation. The project was strongly supported by R&D from Trécé scientists to develop effective, long-lasting lures for PET, PAN, and AA. Various studies were conducted over the course of the study to determine optimal emission rates from lures. At present, a worldwide patent has been filed by New Zealand researchers for these lures. We have no information on the success of this patent or when commercial lures will be available to growers.

The results from each of the first three years of the project have already been presented in previous reports. In 2018, a no-cost extension was granted and several studies continued. In Washington we evaluated again the use of the TRE1379 lure that is loaded with both PET and PAN for our key leafrollers in western North America. In addition, studies were continued in Europe (Hungary and Sweden). Lures were shipped to Dr. Lucia Varela with UC Extension who evaluated them for the exotic pest, light brown apple moth in her area. She found that PET+AA was more effective than PAN+AA which contradicts the initial results from New Zealand. However, again the combination PET+PAN lure appears to provide a similar degree of effectiveness and we would expect that this is favorable for commercial development. At present, only the eye-spotted budmoth is more attracted to PAN+AA than PET+AA and the combination lure can likely still be used for this species.

Additional studies were conducted in 2018 to analyze apple volatiles at different times of the season and to evaluate whether leafroller adults are attracted to potted trees with actively-feeding leafroller larvae. Apple foliage in the field suffers a range of factors that cause micro abrasions on the foliage which then triggers the plant to release key volatiles. Our data does not support previous NZ claims that PET or PAN are con-specific attractants. Injured leaves actually release a large number of compounds and we have shown that some of these are repellent for leafroller adults. Instead, our studies show that only with pristine greenhouse plants do you see any attraction of adults to plants with larval feeding. The more sophisticated analytical techniques used at the ARS Laboratory in Wenatchee allowed us to see that acetic acid is released by plants regardless of leafroller feeding, likely due to microorganisms existing in the phyllosphere of plants and certain species synergized by the exudates from micro wounds. Thus, we have discredited many of the initial claims made by the New Zealand researchers and this will be useful to other scientists following our work.

Several studies were completed in Canada during 2018. Electrophysiological (EAGs) studies were performed on male and female, OBLR and PLR adults to understand the basis for a behavioural interaction (decreased catches) when sex pheromone and kairomone lures were combined in traps. Both OBLR and PLR males exhibited significantly stronger EAG responses when exposed to the sex pheromone component Z11-14:OAc and the aromatic 2-PET simultaneously, than when exposed to either compound alone. Both OBLR and PLR females showed significantly weaker EAG response when exposed to Z11-14:OAc and 2-PET simultaneously, than when exposed 2-PET alone. These EAG differences could explain smaller leafroller trap catches when these compounds are combined in traps. Proboscis Extension Reflex (PER) trials were performed on male and female, OBLR and PLR moths, to better understand the behavioural response to the range of aromatics identified in this study. Both sexes and species exhibited their strongest PER responses to 2-PET, followed by Z3-Hexenyl-benzoate > Benzyl alcohol > Indole > PAN. All other compounds failed to elicit a significant response. In contrast to its importance as an attractant in traps, acetic acid elicited weak PER responses and only when it was diluted to a concentration of 0.01%. The combination of AA + 2-PET elicited PER responses equivalent to PET alone. The PER responses of moths that had had their antennae removed were as strong as those with intact antennae. This indicates that olfactory receptors for these aromatics are present on body parts other than antennae, possibly the maxillary palps. Neither male nor female OBLR and PLR adults exhibited a PER upon exposure to the sex pheromone component Z11-14:OAc and simultaneous exposure to Z11-14:OAc and 2-PET did not significantly reduce the PER response.

Trapping studies in Canada in 2018 showed that leafroller and ESBM aromatic kairomones, 2-PET and PAN, respectively, had no significant affect on the response of adult Apple Clearwing Wing moths to their sex pheromone or kairomones. Multicomponent kairomones consisting of AA-PE-PET-PAN and ethyl butyrate, were used for season long monitoring of six apple pest species simultaneously. Only *Hedya nubiferana*, an invasive bud moth in Canada, showed any significant reduction in catch relative to catches with its own kairomone AA-PE.

During 2018 we developed a prototype device, ***Grey Ghost*** for eventual use in attract and kill of leafrollers (Fig. 1). This was developed by cutting small pieces of the EPA-registered fabric (ZeroFly screen, Vestergaard-Frandsen) impregnated with 0.4% deltamethrin and fashioning a hanger and placing the device in the canopy of the tree. These nets have recently been tested with the brown marmorated stink bug, *Halyomorpha halys*, in the eastern U.S. as much larger ghosts placed around orchards. In our laboratory, we are running various tests to examine whether moths contact the deltamethrin-treated netting, how long they contact it for, and whether they die after contact. Moths are not repelled and will land on the fabric, and bouts of exposure can last from 1-40 s. Moths

exposed for < 10 s will mostly die by 24 h. Sublethal exposure for the moths that survive shows a significant reduction in mating and egg laying. We established three 1-acre plots treated with 24 Grey Ghosts each at our USDA research farm. We compared moth catches of Pandemis and obliquebanded leafrollers in the center of these three plots plus paired untreated plots. Unfortunately, very few leafrollers were caught during 2018 perhaps because of the extensive 2017 mass trapping studies conducted in the same blocks in 2017. Therefore, this approach still needs to be evaluated. Similar trials conducted with codling moth suggested this ghostly approach might be effective and studies are continuing with that key pest.

Fig. 1. The Grey Ghost baited with lures for leafrollers and placed in the canopy of an apple tree, 2018.

Studies were conducted in 2018 to assess whether traps could be baited with the 4-way K for codling moth and with the PET/PAN+AA lure for leafrollers to promote the development of electronic smart traps. Unfortunately, the flight of leafrollers was very low in the orchard where the trial was conducted, and it remains unclear whether this approach is compatible.

EXECUTIVE SUMMARY

The volatile profile from apple foliage was carefully characterized through this project using the ARS facility (Dr. James Mattheis) in Wenatchee's expertise and a scientist (Dr. Valentino Giacomuzzi) in Italy. A survey of the key volatiles presented alone and in combination with acetic acid were evaluated for Pandemis and obliquebanded leafroller in a series of trials. We established that two volatiles, 2-phenylethanol and phenylacetonitrile when used in combination with acetic acid are attractive to both sexes of at least fifteen leafroller species (data supplied by Dr. Marco Tasin in Sweden and Dr. Julia Jósvai in Hungary) including all the important pest species in the western North America. The project was strongly supported by R&D from Trécé scientists to develop effective, long-lasting lures for all three volatiles. Various studies were conducted over the course of the study to determine optimal emission rates from lures. At present, a worldwide patent has been filed by New Zealand researchers (Drs. Ashraf El-Sayed and Maxwell Suckling) for use of these lures. We have no information on the success of this patent or when commercial lures will be available to growers. There remains considerable interest to have these lures made available to monitor orchards treated with sex pheromones in Washington State. Working in orchards with sizeable populations of leafrollers was a major limiting factor impacting this project. Our limited data suggests that these lures would have potential if used for lure and kill technologies, but more field validation following our project is needed.

FINAL PROJECT REPORT

Project Title: Fire blight resistance and fruit quality in new Washington cultivars

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Cooperators: none

Other funding sources
Agency Name: **USDA-NIFA-Specialty Crop Research Initiative**
Amount awarded: **$10M** (Sept 1, 2014 – Aug 31, 2019)
Notes: **Title** 'RosBREED: Combining disease resistance with horticultural quality in new rosaceous cultivars'; Norelli: Co-PI and Team Leader for Pathology; Evans: Co-PI; Peace: Co-Project Director

Total Project Funding: $106,110

Budget History:

Item	Year 1:	Year 2:	Year 3:
Salaries[1]	6,132	6,255	6,380
Benefits	491	500	510
Wages[2]	4,000	9,152	9,518
Benefits	392	897	933
Supplies[3]	6,050	7,050	6,150
Travel[4]	560	1,120	1,120
Plot Fees[5]	4,500	4,000	3,100
Miscellaneous[6]	10,300	3,000	14,000
Total	32,425	31,974	41,711

1: summer student for trait measurement, Kearneysville, **2**: wages for time-slip labor for orchard management and trait measurement, Wenatchee, **3**: combined total orchard, greenhouse and laboratory supplies for both Kearneysville and Wenatchee, **4**: travel to field plots in Wenatchee, **5**: Kearneysville and Wenatchee, **6**: genotyping, Kearneysville and Pullman.

ORIGINAL OBJECTIVES

1. Determine the best sources of fire blight resistance in *Malus sieversii* to incorporate in the WSU apple breeding program.

2. Determine fire blight resistance levels in RosBREED reference germplasm for current use in the WSU apple breeding program.

3. Develop DNA tests to enable the fire blight resistance of 'Enterprise' and 'Splendour' to be efficiently evaluated in seedlings and to evaluate genetic resistance in current elite selections.

The overall goal of this project was to enable selection of new Washington apple varieties that are fire blight resistant and have superior fruit quality, as soon as possible. The specific goal of the first objective was to select among the highly resistant *M. sieversii* accessions the best parents to use in the WSU apple breeding program (**WASB**) for strong fire blight resistance based upon the accession's fruit quality. The goal of the 2nd objective was to determine the fire blight resistance-influencing loci (**FBL**) among the current parents and seedling of the WABP. The goal of the third objective was to develop and evaluate DNA markers for known FBL among select parents that have been used in the WABP.

This research addressed the 2015 Apple Crop Protection High Priority Research topic "Fire Blight" and the Apple Horticulture High Priority Research topic "Improved scion and rootstock genetics".

SIGNIFICANT FINDINGS

- Nine *M. sieversii* accessions were identified as potential sources of high-level fire blight resistance for use in the Washington apple breeding program (**WABP**) that were resistant to fire blight shoot and blossom infection (Obj. 1)
- Crosses were made with four fire blight resistant *Malus sieversii* accessions to incorporate higher levels of fire blight resistance into the WABP (Obj. 1)
- 1650 trees of 556 elite cultivars and their seedlings were challenged with fire blight in 2016 and 2017 to determine their resistance and facilitate the discovery of fire blight tolerance genes among the existing seedlings and selections of the WABP (Obj. 2)
- Two DNA markers were developed for the fire blight resistance locus on chromosome 7 of 'Enterprise'
- Two DNA markers were developed for the fire blight resistance locus on chromosome 5 of 'Splendour'

RESULTS & DISCUSSION

Obj. 1: Determine the best sources of fire blight resistance in *Malus sieversii* (wild progenitor of the domestic apple) to incorporate in the WSU apple breeding program

A previous WTFRC project identified *M. sieversii* accessions that are highly resistant to fire blight shoot infection. This project evaluated 21 shoot blight resistant accessions to for their resistance to blossom blight and fruit quality to determine the best accessions to incorporate into the WSU apple breeding program.

Evaluation of blossom blight susceptibility: Twenty accessions were evaluated for their resistance to fire blight blossom infection. Of these, 6 accessions (PI657054, PI657085, GMAL3552.v, GMAL3608.h, GMAL4211.a, GMAL4211.d) were found to have unacceptably high levels of blossom blight susceptibility in either the West Virginia or Washington trails.

Evaluation of fruit quality: Fruit of the 21 accessions were harvested from the research plot at USDA-ARS Kearneysville, WV at Cornell starch stage 3 and shipped to the TFREC, Wenatchee for evaluation. Fruit quality was evaluated using the full range of instrumental and sensory traits by the WSU apple breeding program (WABP) on arrival and after two months of refrigerated air storage.

None of the *M. sieversii* accessions in this trial have commercially acceptable fruit quality. However, fruit quality characteristics were used to select among the accessions those most suitable for use in the WABP. In 2016 crosses were made with 3 accessions, including GMAL4002.k. In 2017 an additional cross was made with GMAL3688.c. Some of the seedlings from the 2016 and 2017crosses were evaluated for their resistance to fire blight. Selected seedlings are currently being grown to maturity.

Obj. 2: Determine fire blight resistance levels in RosBREED reference germplasm

While Objective 1 focused on identifying the best sources of fire blight resistance for future use as parents in the WABP, the purpose of Objective 2 was to leverage resources previously developed as part of RosBREED 1 to identify genetic factors associated with fire blight resistance/susceptibility. Although complete immunity or resistance may not be available in the current WABP or RosBREED material, we know that there is a gradient of susceptibility among this material that ranges from highly susceptible to "tolerant". An example of a tolerance is 'Delicious', which at times can become infected with fire blight but rarely are the losses due to this disease devastating in nature. On the other hand, individuals like 'Gala' or 'Jonathan' are highly susceptible and losses can be very severe. Although "tolerance" is potentially a useful type of resistance, the genetic factors controlling "tolerance" are not understood/known. Our goal in this project was to identify fire blight resistance-influencing loci by determining levels of resistance to fire blight in the RosBREED apple germplasm, which is a collection of pedigree connected elite cultivars and their seedlings.

Because fire blight is a sporadic disease from year to year and in its distribution within the orchard, reliable evaluation of fire blight resistance requires artificial challenge of test plants with the fire blight bacteria. Trees of the RosBREED reference germplasm have been grafted onto M.111 rootstock and planted at WSU's Columbia View Orchard. The reference germplasm included several cultivars known to be either susceptible or resistant to fire blight that served as controls to ensure that minimum disease pressure thresholds were achieved in the tests. Vigorously growing shoots will be challenged by dipping a pair of scissors in a suspension of the bacteria and then cutting the youngest leaves of the shoot tip. Resistance will be determined by measuring the percent of the current seasons shoot length that becomes infected. Because economic losses from fire blight are the result of the death of young trees and woody tissue, rating cultivar resistance based upon progression of disease in shoot tissue has proven a reliable method of accessing fire blight resistance.

The number of shoots inoculated per tree ranged from 3-10 with an average of about 6 shoots per tree. The resistance response to fire blight inoculation was variable ranging from highly susceptible to resistant. The average proportion of healthy tissue ranged from 0.0 (all inoculated shoots were killed) to 1.0 (all inoculated shoots did not develop symptoms of fire blight infection). Of the 556 individuals evaluated, about 16% had an average proportion of healthy tissue of ≤ 0.25, which is indicative of high susceptibility. In contrast, about 55% of individuals had average proportions of healthy tissue ≥ 0.25 but <0.75 and should be considered susceptible. Approximately, 29% of individuals had average proportions of healthy tissue ≥ 0.75 and should be considered tolerant to fire blight. Average age of

wood infected ranged from 0.0 to 2.5 with average ratings of >1.0 indicating that on average fire blight moved into the previous season's growth. 63.8% individuals had a maximum rating ≥ 2.0, indicating that in at least one challenged shoot the pathogen moved into 2 year or older tissue. Higher maximum ratings (>1.0) indicate more severe infections.

The fire blight resistance / susceptible results for many of the apple varieties grown in Washington State are given in Table 2. Among the cultivars listed in Table 2, Enterprise and Tsugaru were the most resistant to fire blight. Aurora Golden Gala, Cosmic Crisp®, Delicious, Empire, Golden Delicious, and Fuji were classified as moderately resistance in both years, with Fuji performing better than anticipated. GingerGold and Winter Banana were the most susceptible cultivars to fire blight. Gala and Granny Smith were also consistently rated either highly susceptible or moderately susceptible. The most variable cultivars were Piñata® and Spartan which were evaluated as highly susceptible one year and moderately resistant the other. The complete data will be available soon through the WSU Tree Fruit Extension website and the WSU Decision Aid System once the peer-reviewed manuscript* describing the research is accepted for publication.

Although information on the fire blight resistance of apple cultivars is available on the worldwide-web, information on new cultivars has been lacking and much of the available information is anecdotal, based on personal accounts rather than research. This study was the first systematic fire blight evaluation of many new cultivars with controlled pathogen challenge in replicated field trials over multiple years. The results of this study have been written up and submitted for peer review publication in the *Plant Pathology* journal. The information will also be made available to cooperative extension and commercial farm advisors.

Furthermore, the results are currently being analyzed by Sarah Kostick, a graduate student with Kate Evans, using FlexQTL software to identify and predict fire blight resistance-influencing loci. This software was developed and used in the "RosBREED" project utilizing high-resolution genome scans that were previously completed by the project and our analysis will draw upon expertise from the project. The fire blight resistance-influencing loci identified will allow for the future development of additional DNA tests for fire blight resistance.

Table 2. Fire blight resistance and susceptibility of many of the apple varieties grown in Washington State. Resistance was evaluated by the proportion of the current season's shoot length blighted following challenge with the fire blight pathogen (*Erwinia amylovora*). The "control" cultivars used to determine resistance class are highlighted in bold (see sidebar).

Variety	Resistance/Susceptibility Class (2016 / 2017)
'Aurora Golden Gala'	MR / MR
'Cripps Pink' (Pink Lady®)	I / MS
'Cameo'	MS / MR
'Delicious'	**MR/MR**
'Empire'	MR / MR
'Enterprise'	HR / MR
'Fuji'	MR / MR
'Gala'	MS / HS
'GingerGold'	HS / HS
'Golden Delicious'	MR / MR
'Granny Smith	MS / HS
'Honeycrisp'	MS / MR
'Jonagold'	MR / MS
'Jonathan'	**HS/HS**
'McIntosh'	**MS/MS**
'Minnewashta' (Zestar!®)	MS / I
Pinova' (Piñata®)	MR / HS
'Rome Beauty'	MS / MR
Russian seedling #12740-7A	**HR/MR**
'Spartan'	HS / MR
'Splendour'	MR / I
'Tsugaru'	HR / MR
'WA2' (Sunrise Magic®)	MS / MR
'WA38' (Cosmic Crisp®)	MR / MR
'Winter Banana'	HS / HS

Key to resistance / susceptibility class codes:

HR: highly resistance (significantly different from both MS control cultivar (McIntosh) and HS control (Jonathan)).

MR: moderately resistant (significantly different from HS control, but not MS control)

I: intermediate (not significantly different from any of the control cultivars)

MS: moderately susceptible (significantly different from HR control cultivar (Russian seedling #12740-7A), but not the MR control cultivar (Delicious))

HS: highly susceptible (significantly different from both MR and HR control cultivars)

Obj. 3: Develop DNA tests to enable the fire blight resistance of 'Enterprise' and 'Splendour' to be efficiently evaluated in seedlings and to evaluate genetic resistance in current elite selections

Although most of the genetic factors controlling "tolerance" to fire blight in the WABP are currently not known, we do know that some of the parents previously used in the program, such as 'Enterprise', and 'Splendour' are tolerant to fire blight. The goal of Objective 3 was to develop DNA tests for the genetic factors controlling fire blight resistance in these parents to enable efficient evaluation of their progeny seedlings and evaluate the genetic resistance of current elite selections within the WABP.

Before a DNA test could be developed the loci needed to be genetically characterized, and identifying the loci controlling the fire blight resistance of 'Enterprise' and 'Splendour' was beyond the scope of this project. However, this was part of both the USDA-NIFA RosBREED (which targeted 'Splendour') and the European FruitBreedomics (which targeted 'Enterprise') projects. We originally anticipated results from these projects to be available to us to develop DNA tests within the third year of this project. Unfortunately, the loci took longer than anticipated to identify due to unforeseen complexities in the inheritance of fire blight resistance and we therefore requested a no cost extension to complete the development of the DNA in 2018.

A genetic locus controlling fire blight resistance in 'Enterprise' was identified on chromosome 4 (van de Weg *et al.* 2018). Fei Xiong Luo, a graduate student in Cameron Peace's program, developed and evaluated several DNA markers for this locus by comparing DNA test results of seedlings with results from direct challenge of seedlings with the fire blight pathogen. In the end, two distinct seedling populations were used to validate the 'Enterprise' DNA test.

Using new genomics methods candidate loci for fire blight resistance in 'Splendour' were identified in the RosBREED project. Stijn Vanderzande, a post-doctoral RosBREED scientist working with Cameron Peace, similarly developed and evaluated DNA markers for a fire blight resistance locus found on chromosome 5.

The DNA tests for the fire blight resistance loci from 'Enterprise' and 'Splendour' were both used successful in RosBREED experimental crosses this past month and will be available for use in the WABP in 2019.

LITERATURE CITED

Eric van de Weg, Mario Di Guardo, Melanie Jänsch, Didier Socquet-Juglard, Fabrizio Costa, Isabelle Baumgartner, Giovanni A.L. Broggini, Markus Kellerhals, Michela Troggio, François Laurens, Charles-Eric Durel, and Andrea Patocchi. 2018. Epistatic fire blight resistance QTL alleles in the apple cultivar 'Enterprise' and selection X-6398 discovered and characterized through pedigree-informed analysis. Molecular Breeding 38:5. https/doi.org/10.1007/s11032-017-0

EXECUTIVE SUMMARY

Fire blight is a devastating disease affecting the Washington apple industry. The disease can kill young trees outright or result in permanent structural damage to mature trees. Spray application of antibiotics is currently the most effective control for fire blight however its effectiveness is limited by the development of streptomycin resistance in the pathogen and its lack of efficacy in controlling the shoot blight phase of the disease. Breeding for resistance to fire blight offers one of the most effective and sustainable options for managing this disease. The goal of this project was to develop the resources and tools necessary to effectively incorporate fire blight resistance into the WSU apple breeding program (**WABP**).

Four significant accomplishments of this project have made this goal attainable:

1) A DNA marker was developed for the major fire blight resistance locus present in 'Enterprise' which will facilitate the efficient screening of 'Enterprise' progeny within the WABP.
2) A DNA marker was developed for a fire blight resistance locus present in 'Splendour' which will also facilitate the efficient screening of 'Splendour' progeny within the WABP.
3) Four crosses were made to incorporate a high-level fire blight resistance from wild *Malus sieversii* into the WABP. Although several additional crosses will be required before the progeny of these crosses are expected to have superior fruit quality, the process has begun.
4) The fire blight resistance of over 500 elite cultivars and their seedlings was determined under Washington State growing conditions. This information will not only be used for extension purposes and selecting future WABP parents but is also being analyzed to identify fire blight influencing loci within current seedlings of the WABP.

The development of fire blight resistant scion cultivars is a long-term goal. The recent success of fire blight resistant apple rootstocks demonstrates that fire blight resistant cultivars can be developed and that it is an important trait for the apple industry. However, the acceptance barrier for a fire blight resistance scion cultivar will be much higher because unlike rootstocks which are selected based upon horticultural performance, scion cultivars are selected based upon market demand. None the less, the increased efficiency of DNA informed breeding and the growing trend toward new cultivars in the apple industry indicate that the scion cultivars of the future could, and should, be more resistant to fire blight. Incorporating fire blight resistance into the WABP apple breeding program will lead to the release of new cultivars with fire blight resistance similar to or greater than that of 'Delicious' and thereby greatly reduce the occurrence of fire blight and reduce the need for high-priced applications of antibiotics.

CONTINUING REPORT **Year: 2018**

Project Title: 2018 WTFRC apple pesticide residue study

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Since 2011, the Washington Tree Fruit Research Commission (WTFRC) has conducted annual trials to evaluate pesticide residues on 'Gala' apples. This year, we applied seven insecticide/acaricides, four fungicides, and one plant growth regulator with a Rears Pak-Blast sprayer according to either an "aggressive" protocol intended to simulate a worst-case scenario with the highest possible residues while observing label guidelines (maximum label rates at minimum retreatment and pre-harvest intervals) or a "standard" protocol following more typical industry use patterns for rates and timings. Each treatment protocol was sprayed at both 100 (concentrate) and 200 (dilute) gallons of water per acre while holding the rate of pesticide per acre constant. Fruit samples were collected at commercial maturity on August 29 and delivered the next day to Pacific Agricultural Labs (Sherwood, OR) for chemical residue analysis.

TRIAL DETAILS

- 11th leaf 'Pacific' Gala / M.9 Nic.29 trained to central leader/spindle on 3' x 10' spacing
- 2 x 25 gal Rears Pak-Blast sprayer calibrated to 100 or 200 gal / acre
- All pesticides applied with 8 oz Regulaid / 100 gal water / acre
- No measurable precipitation recorded during trial except 0.01" of rain on Aug 25 (4 days before harvest)

Measured residues vs. maximum residue levels (MRLs) for uniformly applied STANDARD industry apple pesticide programs in 100 or 200 gal water/acre utilizing typical rates, timings, and retreatment intervals. 'Gala'/M.9 Nic.29, Rock Island, WA. WTFRC 2018.

Chemical name	Trade name	Application rate	Application timing(s)	100 gal/acre	200 gal/acre	US MRL[1]	Lowest export MRL[1]
		oz per acre	*dbh*	*ppm*	*ppm*	*ppm*	*ppm*
Ethephon	Ethephon 2SL	36	58	**0.22**	**0.35**	5	0.01 (UAE)
Spinetoram	Delegate WG	7	35 & 21	0.022	.031	0.2	0.05 (many)
Cyantraniliprole	Exirel	13.5	35 & 21	0.16	0.22	1.5	0.8 (many)
Spinosad	Entrust	3	35 & 21	0.029	0.091	0.2	0.1 (many)
Tolfenpyrad	Bexar	27	35 & 21	**0.46**	**0.65**	1	0.01 (many)
Myclobutanil	Rally 40WSP	10	35 & 21	**0.27**	**0.41**	0.5	0.01 (UAE)
Novaluron	Rimon	32	35 & 21	0.38	0.52	3	2 (CAN, TAI)
Fluxapyroxad	Merivon	5.5	28	0.054	0.11	0.8	0.8 (Canada)
Pyraclostrobin	Merivon	5.5	28	0.033	0.071	1.5	0.5 (many)
Etoxazole	Zeal	2	28	0.054	**0.089**	0.2	0.07 (many)
Difenoconazole	Inspire Super	12	28	**0.025**	**0.024**	5	0.01 (India)
Cyprodinil	Inspire Super	12	28	**0.061**	**0.054**	1.7	0.05 (INDO)
Ziram*	Ziram 76DF	96	21	1.26	0.36	7	2.5 (Taiwan)
Fenpropathrin	Danitol	18	14	**0.33**	**0.41**	5	0.01 (many)

[1] Top markets for WA apples with established MRLs; 16 October 2018. http://www.nwhort.org/AppleMRLs.html, https://www.globalmrl.com/

* Dithiocarbamate residues cannot be directly measured; total Ziram values are estimates based on analysis of the degradation product CS_2

Measured residues vs. maximum residue levels (MRLs) for uniformly applied AGGRESSIVE industry apple pesticide programs in 100 or 200 gal water/acre utilizing maximum labeled rates, and minimum preharvest and retreatment intervals. 'Gala'/M.9 Nic.29, Rock Island, WA. WTFRC 2018.

Chemical name	Trade name	Application rate	Application timing(s)	100 gal/acre	200 gal/acre	US MRL[1]	Lowest export MRL[1]
		oz per acre	*dbh*	*ppm*	*ppm*	*ppm*	*ppm*
Ethephon	Ethephon 2SL	36	35	**0.42**	**0.57**	5	0.01 (UAE)
Spinosad	Entrust	3	21 & 7	0.085	**0.11**	0.2	0.1 (many)
Etoxazole	Zeal	3	14	**0.081**	**0.13**	0.2	0.07 (many)
Spinetoram	Delegate WG	7	14 & 7	**0.062**	**0.084**	0.2	0.05 (many)
Cyantraniliprole	Exirel	20.5	14 & 5	0.20	0.40	1.5	0.8 (many)
Fluxapyroxad	Merivon	5.5	7 & 1	0.32	0.51	0.8	0.8 (Canada)
Pyraclostrobin	Merivon	5.5	7 & 1	0.30	0.47	1.5	0.5 (many)

[1] Top markets for WA apples with established MRLs; 16 October 2018. http://www.nwhort.org/AppleMRLs.html, https://www.globalmrl.com/

NOTE: Residue results for several materials are not reported in this table due either erroneous application or lack of product.

DISCUSSION

As in the previous 7 years of studies, no residue from a pesticide applied following label-recommended rates and timings exceeded the US Environmental Protection Agency's tolerance. Pesticides which produced residues above MRLs for important export markets included **Ethephon 2SL, Bexar, Rally 40WSP, Zeal, Inspire Super, Danitol, Entrust, and Delegate WG**. In most cases, these potentially problematic findings have less to do with the actual amount of residue generated by these products than with the fact that some nations have very stringent MRLs; these tolerances are frequently set at the limit of quantitation (LOQ), or smallest amount that can be reliably measured by modern analytic methods, essentially creating a *de facto* ban on importation of apples treated with these products. Growers hoping to market their fruit to such nations should consider avoiding use of those materials altogether.

In general, we found higher residues in 2018 from dilute (200 gal water/acre) than concentrate (100 gal water/acre) applications, suggesting that the higher carrier volume improved coverage. This trend is consistent with our results from comparing 200 gal/acre (concentrate) vs. 400 gal/acre (dilute) applications in a 2016 cherry study, but counter to results from our 2017 apple and 2018 cherry studies, where concentrate applications generally produced higher residues than dilute. These contradictions make interpretation of our cumulative data set quite difficult; simply put, our results to date have shown no consistent effect of water carrier volume on pesticide residues.

Reports from previous pesticide residue studies on apple and cherry which provide a broader context for these results are available on the WTFRC website at www.treefruitresearch.com. We encourage growers and consultants to stay abreast of current information on international MRLs, which often

change in response to trade negotiations and/or political developments. For more information, visit the Northwest Horticultural Council website at www.nwhort.org.

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, or a guarantee of similar results regarding residues for any user. Apple growers should consult their extension team members, crop advisors, and warehouses to develop responsible pest control programs.

CONTINUING PROJECT REPORT **YEAR**: 1 of 2
WTFRC Project Number: CP-18-100

Project Title: Data to model apple airblast spraying drift exposure levels

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Cooperators: Dr. Milt Teske (Continuum Dynamics Inc., Princeton, NJ)

Budget: Year 1: $96,108 **Year 2: $66,334**

Other funding sources

Agency Name: U.S. Forest Service
Amt. requested/awarded: $39,315
Notes: equipment cost share

Agency Name: Washington State Specialty Crop Block Grant (SCBG) for FY2017-FY2019
Amt. requested/awarded: $120,000
Notes: To fund the modeling effort (see 'Justification'). Awarded to Washington Tree Fruit Research Commission (Lead: Mike Willett)

Agency Name: USDA Office of Pest Management Policy
Amt. requested/awarded: $187,000
Notes: To fund the modeling effort (see 'Justification').

Agency Name: U.S. Forest Service
Amt. requested/awarded: $23,000
Notes: To fund the modeling effort and canopy characterization (see 'Justification').

Total in-kind and other funding sources: $369,315

Budget 1

Organization Name: WSU-IAREC **Contract Administrator:** Katy Roberts

Telephone: 509-335-2885 **Email address:** arcgrants@wsu.edu

Item	2018	**2019**
Salaries	21,000	21,840
Benefits	11,668	12,134
Wages	14,880	15,475
Benefits		
Equipment	12,000	
Supplies	34,955	15,280
Travel	1,605	1,605
Miscellaneous		
Plot Fees		
Total	96,108	**66,334**

Footnotes: (Year 1) Salaries of $21,000 plus $8,364 benefits will support a postdoctoral researcher who will work closely with the PIs in planning and conducting field experiments, laboratory analysis, data analytics and reporting. $12,318 (including 22.2% benefit) requested to support field work pertinent to field deposition studies (20 trials/crop/season) and canopy characterization (8h/day/person x 7 personnel x 12 days). Additionally, $5,866 requested to support lab work (fluorometry analysis) pertinent to field deposition trials (8 h/day/person x 4-person x 10 days). $1,605 is requested to travel to field sites (100 mile return trip @ 0.535/mile x 15 trips with two vehicles). $34,955 is requested for material and supplies that include procurement of deposit samplers (flat cards, string samplers, artificial foliage samplers), fluorescent tracer, labels, gloves, zip ties, general field supplies, vials, ethanol, chem-wipes and general lab supplies as well as the tractor & sprayer rentals. The material cost also includes $12,675 procurement of 8-m string tower drift poles ($3975+250 S&H/set of 3 x 3 sets). Funds of $12,000 are requested to procure Plant Canopy Analyzer (LAI-2200C) from LI-COR Biosciences.

Year 2 cost includes all the above except the cost of equipment and material supplies available in year-1. Please note that salaries are inflated 4% rate in year-2.

1. OBJECTIVES

The primary objective of this project is to generate data for validation of ***a mechanistic airblast spray drift model*** currently being developed (see 'Justification' of original proposal) to estimate exposure values to assess risk from airblast spraying in 'central leader' apple. Such model would be an improvement over **'worst-case scenario'** estimates currently used by EPA. Overall, measurements will be made up to 600 ft downwind from the apple block (central leader) to assess drift and relate it to key meteorological parameters. Studies have been conducted at dormant (***obj. #1 year-1 efforts***) and will be conducted in full canopy (***obj. #2 year-2 efforts***) growth stages. The fluorometry analysis based deposition data along with the pertinent canopy and environmental conditions will be used to validate the mechanistic model being developed to estimate airblast sprayer drift/exposure levels. Per Table 1, this project is on schedule of what was proposed for year-1.

Table 1. Project activity schedule and quarterly benchmarks (*Planned activities; √ Completed activities).

Objectives	Year 1-2				Year 2-3			
	Q3	Q4	Q1	Q 2	Q3	Q4	Q1	Q2
1. Airblast sprayer drift/exposure levels assessment up to 600 ft downwind from 'central leader' apple blocks during dormant stage								
Task 1.1: Field block scouting and setting up of the field samplers and metrological stations	√	√	√					
Task 1.2: Canopy mapping via standard ground-reference methods, data processing		√	√, *					
Task 1.3: Conduct field trials (20 runs)		√						
Task 1.4: Fluorometry analysis, data digitization and statistical analysis		*	*					
2. Airblast sprayer drift/exposure levels assessment up to 600 ft downwind from 'central leader' apple blocks at full canopy stage								
Task 2.1: Use block from 1.1 and setting up of the field samplers and metrological stations				*	*			
Task 2.2: Canopy mapping via standard ground-reference methods, data processing					*	*		
Task 2.3: Conduct field trials (20 runs)					*			
Task 2.4: Fluorometry analysis, data digitization, processing and statistical analysis					*	*		
Data from obj. #1 & #2 for USDA-FHAAST and EPA team developed drift model validation			*	*		*	*	*
Reports and publication			*	*	*	*	*	*

2. SIGNIFICANT FINDINGS

No major findings to report, as this project work cycle somewhat mismatches to funding cycle and when the field experiments for year-1 needed to be conducted, i.e., dormant stage. Team has conducted the extensive field trials this fall (Actual data collection dates: November 27 to December 2, 2018). From year-1 planned project activities, the major activity of field experimentation has already been completed. The laboratory analysis of the samples and data interpretation are to be completed in the first quarter of 2019.

Per the protocol, we have conducted 20 spray trial runs to collect 2320 deposit samples (# of cards: 880; # of artificial foliage:800; # of horizontal strings: 400; vertical strings: 240). We are in the process of analyzing those samples using fluorometry analysis and pertinent findings along with associated weather data will be reported at the end of Spring 2019.

Fig. 1. Experimental field site.

3. METHODS

Year-1: Below is brief summary of experimental details pertinent to year-1 objectives:

- **Completed:** The field experiments were conducted at cooperating grower (Olsen Brothers) field site (46°18'57.6", N 119°34'36.8" W) located near Benton City, WA. It is ~9.24 acre (760 x 530 ft) 'Gala'(M9-337) apple

block planted in 2005. Rows were spaced at 9-ft and trees trained in 'central leader' canopy architecture were spaced at 3-ft. Downwind the orchard had 22.4 acer (780 x 1250 ft) bare field (see Fig. 1) block making an ideal location for this study.

- **Completed:** Field site was prepared in August-October of 2018 to have transect layouts, metrological stations set-up, base measurements, etc.
- **Completed:** Field was instrumented with three main weather stations (MET) (see Fig. 2). MET 1 was inside the orchard at 60-ft apart from the spraying row at upwind direction. It consisted of a 3D sonic anemometers at 3, 6, 12 and 24 ft above ground level (AGL) (Three 3-axis 81000 from Young R.M. Ultrasonic and a 3-axis Vx probe from Ultrasonic-Applied Technologies Inc.). MET 2 was located downwind at 600 ft away from the orchard in an open field. It consisted of 2D sonic four anemometers at 3, 6, 12 and 24 ft AGL (ATMOS 41, Meters Group). MET3 was located at the same row as MET1 but 120 ft further away to be closer to the end of orchard row. It was fixed with four anemometers (ATMOS 41, Meters Group) similar heights of 3, 6, 12 and 24 ft AGL.

Fig. 2. Weather stations setup inside the field (left) MET 1 at 60-ft upwind from the spraying row; (middle) MET 3 at 120ft away towards the end of orchard from MET 1 in the same row; and (right) open field MET 3 at 600-ft downwind.

- **Completed:** There were four types of drift catching samplers; Myler cards (2"x2"), Artificial foliage (Hedge slats of 1.5" length), Horizontal strings (1 m length), and vertical strings (sectioned at 12, 18 and 24 ft). The string samplers were made from 1.8 mm dia. white color spear gun spectra cord (SGT Knots). The arrangement of card, artificial foliage and the horizontal string samplers in the field is shown in figure 3(left) and the vertical string sampling station is shown in figure 3(middle).

Fig. 3. Arrangement of different samplers at the field setting; (left) horizontal string, artificial foliage and the Mylar card; (middle) vertical string set-up with a telescopic pole; and (right) Mylar card setup for in-field deposition assessment.

- **Completed:** The dormant stage data collection was conducted per the experimental protocol given in the original proposal at leaf drop stage. There were three blank

trails and 17 spray trials. Each trial involved spraying four passes of spray mix that had fluorescent tracer dye (Pyranine 10G, Keystone). Spraying was done in the third row from the edge.

- Completed: Canopies have been characterized (data collection) for estimation of Leaf Area Index, Porosity, Canopy Volume, Leaf Wall Area, etc. using small UAS based RGB imaging as well as via ground based RGB, 3D imaging and PAR sensing systems.
- Ongoing: Collected deposit samples, 2320, are being analyzed using the fluorometry analysis. The details of the fluorometry analysis has been reported in Salyani (2000) and Khot et al. (2012). Briefly, a known volume of deionized water will be added to the plastic bags containing the deposit samples. The sample bags will then be shaken for 1-min using a mechanical shaker, to thoroughly mix the tracer deposit into deionized water in the sample bags. The rinsate will then be transferred into two 10- ml matching cuvettes (Fisher Scientific, Hampton, NH). Each cuvette will be analyzed twice for fluorescence intensity using the fluorometer (Model: 10AU, Turner Designs, San Jose, CA). The fluorometry analysis based deposition results (as amount of tracer per sample in mass/area) will be used to validate an orchard airblast 'spray drift model'. This data will also be utilized in combination with previously collected 'Spray Drift Task Force' data to extend the results and create a more robust analysis.
- Ongoing/Future: Pertinent canopy parameters will be extracted from the imagery and time series datasets. Environmental conditions data will be tableted to extract pertinent stability parameters to be used to validate the mechanistic model being developed to estimate airblast sprayer drift/exposure levels.

Year-2

Similar to year-1, validation measurements will be made up to 600 ft downwind in a commercial 'central leader' apple block to facilitate the assessment of chemical drift/exposure levels as related to key in-field and open-field meteorological parameters. Activities involve a total of twenty (17 plus 3 blank) field trials at full canopy growth stage (obj. #2 year-2 efforts) in an apple block selected for year-1 trials. Experimental trial protocols (Fig. 4 and outlined in year-1 activities) developed in coordination with the USDA Forest Service and EPA will be followed in year-2 as well.

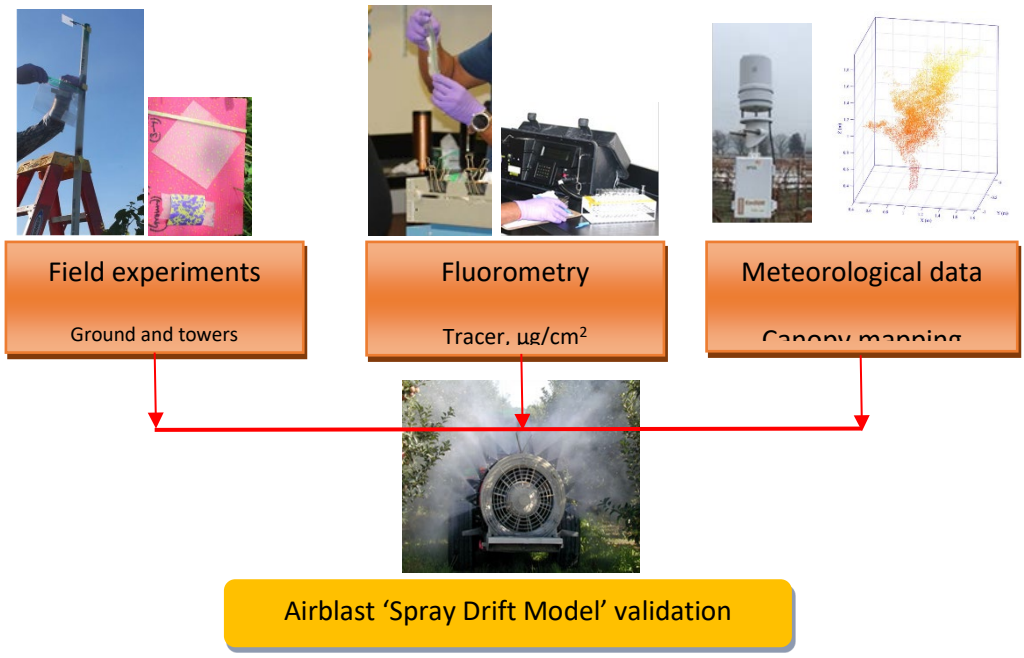

Fig. 4. Depiction of airblast 'Spray Drift Model' validation in 'central leader' apple.

Pertinent to the year-2 field trials, collected samples will be kept in a cooler and later moved to a cooling chamber until the laboratory analysis is completed. Samples will be analyzed using fluorometry analysis.

4. RESULTS & DISCUSSION

Nothing to report. It is expected that this project will generate scientific data of airblast sprayer drift/exposure levels up to 600 ft downwind 1) pertinent to 'central leader' apple canopies present in the Washington state and 2) with relevant meteorological conditions during dormancy and full canopy growth stages. Data will be used to validate and refine the mechanistic model currently being developed. As the study protocols are developed in collaboration with USDA-FHAAST and EPA teams, we anticipate no major pitfalls/problems during this research project though we can't control ambient weather conditions during trial period.

5. LITERATURE REVIEW

Pest and disease management has not lost importance in tree fruit production systems with the rising incidents of invasive insects, pests and pathogenic infestation. Regulatory agencies are also pushing for best management practices and effective spray applications that address worker safety and environmental concerns associated with spray material drifting off-target.

Pesticide exposure from drift is a risk to farm workers if applications are being conducted anywhere nearby. Furthermore, old tree architectures are being replaced with modern vertical and y-trellised tree architectures. Such modernization creates unique challenges pertinent to chemical drift and increased human exposure incidents in the state of Washington. An examination of 'Washington Department of Health' data on drift incidences shows that between 2010 and 2014, 59% of all chemical drift incidences were from an orchard airblast sprayer (Fig. 4). Concerns raised by these reported events create high levels of concern regarding drift, leading to weakly supported evidence assumptions regarding the extent of drift occurring with the use of airblast sprayer applications and the consequent adoption of worst case scenarios in the pesticide risk assessment process.

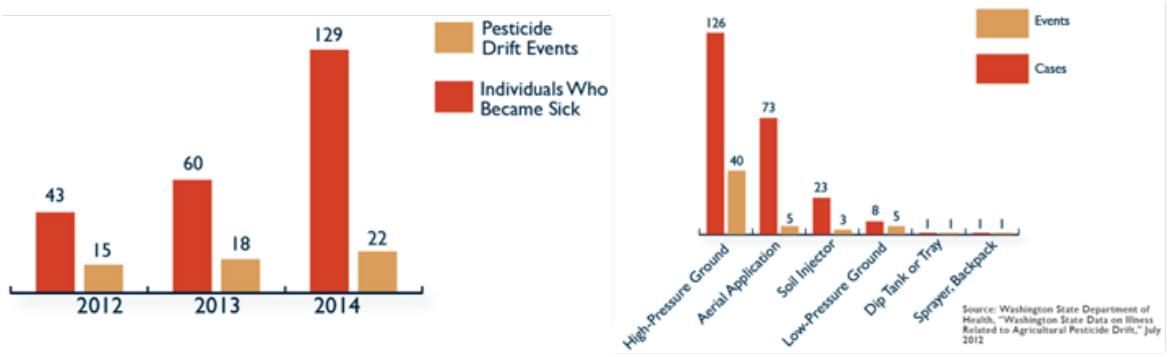

Figure 4. Pesticide drift events and affected number of individuals (left) data pertinent to the application type (right) in the state of Washington (Source: Ford, 2017).

Innovative sprayer designs, nozzles, spray adjuvants, and several machine-related factors can reduce off-target drift and improve spray application efficiency. Simple changes to sprayers such as using low-drift nozzles can reduce airborne drift while providing adequate coverage (Derksen et al., 2007). Sensors mounted on conventional airblast sprayers can be used to target spray delivery to the canopy, significantly reducing ground deposits and the potential for pesticide runoff into surface waters (Brown et al., 2008; Landers, 2008). Recently, stereovision (Kise and Zhang, 2008; Rovira-

Mas et al., 2005, 2006), LiDAR (Rosell-Polo, 2009; Khot et al., 2012; Gil et al., 2014), and ultrasonic sensors (Llorens et al., 2011a,b; Gil et al., 2013) have been shown to characterize canopy shape, which was then used to modify the amount of spray volume applied. Such variable rate application can result in a 30% decrease in total volume applied (Gil et al., 2007; Llorens et al., 2010). However, no current official guidance is available from EPA to allow registrants to encourage the adoption of these drift reduction technologies. Weather can also affect spray quality. High heat and low humidity can cause droplets to evaporate and reduce in size. Small droplets tend to drift farther off-target and are more affected by wind speed. Studies of airblast applications show differences in canopy deposition and off-target drift under changing wind speed and direction as it relates to canopy row orientation (Armstrong et al., 2013a,b; Tsai, 2007). Finding ideal weather conditions for the entire duration of a spray can be quite challenging. Also, standard orchard or farm operations can interfere with spray applications. For example, large farm crews often need to be in the field working on horticultural issues such as pruning, thinning fruit, or other maintenance.

Due to these complexities, a more realistic, data driven, mechanistic model to predict off-target drift is being developed. This is a critical need to ensure the regulations and buffer zones are accurately developed. ***This project will generate data to validate those modeling efforts.***

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CONTINUING PROJECT REPORT **YEAR**: 2 of 3
WTFRC Project Number: CP-17-100

Project Title: Rapid lab and field detection of two major apple quarantine pathogens

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P.O Box 646430
City/State/Zip: Pullman/WA/99164

Cooperators: Stemilt Growers LLC.

Total Project Request: **Year 1:** $35,211 **Year 2:** $15,429 **Year 3: $3,800**

Other funding sources None

WTFRC Collaborative Expenses: None

Budget 1 (Achour Amiri)
Organization Name: Washington State University **Contract Administrator:** Katy Roberts/Kim Rains
Telephone: 509-335-2885/509-293-8803 **Email address:** katy.roberts@wsu.edu/kim.rains@wsu.edu

Item	2017	2018	2019
Salaries[1]	12,635	9,371	
Benefits	4,449	3,358	
Wages			
Benefits			
Equipment[2]	13,735		
Supplies[3]	4,000	2,000	3,000
Travel[4]	392	700	800
Miscellaneous			
Plot Fees			
Total	35,211	15,429	**3,800**

Footnotes:
[1] Salaries are Rachel Bomberger (30% FTE in 2017 and 10% FTE in 2017) and for 6 months 20% FTE for Research Intern (Amiri lab) for 2018.
[2] Equipment will include costs for portable Genie II instrument to be used in and outside lab environments.
[3] Supplies include reagents for LAMP assay optimization and field use.
[4] Travel to Wenatchee for Rachel Bomberger and for Amiri lab to field and packinghouse samplings and testing.

OBJECTIVES:

1. Laboratory development and optimization of the LAMP assay to:

   -Identify and distinguish the different species causing in the bull's eye rot complex, including *Neofabraea perennans*, *N. malicorticis*, *N. alba* and *N. kienholzii.*

   - Identify the causal agent of speck rot (*Phacidiopycnis washingtonensis*).

2. Evaluate the sensitivity and reliability of the LAMP assay for early detection of the aforementioned pathogens on:

   a- Apple trees (cankers) and crabapple trees in orchards.
   b- Healthy and decayed fruit before and after several months of storage.

SIGNIFICANT FINDINGS:

Objective 1.

- The primer sets developed initially to detect the four *Neofabraea* species (bull's eye rot) could not provide specific detection due to high sensitivity to the LAMP reaction.
- Five more primer sets have been designed and have been tested under laboratory conditions to detect the *Neofabraea* species.
- Two sets have been selected among the six sets and are being optimized in the laboratory.
- The primer sets developed to detect *Phacidiopycnis washingtonensis* (speck rot)were able to detect the fungus in 30 min to 45 min.
- A Gennie II portable instrument (Figure 2) which is battery-powered has been acquired and is being tested for accuracy in the lab.

Objective 2.

- One commercial conventional and one organic orchard (cv. Pinata), with known history of bull's eye rot, have been used in 2018 to sample fruits and cankers throughout the season for LAMP detection.
- Trials were conducted in a 'Golden Delicious' block at Sunrise to inoculate fruit with *Neofabraea perennans*. Fruit were sampled at different timings and are being processed for LAMP detection to verify how long before harvest the pathogen can be detected.

METHODS:

Objective 1. Laboratory development and optimization of the LAMP assay to detect bull's eye rot species and speck rot.

1-a&b. Lamp primers design and LAMP assay optimization. Three set of primers have been designed for each species using the Beta-tubulin gene.

Pure culture of isolates previously characterized as *N. perennans*, *N. alba*, *N. malicorticis*, *N. kienholzii* and *P. washingtonensis* were used to extract DNA. Ongoing work consist of testing the primers and the sensitivity of LAMP assay will be tested by using different DNA concentrations (1, 2, 4, 8, 16 and 32 ng/µl).

Once primer accuracy and LAMP conditions have been set, we will move to detection on inoculated fruit. Here, the LAMP assay will be tested on fruit previously inoculated with each species. Fruit will be inoculated by sprays of spore suspensions at 10^2, 10^3, 10^4, and 10^5 spores/ml then incubated for 2 and 4 months at 33°F at room atmosphere. To detect latent infection, fruit will be crushed and homogenized and a sample will be used to run the LAMP reactions. For pathogen detection from a rotted fruit, small samples will be taken directly from the margin of lesions, suspended in water or lysate buffer and used for LAMP reactions. Eventually, branches of apple trees (cv. Fuji) will be wounded and inoculated with *P. perennans* and *P. washingtonensis* in mid-spring of 2018 at Sunrise Orchard and infected wood will be disrupted in a mortar or mesh-plastic bags and used in fall of 2017 for LAMP assay optimization. Experimental trees will be pruned and sprayed with Topsin-M to insure complete inoculum removal.

1-c LAMP assay reactions

For laboratory use, LAMP identification will be assessed by comparing the turbidity of different samples (which is visible to the naked eye) or by fluorescence using intercalating dyes such as SYTO 9 or SYBR green that can be used to create a visible color change that can be observed with a fluorimeter, UV-excitation, or by naked eye (Figure 1). While these approaches may work for lab samples with known and optimized DNA concentrations, confusing and ambiguous results can occur with samples in the orchards because of disparity in DNA concentration.

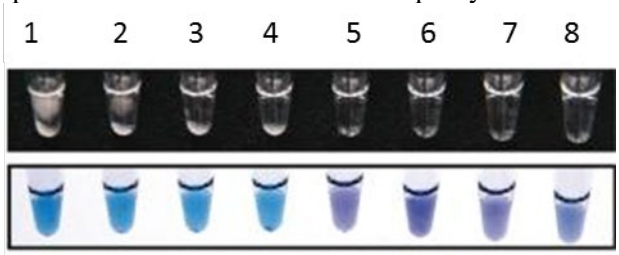

Figure 1. Example of detection sensitivity using LAMP assay based on tubidity (top) (note differences between samples 1 to 4) and fluorescent dye (bottom).

To avoid potential pitfalls from turbidity characterization, we will use Genie II instrument (Figure 2) for orchard identifications. In order to use the LAMP technology in an orchard, a powered heat block or water bath is required-neither instrument is convenient to under field conditions unless they are battery operated. The Genie II is a battery-powered instrument consisting of a thermocycler necessary for the LAMP assay and a screen that show real-time amplification of positive DNA if present in the sample. In addition, Genie II has the ability to target RNA as well, which could be used to quantify the pathogen or study the expression of certain genes during the infection process.

Objective 2. Evaluate the sensitivity and reliability of the LAMP assay for early detection of pathogens on trees and latent infections on fruit at harvest and after several months of storage.

2a. *Detection of Neofabraea and Phacidiopycnis inoculum on trees in orchards using the portable Genie II instrument*.

The disease monitoring survey conducted in the 2016 season has allowed us to locate a number of orchards with higher risk for the two pathogens. We will select four orchards with a high bull's eye rot frequency and four others with a high speck rot frequency. Trees at the selected orchards will be scouted between March and October of 2018 and 2019 for cankers and other typical

symptoms of the two diseases. For Phacidiopycnis, if crabapple trees are present in the same orchards, they will be checked for twig dieback and rotted or mummified fruits.

To detect the pathogens, we will first look for fruiting bodies (acervuli and conidia) of the fungi on the cankers and if present conidia will be collected with a sterile swab and re-suspended in a 5 ml of lysing buffer. If conidia are not observed, a small piece of the bark at the margin of cankers will be cut with a sterile scalpel, homogenized in plastic mesh bags containing 5 ml of lysing buffer and the lysate will be transferred to test tubes. The lysate will be used for pathogen detection in 0.5 ml tubes using the portable Genie II instrument (Figure 2). A negative control (water or DNA of *Alternaria alternata*, a common fungal contaminant) and a positive control consisting of DNA of one of the target pathogens will be used. A minimum of 10 sample trees will be checked and tested at once in each orchard. We expect the reaction to run for 30 min before a diagnosis can be made.

2b. Detection of Neofabraea and Phacidiopycnis on fruit before harvest and after several months of storage.

Thirty fruit will be collected monthly from 0.5 inches (10-15 mm) stage until harvest from the same orchards used for field detection (objective 2a). In addition to healthy trees, trees with visible cankers and twig dieback will be marked and used to harvest fruit. Fruit will be transferred to the Pathology Lab at WSU-TFREC in Wenatchee and will be used immediately for pathogen detection or kept in cold storage (33°F) for no more than a week. Fruit will be also sampled after 2, 4, 6, and 8 months of storage from different packinghouses, ideally using fruit from the same orchards sampled in objective 2a. The postharvest sampling will include asymptomatic fruit (no signs of rotting) to check for latent infections and decayed fruit showing symptoms of the two pathogens to detect the exact species that causes the disease.

For detection, the whole fruit (asymptomatic) will be crushed, lyophilized, and homogenized and 100 to 200 mg will be suspended in a lysate buffer. For decayed fruit, a small piece (using the same procedure described above for lab optimization) of rotted tissue will be transferred to a lysate buffer. All samples will be subjected to LAMP reaction in the Genie II Instrument (Figure 2).

RESULTS AND DISCUSSION

Objective 2: Primer design and LAMP assay optimization

The first sets of primers did not provide specific detection and results were not consistent through the different runs (Fig. 1).

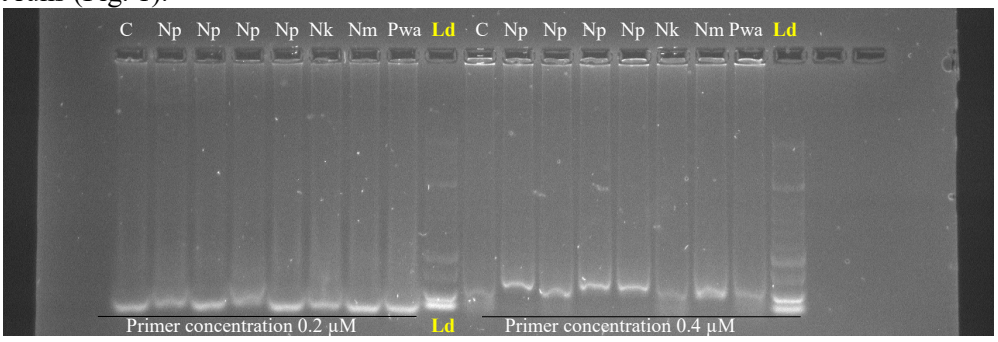

Fig. 1. Example of non-amplification of the target gene (b-tubulin) using the first primer set. C= control, Np = *Neofabraea perennans*, Nk = *Neofabraea kienholzii*, Nm= *Neofabraea malicorticis*, Pwa = *Phacidiopycnis washingtonensis*, Ld= molecular ladder (used for comparison).

In a follow-up step, we have designed 5 other primer sets at different regions of the B-tubulin gene to increase the chance of specific and consistent detection. As shown on Figure 2 below, the primer sets 1, 3 and 4 are specifically (yellow arrows, this is what is needed) amplifying *N. perennans* but not *P. washingtonensis.* While Set 5 did detect *N. perennans*, the positive reaction seen in the control (no fungal DNA) requires further optimization. The primer set #2 does not seem to be appropriate as it gave a positive reaction in the control and for *P. washingtonensis* (which is not wanted).

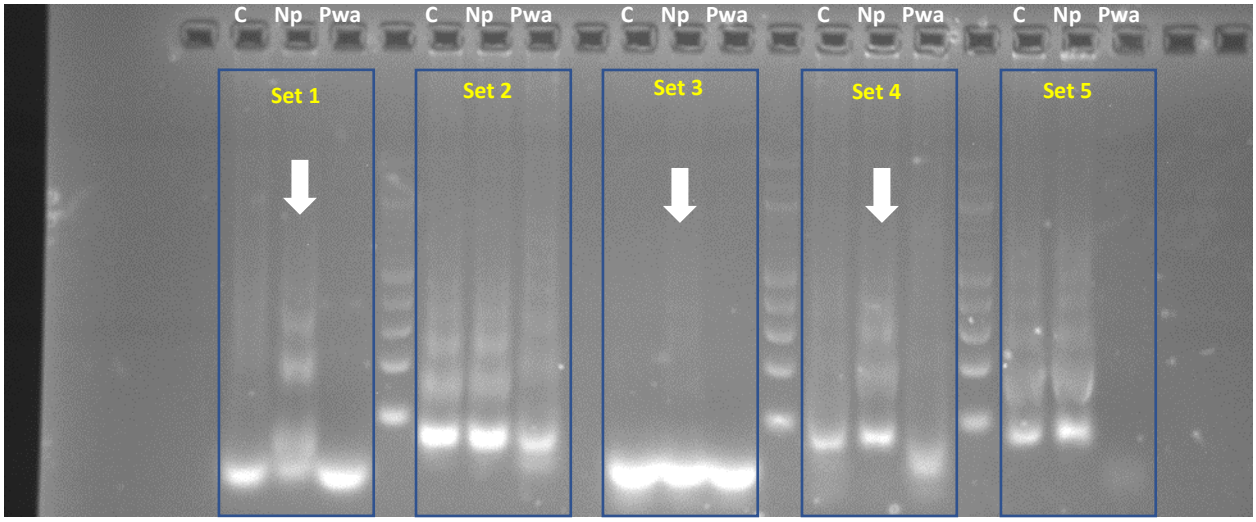

Fig. 2. Amplification of B. tubulin gene using 5 new primer set. C = control (no DNA), Np = *Neofabraea perennans*, Pwa = *Phacidiopycnis washingtonensis*, Ld= molecular ladder (used for comparison).

Acquisition of the portable Genie II instrument for orchard and Packhouse use

The LAMP assay requires only one constant temperature to be used during detection compared to 5 to 6 different temperatures for regular PCR. Therefore, only heavy thermocycler machines can be used for PCR a heat block or a water bath is sufficient for LAMP. In order to conduct detection outside lab conditions where a power source is not available, the Genie II instrument with a potable battery (4 hours while LAMP can be done in 30 min) is a valuable useful tool. We acquired the Genie II (Figure 2) thanks to funds provide by the WTFRC and it is currently being tested in the lab to test correct functioning and its use for pathogen detection.

Figure 2. Portable Genie II instrument, battery-enabled aquired by Pathology lab at WSU-TFREC to be used for orchard and warehouses detection of pathogens.

Objective 2. Field detection of *Neofabraea* species using LAMP

Detection of Neofabraea species in artificially infected fruit. Trials were conducted at Sunrise in a 'Golden Delicious' block in 2018. Fruit on trees were inoculated with spore suspensions of N. perennans at ta concentrations of 5,000; 50,000 and 500,000 spores/ml, in addition to a control sprayed with water, one month before commercial maturity. Different concentrations are meant to test the sensitivity (lowest inoculum size for a good detection) of LAMP detection. Fruit were harvested at commercial maturity in sperate bags for each concentration and transported to the TFREC pathology lab. Fruit at stored at -4°F waiting for LAMP detection to be conducted

Detection of Neofabraea species in naturally infected fruit. Two commercial orchards of the cultivar Pinata (susceptible to bull's eye rot) located in the Quincy area were used; one orchard was conventional and the second one was organic. Fruit were sampled at 3 times, including two and one month before commercial maturity and at harvest. Additional fruit were also harvest and stored in a regular atmosphere at 34°F and sampled after 1, 2 and 3 months in storage (ongoing), to detect the fungus while fruit are in a cold storage. Fruit were homogenized in a blender and 10 g is frozen in sterile plastic tubes at -4°F until LAMP detection is conducted.

Ongoing and future work:

Objective 1.

- Complete the LAMP optimization for Neofabraea species in the lab and test for specificity.
- Complete the LAMP optimization for Neofabraea species in the lab and test for specificity.
- Complete visual assessment methods for the LAMP assay using turbidity or specific dyes
- Optimize the use of Genie II devices for portable detection of pathogens

Objective 2.

- All field experiments have been conducted and samples were collected
- Once the LAMP method is complete and running accurately (Objective 1), field samples will be subjected for LAMP detection.

CONTINUING PROJECT REPORT **YEAR**: 2 of 3
WTFRC Project: CP-17-104

Project Title: Implementation of alternative methods to control replant disease

PI:	S. Tianna DuPont	**Co-PI**:	Mark Mazzola
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Cooperators: Mike Robinson, BMR Orchards; Jim Baird, Baird Orchards; Sam Godwin, Box Canyon Orchard

Total Project Request: **Year 1:** $60,577 **Year 2:** $34,163 **Year 3: $35,248**

Other funding sources

Agency Name: USDA Crop Protection
Amt. awarded: $195,713
Notes: USDA Crop Protection Grant funds two additional sites. Thank you to in kind support from Progene Seeds, Trident Fumigation, Farm Fuel, Baird/BMR Orchards, Box Canyon Orchard.

Budget 1
Organization Name: Washington State University **Contract Administrator:** Katy Roberts/Kim Rains
Telephone: 509-335-2885/509-293-8803 **Email address:** katy.roberts@wsu.edu/kim.rains@wsu.edu

Item	2017	2018	**2019**
Supplies[1]	23,609	2,000	2,000
Travel[2]	1,037	1,037	1,037
Total	24,646	3,037	**3,037**

Footnotes:
[1]Plot set up and maintenance supplies over and above normal horticulture, i.e. seed meal, virtually impermeable plastic, etc.
[2]Travel to Othello for plots set up, maintenance and sampling.

Budget 2
Organization Name: USDA **Contract Administrator:** Chuck Myers
Telephone: 510-559-6019 **Email address:** chuck.myers@ars.usda.gov

Item	2017	2018	**2019**
Salaries	19,800	20,592	21,416
Benefits	6,283	6,534	6,795
Supplies[3]	9,848	4,000	4,000
Travel[4]			
Miscellaneous			
Plot Fees			
Total	35,931	31,126	**32,211**

Footnotes:
[1]Wages for a 33% scientific assistant to conduct microbial analysis of rhizosphere.
[2]Benefits at 31.7% for scientific assistant.
[3]Microbial analysis supplies.

OBJECTIVES

1. Conduct field scale experiments to test the efficacy of bio-renovation and anaerobic disinfestation as alternatives to soil fumigation for the control of apple replant disease. At each on-farm site four treatments (mustard seed meal bio-renovation, anaerobic soil disinfestation, fumigated control and non-fumigated control) will be applied in randomized strips in each of four blocks (four replicates each). Plant response to treatments will be assessed by measuring trunk cross sectional area and yield. In addition, microbial analysis of roots and soil will be conducted to determine treatment effects on target replant pathogens and overall composition of the microbiome including potential beneficial microbes.
2. Use field scale experiments to demonstrate to growers the steps to bio-renovation and anaerobic soil disinfestation. Each step will be documented with photos and video to create Extension factsheets explaining the process and lessons learned. Conducting trials at a large plot scale will allow us to use the same equipment growers would use, develop practical expertise, and work out the inevitable kinks with a new technique.

SIGNIFICANT FINDINGS

- The cumulative anaerobicity (cumulative mV hours with reduction potential (*Eh*) reached the target level of 50,000 mV hr for Tonasket, Rock Island and Othello re-do sites (Figure 1&4).
- Analysis of bacterial and fungal populations showed good separation for *Brassica* seed meal treated, anaerobic treated soils and control soils for Tonasket and Rock Island sites (Figure 2) and Othello redo sites (Figure 5).
- Tree cross sectional area measurements for Othello site do not show significant differences between mustardmeal treatment and fumigated control, or control 1 year after treatments were implemented (Figure 3).

METHODS

Experimental site and design:
The experiment was implemented at three field sites including a 12-acre grower field in Othello WA (46.933876, -119.392096), a 1.5-acre field at Washington State University Sunrise research orchard at Rock Island, WA (47.31988,-120.0663747) and a grower 1 acre field in Tonasket WA (48.810692, -119.505724). All sites had a history of replant disease. Soil type at the Rock Island site is a Pogue fine sandy loam, at the Tonasket site are a Nighthawk loam, and Adkins is a very fine sandy loam, 0-5 percent slope. Sites were split into experimental blocks of 40 ft by 200 ft with 5 experimental blocks in Rock Island and 4 in Tonasket and Othello. In Tonasket and Rock Island each of four soil treatments including anaerobic soil disinfestation (ASD), brassicaceae seed meal (BSM), fumigation, and no-treatment control were randomly assigned to 10 ft by 200 ft plots within each 8,000 square foot experimental block. In Othello BSM, ASD and fumigated treatments were assigned to 0.8 acre plots (3,300 to 3,600 tree row ft) in each block. The non-fumigated control treatment was assigned to an 80 by 5-foot plot nested within the fumigated control. Plots in Rock Island were further split based on the rootstock that will be planted into the soil (M9 or G41).

Site preparation: All sites were prepared by deep ripping, rock removal and three discing events. Soils in Othello were amended per soil test recommendations with 169 units of N, 30 units of sulfur and 1 unit of boron (urea, ammonium sulfate and borax). The field site was planted to triticale (*Triticosecale)* cultivar Tritical 141 at 100 lbs per acre using a Great Plains seed drill on April 19, 2017 seeded to moisture (~1”). At anthesis on June 26, 2017 triticale was 4.5 to 5.5 feet tall with an

average of 3.5 ton/A dry biomass. Triticale was swathed and baled removing material from mustard seed meal and control plots.

Treatments:

ASD treatment Rock Island and Tonasket: Soil moisture of the ASD plots was brought up to 17% volumetric moisture content (VMC) and 20.3% gravimetric moisture content using a sprinkler irrigation system and big gun irrigation prior to biomass application. Timothy hay was applied to the ASD plots at 8 tons/A, flailed to chop into small particles and sampled for particle size and nutrient levels. Biomass was incorporated into soil with a rototiller in two to four passes (July 5, 2018 Rock Island; August 8, 2018 Tonasket). Totally impermeable plastic film (Rock Island: TIF, 1.2 ml; clear, Vaporsafe, Trical, Gilroy, CA; Tonasket: TIF, Tri Est, Black, 1.2 ml) was applied using a plastic layer (Mechanical Transplanter) covering ASD plots. Soil moisture was then brought to and maintained above 30% VMC using a double drip line running constantly under the impermeable film. Treatments were maintained for 4 weeks. At the end of 4 weeks plastic was removed and irrigation turned off. Plots were allowed to aerate to reduce phytotoxicity for 4 weeks before samples were taken.

ASD treatment Othello: On June 28, 2017 triticale was cut and swathed into four-foot windrows using a John Deere R450 swather where swaths were lined up on future tree rows. Swathing concentrated plant biomass produced on a ten-foot width into a four-foot width area. As such 3.5 T/A field grown biomass averaged 10 ton/A applied biomass to the tree row. Six days (July 3) after cutting at 20% moisture, triticale was flail chopped using a Pak flail which left a six-foot wide swath of chopped biomass. A hay rake was used to re-concentrate chopped material into the four-foot wide treatment areas. Biomass was incorporated with a Celli rototiller to an eight-inch depth. Three-acre inches of irrigation (0.28 in/hr) were applied using hand lines with 6 gal/ min sprinkler heads to thoroughly wet the soil. Four to eight hours after irrigation, plastic was laid to seal the treatment area. Soil moisture averaged 24% in the top 5 cm and 25% at 15 to 20 cm at the time plastic laying began and 24 % (0-5cm) and 19% (15-20 cm) by the time plastic laying was finished in reps A&B. Soil moisture averaged 30% (0-5cm) and 32% (15-20 cm) as plastic laying commenced in reps C&D. Plots were irrigated with an additional 3 acre inches of water (11 hrs) resulting in soil moistures averaging 26% (0-5cm) and 34% (15-20 cm). One week after initial irrigation plots were re-wetted with an additional 1.7 acre inches of water.

Mustard meal treatment (BSM) Rock Island and Tonasket: Initial soil moisture in mustard treated plots was 25% and temperature was 81℉ (27℃) in Rock Island. Soil moisture was brought up by using both big gun and sprinkler irrigation systems (July 2-4, 2018). At soil moisture appropriate for tractor operation, Pescadero Gold Mustard meal (1:1 formulation of B. juncea and S. alba) (Farm Fuels Inc., Watsonville, CA) was applied using a Whatcom spreader at 1.7 lb per tree-row-foot (1.6 lb per tree-row-foot target) and raked to form 4-foot-wide strip (July 6, 2018 Rock Island; August 9, 2018 Tonasket). Mustardmeal was incorporated and mixed thoroughly into the soil using a rototiller to an 8 to 10-inch depth. The plots were sealed with totally impermeable film using a plastic layer within 20 minutes of incorporation.

Mustard meal treatment (BSM) Othello On July 15, 2017, 3.4 inches of irrigation water was applied (0.28 in/hr). On July 19 and 20, 2017 when soils had drained and dried to moisture appropriate for tractor implements Pescadero Gold Mustard seed meal (Farmfuels) was applied using a Whatcom compost spreader at 1.6 lbs per tree-row-foot in a four-foot swath (6.8 T/treated acre, or 3.4 T/orchard acre). Mustard seed meal was incorporated into soil using a Celli rototiller within a maximum of 3 hours of spreading (average 30 minutes) and sealed with Totally Impermeable Film (TIF, Vaporsafe, Trident Inc) within ten to thirty minutes of incorporation. Soil temperature averaged 24˚ C (75 °F) at the time of treatment application.

Table 1. Particle size hay carbon inputs (%)

	>4 mm	<4mm->2mm	<2mm->1mm	<1 mm
Othello	49	30	14	7
Rock Island	59	16	5	20
Tonasket	40	30	21	9

Table 2. Field Operations Rock Island WA

Anaerobic Soil Disinfestation				
Operation	**Implement/Equipment**	**details**	**hrs/A**	**Date**
Pre-Irrigation	Sprinkler system (R5 sprinklers)/ Big gun system (8 mm nozzle)	1.5 acre-inches applied	3	July 2-4th 2018
Hay distribution	By hand	8 Ton/A	15	July 4, 2018
Hay chopping	Flail mower		1	July 4, 2018
Incorporation	Maschio Rototiller	8-inch depth	2	July 5, 2018
Tarping	Mechanical Transplanter	N/A	2	July 5, 2018
Saturation	Drip irrigation to flood soil	0.44 acre-inches per hour	Constantly applied	July 6-27, 2018
Mustardmeal treatment				
Operation	**Implement/Equipment**	**details**	**hrs/A**	**Date**
Mustard application	Whatcom compost spreader	2 for belt and 2 for floor	2	July 6, 2018
Incorporation	Maschio Rototiller	8-inch depth	2	July 6, 2018
Tarping	Mechanical Transplanter	Within 20 min of mustard incorporation.	2	July 6, 2018

Table 3. Field Operations Tonasket WA

Anaerobic Soil Disinfestation treatment				
Operation	**Implement/Equipment**	**details**	**hrs/A**	**Date**
Pre-Irrigation	Big gun system (8 mm nozzle)	5 acre-inches applied in 12-hour sets.	3	Aug 2-6, 2018
Hay distribution	By hand	8 Ton/A	15	Aug 8, 2018
Hay chopping	Flail mower		1	Aug 8, 2018
Incorporation	Maschio Rototiller	8-inch depth	2	Aug 8, 2018
Tarping	Mechanical Transplanter	N/A	2	Aug 8, 2018
Saturation	Drip irrigation to flood soil	0.44 acre-inches per hour	Constantly applied	Aug 8-29, 2018
Mustardmeal treatment				
Operation	**Implement/Equipment**	**details**	**hrs/A**	**Date**
Mustard meal application	Mill Creek mulch spreader	1.7 lbs/ tree row ft Setting 4 floor; 4 belt	2	Aug 9, 2018
Incorporation	Maschio Rototiller	8-inch depth	2	Aug 9, 2018
Tarping	Mechanical Transplanter	Within 20 min of mustard incorporation.	2	Aug 9, 2018

Table 4. Field Operations Othello

Anaerobic Soil Disinfestation treatment				
Operation	**Implement/Equipment**	**details**	**hrs/A**	**Date**
Fertilize			0.25	
Tillage	John Deer 7200/ 15 foot disc		0.25	
seed triticale	John Deer 7200/Great Plains seed drill	95 lbs per acre	0.25	
Irrigation	Hand lines (R33 sprinklers)	6 gal/min, .28"/hr	4	
cut and swath	John Deere R450 swather	4 ft windrow	0.5	

chop	Pak flail	0.7 mi per hour	1	
Incorporation	John Deer 7200/ Celli rototiller	all the way down (8" depth)	2	
Tarping	Kubota M8540 / Mulch later (Mechanical Transplant Co Model 90)	N/A	2	
Brassica Mustard Meal Disinfestation				
Operation	**Implement/Equipment**	**Setting**	**Hrs/A**	
Pre-irrigation	Hand lines (R33 sprinklers)	6 gal/min, .28"/hr	12	
Mustard meal application	John Deer 5083/ Whatcom mulch spreader	4 low, 1700 rpms, belt 5, floor 4, gate 12.5 inches	2	
Incorporation	John Deer 7200/ Celli rototiller	all the way down (8" depth)	2	
Tarping	Kubota M8540 / Mulch later (Mechanical Transplant Co Model 90)	N/A	2	

Sampling

Soil sampling: Post-treatment soil was sampled in ASD, BSM, and control treatments with a soil core (20 cores per plot) at the end of the treatment period of three weeks, well mixed and immediately transferred to a cooler. Half of the soil was placed at 4℃ for soil pH analysis and the other half was placed at -80℃ for DNA extraction to conduct soil microbial profiling.

Soil moisture: Volumetric water content (VWC) attained after/during application of irrigation for ASD treatments was determined by measuring the dielectric constant of the media using capacitance/frequency (EC-5 Decagon Devices). In Othello soil moisture was measured at the commencement of laying plastic, as plastic laying finished, and two hours after blocks were re-wetted. In Rock Island and Tonasket measurements were performed on a weekly basis. Final soil moisture was measured at the dissembling of the treatment at three weeks.

ORP: Soil oxidation reduction potential (ORP) was measured as a parameter to assess accumulation of anaerobic conditions. ORP probes (SC500C-ORP, Sensorex, Garden Grove, CA) were buried 6 inches below the soil surface and measurements were recorded every 1 minute and logged in a data logger (CR1000, Campbell Scientific Inc., Logan, UT). At least two probes were placed in each ASD plot. ORP values were converted to *Eh*, by adding 199 mV to the ORP reading and the average value obtained from all sensors of the same treatment was calculated. *Eh* values below 200 mV were identified and cumulative *Eh* was calculated for the period of 3 weeks to 4 weeks of tarping.

Tree growth: A total of 25 trees were selected per plot in a checkerboard pattern in the central 2 rows of each acre block (15 inner trees in control plots). Tree cross sectional area was measured at 20 cm above the graft union.

RESULTS & DISCUSSION

Tonasket and Rock Island sites: In anaerobic treatments soil oxidation reduction potential (ORP), a parameter to assess accumulation of anaerobic conditions, reached target totals of 50,000 mV hr for Rock Island, Tonasket and Othello redo plots (Figure 1&4).

Terminal-restriction fragment length polymorphism (T-RFLP) analysis was used to detect changes in fungal and bacterial community composition in response to soil treatment. The analysis demonstrated

that bacterial and fungal populations were transformed in composition in response to the *Brassica* seed meal and anaerobic treatments relative to the no treatment control (Figure 2).

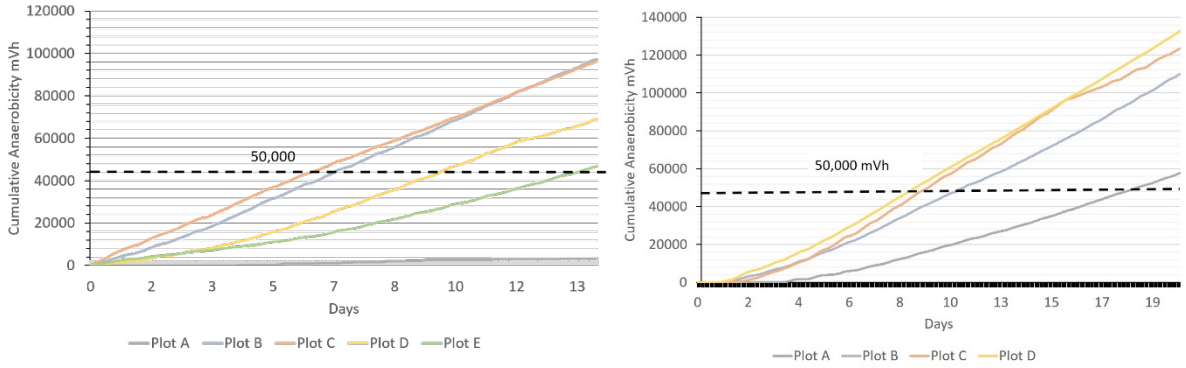
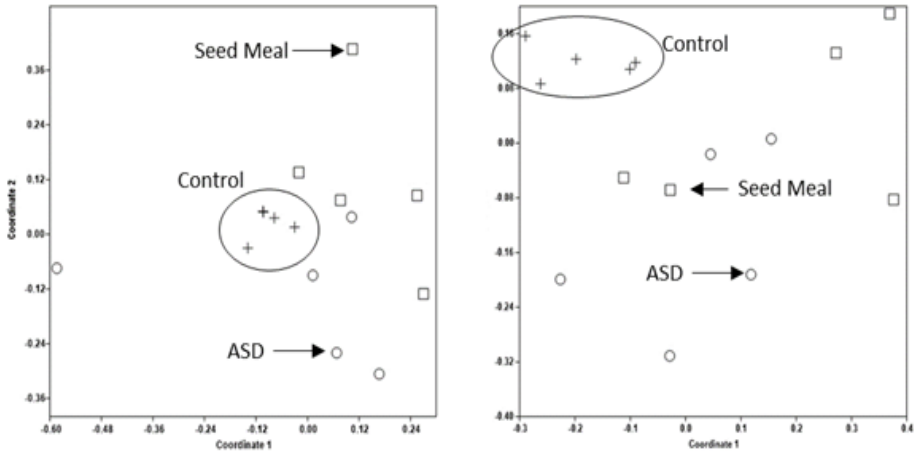
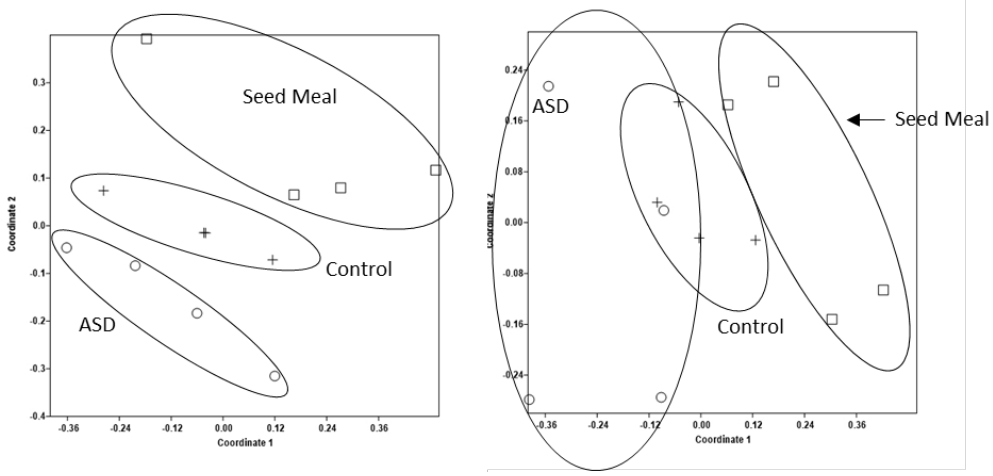


Figure 1. Cumulative anaerobicity (mVh) for Rock Island (left) and Tonasket (right) sites.



a. Rock Island



b. Tonasket

Figure. 2. Effect of soil treatment on relative similarity of bacterial (right panel) and fungal (left panel) communities recovered from orchard field soils for (a) Rock Island and (b) Tonasket sites. Communities were characterized by terminal restriction fragment length

polymorphism analysis of amplicons generated targeting the bacterial 16S rRNA gene and the internal transcribed spacer region of fungal rRNA genes. Treatments: Control, *Brassica* Seed Meal and Anaerobic Soil Disinfestation (ASD).

Othello site: Initial tree growth measurements for trees planted in spring 2018 were not different between the fumigated control, brassicacae seedmeal, and no-treatment control for 2017 treated plots. Diameter was smaller for the anaerobic treatment which had not reached anaerobicity in 2017 (Figure 3). In 2018 the anaerobic treatment was re-done in order to have treatments reach anaerobic levels. Plots reached target goal 50,000 mV hr (Figure 4). T-RFLP analysis demonstrated that bacterial and fungal populations were transformed in anaerobic treatment compared to no-treatment controls (Figure 5).

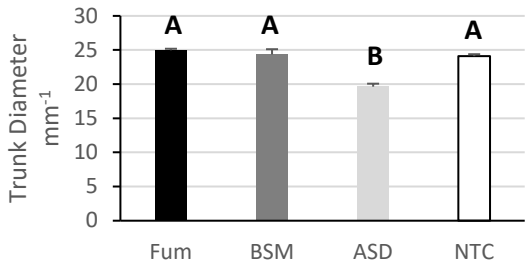


Figure 3 Tree cross sectional area of trees at Othello site (2017 treatments).

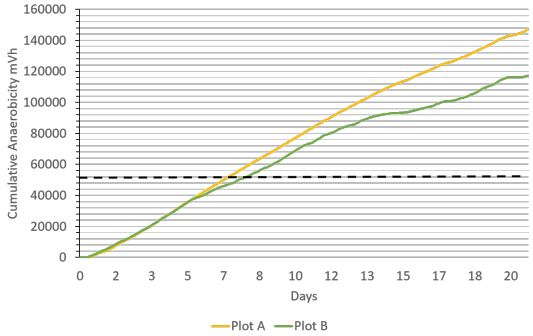


Figure 4 Cumulative anaerobicity (mVh) Othello re-do plots 2018.

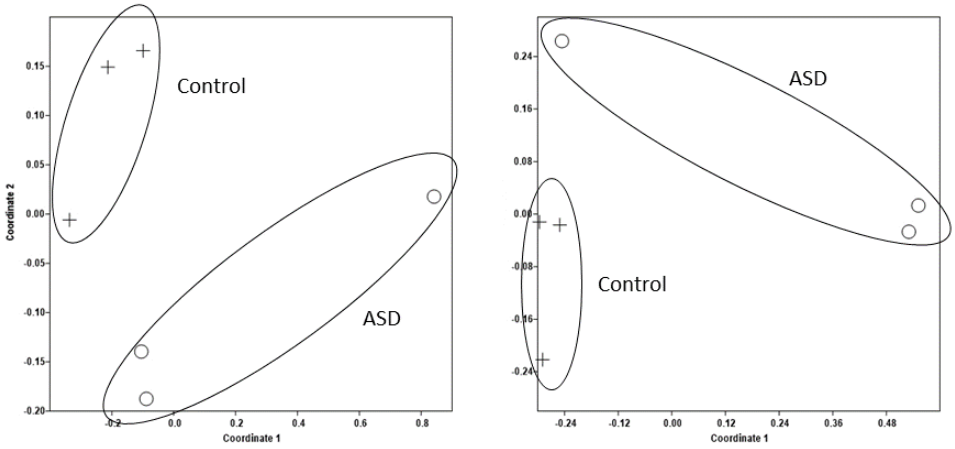


Figure 5. Effect of soil treatment on relative similarity of bacterial (right panel) and fungal (left panel) communities recovered from orchard field soils for Othello re-do plots. Communities were characterized by terminal restriction fragment length polymorphism analysis of amplicons generated targeting the bacterial 16S rRNA gene and the internal transcribed spacer region of fungal rRNA genes.

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

Project Title: Refinement of practical fire blight control: Non-antibiotic and SAR

PI: Kenneth B. Johnson
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Total Project Request: Year 1: $39,980 **Year 2**: **$40,779** (2% inflation)

Other funding sources: None

WTFRC Collaborative expenses: None

Budget

Organization Name: OSU Agric. Res. Foundation **Contract Administrator:** Russ Karow
Telephone: (541) 737-4066 **Email address:** Russell.Karow@oregonstate.edu

Item	**2018-19**	**2019-20**
Salaries Faculty Res. Assist. 3.5 mo	17,339	17,686
Benefits OPE 61%	10,577	10,788
Undergraduate labor (&OPE 12%)	1,064	1,085
Equipment	0	0
Materials and Supplies	1,500	1,530
Local Travel	1,500	1,530
Plot Fees	2,000	2,040
Medford russet trials (plot fees and labor)	6,000	6,120
Total	$39,980	**$40,779**

OBJECTIVES:

1) In integrated programs with Blossom Protect + Buffer Protect, evaluate EPA-registered, NOP-approved materials with demonstrated anti-microbial activity (e.g., Previsto, Jet Ag, and lime sulfur) for their ability to suppress fire blight and to induce fruit russeting on apple and pear trees.

2) In integrated programs with Blossom Protect + Buffer Protect, evaluate the mineral material, alum ($KAl(SO_4)_2$) and an alum-containing stone powder for their ability to suppress fire blight and to induce fruit russeting on apple and pear trees.

3) Evaluate alternative yeasts for their ability to suppress fire blight.

4) Evaluate amount and placement of Actigard trunk paints for the purpose of protection from fire blight during primary bloom.

SIGNIFICANT FINDINGS:

- Fire blight pathogen populations on pear and apple flowers continues to increase during the post-petal fall period.
- Blossom Protect (yeast) and Previsto (copper) were outstanding non-antibiotic materials for fire blight control.
- Alum, an effective fire blight control material, reduces the pH of floral surfaces.
- Lime sulfur or Jet Ag (H_2O_2 in peracetic acid) sprayed near petal fall provided outstanding suppression of yeast populations on flowers.
- Pear and apple showed differential fruit russeting responses to non-antibiotic materials. E.g., fruit russeting on Comice pear was severe after 4% lime sulfur but russeting on Fuji apple after lime sulfur was negligible (less than observed on the water control).
- Treatments of several alternative yeast strains suppressed fire blight but not to the same degree as observed with Blossom Protect.
- Concentrated Actigard applied to the trunks of apple trees at 10% bloom suppressed fire blight but not to the outstanding level observed in 2017.

METHODS:

Rationale. In recent years, there has been a rapid increase in the number of biopesticide materials available for non-antibiotic fire blight control. Many have become EPA-registered with only a limited number of field trials that demonstrate efficacy. Consequently, we are continuing comparative investigations of various registered and unregistered materials for fire blight control in organic systems. We seek to understand on a comparative scale: the effects of these materials on epiphytic pathogen populations, their ability to suppress infection, their effect on the PH of floral surfaces, and their effect on fruit finish (russeting).

Experimental design. Objectives 1-3 were addressed in experimental orchards located at Oregon State University's Botany & Plant Pathology Field Laboratory near Corvallis (pathogen-inoculated), and at the OSU Southern Oregon Research and Extension Center near Medford, OR (fruit finish only) . Experiments were arranged in a randomized complete block designs with 3 to 4 replications. Treatment suspensions were sprayed to near runoff with backpack sprayers during early morning hours. To enumerate pathogen and yeast populations on flowers, five flower clusters were sampled from each replicate tree at full bloom, petal fall, and one-week post-petal fall, which was followed by washing the flowers, recording the pH of the wash, and dilution plating the wash on a selective culture media. In Corvallis trials, incidence of fire blight was determined by counting and removing the blighted flower clusters on each tree at 2- to 4-weeks after bloom. Number of blighted clusters per tree were divided by total clusters, which were counted before bloom. In Medford, in late

August, the percent of the fruit surface with symptoms of russeting was scored with a modified Horsfall-Barratt rating scale.

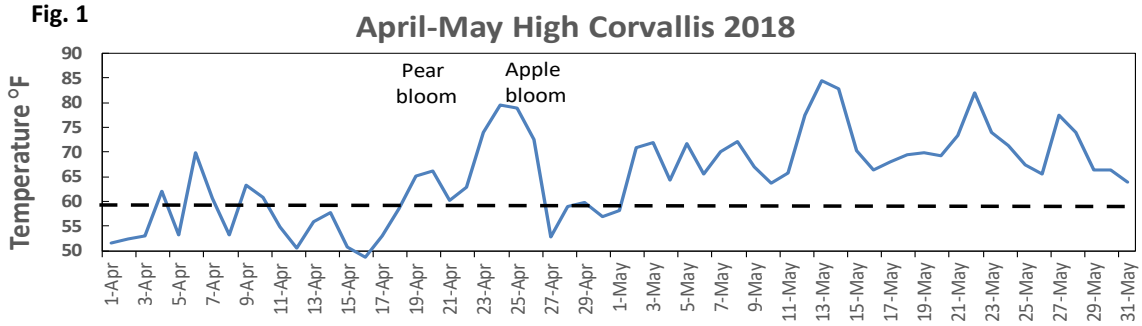
For objective 4, the experiments were conducted in 4-yr-old 'Gala' and 2-yr-old 'Cripp's Pink' apple blocks located near Corvallis; experiments were arranged in a randomized, complete block designs with 6 to 7 replications. Concentrated trunks paints of systemic acquired resistance inducing materials were applied at 10% bloom. Inoculation and measurement of disease incidence was as described above.

Data summary and analysis. Measured population sizes for *E. amylovora* and the yeast, *Aureobasidium pullulans,* were $\log_{10}$-transformed and plotted in graphical arrays. The effect of the treatments on the pH of floral surfaces were plotted similarly. The effect of the fire blight control treatments on incidence of fire blight were subjected to analysis of variance (ANOVA).

RESULTS

Weather in spring 2018. During pear bloom, temperatures were generally unfavorable for fire blight development until after petal fall. Consequently, epiphytic pathogen populations were low during most of bloom. Nonetheless, fire blight incidence was remarkably high owing to a heavy bloom and warm temperatures after petal fall when pathogen populations increased to > 1 million cells per flower. In apple, temperatures were most favorable for fire blight development during the early and late portions of the bloom period. Maximum daily high between 9 April and 10 May averaged 63ºF; maximum daily high from 23 to 26 April averaged 76ºF. Fire blight risk as determined by the heat unit model, COUGARBLIGHT, was 'high' to 'exceptional' from 24 to 27 April. For objectives 1-3, the water-controls averaged 251 infections per tree in Bartlett pear (30% of total clusters) and 114 infection per tree in Golden Delicious apple (36% of total clusters).

Fig. 1



Objectives 1, 2 and 3:

Most of the treatments in the trials were integrated biological and chemical programs beginning with Blossom Protect + Buffer Protect, followed by two treatments of a non-antibiotic chemical (Table 1).

Infection suppression. **In pear**, outstanding suppression (> 83%) was observed with FireWall 50, and Blossom Protect plus Buffer Protect (twice), and Blossom Protect plus Buffer Protect (once) followed by Previsto (twice). Most other treatments in the pear trial also reduced significantly ($P <$ 0.05) the incidence of infection when compared to the water-treated control; exceptions were alum as a solitary treatment, Blossom Protect/Buffer Protect followed by Serenade Opti (once) then lime sulfur (once), and the two treatments of lab-grown *Cystofilobasidium infirmominiatum* (strain YY6) and *Cryptococcus spp.*(strain C16). **In apple,** excellent suppression (> 90%) was observed with all treatment programs except *Cystofilobasidium* (strain YY6) and *Cryptococcus spp.* (strain C16), which provided high levels of suppression (> 80%).

Pathogen populations in flowers. **In pear**, with cool weather for most of bloom, pathogen populations on flowers were initially in the range of 100 to 10,000 CFU/flower but increased by three orders of magnitude (>1000-fold) with the warmer weather in the post-petal fall period. Integrated programs of Blossom Protect followed by alum or Previsto showed suppression of pathogen populations on flowers that were similar to streptomycin (Fig. 2A). **In apple**, an integrated program

of Blossom Protect followed by Previsto (Fig. 2A) showed suppression of pathogen populations on flowers that was similar to streptomycin (Fig. 2B).

Yeast populations in flowers. **In both pear and apple,** the alternative yeast treatments (Fig. 3 A&B) developed floral populations as high as or higher than Blossom Protect strains of *A. pullulans.* For trees that received Blossom Protect, integrated programs ending with 4% lime sulfur treatment or Jet Ag at petal fall had comparatively low yeast populations on developing fruitlets as the trees entered the post-bloom period (Fig. 3A&B).

Floral pH. **In both pear and apple,** relative to other treatments, lower floral pH measurements were associated with spray programs that included two treatments of alum or alum-containing, VP20 (Fig. 4A&B).

Table 1. Evaluluation of non-antibiotic materials for fire blight control in Bartlett pear and Golden Delicious apple, Corvallis, 2018.

Treatment	Rate per 100 gallons water	Stage treatment applied* 70% bloom	Full bloom	Petal Fall	PEAR Percent blighted floral clusters **		APPLE Percent blighted floral clusters**	
Water		**---** §	**X**	**X**	29.7	**a**	35.9	**a**
FireWall	8 oz.	---	X	---	4.5	ef	0.8	d
Alum 1%	133.5 oz.	---	X	X	16.9	abc	1.7	cd
VP20	144 oz.	---	X	X	16.2	bc	1.1	cd
Blossom Protect	**21.4 oz.**	**X**	**X**	**---**	**5.8**	**def**	**2.2**	**bcd**
Buffer Protect	**150 oz.**	**X**	**X**	**---**				
Blossom Protect	21.4 oz.	X	---	---	9.9	cde	0.5	d
Buffer Protect	150 oz.	X	---	---				
then Alum 1%	133.5 oz.	---	X	X				
Blossom Protect	21.4 oz.	X	---	---	11.1	cd	3.1	bcd
Buffer Protect	150 oz.	X	---	---				
then VP20	144 oz.	---	X	X				
Blossom Protect	21.4 oz.	X	---	---	4.2	ef	0.9	d
Buffer Protect	150 oz.	X	---	---				
then Previsto 1%	96 fl. oz.	---	X	X				
Blossom Protect	21.4 oz.	X	---	---	22.6	ab	1.0	cd
Buffer Protect	150 oz.	X	---	---				
then Serenade Opti (Biolink)	20 oz.	---	X	---				
then Lime sulfur 4%	512 fl. oz		---	X				
Blossom Protect	21.4 oz.	X	---	---	13.0	bcd	2.5	bcd
Buffer Protect	150 oz.	X	---	---				
then Serenade Opti (Biolink)	20 oz.	---	X	---				
then Jet Ag	167 fl. oz		---	X				
Blossom Protect	21.4 oz.	X	---	---	14.6	bc	-	
Buffer Protect	150 oz.	X	---	---				
then Serenade Opti (Biolink)	20 oz.	---	X	X				
Blossom Protect	21.4 oz.	X	---	---	15.1	bc	-	
Buffer Protect	150 oz.	X	---	---				
then Aleo (Brandt 719)	6 oz.	--	X	X				
ALTERNATIVE YEASTS								
C. infirmominiatum YY6	10^7 cfu/ml	X	X	---	17.8	abc	3.9	bcd
Buffer Protect	150 oz.	X	X	---				
C. neoformans C16	10^7 cfu/ml	X	X	---	17.9	abc	5.5	b
Buffer Protect	150 oz.	X	X	---				
A. pullulans AP3	10^7 cfu/ml	X	X	---	17.1	bc	-	
Buffer Protect	150 oz,	X	X	---				

*** Trees inoculated with *Erwinia amylovora* strain Ea153N (streptomycin-sensitive) at 1 x 10^6 CFU/ml on 12 April (pear) and 25 April (apple). ** Trees used in the experiments averaged 841 and 256 flower clusters per tree for pear and apple, respectively. For each treatment, percent blighted flower clusters was transformed arcsine($\sqrt{x}$) prior to**

analysis of variance; non-transformed means are shown. § X indicates material was sprayed at that bloom stage date; --- indicates material was not applied at that bloom stage. Means within a column followed by the same letter are not significantly different according to Fischer's protected least significance difference at *P* = 0.05.

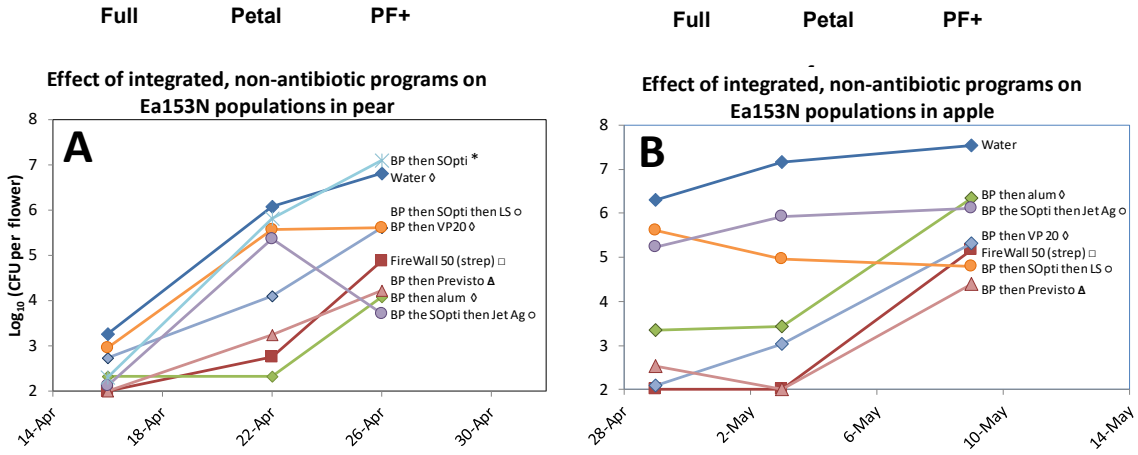


Fig. 2. Effect of treatments applied to A) Bartlett pear and B) Golden delicious apple trees to suppress fire blight on the population size of *E. amylovora* strain 153N on flowers during April and May 2018. Pathogen populations were determined by washing five flower clusters (~25 flowers, bulked) from each replicate tree, and plating the wash onto a selective culture medium. Log_{10} = 2.0 was the detection limit of the assay. Data depict mean of each treatment program on each sampling date.

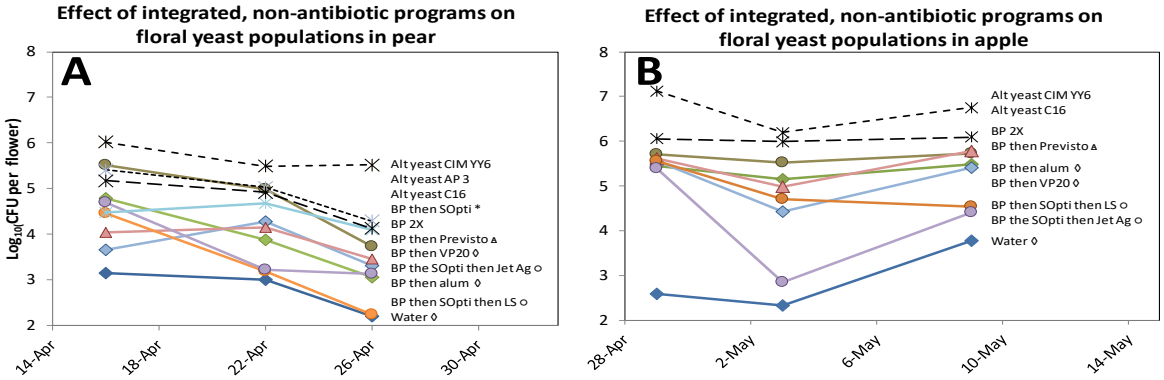


Fig. 3. Effect of treatments applied to A) Bartlett pear and B) Golden delicious apple trees to suppress fire blight on the population size of *Aureobasidium pullulans* (the yeast in Blossom Protect) on flowers during April and May 2018. *A. pullulans* populations were determined by washing five flower clusters (~25 flowers, bulked) from each replicate tree and plating the wash onto a selective culture medium. Data depict mean of each treatment program on each sampling date.

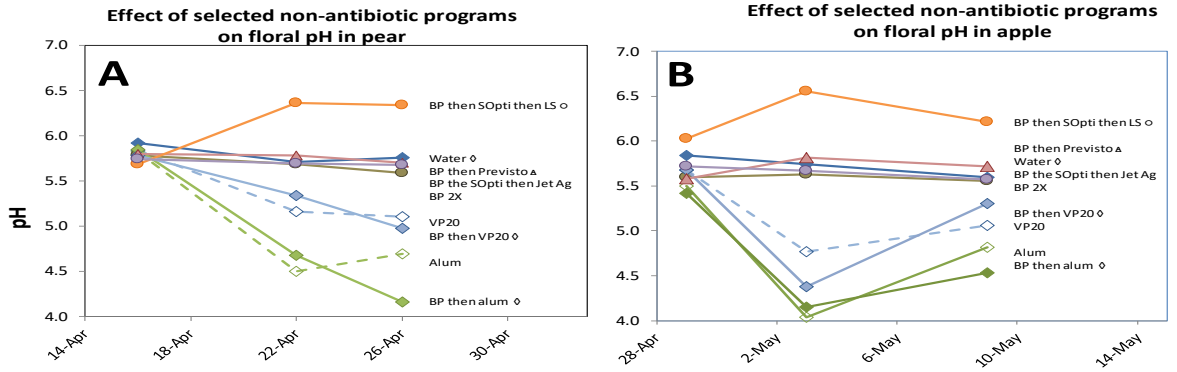


Fig. 4. Effect of treatments applied to A) Bartlett pear and B) Golden delicious apple trees to suppress fire blight on the pH of floral surfaces during April and May 2018. A hand-held pH-probe was placed in a deionized-water wash of five flower clusters (~25 flowers, bulked in 25 ml of water) from each replicate tree. Data depict mean of each treatment program on each sampling date.

Fruit russeting. Application of non-antibiotic fire blight materials to Comice pear and Fuji apple resulted in significant differences ($P \leq 0.05$) in fruit russeting severity, although the specific material that caused the most russeting differed among the trials. For example, Serenade Opti (full bloom) then 4% lime sulfur (petal fall) was the most injurious material treatment applied to Comice pear, but this same treatment resulted in the least amount of russeting on Fuji apple (less than the water control). In contrast, Previsto copper elevated fruit russeting on pear by two-fold compared to the water control but the same material elevated russeting on Fuji apple by six-fold compared to the water control. In both trials, alum and the alum-containing material, VP 20, elevated russeting severity by two- to three-fold compared to the water control.

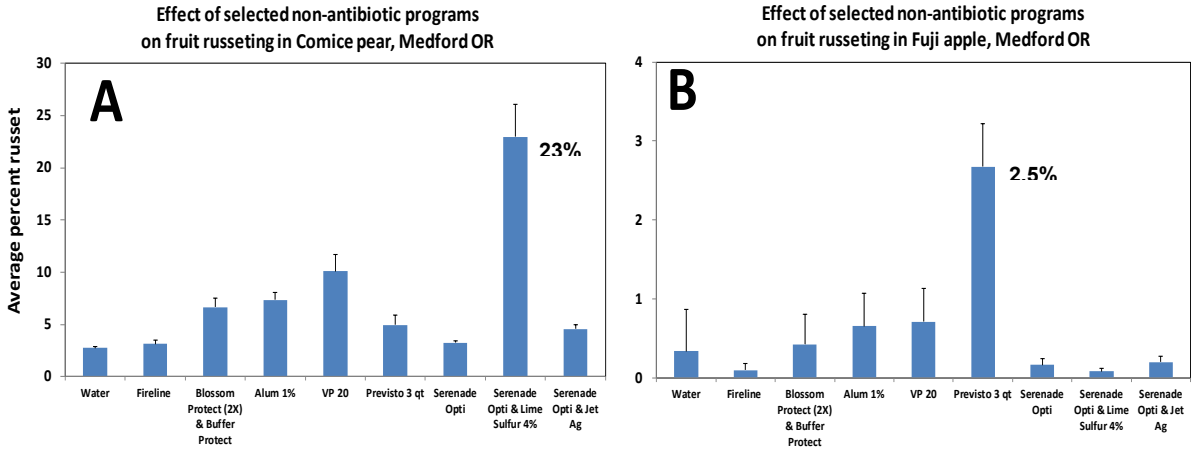


Fig. 5. Effect of non-antibiotic fire blight control materials applied to A) Comice pear and B) Fuji apple trees on severity of russeting injury (%) of the fruit surface. Orchards were located near Medford, OR. Treatments were applied to trees at full bloom and at petal fall (April). In late August, 30 fruit from each replicate tree were rated for russeting severity. Data depict mean and standard error from four replicate trees that received each treatment.

Objective 4. In 2017, a concentrated Actigard (50WG, acibenzolar-S-methyl, 50% a. i., Syngenta Crop Protection, Greensboro, NC) trunk treatment applied once at 30% bloom provided outstanding (> 85%) suppression of floral infection by the fire blight pathogen. Consequently, the purpose of the 2018 trials was to determine 1) if this response was reproducible and 2) if the amount of Actigard required to observe the response could be reduced. In the 2018 trials, disease intensity was low to moderate with fire blight infections on water-treated or no-treatment control treated trees, which averaged 5 to 15 strikes per tree (11 to 38% of total clusters). Only the full rate of Actigard (i.e., the concentrated treatment applied to both sides of a 1-m length of the central leader) provided a significant response ($P \leq 0.05$), and only when compared to the water-treated control in the Cripps Pink trial (Table 2). In this trial, the Actigard-full trunk treatment averaged 8% infected clusters, which was significantly lower ($P \leq 0.05$) than the water-control (24%) but not the no-treatment control (11.4%). In the Gala trial, all treatments were similar statistically, and none of the treatments had a significant effect ($P > 0.05$) on the incidence of infection when compared to the no-treatment control. Nonetheless, the trend in the data was that the full and ½ rates of Actigard had 35% less infection than the control (and the other treatments in the trial, which were 1/4 rate of Actigard, aspirin *(acetylsalicylic acid, Kroger, Cincinnati, OH)* , or salicylic acid (SAR Activator SA (10% salicylic acid, Growth Products, White Plains, NY)).

Discussion. Since 2015, we have measured fire blight pathogen populations in flowers at the growth stages of full bloom, petal fall and a week post-petal fall. In each season, we consistently observe pathogen populations continuing to increase into the post-petal fall period, often increasing greatly after the residual effects of the petal fall-treatment timing has begun to dissipate (Fig. 2). Because populations > 10^5 CFU per flowers are the threshold population required for infection, it is likely the increased populations after petal fall account for at least some of the observed infections. The

Table 2. Evaluluation of trunks paints of systemic acquired resistance inducing materials for fire blight control in Gala apple, Corvallis, 2018.

		Date treatment applied*	GALA		CRIPPS PINK	
Treatment	Rate per quart water	18 Apr 10% bloom	% blighted floral clusters**		% blighted floral clusters**	
Water-treated	**-**	**X[§]**	**---**		**23.9**	**a**
Non-treated	**-**	**---[§]**	**38**	**a**	**11.4**	**ab**
Actigard - full	1 oz.	X	25	a	7.9	b
Actigard – ½ one side	1 oz.	X	24	a	13.1	ab
Actigard – ¼ low ring	1 oz.	X	36	a	22.2	a
aspirin - full	1 oz.	X	41	a	23.1	a
SAR Activator SA - full	16 fl. oz.	X	47	a	14.7	ab

*** Trees inoculated on 24 April with 1 x 10^6 CFU/ml *Erwinia amylovora* strain Ea153N (streptomycin- and oxytetracycline-sensitive fire blight pathogen strain).**
**** Transformed arcsine($\sqrt{x}$) prior to analysis of variance; non-transformed means are shown.**
[§] 'X' indicates material was applied on 18 April '---' indicates material was not applied on that date.
[#] Means within a column followed by same letter do not differ significantly ($P = 0.05$) based on Fischer's protected least significance difference.

duration of the effective residual of specific non-antibiotic materials after a spray treatment (e.g., at petal fall) is a poorly understood part of fire blight suppression.

Collectively, the charts (Fig. 2-4) illustrate how the various materials effect infection, pathogen populations and fruit finish, and highlight the difficulty (and frustrations with) non-antibiotic fire blight control. A primary frustration is that most effective materials (copper, alum) also elevate the risk of fruit russeting. In addition, other effective materials (e.g., Blossom Protect) do not have a great impact on pathogen populations, which results in greater inoculum carryover into the post-petal fall period. For apple, lime sulfur continues to look like a useful material for late bloom suppression of pathogen populations with limited fruit russeting risk. Trials in 2019 will examine lime sulfur treatment after use of effective materials at full bloom (Previsto, alum). We also want to determine if higher rates of fruit-safe materials such as Serenade improve control. These rates would be above the current Serenade label, but the data could potentially be a basis for label modification.

On Objective 3 (yeast biocontrol), our initial hypothesis is that *A. pullulans*-based biocontrol would not be strain specific. We adopted this hypothesis because the strains in Blossom Protect were selected originally to suppress postharvest fruit rots of pome fruit and not fire blight. Somewhat surprisingly, the alternative yeasts attained higher populations on flowers than the Blossom Protect

strains of *A. pullulans*. They also suppressed fire blight, but not to the same degree as the strains in Blossom Protect. We will evaluate this objective again in 2019.

Other than Actigard, we have not identified another material that consistently induces SAR in apple trees. Also, trunk paints of Actigard were not as effective in 2018 as in 2017. In 2018, both orchard blocks experienced a relatively light bloom and high tree-to tree variability in bloom density, which confounded the experimental design. Some of this unexplained variability was apparent with the low amount of disease that developed in some of the control treatments.

CONTINUING PROJECT REPORT **YEAR**: 1 of 3
WTFRC Project Number: CP-18-102

Project Title: Understand the epidemiology of Botrytis to curb gray mold postharvest

PI: Achour Amiri
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PI: Tobin Peever
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City/State/Zip: Pullman/WA/99164-6230

Cooperators: Chelan Fruit, Gebbers Farms, Allan Brothers

Total Project Request: **Year 1:** $32,360 **Year 2:** **$34,943** **Year 3:** $33,371

Other funding sources
Agency Name: WSDA-Specialty Crop Block Grant program
Amt. awarded: $170,195
Notes: "Strategies to enhance pre- and postharvest management of gray mold in pome fruit" PI: Amiri, co-PI: Tobin Peever

WTFRC Collaborative Expenses: None

Budget 1: (Achour Amiri)
Organization Name: WSU Contract Administrator: Katy Roberts/Kim Rains
Telephone: 509-335-2885/509-293-8803 Email address: arcgrant@wsu.edu / kim.rains@wsu.edu

Item	2018	2019	2020
Salaries[1]	14,400	14,976	15,575
Benefits[1]	6,385	6,640	6,906
Wages[2]	5,760	5,990	6,230
Benefits[2]	545	567	590
Equipment			
Supplies[3]	1,500	3,000	3,000
Travel[4]	1,070	1,070	1,070
Miscellaneous			
Plot Fees[5]	2,700	2,700	
Total	32,360	**34,943**	33,371

Footnotes:
[1] Salaries are for a Research Intern (0.3 FTE) at 44.3% benefit rate.
[2] Wages are for non-student temporary employee for summer help at 9.5% benefit rate.
3 Supplies include reagents for PCR and qPCR, agar media, plates and sampling materials.
[4] Travel to commercial and experimental orchards and packinghouses in WA for trials set -up, sampling and data collection.
[5] Plot fees for a block to be used for preharvest trial on gray mold in years 1 and 2.

OBJECTIVES:

Objective 1. Investigate infections timing of fruit by *Botrytis* in Washington apple orchard to determine critical timing of infections and subsequent disease expression in storage.

Objective 2. Evaluate the effect of preharvest weather conditions (rain and temperature) on infections and gray mold development in organic and conventional orchards.

Objective 3. Define the genetic structure of the *Botrytis* populations in pre- and postharvest environments and its impact on fitness, pathogenicity and resistance to commonly used fungicides.

SIGNIFICANT FINDINGS:

- *Botrytis* was detected on flowers and fruit collected throughout the season from bloom to harvest.
- *Botrytis* was detected in the airs of orchards throughout the season from bloom to harvest.
- The size of *Botrytis* inoculum was greater in organic orchards compared to conventional orchards.
- The inoculum size decreased from bloom to fruit set in conventional but then increased toward maturity and harvest. In organic orchards, the inoculum size increased throughout the season.
- Variabilities in inoculum size and dynamic throughout the season has been observed between orchards located in different districts.

METHODS

Objective 1. Investigate infections timing of fruit by *Botrytis* in Washington apple orchard to determine critical timing of infections and subsequent disease expression in storage.

In spring of 2018, 60 apple blossoms were collected from four orchards throughout WA including two conventional and two organic orchards. Orchards were located in Okanogan county (Pateros), Chelan County (Orondo), and in Grant County (Othello and Mesa). In the summer, 60 fruit were collected from the same trees and orchards used for flowers sampling on a monthly basis from fruit setting to harvest. Blossom and fruit samples were transported in separate clean bags to the Pathology Lab at Washington State-Tree Fruit and Extension Center (TFREC) in Wenatchee. Flowers were freeze-dried and stored at -80°C. Fruit were peeled and the peel and the flesh of the fruit will be freeze-dried separately and stored at -80°C. The separation of the peel from the flesh will help separate between infestation (spores present on the surface) from endophyte infections (present inside the fruit). DNA was extracted from freeze-dried samples and the presence of *Botrytis* were detected using a quantitative polymerase chain reaction (qPCR) assay (Diguta et al. 2010). Alternatively, spores of *Botrytis* were enumerated from fresh (non-dried samples) on a *Botrytis* semi-selective artificial agar medium (Edwards and Seddon 2001).

Objective 2. *Evaluate the role of preharvest weather conditions (rain and temperature) on infections and gray mold development in organic and conventional orchards.*

Temperature and wetness are the two main factors that drive *Botrytis* infections. In central Washington, wetness (>10 hours) can be primordial in early season and at bloom when flowers can be very susceptible to *Botrytis* infections and between September and November for fruit, whereas temperatures

of late spring and early summer (65 to 78°F) and at harvest in September through October combined with rain can be very conducive for fruit infections. The objective is to make a correlation between temperatures and wetness and *Botrytis* infections throughout the season. Protocols and trials described in Objective 1 will be used for this objective. Weather data (rain and temperature) will be collected from the Washington State University-AgWeaterNet (http://www.weather.wsu.edu/) in way to obtain data for all and each sampled orchard from the closet weather station. A correlation between rain and temperature occurring at bloom and at each period of fruit sampling and *Botrytis* incidence on flowers and fruit from the orchards will be analyzed. We will conduct trials for 3 years and we aim to collect enough consistent data that would help construct a predictive model for *Botrytis* infections in the future (not part of this project).

Objective 3. *Define the genetic structure of the Botrytis populations in pre- and postharvest environments and its impact on fitness, pathogenicity and resistance to commonly used fungicides.*

Botrytis isolates collected from flowers (early season: overwintered populations and those collected at bloom and throughout the growing season) and fruit in the orchard (summer and late-season) as well as from decayed fruit (after several months of storage) will be DNA fingerprinted using molecular markers. A set of 12 microsatellite markers developed by Fournier et al. (2002) and in the laboratory of Co-PI Peever will be used to fingerprint isolates. These markers are currently being used to fingerprint Pacific Northwest isolates of *B. cinerea* from small fruit in Peever lab. We will test the hypothesis that gray mold infections initiated in the orchard contribute substantially to packinghouse infections. The alternative hypothesis is that packinghouse infections although initiated in the orchard undergo a long period of cold temperature and controlled atmospheres (Low O_2 and high CO_2 concentrations) and may be genetically impacted. The use of these molecular markers will allow us to verify these hypotheses. We will also use these markers to investigate the impact of fungicide sprays (Organic vs. conventional orchards) and resistance levels on the evolution of *Botrytis* populations in apple orchards and packinghouses, as well as on their fitness and pathogenicity. Gray mold of fruit crops is known to be mainly caused by the species *Botrytis cinerea*. However, recent studies from strawberry, blueberry, blackberry, and grape have reported that gray mold can also be caused by species other than *B. cinerea*. The importance of these other species, including *Botrytis pseudocinerea, B. mali, B. group S, and B. californicae*, as decay agents in Washington apple storage facilities will be determined using genetic markers specific to these species (Dowling and Schnabel 2017; Fournier et al. 2003; Leroch et al. 2013; Li et al. 2012; Saito et al. 2016).

RESULTS AND DISCUSSION

Objective 1. Dynamic of *Botrytis* population size through the preharvest season

As shown on Figure 1 below, *Botrytis* was detected in orchards regardless on the management type. However, the incidence of *Botrytis* was greater in organic orchards compared to the conventionally-managed orchard. There seem to be a carry-over from bloom to fruit set in organic orchard whereas the incidence of Botrytis in conventional orchards decreased slightly between bloom and fruit set stage before picking-up again towards commercial maturity. Fungicide spray programs in conventional orchard are being analyzed to correlate with potential fungicide spray effect. While similar *Botrytis* patterns were generally observed between the two conventional orchards (Figure 1B), the amplitude of the progression in *Botrytis* populations change was different in the two organic orchards as more inoculum was seen in the orchard located in Pateros. The age of the two orchards and the weed management aspect could account for this difference.

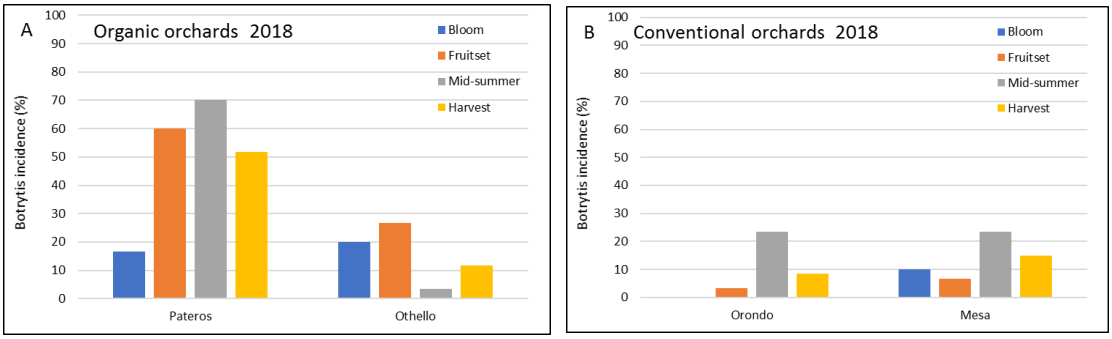

Figure 1. Evolution of *Botrytis* incidence throughout the preharvest growing season in organic (A) and conventional (B) orchards.

Infections by *Botrytis* were observed in all parts of the fruit (cuticle, stem-end, calyx and inner flesh) at harvest with Botrytis incidence varying from an orchard to another. This observation indicates that not only the external parts (calyx, cuticle and stem-end) of the fruit contains *Botrytis* inoculum at harvest, but also the flesh which indicates latent (dormant) infections from previous infections in the orchard.

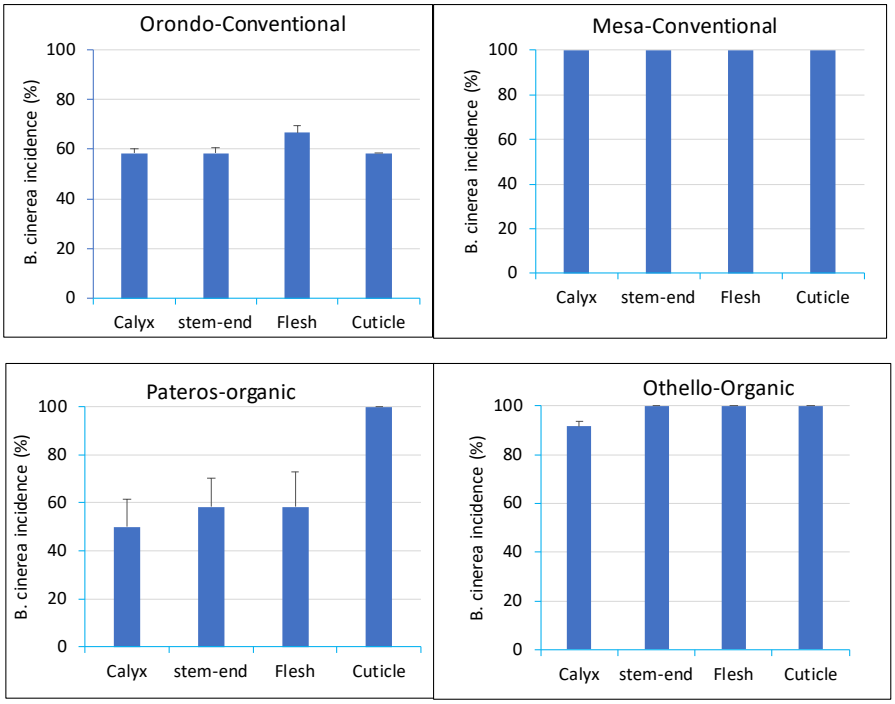

Figure 1. Incidence of *Botrytis cinerea* on different parts of the fruit at commercial maturity (harvest time) in organic and conventional orchards.

Progression of *Botrytis* infections in storage (ongoing): Fruit were harvested at commercial maturity from each of the four orchards and are stored in same storage facilities where all fruit the same orchards are stored. Fruit will be analyzed for *Botrytis* presence in March of 2019 (6 months of storage).

Impact of weather conditions on Botrytis incidence preharvest (ongoing): Weather parameters including temperature, wetness, and rainfall from all the four orchards used in the study we collected

and are being analyzed to detect any correlation with variability in Botrytis incidence between locations and throughout the season.

Genetic population structure: In total, 150 isolates of *Botrytis* were collected from the orchards used in this study. The isolates are stored in glycerol at -112°F until more isolates from storage rooms are collected in March of 2019 to conduct a genetic analysis in order to detect the presence of species other than B. cinerea in populations from Washington.

Future work:

2019-2020: Analyze weather data and impact on botrytis incidence

Obtain data from cold storage facilities on samples collected from previous season

Conduct a 2nd year of field trials in the same orchards used in Year 1 for comparison

Conduct the genetic analyses of Botrytis collected during the 2018-19 season.

CONTINUING PROJECT REPORT **YEAR:** 1 of 3
WTFRC Project: CP-18-105

Project Title: Using cold storage to increase the stability of honey bee supply

PI:	Brandon Hopkins	**Co-PI:**	Walter Sheppard
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Address 2:	PO Box 646382	**Address 2:**	PO Box 646382
City/State/Zip:	Pullman WA 99164-6382	**City/State/Zip:**	Pullman WA 99164

Cooperators: Olson's Honey, Idaho Bee Storage

Total Project Request:
(Original Request) - Year 1: $209,822 Year 2: $17,754

Awarded - Year 1 (2018): $100,000

Modified Request – Year 2 (2019): **$28,314** Year 3: $31,864

Other funding sources

Agency Name: California Almond Board
Amt. awarded: $97,000
Notes: The award from the Almond Board was made possible by leveraging the money provided by the WTFRC to secure the remainder of the funding required to complete the construction of the research equipment needed for the research described in this proposal

Agency Name: USDA-NIFA
Amt. requested: $499,800
Notes: The proposal included research that is only possible because of the funding awarded by the WTFRC and the Almond Board of California. We should hear from the funding agency before the Research Review in January

Organization Name: WSU **Contract Administrator:** Katy Roberts
Telephone: 509-335-2885 **Email:** arcgrants@wsu.edu

Item	2018	**2019**	2020
Salaries		15,290	18348
Benefits		2464	2956
Wages		6000	6000
Benefits		1560	1560
Goods and Services	100,000		
Shipping			
Supplies		3000	3000
Travel			
Plot Fees			
Miscellaneous			

Total	100,000	**28,314**	31,864

OBJECTIVES

Goals
The initial goal of this proposal is to construct three controlled atmosphere rooms capable of holding a significant number of honey bee colonies for winter and summer experiments. The second goal is to utilize the controlled atmosphere facilities to address the following objectives:

Objectives:
1. Optimize controlled atmosphere storage conditions (CO_2, humidity, temperature) to maximize Varroa mite mortality while producing healthier bees following the winter storage period.
2. Determine ideal timing and storage conditions for mid-season (summer) honey bee colony cold-storage to create a break in the brood cycle that allows beekeepers to more efficiently and effectively control Varroa.
3. Utilize the combined findings from the objectives above to develop a whole season IPM strategy for commercial tree fruit pollinators. Implement that strategy in collaboration with a commercial beekeeping operation to demonstrate the feasibility and economic benefit to the commercial beekeeping industry.
4. Leverage the new facilities to attain additional funding to expand the research/utility of indoor controlled as it applies to the stabilization of the beekeeping industry for the benefit of the tree fruit industry

SIGNIFICANT FINDINGS
Provide a bulleted list of significant findings during the prior year(s) of the project.

- The most significant progress is related to Objective 4. We were able to leverage funding provided by the WTFRC to secure funding from the Almond Board of California to get enough funds to move forward with the facilities needed. The time to get these funds and the time for design and planning with WSU facilities has pushed the timeline of the original grant back approximately 1 year (updated in methods below).

- With the combined funds from WTFRC and the Almond Board for the facilities, we were able to apply for USDA-NIFA funding to expand the research associated with this project

- Continued to gather preliminary data regarding objective 2. In that we completed an observational study with a commercial beekeeper in Idaho where we were able to force colonies into a broodless state and significantly improve varroa control in those colonies compared to colonies with capped brood (Fig 1).

METHODS
The research laid out in this proposal is dependent on an initial acquisition of three 1280 ft^3 controlled atmosphere chambers. Each chamber will be capable of holding 80 colonies at adjustable temperature, CO_2 and humidity levels. Temperature adjustments will range from 35-55° F with a range of CO_2 levels from normal atmospheric (300 ppm) to 100,000 ppm (10%). The design has been started and WSU facilities has placed the project on the list of projects. The goal for completion has been set for August 2019

Year 1 (2018) – Procurement of additional funding and design of research chambers - **Completed**

Controlled atmosphere honey bee wintering-

Year 2- indoor wintering - 2019

July – September - Construction and placement of CA experimental chambers,
WSU facilities will prepare the site close to the existing WSU bee facility (Pullman) and prefabricated CA experimental chambers will be set up and operational by late September 2019.

October -160 honey bee colonies will be assessed. The number of frames of bees and brood will be recorded. Samples of bees will be collected in alcohol to determine the initial Varroa mite load in each colony. Those sample will also be used to determine tracheal mite and nosema infection (two economically important pests besides Varroa mite). Each colony will be weighed before and after the trial period. Four colonies in each treatment will be fitted with a hive scale to continually log the weight change through the trial period.

November – Using information about the initial Varroa mite loads for each colony; colonies will be assigned to one of three controlled atmosphere rooms and a set of 40 colonies will remain outdoors for the winter as an additional control. The colonies will be distributed so that each group contains, on average, the same Varroa mite load. All rooms will be set at 40°F and the manipulated variable will be CO_2. One room will be held at normal atmospheric level, room 2 at 4% CO_2 (40,000 ppm) and room 3 will be set at 8% CO_2 (80,000 ppm). Each hive will be fitted with a screened bottom (allows falling debris/mites to fall through) and a "sticky card" will be swapped out fresh each week for the 65 day trial. Each of the sticky cards will be assessed to determine mitefall. At the end of the 65 day storage period the colonies will be removed and placed outdoors where a miticide treatment will applied along with a fresh sticky card. The number of mites remaining after storage will be compared to the total number of mites gathered on all sticky cards during storage to determine Varroa mite mortality caused by the treatment period. All treatments will be compared for effects on population size, survival and mite loads.

Year 3 – Indoor wintering - 2020

October – The experimental set up from winter of 2019 will be duplicated in year 3, except the CO_2 level that provided the best control of Varroa will be used in all three chambers. In year 3, we will evaluate the effects of humidity at three levels (45%, 65%, 80%) to enhance mite mortality. Previous research has suggested that Varroa mite mortality in the winter is increased due to dehydration because mites have a much larger surface area to volume ratio than honey bees. Carbon dioxide interferes with control of respiratory openings that has a major effect on controlling water loss. It is our hypothesis that by increasing CO_2 and decreasing humidity we can increase mite mortality while bees are stored indoors for the winter months.

Controlled atmosphere Mid-season brood break for enhanced Varroa control
Year 3- Summer brood break – 2020

August – 160 honey bee colonies will have the honey crop removed and colonies assessed prior to the onset of the trial. The number of frames of bees and brood will be recorded. Samples of bees will be collected in alcohol to determine the initial Varroa mite load and tracheal and nosema infection in each colony. Each colony will be weighed before and after the trial period. Four colonies in each treatment will be fitted with a hive scale to continually log the weight change through the trial period. Those same colonies will be fitted with traps at the entrance to monitor the number of bees that die and are removed from the colony during the trial period.

With information about the Varroa mite loads for each colony; colonies will be assigned to one of three controlled atmosphere rooms and a set of 40 colonies will remain outdoors for the 3-week trial as an additional control. The colonies will be distributed so that each group contains, on average, the same Varroa mite load. They will be placed in the controlled atmosphere chambers for 18 days. One room will be set at 40°F and in complete darkness. The second room will be held at 50°F in complete darkness. The third room will be held at 40°F with a season normal diurnal light cycle. Each hive will be fitted with a screened bottom (allows falling debris to fall through) and under the screened bottom a "sticky card" will be placed. At the end of the 18-day storage period the colonies will be removed and placed outdoors where a miticide treatment will applied along with a fresh "sticky card". The number of mites gathered following the miticide treatment will be compared to the total number of mites gathered on all sticky cards to determine the Varroa mite mortality caused by the treatment period. All colonies will be assessed again as they were at the start of the experiment and health, colony size, mite loads will be compared. These colonies will then be followed through the rest of the season until it becomes too cold to work bees (below 50°F). They will be sampled and assessed at one and two months post-treatment date. They will again be assessed when they are moved to California for almond pollination the following year

Year 4 - 2021

Using findings from the experiments described above we will engage with commercial beekeeper collaborator to follow and study 400 honey bee colonies using the CA overwintering facilities of our collaborator (Olson's Honey) in Yakima WA. We will assess all 400 as described in methods above during the period of almond pollination in California. All colonies will be given a numbered tag. At the end of the almond pollination season, all colonies will be treated with the most widely used registered commercial product for Varroa control (Apivar). Hives will again be assessed while colonies are in apple orchards during pollination. Any colony issues (queenless, bacterial or fungal disease) other than those caused by Varroa mite will be remedied and recorded. Nutritional issues and swarm control will be decided on by the beekeeper, consistent with his normal operating procedures. Colonies will be assessed again in the summer after canola seed pollination during which time the honey crop will be removed. After this assessment colonies will be divided into 4 treatment groups with the average Varroa load and colony strength equally distributed across all 4 treatment groups. Treatment group 1 (commercial standard control) will be treated with industry standard miticide treatment in conjunction with rest of the commercial operation and will be moved to California "holding yard" in November when the rest of the groups are moved to controlled atmosphere storage for the winter. Treatment group 2 will be treated the same as group 1 except that it will be placed in controlled atmosphere storage for the winter months before almond pollination begins. Treatment group 3 will be moved into a controlled atmosphere facility for 18 days to create a break in the brood cycle. After which it will be treated with a single application of miticide. Treatment 4 will remain outside isolated from the rest of the commercial operation and left untreated. All colonies will be assessed again in October as they are prepared for winter. Colony strength, Varroa loads and survival will be compared between treatment groups after the October sampling and again in January as they are prepared for placement in almond orchards.

RESULTS & DISCUSSION

Focus on the findings during the prior year(s) of the project. Discuss significance to the industry and potential economic benefits. Use summary graphics.

The work on this project has been focused on securing additional funding and planning with WSU facilities to prepare for arrival of the new equipment. We utilized the funding provided by the WTRC to secure the additional funds need for the controlled atmosphere chambers from the Almond Board

of California ($100,000). The chambers will provide a wealth of research potetial moving forward and the combined funding that provided these chambers allowed us to persure USDA-NIFA funding that utilizes the future chambers for honey bee research. All preliminary evidence suggests that this line of research will provide valuable information for the commercial beekeeping industry to help keep more colonies alive and stabilize pollination supplies.

One of the comments/feedback we received about the concept of forcing period of broodlessness was that our preliminary work was done in August and many beekeepers are still producing honey at that time. We performed an additional observational study with a commercial beekeeper in southern Idaho who allowed us to follow a set of his colonies after almond pollination (April). We demonstrated the ability to stop brood rearing and significantly increase Varroa mite control.

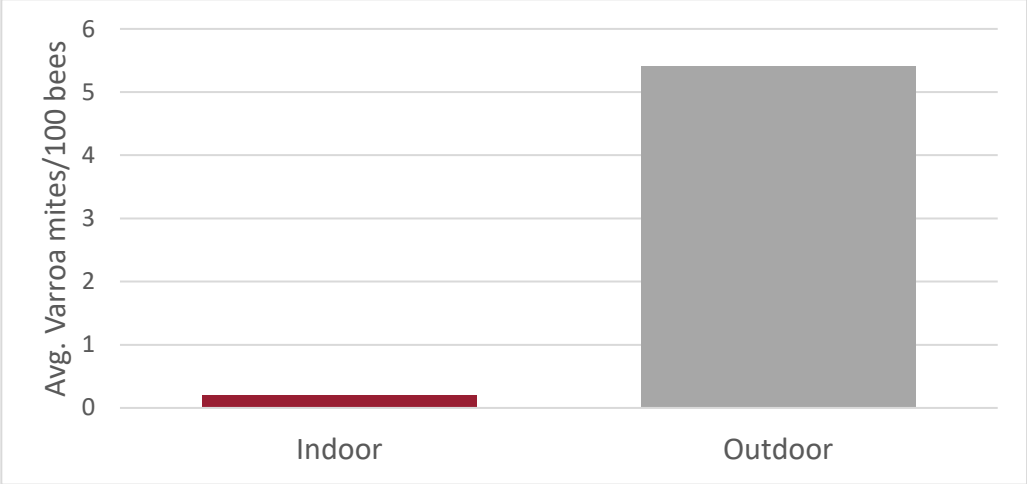

Figure 1: Average Varroa mite populations in colonies that experienced a break in brood production compared to colonies outdoors that continued brood rearing. All colonies experienced the same varroa treatment. The only difference being whether they were moved indoors for 18 days or remained outdoors.

CONTINUING PROJECT REPORT **YEAR**: 2 of 3
WTFRC Project Number: CP-17-102

Project Title: Optimizing control for leafrollers and Western tentiform leafminer

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Cooperators: Nick Stephens, Columbia IPM; Dave Gleason Domex Superfresh Growers

Total Project Request: **Year 1:** $78,428 **Year 2:** $81,565 **Year 3: $38,934**

Other funding sources: None

Budget 1
Organization Name: Washington State University **Contract Administrator:** Katy Roberts/Kim Rains
Telephone: 509-335-2885/509-293-8803 **Email address:** katy.roberts@wsu.edu/kim.rains@wsu.edu

Item	2017	2018	**2019**
Salaries[1]	45,000	46,800	22,725
Benefits[2]	17,069	17,751	7,817
Wages	9,600	9,984	4,800
Benefits[3]	259	270	77
Equipment			
Supplies[4]	2,500	2,600	1,352
Travel	4,000	4,160	2,163
Miscellaneous			
Plot Fees			
Total	78,428	81,565	**38,934**

Footnotes:
[1] new position
[2] 34.1%
[3] 2.7%
[4] includes lab and field supplies
[5] w/in state travel

Objectives:

1. Evaluate different timing strategies for leafroller management using *Bt* and/or Entrust in organic orchards

2. Evaluate control strategies for leafroller in conventional orchards

3. Determine the causes of western tentiform leafminer (WTLM) outbreaks and evaluate how to optimize management to reduce effects on parasitoids.

Significant Findings:

- For the past 2 years, we have put out the treatments for OBLR laid out in objectives 1 & 2, but populations in previously infested areas have not developed. We have decided to drop these two objectives (with approval of Dr. Hanrahan), reduced the budget in the final year, and will focus on objective 3.
- Comparison of conventional and organic orchards showed that western tentiform leafminer (WTLM) pheromone trap catches were much higher in the organic blocks, and leaf samples showed all the leafmining activity was restricted to the organic blocks.
- Parasitism of the leafminers occurred primarily at between 22 Aug and 11 Sept, with 91% of the parasitoids captured on sticky cards occurring during this period and only small numbers before that.
- The parasitoid *Pholestesor ornigris*, which was not previously reported as occurring in North Central Washington apple production areas, was about 10x more common than *Pnagalio flavipes,* which was previously recorded as the dominant parasitoid.
- Phenology of adult trap captures of WTLM appears consistent with previous data but is more variable at the organic locations in general.
- WALH was also found in much higher levels in most commercial organic sites compared to the conventional blocks adjacent.

Objectives 1 & 2: *(1) Evaluate different timing strategies for leafroller management using Bt and/or Entrust in organic orchards (2). Evaluate control strategies for leafroller in conventional orchards*

Methods: This experiment was designed to assess spray timings for managing obliquebanded leafroller (OBLR) using organic and conventional insecticides. Objective 2 was not implemented because no conventional blocks of apples containing significant OBLR populations were found.

The basis of this study comes from computer simulations that show that there are only four windows in time where sprays can significantly depress OBLR population levels, with two of those windows having the biggest impacts on final population levels. In the *Bt* (Deliver, Certis USA) treatments, we targeted different combinations of the four windows in field trials to evaluate the model predictions and how much control is improved with each window. It is likely that only 2 of the windows need to be targeted.

Results. Sprays were applied to mature, organically grown Golden Delicious apple trees in a site in Quincy that has historically had problems with OLBR. The sprays were applied using a gas-powered backpack sprayer calibrated to deliver 50 GPA (Table 1). Treatments were assessed on 18 May by visually examining all treated and control trees for evidence of live and/or dead OBLR. No signs of OBLR were evident during that assessment, thus, the experiment was discontinued, and additional sprays targeted for 900 DD were not applied.

Table. 1. Treatment list, rate/A, target DD timings and (date of application)

Program	Insecticide	Rate/Acre	Timing 1	Timing 2	Timing 3	Timing 4
1	*Bt* (Deliver)	1 lb	90 DD (4/20)	+1 wk (4/27)	+1 wk (5/4)	
2	oil	1 gal	900 DD	+1 wk		
3	Oil + *Bt*	1 gal, 1 lb	900 DD	+1 wk	+ 1 wk *Bt* only	+ 1 wk *Bt* only
4	Trt 1 + Trt 2		4/20			

Objective 3. Determine the causes of western tentiform leafminer (WTLM) outbreaks and evaluate how to optimize management to reduce effects on parasitoids.

Methods: Adult male WTLM were monitored in 6 WA apple blocks using pheromone baited sticky traps in 2018. Additional population estimates of larval stages were obtained from weekly leaf samples. Three sites were in Quincy, two near Sunrise Research Orchard and one site was located about 10 miles north of Orondo. Two of the three Quincy blocks were Honeycrisp apples that were planted adjacent to each other; one was farmed organically and the other was farmed conventionally. The third Quincy site was a block of organic Red Delicious. The two blocks near Sunrise Research Orchard were adjacent to each other consisted of Red and Golden Delicious. One block was organic, the other was conventionally farmed. The site north of Orondo was a block of organically farmed Gala.

As in 2017, levels of WTLM were the highest in the organic blocks compared with levels observed in conventional blocks. Similarly, adult WALH were more abundant on yellow sticky traps in organic blocks compared with levels observed in conventional blocks.

Three pheromone traps were deployed in each orchard before the start of the first WTLM generation and checked frequently to determine first catch. Afterwards, the traps were checked weekly. Lures were replaced every four weeks. The pheromone traps are highly active and attract extremely high numbers of males.

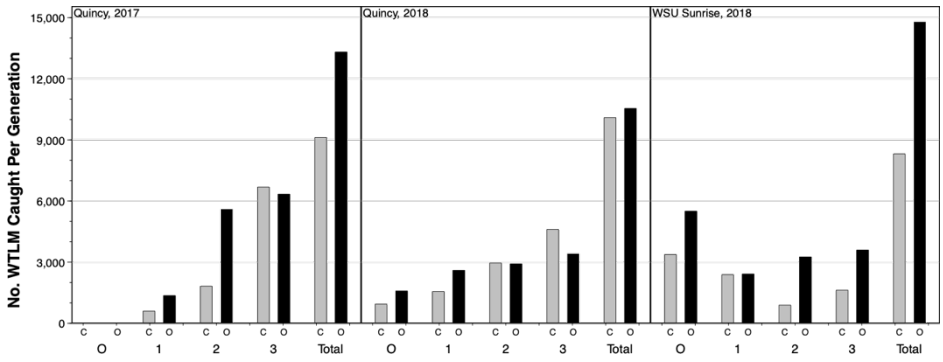
Leaf samples consisting of 75 leaves per orchard were collected weekly starting in mid-May and examined for WTLM mines in the field until after harvest (leaf sampling ended 11 Oct). Leaves with mines were brought back to the laboratory and the mines were examined under a dissection scope to whether they contained live or dead WTLM, parasitoids or whether there was evidence that WTLM or parasitoids had emerged.

Nine yellow sticky card traps were also placed in each of the blocks in Quincy and at Sunrise Research Orchard and changed weekly from early April until 9 Oct. They were assessed in the laboratory under a dissection scope for adult parasitoids of WTLM, primarily *Pnagalio flavipes* and *Pholestesor ornigris*. Adult white apple leafhoppers, *Typhlocyba pomaria* McAtee (WALH) and their *Anagrus sp*. (Mymaridae) parasitoid were also counted on the collected yellow sticky cards when it became apparent that there were outbreaks of this pest in organic blocks.

Spray records from several orchards and years were also examined and rates and timings of insecticides known to have detrimental impacts on natural enemies were noted.

Results and Discussion:
Monitoring WTLM. The pheromone for the WTLM is highly active, which would tend to make adjacent plots very similar in numbers. However, our data for 2017 (at Quincy) and 2018 (at Sunrise)

Fig. 1. Trap catch per generation at paired organic/conventional sites in 2017 and 2018. Numbers at the bottom of the graph represent the generation (O=overwintering).

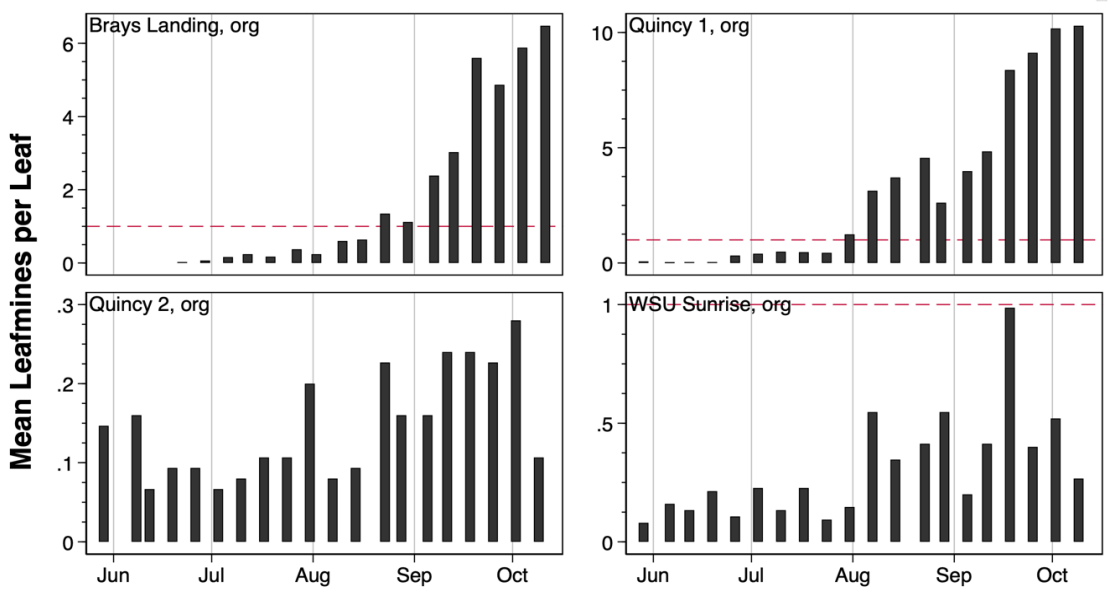


showed marked increases in the organic block of our paired (conv/organic) blocks; the paired blocks in Quincy in 2018 did not show much difference in trap catch (Fig. 1).

In spite of the large number of WTLM males caught in traps in the adjacent conventionally managed orchards, leafmines were never recorded in the leaf samples taken in the conventionally managed orchards, but very large numbers of mines were found in the organic blocks (Fig. 2).

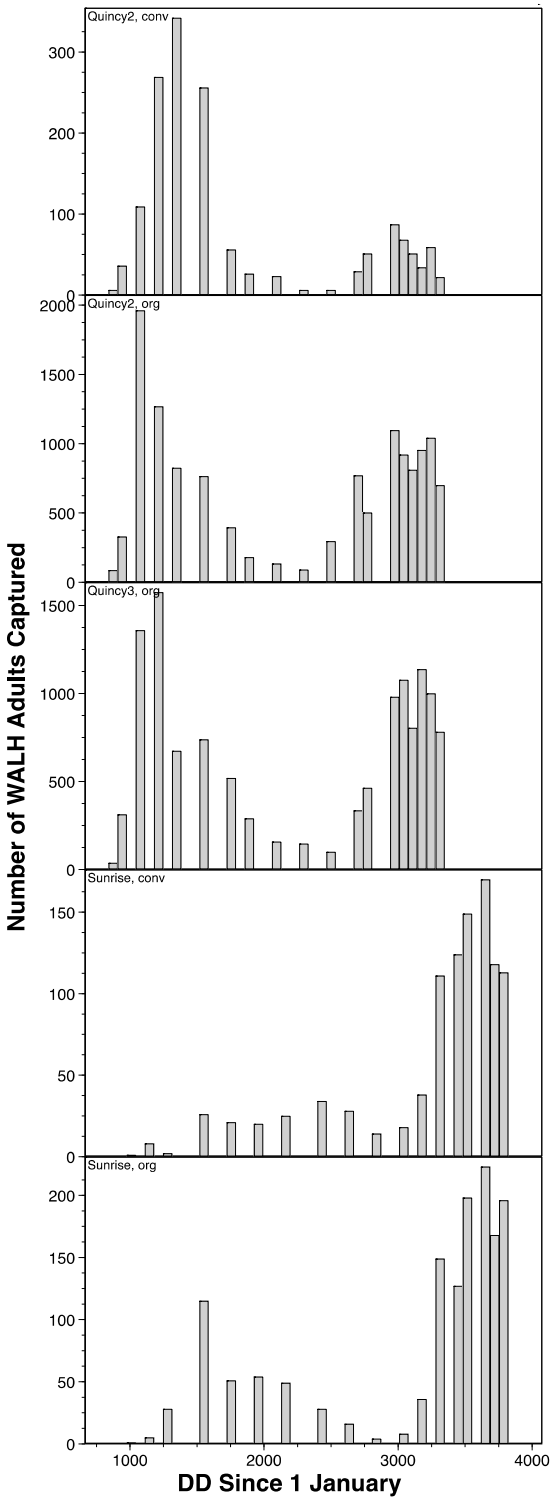
Yellow panel traps in the paired orchards did show WTLM parasitoids were present in both areas, but were considerably higher in the organic blocks, especially in the Quincy paired block where *Pnagalio flavipes* was relatively rare in the conventional area (78 captured season-long) compared to 1,591 in the organic block. The dominant parasitoid (10x higher captures than *P. flavipes*) at both Quincy sites was *Pholestesor ornigris*, a parasitoid that was not recorded in central Washington in Barrett's 1988 dissertation on WTLM and *P. flavipes*. In the paired orchard block in Quincy, there were 428 *P. ornigris* in the conventional block versus 16,196 that were captured in the adjacent organic block. The vast majority (≈91%) of *P. ornigris* was captured between 22Aug and 11 Sept in both areas. *P. ornigris* was also captured at the Sunrise orchard, so its distribution is not restricted to just a small area in Quincy, although its full distribution is not known at this time. The highest number of *P.*

Fig. 2. Mean number of mines per leaf at the organic sites in 2018. Dashed horizontal line is 1 mine per leaf is the threshold for treatment used in the 1990's.



flavipes was captured at the Quincy site at roughly the same times as when *P. ornigris* was present and this period corresponds to the timing of the larvae of the 3rd summer generation and the beginning of the fourth flight of the adults. The literature suggests that there is an alternation of importance of leafminer parasitoids that are endoparasitoids (*P. ornigris*) and ectoparasitoids (*P. flavipes*), with the former being important in the first and third larval generations and the latter being more important in the 2nd and 4th generations. Our data do not show this shift in importance between the two species, instead the populations just tend to increase particularly near the end of the season with relatively few parasitoids found in the early in the season at any site. The leaf samples also recorded parasitism as well as host feeding on the sap and tissue feeding stages. As with the yellow panel data, the leaf samples showed that sap feeding WTLM larvae were the most heavily impacted by host feeding and parasitism (the stages attacked most by *P. ornigris*) and its occurrence increased through the last sample that was taken in mid-October.

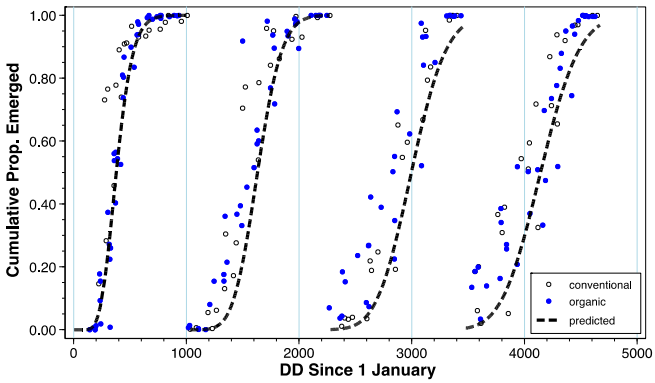
Fig. 3. WALH trap captures throughout the season in 2018. Notice the difference in y-axis numbers between each graph.



The spray records we have for 2017 show the spray programs were relatively soft although the organic blocks all had a single Entrust® application in mid-April. The timing for this would have been near the end of the overwintering adult flight, so it would correspond to roughly the time when the older WTLM tissue-feeding stages would be present. This is the stage that is most heavily parasitized by *P. flavipes*, whereas *P. ornigris* tends to parasitize mostly the younger sap-feeding stages of the WTLM larvae. The timing of the spray would suggest that it may have caught the latter part of the adult *P. flavipes* overwintering population emergence, but until we can do more evaluation of the emergence curves of both *P. flavipes* and *P. ornigris*, it is hard to definitively say that adult parasitoids were affected, but if because Entrust® does have good activity on larval WTLM, it is likely that the parasitoid larvae of both *P. flavipes* and *P. ornigris* were affected. The 2018 data should help pin this down further when we receive the spray records; for 2018 we have: adult trap counts of WTLM and the two different parasitoid species, leaf samples where we recorded parasitism and host feeding, as well as phenology of the sap and tissue feeding stages.

White apple leafhopper (WALH) evaluation. In addition to the blow up of the WTLM populations in the organic blocks, WALH populations also were extremely high in the Quincy organic blocks and exhibited similar trap catch curves to the conventional blocks, but at about 7-fold higher levels (Fig. 3). The parasitoid of the WALH *Anagrus* spp. was also caught on the yellow panels and was 4-10 x higher in the organic blocks where the WALH population had increased dramatically. We will have a better picture of this when we are able to match the 2018 spray records with our population curves.

Fig. 4. Phenology of adult WTLM males caught in pheromone traps. Data from organic blocks (filled circles) was more variable than that collected from conventional orchards.



Phenology of WTLM adult males. The phenology of adult males was similar from data collected from Bruce Barrett's thesis (1985 & 1987), samples that were collected in 2013 on natural enemy attractive traps, and data last year and this year (Fig. 4). However, the greatest amount of variation occurred in the organic orchards in each generation, possibly because of the Entrust® sprays that occurred in the organic blocks.

Work next year. We plan to focus the work on the paired plots where outbreak conditions occurred and evaluate how the parasitoid population dynamics are affected by the spray programs. We will also continue the leaf samples so that we can define the phenology of the immature stages and the adult parasitoid phenology and evaluate the parasitoid impact through host feeding. Combined with the spray records, this should allow us to have a better understanding of how the timing impacts natural enemies of the WTLM and sensitive periods that can cause outbreaks of WTLM and WALH.

Literature Cited:

Barrett, Bruce. 1988. The population dynamics of *Pnigalio flavipes* (Hymenoptera: Eulophidae), the major parasitoid of *Phyllonorycter elmaella* (Lepidoptera: Gracillariidae) in central Washington apple orchards. Ph.D. Dissertation. Washington State University. 136 pp.

CONTINUING PROJECT REPORT **YEAR**: 2 of 3
WTFRC Project Number: CP-17-101

Project Title: Evaluating and improving biological control of WAA

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Cooperators: None
Total Project Request: **Year 1:** $54,301 **Year 2:** $85,049 **Year 3: $84,478**

Other funding sources
Agency Name: WSDA-USDA SCRI Block Grant
Amt. awarded: $194,910
Notes: (expired early 2017)

WTFRC Collaborative Expenses: None

Budget 1
Organization Name: Washington State University **Contract Administrator:** Katy Roberts/Kim Rains
Telephone: 509-335-2885/509-293-8803 **Email address:** katy.roberts@wsu.edu/kim.rains@wsu.edu

Item	2017	2018	**2019**
Salaries[1]	31,146	51,612	48,041
Benefits[2]	7,439	17,368143	17,167
Wages	8,000	8,320	8,653
Benefits[3]	216	225	234
Equipment			
Supplies[4]	3,500	3,640	3,786
Travel[5]	4,000	4,160	4,326
Miscellaneous			
Plot Fees		2,184	2,271
Total	54,301	85,049	**84,478**

Footnotes:
[1] Project Assistant (Y1 -12 months, Y2 – 3 months); Y2-3 Matt Jones 50% FTE; Tawnee Melton 30% FTE Y1-Y3.
[2] Project Assistant 11.7%, U Chambers (32.1%), T Melton (47.5%)
[3] 2.7%
[4] includes lab and field supplies
[5] w/in state travel

Objectives

1. Evaluate the effect of augmenting/reducing earwigs on woolly apple aphid population levels and earwig-related fruit damage
2. Use molecular methods to evaluate the gut contents of earwigs to assess feeding habits
3. Use HD video monitoring to observe natural enemy attack rates on WAA in a field situation
4. Evaluate changes in biological control of WAA when natural enemy lures are placed in the field

Significant findings

- Objectives 1-2 were completed last year and reported in last year's report.
- Video monitoring was unsuccessful at the WSU Sunrise orchard this year because of predation and parasitism in early season that started before lacewings emerged and high mid-season temperatures which crashed WAA population levels.
- Our plots set up to evaluate lures effect on biological control in 4 different locations all had populations crash caused by a combination of an extraordinary high level of predation from ladybird beetles, parasitism from *Aphelinus mali*, and high temperatures.
- A model developed under a technology grant suggests that there are two different types of locations based on the number of hours per day over 92°F in mid-summer and the length of that period. Warmer sites tend to have lower populations that drop sharply in July and peak in the fall in September. Cooler locations have higher population levels that slow in mid-summer (but don't crash) and rely more on biological control. We will use these insights to change our experimental plans next year evaluate lure effectiveness.

Objective 1. Evaluate the effect of augmenting/reducing earwigs on woolly apple aphid population levels and earwig-related fruit damage

This objective was completed last year.

Objective 2. Use molecular methods to evaluate the gut contents of earwigs to assess feeding habits

This objective was completed last year.

Objective 3. Use HD video monitoring to observe natural enemy attack rates on WAA in a field situation

Methods. Four video cameras were set up at Sunrise and focused on WAA colonies to record natural enemy activity at the WAA colonies. Recordings were done daily between 4 a.m. and 11 p.m. The cameras were moved when any of the WAA colonies disappeared.

Results. Video recording of WAA colonies where lures were nearby showed a total of only 58 lacewings in ≈2,800 hours of recording between 7 June and 5 September. As mentioned below, this was related to low counts of WAA (it was very difficult to find colonies to video) and high populations of ladybird beetles bolstered by a massive outbreak of rosy apple aphid and parasitism by the WAA parasitoid, *A. mali*. The problems were exacerbated by issues with the video system that prevented recording in July. During the July and August periods, we attempted to transfer WAA colonies to build the population in the block, but they were unsuccessful.

Work next year. Sunrise is a high temperature site that has a mid-summer population crash related to temperature and amplified by natural enemies. We will concentrate the video portion in mid-May

through June and then start again in the fall between mid-August and the end of September. This is before we started last year and later than we recorded this year.

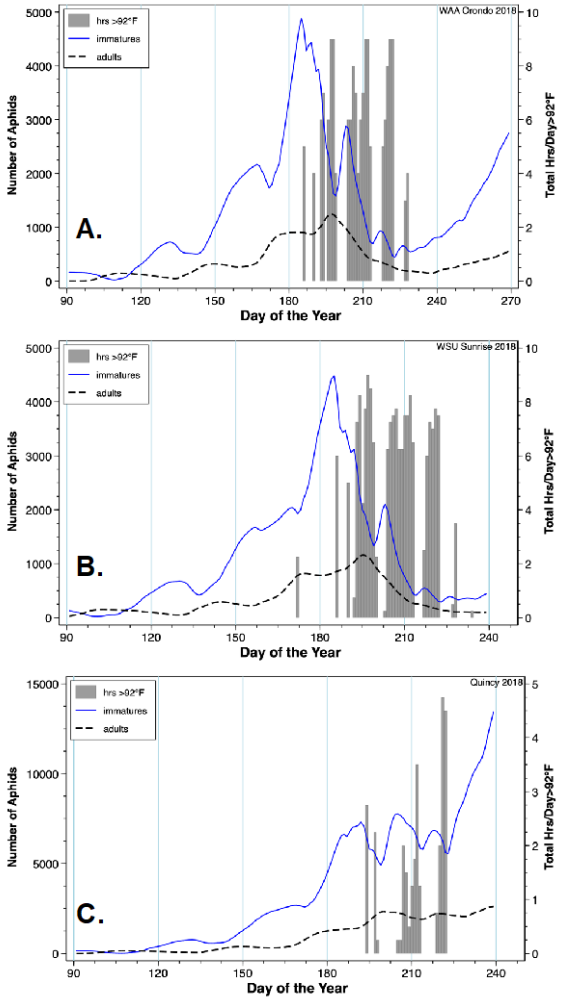
Objective 4. Evaluate changes in biological control of WAA when natural enemy lures are placed in field situations.

*Methods***.** Trials to study the effect of lures on the biological control performance of lacewings on WAA colonies were set up in four orchards in 2018. Trials were set up at Sunrise and near Quincy on 27 June to coincide with the second lacewing generation. However, by 3 July, it became obvious that most of the colonies at both sites were heavily parasitized and not suitable for the experiment. Even netting WAA colonies to exclude predators and parasitoids as well as attempts at transplanting WAA colonies did not improve the infestation levels in the experimental plot at Sunrise.

An additional orchard near Orondo with high WAA infestation was then included in the study, and 30 plots were set up on 12 July: 10 for the untreated control, 10 for treatment with acetic acid + methyl salicylate + 2-phenylethanol (AMP) lures, and 10 with squalene (SQ). The order of the plots within the orchard block was randomized. Each plot consisted of a 15-feet section of a tree row, and the plots were approximately 60 feet apart to reduce interference of the lures. In each treatment plot, 6 lures were placed 3 feet apart and between 3-6 feet above the ground near WAA colonies. The number of WAA colonies was recorded once per week within a 1.5-foot radius around each lure. The monitoring included the number of WAA colonies, classified into colony length categories, the approximate percentage parasitism, the number of single (*C. plorabunda*) or clustered lacewing eggs (*C. nigricornis*) was recorded. The color of the lacewing eggs was noted as that indicates the age and hatch status of the eggs (green – new eggs, darker-grey – near hatching, white – hatched). The lacewing eggs were then marked to avoid recount in the following weeks. The presence of any other natural enemies was also recorded. The same parameters were monitored in the control plots, where no lures were placed, in the 1.5-foot radius around random 6 locations with WAA colonies.

Results. Although the experiments were well designed, the unpredictable nature of WAA and hindered our best efforts to evaluate the effect of lacewing lures on lacewing oviposition and WAA predation. WAA infestation had been sufficient at our study sites in the past years. However, an unprecedented population explosion of ladybeetles at Sunrise, likely due to the extremely high infestation with rosy apple aphid, as well as high parasitism rates early in the season prevented the resident WAA population from ever reaching sufficient levels. WAA numbers in two other grower orchards also did not increase as expected from previous years. Therefore, the experiment was relocated in the second half of July, when lacewings were still active, to a block in Orondo that had a very high infestation level where the consultant reported that it was due for a pesticide application to get the WAA under control.

Fig. 1. WAA model runs based on temperatures at: **A**. Orondo grower's field **B.** WSU Sunrise, and **C**. Quincy. Notice the difference in y-axis scales and the number of hours per day when temperatures were >92°F



At the site near Orondo, WAA colonies disappeared after only 3 weeks into the trial. With only two dates of data no statistical analysis is possible as to the effects of lures on the number of lacewing egg clusters or number of WAA colonies, and the data showed no differences in the number of egg clusters or WAA colonies. No lacewing eggs were found during that time.

Model Evaluation. A model for WAA based solely on temperature effects on reproduction, survival, developmental times was completed by Jones in late September (as usual, too late to help last seasons' work). More detail is provided in the technology report (TR-17-102a), but briefly, the model is based on a synthesis of studies going back to the 1930's. The model updates every 5 DD throughout the year using the average temperatures observed in the field during each 5 DD period and tracks the age and abundance of individuals in the immature and adult stages. The model is not intended to predict exact numbers seen in the field, but instead to provide us with an understanding of how temperature affects population growth. The model demonstrates that much of the population dynamics of this insect is driven by the temperature and are rendered more unstable by predation (which is not tracked by the model). Temperatures above 92°F are especially telling on the WAA abundance and these factors show that in 2018 the sites where we were attempting the studies were going to crash significantly based solely on the temperatures. However, it is mentioned here because evaluating the temperature profiles at the sites of Sunrise and Orondo, the population would have crashed regardless of the presence of natural enemies. The Quincy location had much less temperature-driven mortality and the peak population size was much higher than at the other two sites. Quincy would exemplify a location where biological control is much more important even during mid-season where the population is suppressed during the heat, but the heat is not enough to crash the population on its own.

Work next year. Our models and some of the work of a previous master's student (Connor O'Leary) suggest that we need to expand our work with the lures to two different time periods in warm locations to start much earlier in the season and then starting in early August so that we can prevent population growth associated with cooler fall temperatures. Lures during these periods may provide

us with a way to reduce the size of the early season peak, and to slow the growth during the fall when conditions are conducive to building the overwintering population level.

We intend to focus on these two periods at four separate locations; two historically cooler locations and two warmer locations. Lure placement will start earlier and go later at all locations and continue through the mid-summer temperature-mediated population drops.

CONTINUING PROJECT REPORT **YEAR**: 3 of 3 (No Cost Extension)
WTFRC Project number: CP-16-101

Project Title: Brown marmorated stink bug control in Washington

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Cooperators: None

Total Project Request: Year 1: $70,798 **Year 2**: $90,327 **Year 3**: $93,668

Other funding sources

Agency Name: NIFA-Specialty Crop Research Initiative (SCRI); Washington State Commission on Pesticide Registration

Amt. Received: SCRI grant: $9,164,909 (funded); WSCPR: $16,356 (#16PN25, funded); $18,733 (#17AN029; funded); $21,851 (#18AN011, funded).

WTFRC Collaborative Expenses: None

Organization: WSU **Contract Administrator:** Katy Roberts/Kim Rains
Telephone: 509-335-2885/509-293-8803 **Email:** arcgrants@wsu.edu/kim.rains@wsu.edu

Item	2016	2017	2018	2019
Salaries[1]	44,564	59,716	62,104	
Benefits[2]	9,435	14,973	15,572	
Wages[3]	8,042	8,364	8,699	
Benefits[4]	431	448	467	
Equipment	0	0	0	
Supplies[5]	3,000	1,500	1,500	
Travel[6]	3,326	3,326	3,326	
Miscellaneous	0	0	0	
Plot Fees[7]	2,000	2,000	2,000	
Total	70,798	90,327	93,668	**0**

Footnotes:
[1]Research Intern, 7 months (year 1), 12 months years 2 and 3, 0.60 FTE. Ph.D. student 3 years.
[2]Benefits for Research Intern 38.6%, Ph.D. student 9.37%.
[3]Wages for Ph.D. student (summer only), 1 time-slip help, 0.5 FTE, summer.
[4]Benefits for Ph.D. student 2.4%, time-slip 10%.
[5]Supplies – office and lab supplies, electronics, statistical consulting.
[6]Travel to plots – motor pool rental.
[7]Two acres apple (WSU Sunrise)/yr x $1,000/acre, 3 years.

Objectives:

All the objectives listed address brown marmorated stink bug (BMSB), identified as a 'Critical' priority

1. Determine distribution of *Trissolcus japonicus* in Washington
2. Maintain a laboratory culture of *T. japonicus* in preparation for release
3. Evaluate IPM-friendly management strategies for BMSB
4. Document the spread of BMSB within the state
5. Determine suitability of native shrub-steppe plants as hosts for BMSB

Significant Findings:

- BMSB has been detected in 24 counties in Washington State, with Clark, King, Pierce, Snohomish, and Thurston counties having the highest number of reports in 2018.
- *T. japonicus* were recovered from three new sites in the state in 2018.
- *T. japonicus* were released in Prosser, Walla Walla, White Salmon, and Yakima.
- The majority of stink bugs fly into orchards below 12 ft, showing promise for physical barriers.

Objective 1: Determine distribution of *Trissolcus japonicus* in Washington[1]

Obj. 1 Methods. The first adventive *Trissolcus japonicus* population (Fig. 1) on the west coast was discovered in Vancouver, WA in 2015. Persistent populations in Vancouver were found in 2016 and 2017, and the first detection of *T. japonicus* in Eastern Washington occurred in 2017. These detections, and the apparent range expansion of *T. japonicus* in the state warranted further exploration. Surveys in 2018 were conducted in five cities (2 sites each: Prosser, Walla Walla, White Salmon, Vancouver, Yakima) to determine both current distribution and success of parasitoid releases made in 2017-2018.

Fig. 1. *Trissolcus japonicus* ovipositing

Egg masses for the survey were taken from a colony of BMSB maintained in small insect cages on a diet of mixed seeds and vegetables. Egg masses were removed daily, ensuring maximum attractiveness to the parasitoid. They were glued to card stock, labeled, and transported to the survey sites. The pieces of card stock were pinned to the lower surface of known host plants (deciduous trees). The masses were retrieved 3-4 days after deployment, returned to the laboratory and held at 22 °C (72 °F) until host or parasitoid emergence was complete.

Obj. 1 Results. A total of 232 BMSB sentinel egg masses, including two wild egg masses, were deployed in in 2018. *T. japonicus* was found at two locations in Walla Walla (one a repeat find from 2017), and a single location in Yakima (the site of the sole 2017 release). This shows the likely persistence of the population in Walla Walla, and the success of release efforts made in Yakima in 2017. The BMSB egg masses deployed in Vancouver yielded *T. japonicus*, bringing it to four consecutive years being detected in Vancouver. Other parasitoids detected by the survey were *T. brochymenae*, *T. euschisti*, and *Trissolcus* sp.

[1] Sentinel egg mass survey protocols were developed by Dr. Kim Hoelmer, USDA-ARS

Obj. 2. Maintain a laboratory culture of *T. japonicus* in preparation for release (this objective now includes releases)

Obj. 2 Methods. Adult *T. japonicus* found in the Vancouver sites were returned to the laboratory to rear for release. Adults were kept in Petri dishes with honey water. When BMSB egg masses were available, a pair of *T. japonicus* was transferred to a small plastic cup containing the egg mass, and the female allowed to oviposit. After oviposition was complete, the egg mass was removed and incubated at 20 °C (72 °F) for three weeks until new adults emerged. Adults held with only honey-water were quite long-lived, and the colony could be perpetuated whenever egg masses were available.

For releases, two parasitized egg masses were placed in a small closed container with honey-water until all adults had emerged. They were transported to the selected release sites, and the lid removed to allow adults to escape (Fig. 2). Releases were repeated 4-5 times during the growing season (late June-early October) at 8 pre-selected sites in 4 cities (2 sites/city).

Obj. 2 Results. A total of 1,827 adults were released (112 male, 1,715 females) in two sites each in Prosser, Walla Walla, White Salmon, Yakima.

Objective 3. Evaluate IPM-friendly management strategies for BMSB
Obj. 3a. Physical exclusion, large field cages (completed in 2017).

Obj. 3a. Methods. The direct pest exclusion and non-target effects experiments in 2016 and 2017 investigated shade net enclosures as a measure for reducing stink bug and codling moth damage. Because cages can exclude natural enemies as well as pests, the 'non-target' effects of the cages was determined on secondary pests and their natural enemies.

The plots used in this experiment were built over mature trees in a 1.2 acre apple orchard at the WSU Sunrise Orchard. Cages were built over mature trellised apple trees planted at 3 x 10 ft spacing. Cage frames were built from dimensional lumber supported with posts and guy wires, and covered on all sides and top by white shade net (pearl leno 20% shade, Green-Tek West, Dinuba, CA). Each cage was 40 x 50 ft, and enclosed 4 rows x 12 trees (total 48 trees/cage). Each row was a different apple cultivar (Jonagold, Gala, Granny Smith, and Golden Delicious).

Three treatments were tested: 1) cages made from shade netting (plus supplemental sprays), 2) uncaged, conventional management (routine airblast sprays), and 3) an uncaged, unsprayed check. Treatment 2 received routine sprays for codling moth, but none specific to secondary pests (mites, aphids). All plots received routine applications of herbicides, fungicides and fertilizer. With the exception of stink bugs, all pest and beneficial populations were naturally occurring in the block. Because the orchard had no history of stink bug damage, artificial pressure was created by collecting consperse stink bug, *Euschistus conspersus*, and releasing them in the block. The ability of pests and natural enemies to penetrate the cage barrier was measured with visual observations (timed counts), traps baited with pheromones or kairomones, or behavioral traps (yellow sticky cards, earwig shelters). Counts were made every 2 weeks throughout the season, and a single index of the seasonal counts for each insect was calculated (cumulative insect days, or CID). This index is the average of two successive counts multiplied by the number of intervening days and summed over the season. The CID were analyzed using analysis of variance (SAS 2017, PROC GLIMMIX). Fruit damage was sampled in mid-summer and again just prior to harvest, analyzed using logistic regression with a binomial distribution (PROC GLIMMIX).

Obj. 3a. Results. Very few stink bugs were recaptured in pheromone traps, and stink bug fruit damage was correspondingly low in both years (Fig. 3); however, the damage was lowest in the cage treatment in both 2016 and 2017. Other direct pests such as codling moth (Fig. 4) and leafrollers (data not shown), were also excluded to a marked degree by the cages. Woolly apple aphids reached outbreak levels inside the cages (Fig. 5A) but were present in very low numbers outside the cages. The specialist parasitoid of woolly apple aphid, *Aphelinus mali*, was similarly high inside the cages (Fig. 5B), likely in response to the high aphid populations. It is probable that the cage netting is permeable to this tiny wasp, but also possible that the parasitized aphids present when the trees were caged simply continue to reproduce inside the cages. Despite the high numbers of *A. mali*, the populations of woolly apple aphid were not sufficiently suppressed. Unlike the parasitoid, the macropredators of woolly apple aphid (lacewings and syrphids) were much lower inside the cages, indicating the winged adults were prevented from entering the cage (Fig. 6A, B). Earwigs, another woolly apple aphid predator (Fig. 7), tended to be lower inside the cages (2016), but this effect was less pronounced than with the winged predators.

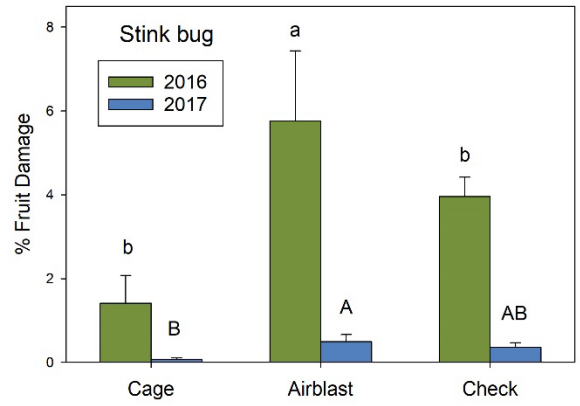

Fig. 3. Stink bug fruit damage, 2016-2017.

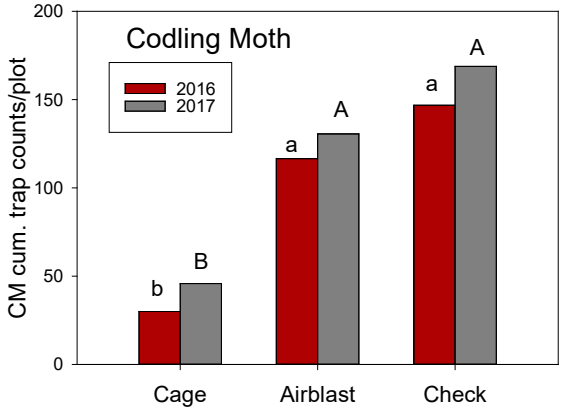

Fig. 4. Codling moth trap counts, 2016-2017

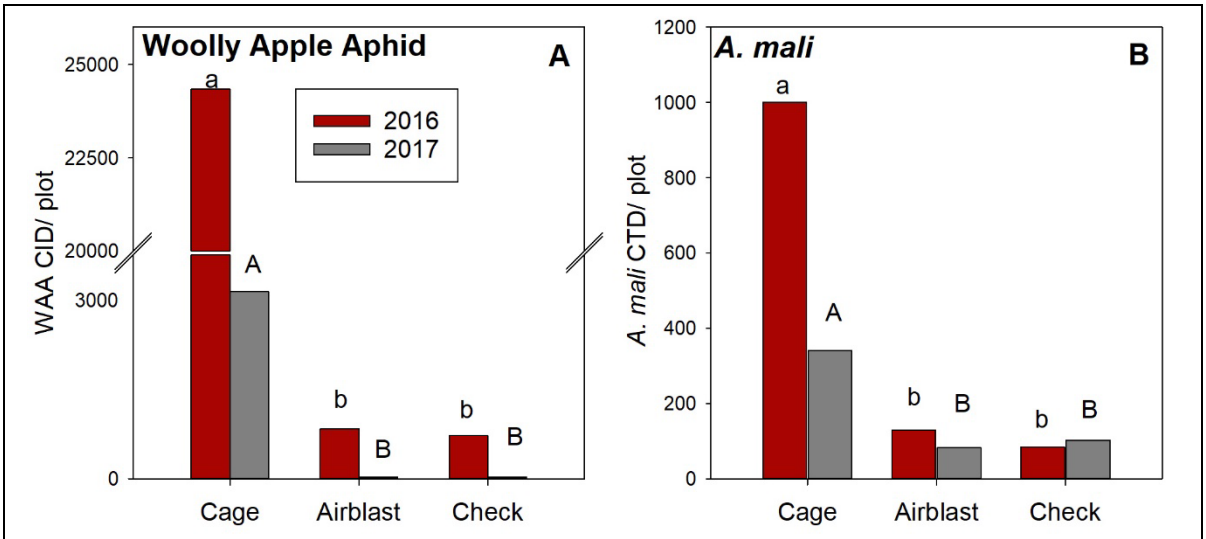

Fig. 5. Seasonal populations (Cumulative Insect Days, CID) of woolly apple aphid (A) and *Aphelinus mali* (B).

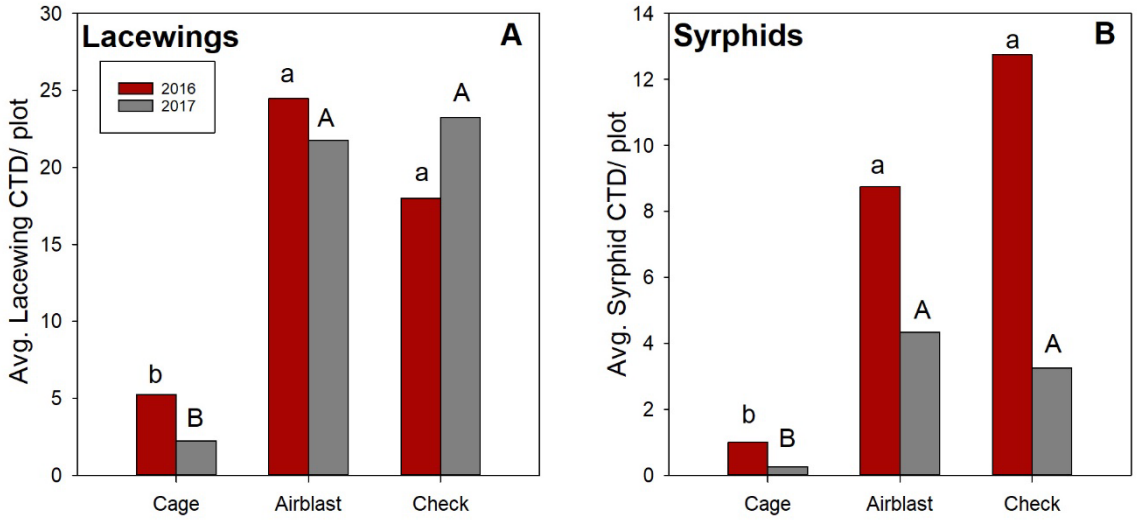

Fig. 6. Seasonal populations (Cumulative Trap Days, CTD) of lacewings (A) and predatory syrphids (B).

Obj. 3b. Physical exclusion, small field cages.

Obj. 3b. Methods. The experimental design of the small cage experiment was similar to the large one, except that the plots were three 'Golden Delicious' trees (single row), and the cages were 10 x 10 x 5 ft. The same treatments were used, but each had 10 replicates in a randomized complete block design. Because of the smaller plot size, only 15 leaves were taken for the mite samples. All cages had a pheromone trap for the three tortricids, but the other two treatments were sampled with 2 traps/ species placed in buffer rows to avoid inter-trap competition. Sampling was done as in the large cage experiment, except that stink bug releases were not made (on the assumption that the large cages represented a more realistic commercial scale). The CID calculation and data analysis were the same as for the large cages.

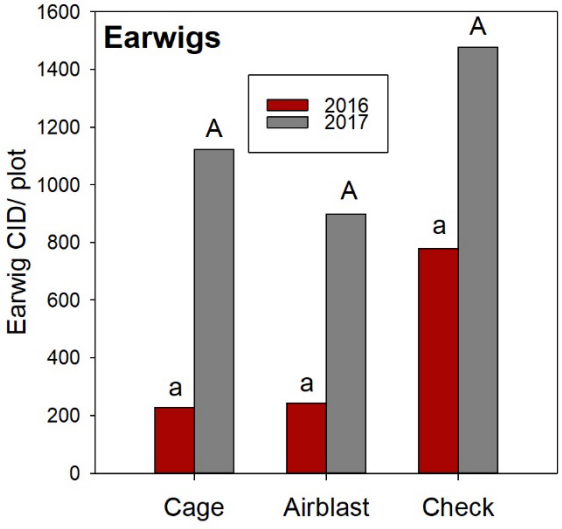

Fig. 7. Seasonal earwig populations, 2016-2017 (Cumulative Insect Days, CID).

Obj. 3b. Results and Discussion. Secondary pests. Woolly apple aphid densities were 400-500 fold higher inside the cages than in the airblast and check treatments, respectively. Spider mites were significantly higher inside the cages; 95% of the mites found were brown mite. Earwigs were not significantly different between the treatments. Lacewing and syrphid adults were effectively excluded by the cages. Thrips damage was significantly less inside the cages (0.21%).

Fruit damage, sunburn. Codling moth pheromone trap captures were greatly reduced inside the cages (15 moths/trap) vs outside (240 moths/trap). Likewise, fruit damage by codling moth was 8.4% inside the cages, vs. 15.9% (airblast) and 58.2% (untreated). Sunburn was significantly reduced inside the

cages (4.23%); however, as in the large cages, the airblast treatment (25.44%) also reduced sunburn relative to the check (39.96%).

3c. Physical exclusion, single-wall barriers.

Fig. 8. Attaching deltamethrin netting to flaps.

Obj. 3c. Methods. For the exclusion study, three commercial apple orchards in the Manson, WA area with a history of stink bug damage were used. The barriers (150 ft long x 15 ft high) of commercial shade netting (20% pearl leno [white] net; Green-Tek West, Dinuba, CA) that were constructed in 2016 were retro-fitted with deltamethrin infused ZeroFly netting (Vestergaard-Frandsen, Washington, DC) in 2018 (Fig. 8). The insecticide infused netting was attached to the inside of the vegetation facing flaps on half (75 ft) of each barrier. Three treatments were tested at each site: 1) net barrier with deltamethrin flaps; 2) net barrier with shade net flaps; and 3) a no-barrier check. Stink bug populations were assessed with a beating tray in the natural vegetation and in the orchard throughout the field season. Fruit damage was determined through visual inspection in late August/early September. A complimentary study was conducted from June - September to determine the height at which stink bugs migrate into orchards. A sticky barrier (13 ft high x 6 ft wide; Fig. 9) was constructed using dimensional lumber and clear sticky panels, 1 x 6 ft (Alpha Scents, West Linn OR), at the orchard boarder in five locations in Manson. Stink bugs on the sticky panels were removed and their height of interception was recorded every week. A single index of the seasonal counts for adult counts was calculated (CID) as average of two successive counts multiplied by the number of intervening days and summed over the season, and a cumulative trap-day CTD index was used for the sticky panel traps. All data were analyzed using analysis of variance (SAS 2018, PROC GLIMMIX).

Fig. 9. Sticky barrier to test stink bug immigration height.

Obj. 3c. Results and Discussion. *Net barriers*. Stink bug damage levels were extremely low with none of the treatments reaching levels higher than 0.2%. Beat tray samples of the surrounding vegetation behind both net treatments resulted in substantially higher counts than samples in the check, and there was no significant difference in the amount of stink bugs found in the orchard between the three treatments (Fig. 10A). While the orchard counts were not significantly different, the netted plots received a much higher pressure of stink bugs migrating from the natural vegetation. There is a greater difference in the amount of stink bugs in the orchard compared to the vegetation in the netted plots (>80% reduction) than in the control plot (40% reduction), indicating the barrier prevented most of the stink bugs from reaching the orchard.

Obj. 3c. Results and Discussion. *Sticky barriers.* There were no statistical differences in interception height for the sticky barriers for stink bugs moving *out* of the orchard (data not shown). However, there was a significant difference in height of movement *into* the orchard (from of the surrounding vegetation). The highest counts were at 4.5 ft (7.20 stink bugs) and the lowest at 12.5 ft (1.20 stink bugs) (Fig. 10B).

Obj. 3 Conclusions. The preliminary information from both the large and small cage experiments indicates there is potential for complete exclusion of codling moth. The wild moth pressure in the

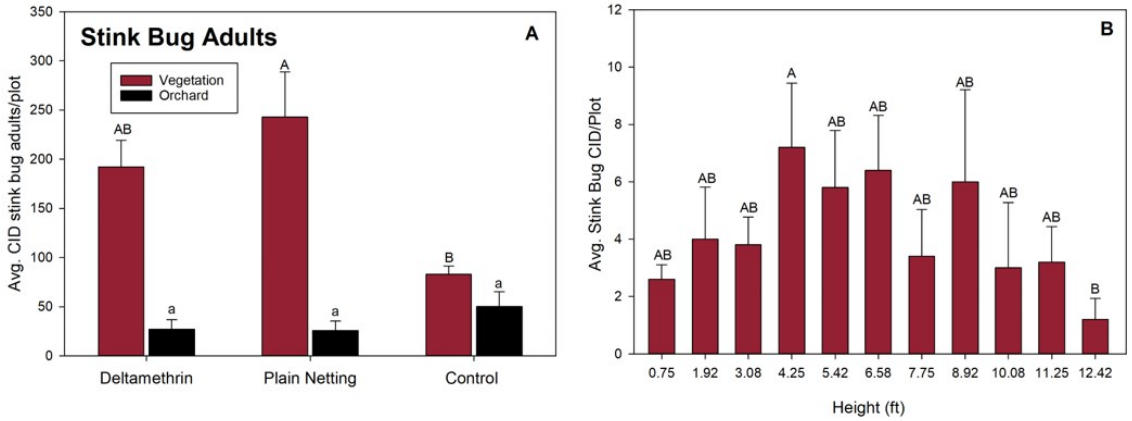

Fig. 10. Stink bug counts in the orchard and vegetation for the net barrier trial (A), and trap capture by height for the sticky barrier (B).

large cage research blocks was high, and late season pheromone trap captures indicate a high degree of success in excluding adults. The small cages trials show that shade netting can be an effective barrier to external moth populations through the use of fluorescently marked moths. Interestingly, a larger proportion of moths were able to escape the cages than enter them.

Sampling stink bugs captured on the net barriers, in the orchard, and in the surrounding vegetation in the Manson trials provided insight into stink bug seasonal migration habits. Stink bugs densities were higher in the surrounding vegetation for both net treatments than the check, yet numerically fewer stink bugs were found in the orchard behind the net treatments than the check. This indicates that the net barrier may be preventing a large portion of the migrating stink bugs from reaching the orchard. The sticky barrier trial further confirms that the majority of adult stink bugs fly into orchards below 12 ft, which implies a barrier may only need to be 12 ft to provide successful control.

Objective 4. Document the spread of BMSB within the state

As BMSB continues to spread, efforts in 2018 focused on sampling in location gaps between know populations and in new cities within important fruit growing regions. We used direct surveys (beating trays, pheromone traps) to determine the presence and relative abundance of BMSB in the state. We also solicited input from homeowners and Master Gardeners. Verified finds were recorded in a database available to BMSB researchers, and the results available in map form (http://tfrec.cahnrs.wsu.edu/beers-tfentomology/bmsb/bmsb-wa/).

Obj. 4. Results and Discussion. A total of 24 counties have reported detections of BMSB (Fig. 11). The highest numbers of BMSB reports came from Clark, King, Pierce, Snohomish, and Thurston counties in 2018 likely reflecting the large amount of vehicular traffic that could help spread this invasive species. Conversely, Chelan county has a (relatively) smaller human population and vehicular traffic, but a keen degree of awareness and interest in this species. Trapping studies and citizen reports discovered the first BMSB in both Okanogan county, and the city of Chelan in early October of this year. This is a major concern, as those are both major fruit growing areas. In general, it appears that the arid climate of eastern Washington will not effectively limit the establishment of BMSB, given the growing number of reports in this part of the state, and establishment to the north in the Okanagan Valley of British Columbia.

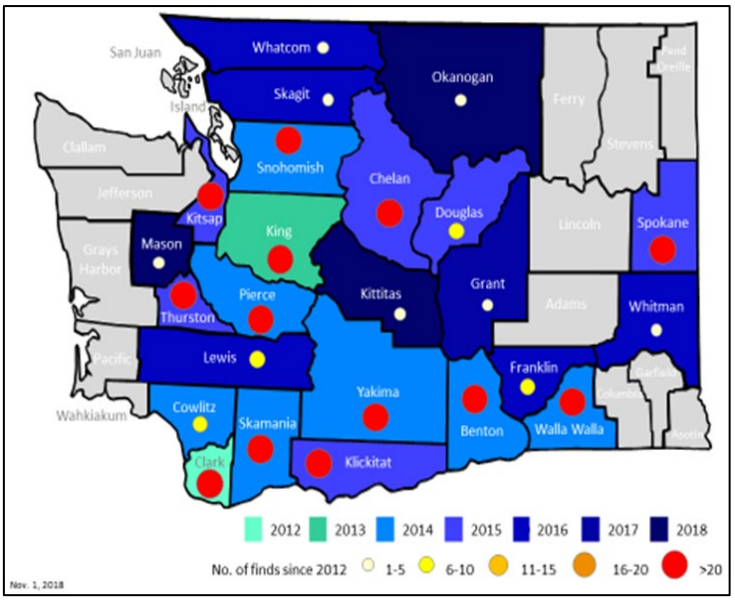

Fig. 11. BMSB finds by county, Washington State, Nov. 2018

Objective 5. Determine suitability of native shrub-steppe plants as hosts for BMSB

The brown marmorated stink bug has usually been associated with humid temperate environments such as the deciduous hardwood forests in northeast Asia, the Mid-Atlantic States in the American Northeast, and the Pacific Northwest west of the Cascades. However, BMSB has shown itself to be remarkably adaptable. It rapidly colonized many areas of Washington State, including population centers in the arid Columbia Plateau. As its range continues to expand and its populations grow, there is legitimate concern that this highly mobile landscape-level pest will be able to build up in the shrub-steppe habitats that border Washington's tree fruit orchards. Knowledge of the landscape ecology of BMSB in the arid PNW is critical for effective scouting and area-wide IPM efforts.

Obj. 5a. Laboratory feeding studies. Methods. In 2018, we evaluated the relative suitability of two common shrub-steppe plants (bitterbrush and sagebrush) for BMSB feeding and oviposition in comparison to Lima bean seedlings (a standard BMSB colony diet), which served as the check. Using a randomized complete block design (3 treatments, 10 reps), we placed a male-female BMSB pair into arenas containing one of the three host plant treatments. Arenas were made from 6-inch pots containing potting soil and 4 x 16-inch cylindrical acetate tops ventilated with screen panels. The acetate tops were fitted over plant cuttings in vases (bitterbrush and sagebrush) or ~10 bean seedlings growing in the potting soil; the bottom edge of the acetate tops were pushed into the soil to form a seal. Insects were weighed at Day 0, 7, 15, and 22 to monitor weight loss or gain as a metric of host plant suitability. At the same intervals, adult mortality was assessed, eggs were counted, and plant material was replaced.

Obj. 5a. Results and Discussion. Due to high mortality in our check treatment, no significant differences were observed in any fitness parameters measured. This mortality is likely due to insufficient ventilation in the arenas and the regular stress experienced by the insects during handling from the frequent weight measurements. In 2019, this trial will be repeated using bug dorms with multiple plant species. The design is based on an observational preliminary study where BMSB were able to complete development from egg to adult on four native plant species in a bug dorm. Using

native plant assemblages will be more applicable to the conditions experienced by insects in the field that regularly move between multiple host plant species.

Obj. 5b. Gut content analysis. Recent developments in "deep sequencing" technology for genetic analysis enable detailed, species-level feeding histories of individual insects to be determined by extracting and sequencing plant genetic material from the guts of insects. This approach has been successfully used on various psylla species by Dr Rodney Cooper (USDA-ARS, Wapato, WA) and we are refining the technique for BMSB in collaboration with Dr. Cooper. This will enable accurate analyses of landscape-level host plant usage by field-caught BMSB. It would also allow us to rapidly screen many potential novel host plants in the lab by exposing BMSB to plant material and then sequencing their gut contents to determine whether feeding has occurred.

CONTINUING PROJECT REPORT **Year**: 1 of 3
WTFRC Project: CP-18-104

Project Title: Optimizing sterile insect release of codling moth in Washington

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Cooperators: Bill Brauchla (NW Wholesale), Jay Brunner (WSU Professor Emeritus), Larry Gut and Chris Adams (Michigan State University), Melissa Tesche (OK-SIR)

Total Project Request: Year 1: $98,947 **Year 2: $98,359** **Year 3: $**102,711

Other funding sources
In-kind contribution from NWW and cooperators ($22,653 for irradiated moths, transport, lures, traps, shipping, and labor to hand-carry across the border); OK-SIR ($4,400 for providing moths at cost vs projected retail price).
Agency Name: Western IPM program
Amount requested: $29,724

WTFRC Collaborative Expenses: None

Organization Name: WSU **Contract Administrator:** Katy Roberts/Kim Rains
Telephone: 509-335-2885/509-293-8803 **Email:** arcgrants@wsu.edu/kim.rains@wsu.edu

Item	2018	**2019**	2020
Salaries[1]	58,940	61,298	63,750
Benefits[2]	20,046	20,847	21,681
Wages[3]	6,240	6,500	7,020
Benefits[4]	591	616	665
Equipment			
Supplies[5]	5,767	2,200	2,200
Travel[6]	7,363	6,898	7,395
Miscellaneous			
Plot Fees			
Total	98,947	**98,359**	102,711

Footnotes: [1]Salaries for project manager (1 FTE) and technician (0.075 FTE); [2]benefits at 33.5% (project manager) and 41.8% (technician); [3]Wages for time slip: $12/hr (yr 1), $12.50/hr (yr 2), and $13.50/hr (yr 3) for 13 weeks/summer; [4]benefits at 9.5%; [5]Supplies: computer, printer/software; lab/office supplies, electronics; video camera/accessories, sterile moths and release stations, bands. [6]Travel to plots, motor pool rental, fuel, per diem, travel to industry meetings to present results

Objectives:

1. *Determine the effect of fixed vs variable release rate on efficacy of SIR*.
   We have completed plot setup and the first year of releases in the replicated plot study in the Tonasket/Malott area. We will continue releases in 2019 using the same treatment scheme and sampling methods as 2018, but with increased sampling intensity for fruit damage at the end of the first generation. Perimeter traps may be dropped, especially in cases where neighboring blocks are treated with SIR.
2. *Compare the non-target effects of broad-spectrum pesticide use versus SIR as a supplement to mating disruption in organic orchards*.
   A broad range of secondary pest and natural enemy samples were taken during the season; however, spray records for the 2018 season which are necessary to conduct analyses, are incomplete at this time. If there are no treatment-based differences in spray programs, this objective should be discontinued.
3. *Examine the synergy between SIR and other tactics using modeling techniques*.
   Models that look at the interaction of multiple mortality/fecundity factors must first be underpinned with field or laboratory estimates of the effects. Initial attempts to investigate the complementarity of mating disruption and SIR using mating tables failed, and an alternative method using molecular markers will be assessed in 2019. If this method proves reliable, field testing will commence in 2020.

Significant Findings

- Recapture of sterile moths peaked during July and August, but was much lower in spring and fall.
- More sterile moths were recaptured at the 3x release rate than the 1x release rate by a factor of 1.8x.
- Sterile:wild (overflooding) ratios ranged from 2-20 depending on treatment and time of season.
- Codling moth fruit damage did not differ among SIR treatments at the end of the first generation, but all treatment means were <0.5% damage.
- Codling moth fruit damage (stings+entries) was significantly lower in the two SIR treatments compared to the check in preharvest samples; the number of entries was significantly lower in the 3x rate than the 1x rate at this time.
- Fruit damage maps revealed the spatial pattern of damage, with the possibility of treating 'hotspots' with higher rates of SIR in 2019.

Methods

Obj. 1. Determine the effect of fixed vs variable release rate on efficacy of SIR
The plots identified in 2018 will be used in the remaining years of the project, maintaining the same treatment rates (Table 1) in the same plots. Moths will be transported from the Osoyoos facility and released by an unmanned aircraft system (UAS) by M3 Consulting of Phoenix, AZ weekly for 22 weeks (late April-mid-September).

Table 1. Treatments tested for codling moth SIR

Trt.	Description	Sterile moth rate
1	1x SIR	Std. CM program + std. rate of SIR (800 sterile moths/acre/week)
2	3x SIR	Std. CM program + gradated rate of SIR Base rate increased to 2x (1,600 moths) and 3x (2,400 moths) rate as CM activity increases
3	Check	Std. CM program + insecticides; no SIR moths

All orchards are organic apple orchards in the Tonasket/Malott area. Plots range from 4-8 acres in size, with range of 0.1 to 1.8 miles between plots to minimize moth spillover. All plots use codling moth mating disruption and will receive a complete organic control program (petroleum oil, CM virus, and optionally, Entrust) at the grower's discretion.

Fig. 1. Unmanned aerial system (UAS) for

Moths will be distributed in the 8 SIR-treated plots by a UAS (drone) (Fig. 1) with a release device calibrated to deliver the specified per-acre rate of moths.

Moth densities and distribution will be sampled using plastic Delta traps baited with the CM-DA-AA lure (codlemone, pear ester, and acetic acid) (Fig. 2A). Traps are deployed at a density of 1/acre in a grid pattern. Liners are changed weekly, and moths categorized by sex (male, female) (Fig. 2B,C) and origin (sterile, wild) using the internal red dye used in the larval diet. Lures will be changed every 6 weeks as per manufacturer's recommendations. Trapping will encompass the entire release season. Trap results will be summarized and mapped using GIS software (Fig. 3) and sent to grower-cooperators weekly along with information on the sterile:wild ratio.

Fig. 2. Plastic delta trap with CM+DA+AA lures (A); codling moth genitalia, male (B) and female (C); note

The success of the treatments will be measured with fruit damage samples (codling moth stings and entries) after the completion of the first generation (ca. late June), then again pre-harvest for each variety (August-October). The location (latitude/longitude) of each damaged fruit will be recorded and mapped to determine spatial patterns and correlated with trap captures. A sample of trees in each plot will be banded with cardboard strips to determine the presence of surviving larvae.

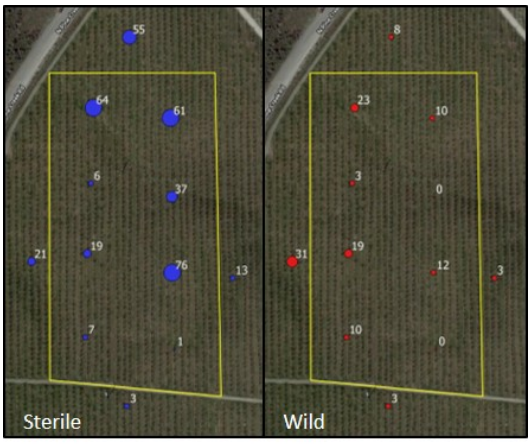

Fig. 3. Sterile and wild codling moth captures in

Obj. 2. Compare the non-target effects of broad-spectrum pesticide use versus SIR as a supplement to mating disruption in organic orchards

The plots in Obj. 1 will be monitored periodically to determine if the treatments differentially affect either the densities of key natural enemies, or problems with secondary pests. Measurement of non-target effects on secondary pests

and beneficials will occur throughout the season, including plant volatile traps for syrphids and lacewings, leaf brushing for pest and predatory mites, and sticky traps for *Aphelinus mali*. These will be summarized in light of spray programs once records are available.

Obj. 3. Examine the synergy between SIR and other tactics using modeling techniques.

In order to construct a predictive model of the interacting effects of multiple control tactics, the magnitude of those effects must first be estimated from laboratory or field data. Development of predictive models dating back to the 1950's have suggested that a critical component to SIR success is the ability of sterile males to compete with wild males for mates. One complicating factor for application to tree fruit pests is the interaction between SIR and mating disruption, which both reduce successful matings, but by very different methods. While pheromone trap captures are frequently used as a proxy for mating success (the ability to locate a phermone source), there may be great disparities between the total trap capture and the male who mates first with a wild female. Because moths typically only mate once, this order of arrival is critical, and ultimately determines whether mating of wild females leads to fruit damage by larvae. Mating tables are considered the most accurate measure of this rate of larval production, but are laborious to deploy. Tethered females can be used to inform these tables, but the low proportion of mating of tethered females on a given night makes sufficient replication challenging. We propose a more direct measure, that of examining the spermaophore in mated wild females, and determining whether it came from a wild or sterile male. This is based on the assumption that wild and sterile moths have genetic differences that can be detected using molecular methods.

In 2019, we will screen wild and sterile moths at different points during the season to search for a stable marker differentiating the two populations. The Osoyoos colony will be sampled periodically using excess moths from the release plots, and wild moths will be sampled from different growing regions around the state using pheromone traps. If a suitable marker can be found, we will use the methodology developed to test mating success under mating disruption, SIR, and a combination of the two.

Results & Discussion

Obj. 1. Determine the effect of fixed vs variable release rate on efficacy of SIR

Sterile moths were deployed at two rates over a period of 22 weeks from 26 April – 20 September, 2018. Moths were obtained from the OK-SIR facility in Osoyoos BC, and transported across the US border and released by an unmanned hexacopter (Hermes V2 UAS) (Fig. 4) operated by M3 Consulting (Phoenix, AZ). Trap liners (interior to plot, 1/acre; perimeter, 4/plot) were collected and changed each Tuesday during the release period, and moth release occurred each Thursday. Unless delayed by weather or equipment issues, all plots were treated on the same day, with an elapsed time of 6-8 hours between retrieval at the Osoyoos facility and release at the

southernmost plot (total of 8 plots, 57 acres). On a per-acre basis, the 1x (constant) treatment received 22 dishes (17,600 moths) during the season, and the 3x (varying) treatment received 52 dishes (41,600 moths); because of the varying rate, the 3x treatment actually received only 2.4-fold the number of moths as the 1x treatment.

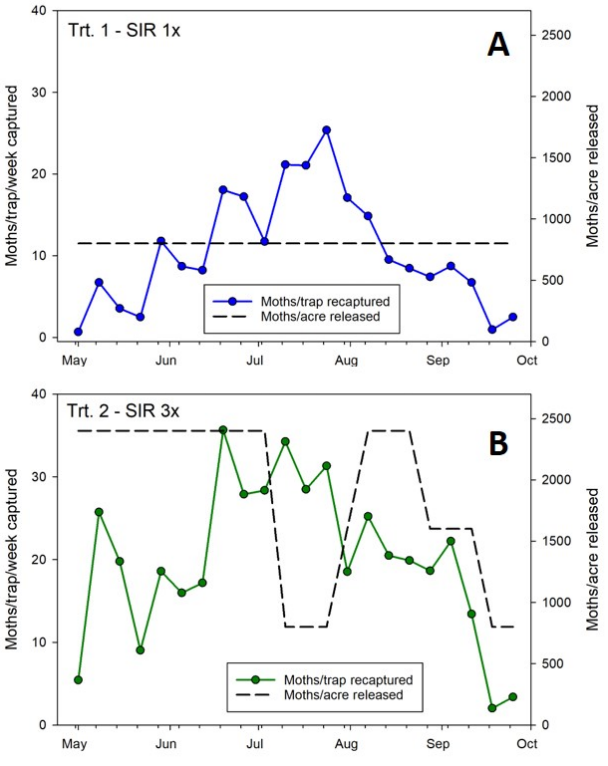

Fig. 5. Sterile moth capture (colored solid lines) relative to release rate (dotted lines) for 1x rate (A) or 3x rate

Sterile moth recapture peaked in mid-summer (July-August) irrespective of release rate (Fig. 5A, B). This pattern is most clearly seen in the 1x release rate, where the rate was constant over the entire season. Studies by Canadian researchers identified the issue of relatively poor performance of the sterile moths at cooler temperatures, possibly related to rearing conditions. Overall, the interior traps in the 3x treatment recaptured 1.8-fold as many moths as the 1x treatment. The percentage recapture of sterile moths (0.19 and 0.13% for the 1x and 3x treatments, respectively) were similar for the two release regimes, although the absolute number varied. The sterile:wild ratios fluctuated during the season, influenced by the response of sterile moths to traps and the generational peaks of the wild population. Ratios were generally highest during mid-summer, and somewhat higher in the 3x rate (2-24, mean 13.8) than in the 1x rate (2-36, mean 11.9); however, the 3x rate had higher wild codling moth numbers that offset the higher number of released sterile moths. The proportion of moths captured by the interior traps were consistently higher over time, irrespective of treatment (data not shown); the same is true if the proportions are calculated by block. In general, the interior traps caught 60-70% of all moths recaptured, and the perimeter traps 30-40%.

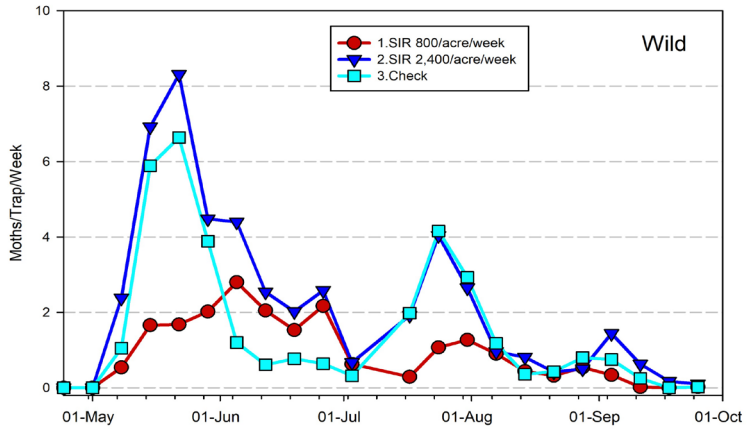

Fig. 6. Wild moth capture in SIR and untreated plots, 2018.

Wild moth densities showed generational peaks, with the strongest peak (late May) in the 1st generation (Fig. 6). There were <10 moths/trap/week throughout the season. This is in contrast to the 2017 season, when the 2nd generation peak was the highest (data not shown). Codling moth bands caught zero larvae at the end of the first generation, and averaged <0.15/band in all treatments in late September. and <0.6/band in any given plot.

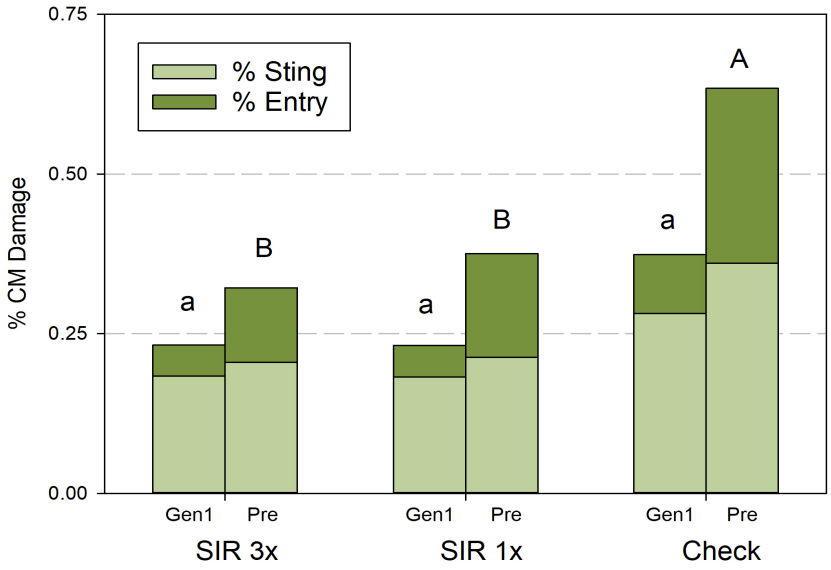

Fig. 7. Codling moth fruit damage after the first generation (Gen1) and preharvest (Pre) in CM SIR treated and check plots, 2018.

Codling moth damage was <0.5% at the end of the first generation (late June), with no significant differences among treatments. At this point in time, however, there was already a trend for higher damage levels in the check. The more intensive preharvest fruit sample revealed significantly lower damage in the two SIR treatments in comparison to the check (Fig. 7). This is a positive indicator that SIR treatments are providing a measurable additional suppression factor for codling moth in high pressure orchards. It is surprising in that the effect was evident at the end of a single season of releases, when the expectation was the 2-3 seasons would be necessary to see population reductions.

The intensive, spatially referenced fruit damage sample at preharvest provides confirmation of trap data regarding areas of higher damage in some of the plots. Many of the plots had damage fairly evenly distributed (Fig 8A), while in others, damage was concentrated in one area of the orchard (Fig. 8B). Information of this type will allow future moth releases to be increased in high-damage areas in an effort to reduce overall damage.

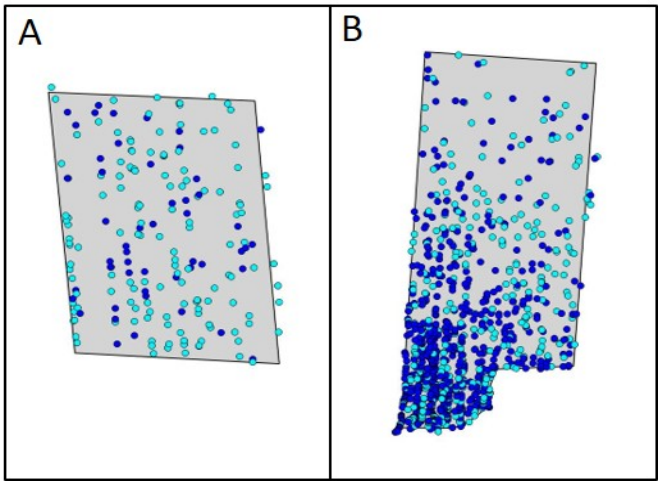

Fig. 8. Spatial patterns of codling moth fruit damage (light blue=sting, dark blue=entry). A – damage evenly distributed;

Obj. 2. Compare the non-target effects of broad-spectrum pesticide use versus SIR as a supplement to mating disruption in organic orchards

Earwig densities were significantly higher (4.1/shelter) in the 1x SIR treatment than the other two (0.06 and 1.4 for the 3x SIR and Check, respectively). *Aphelinus mali*, a specialist parasitoid of woolly apple aphid, was trapped using yellow sticky cards for two 3-4 week deployment periods. In both periods, the 1x SIR treatment had lowest numbers of *A. mali* adults (6.4-8.4/trap), the Check had

the highest numbers (39.9-93.1), with the 3x SIR intermediate (18.6-42.9). Spider mite counts were low (<0.5/leaf), with comparable numbers of predatory mites. Generalist predator (syrphids and lacewings) numbers captured on plant-volatile-baited sticky traps were low, with 2-9/trap over a 6-week period. Secondary pest densities will be assessed as spray records become available.

Obj. 3. Examine the synergy between SIR and other tactics using modeling techniques.

Methods: Mating tables were deployed on four nights during July 2018. Wild moths were collected from a research block on the campus of WSU-TFREC by banding trees, and rearing adults from the diapausing pupae. Sterile moths were obtained from excess occurring between generations when the variable rate treatment was the same as the 1x rate.

Wild virgin females were tethered by tying a thread around the base of the wing and attaching the other end to the table (Fig. 9). The tables were wired onto an apple limb in the upper 1/3 of the canopy and inspected ca. every half hour using ladders. Deployment occurred before dusk, when female codling moths call and mate, and continued until ca. ½ hour after sunset, when activity drops. Inspections during the first part of the mating period were made without any supplemental light, but later inspections employed a red flashlight to help see the moths without disrupting their activity. Mating pairs remain *in copulo* for at least ½ hour, and the technique involves placing a Petri dish lid over the pair, returning them to the lab, and determining if the male is wild or sterile by crushing the abdomen, and looking for the internal red dye of the sterile males.

Fig. 9. Mating table with tethered female codling moth.

We tested four combinations of mating disruption and SIR: 1) MD alone; 2) SIR alone; 3) MD+SIR; and 4) an untreated check (normal mating). Each treatment was deployed inside a 40 x 50 ft net cage in WSU's Sunrise research orchard to prevent spillover between treatments and replicated over time. Ten tethered females on mating tables were placed in the cages, and the requisite numbers of wild and sterile males released to achieve a 20:1 sterile:wild ratio.

Results: No matings were observed in any treatment (including the check) on any replicate night. This lack of matings was unexpected, as previous experiments typically saw about 40% of the females mating in a given evening. The influence of the cage or improper pre-conditioning of the wild moths may have suppressed mating; previous experiments were conducted in open orchards, using the ambient wild male population.

Additional tests were made of different types, heights, and orientations of mating platforms, including placing the female in a small cage, using the research orchard on the WSU campus. Again, no matings of the tethered female were observed. Based on these negative results, a molecular approach to determine mating is being pursued (see Obj. 3 Methods) to answer this critical question.

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

Project Title: Can we get codling moth females to stop laying eggs on apple?

PI: Stephen F. Garczynski
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City/State/Zip: Wapato, WA 98951

Cooperators: Dr. William Walker, Alnarp Sweden; Dr. Man-Yeon Choi, ARS Corvallis, OR

Total Project Request: **Year 1:** $56,110 **Year 2: $58,817** **Year 3:** $61,610

Other funding sources

Agency Name: USDA-ARS, Pacific West Area Office
Amt. awarded: $35,000
Notes: Area Office awarded money to purchase a flight tunnel and Track3D system. The Track3D system is comprised of cameras and software to monitor insect behavioral responses in a flight tunnel.

Budget 1
Organization Name: **USDA-ARS** **Contract Administrator:** Chuck Myers
Telephone: (510) 559-5769 **Email address:** Chuck.Myers@ars.usda.gov

Item	Year 1	Year 2	Year 3
Salaries	$37,306	$39,282	$41,321
Benefits	$13,804	$14,535	$15,288
Wages			
Benefits			
Equipment			
Supplies	$5,000	$5,000	$5,000
Travel			
Miscellaneous			
Plot Fees			
Total	$56,110	**$58,817**	$61,610

Footnotes: Salaries and benefits are requested for a full-time GS-6 Lab Technician. Supplies are for cloning ORs, oligonucleotide primer synthesis, Cas9 mRNA and kits for making CRISPR guide RNAs.

OBJECTIVES

1) Identify and clone Odorant Receptors expressed in the abdomen tip of codling moth females. More than twenty odorant receptors (OR) were identified in a transcriptome generated from abdomen tips of codling moth females. Ten of these receptors have been cloned this past year. In year two, the remaining ORs will be cloned. The cloned ORs will be used in expression assays (not funded by this proposal) to determine activating plant compounds.

2) Knock-out OR genes using CRISPR/Cas9 genome editing. CRISPR/Cas9 genome editing is fully developed for codling moth and will be used to knock-out genes expressed in the female abdomen tip. In the past year, materials to knock-out ORs using CRISPR have been generated or obtained from commercial sources. Codling moth eggs have been injected with different guide RNAs targeting five ORs and are currently being analyzed for effectiveness. This year, laboratory populations of codling moth with inactive OR genes will be generated for use in oviposition assays.

3) Determine which genes are essential for oviposition site selection. The hypothesis tested here will be that inactive OR genes for specific plant volatiles will alter female codling moth oviposition site selection. An oviposition assay for codling moth, developed by researchers in Sweden, will be used to assess the effects of OR gene knock-out populations. In year one, initiation of testing and adapting the oviposition assay was done. Because of the unsatisfactory results, we will continue to develop bioassays to assess the behavioral effects of CRISPR gene knock-outs. In September, ARS Pacific Area Office provided $35,000 for a flight tunnel system that will monitor behaviors using cameras and specialized behavioral analysis software. That system will be set up in January 2019.

SIGNIFICANT FINDINGS

- Transcripts for three of the 10 OR genes cloned are produced by alternative splicing (multiple transcripts/proteins produced by a single gene).
- In collaboration with Dr. William Walker (SLU Alnarp, Sweden), a female expressed OR that is activated by a male produced pheromone has been identified.

METHODS

The CRISPR (clustered regularly interspaced palindromic repeats)/Cas9 (CRISPR associated protein 9) genome editing system has been used extensively in the past few years to study protein function in many organisms. Interest in the CRISPR/Cas 9 system to control insects or to overcome insecticide resistance is now coming of age with substantial investments in this technology by Bayer Crop Sciences. A CRISPR/Cas 9 genome editing system for use in codling moth (refer to Figure 1 for a summary) is fully developed and has been successfully used to knock-out an OR gene thought to be involved in codlemone detection (Garczynski et al., 2017). These methods will be used to knock-out OR genes expressed in codling moth females to evaluate protein function and determine which genes are critical for oviposition site selection by codling moth females.

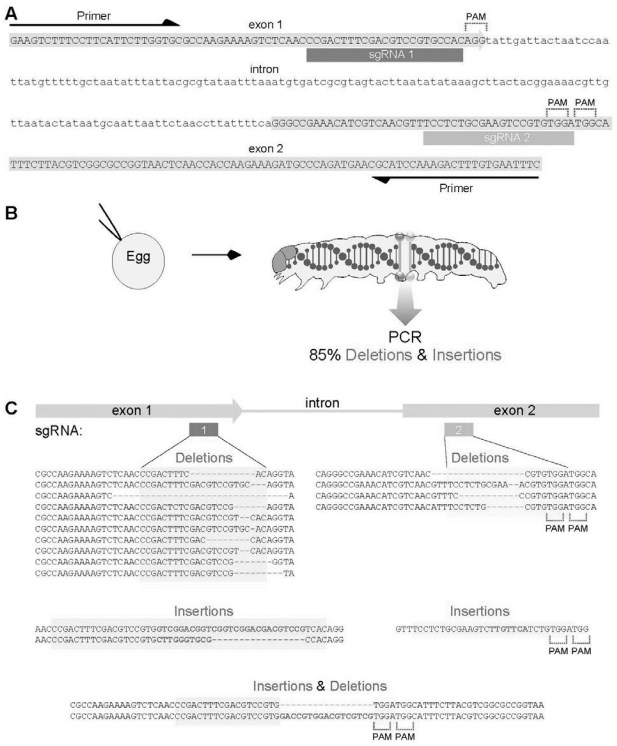

Figure 1. CRISPR/Cas9 genome editing of the *maleless* gene in codling moth. **A)** The region of the codling moth *maleless* gene (parts of exons 1 and 2 and the intron that connects them) targeted for genome editing. Primer sequences are denoted by single-headed arrows, regions targeted by single guide RNAs are denoted by dark grey (exon 1) and light grey (exon 2) boxes and coding sequences are highlighted in light grey. **B)** Pictorial representation of CRISPR/Cas9 injection and analysis. Eggs are injected using a microinjection needle containing Cas9 mRNA and sgRNAs, which are targeting exons 1 and 2 (saline used as control). Emerging neonate larvae are collected, their DNA is extracted and amplified using PCR with primers flanking the targeted exons. PCR products are cloned and 10 representative clones are sequenced to identify insertions and deletions in the targeted region generated by CRISPR/Cas9. **C)** Pictorial representation of the codling moth *maleless* gene and examples of insertions and deletions generated from CRISPR/Cas9 genome editing. Targeted regions of the *maleless* gene are denoted by dark grey (exon 1) and light grey (exon 2) boxes. The edited sequences are expanded and deletions are denoted by hyphens (-) and insertions are denoted by letters above or below hyphens.

1) Identification and cloning ORs expressed in the abdomen tips of codling moth females.

Gene transcripts encoding ORs expressed in antennae of codling moth males and females have been identified in transcriptomes (Walker et al., 2016). Recently, it was discovered that an OR was expressed in the abdomen tip of codling moth females (Garczynski et al., 2017). To determine the extent of OR expression, a female abdominal tip transcriptome was prepared late in 2017. Initial analysis of the transcriptome revealed that 38 potential OR transcripts are present, 28 of which are also found in the antennae (Walker et al., 2016, Garczynski and Walker, Unpublished data). Many of the ORs expressed in the codling moth abdomen tip are related to those activated by plant volatiles in other moths (de Fouchier et al., 2017). To confirm the initial identifications, PCR will be performed with oligonucleotide primers designed to amplify each individual OR found in the transcriptome. Once confirmed, the full-length transcript sequence will be determined using a PCR amplification technique. DNA sequence information gained from this step will be used in subsequent steps to design guide RNAs (see below).

2) Design and production of single guide RNAs.

Single guide RNAs (sgRNA) contain a target-specific nucleotide sequence of the gene of interest along with nucleotide sequence necessary for Cas9 (a protein that cleaves DNA) binding. Using the DNA sequence information above, the genome region containing target sites of the codling

moth OR genes of interest will be identified. With the gene sequence information, a minimum of three regions containing the features needed for CRISPR/Cas9 genome editing will be targeted for sgRNA design and production. Working in collaboration with a Chinese research group that has recently completed sequencing the codling moth genome, the genes for all the ORs expressed in the abdomen tip transcriptome have been identified. Knowing OR gene structure from the codling moth sequenced genome makes designing sgRNAs a relatively easy task. To generate sgRNAs specific to the selected codling moth OR genes, overlapping oligonucleotide primers containing the appropriate nucleotide sequence features will be designed and synthesized. These oligonucleotide primers will then be amplified in PCR reactions to generate a DNA template that will be used to produce sgRNAs in a test tube reaction. The sgRNAs are generated from the DNA template with an *in vitro* transcription kit which makes RNA from a DNA template. Once the sgRNAs are produced they are ready for use in genome editing experiments. The procedures for generating sgRNAs are already developed in the laboratory (Garczynski et al., 2017).

3) CRISPR/Cas9 genome editing of codling moth OR genes.

CRISPR/Cas9 genome editing takes place when an appropriate sgRNA and Cas9 protein are present in the same cell. For this to occur, sgRNA and Cas9 mRNA are co-injected into early stage eggs, and the sgRNAs are transported to the cell nucleus by the Cas9 protein. Once in the cell nucleus, the Cas9 protein/sgRNA complex binds to the target gene and the double-stranded nuclease of Cas9 cleaves the gene creating a mutation that can ultimately result in the loss of protein function. Procedures for performing CRISPR/Cas9 genome editing in codling moth have already been developed (Garczynski et al., 2017). To knock-out our selected OR genes, target specific sgRNAs along with Cas9 mRNA will be co-injected into freshly laid eggs. The genome editing takes place shortly after injection. At this stage, DNA will be extracted from neonate larvae to analyze the effectiveness of the genome editing (see below). It is important to note, that this initial analysis step is necessary because the efficiency of sgRNAs are not equal, and why three sgRNAs are designed for each target gene. The results of this initial analysis will identify the best sgRNAs for large scale knock-out experiments.

4) Analyzing the effectiveness of CRISPR/Cas9 genome editing using molecular techniques.

Two molecular techniques, DNA sequencing and high-resolution melt (HRM) analysis, are used to determine the effectiveness and extent of CRISPR/Cas9 genome editing, and to verify that the mutations made will knock out protein production (Garczynski et al., 2017). For each molecular technique, oligonucleotide primers surrounding the region of the gene being targeted are designed for PCR amplification. These oligonucleotide primers are first used in PCR reactions to amplify genomic DNA of treated and untreated insects, then the PCR products are cloned and sequenced to verify CRISPR/Cas9 generated mutations. Once success of the CRISPR/Cas9 genome editing technique is verified by cloning and sequencing, DNA from larger numbers of injected insects are analyzed with HRM analysis. To perform this assay, PCR amplifications of the targeted gene are done using a real-time PCR machine and the resultant PCR products are subjected to HRM analysis. HRM analysis is a high-throughput technique that is used to detect minor changes in the mutated gene when compared to its unaltered counterpart. Once CRISPR/Cas9 genome editing is verified molecularly, analyses of the effects of these mutations will be assessed using bioassays.

5) Assays to determine the effects of CRISPR/Cas9 genome editing on egg laying.

Oviposition in moths is a two-step process; females must first find their host plant, and after host recognition, oviposition sites need to be identified (Honda 1995). Ovipositing females use plant volatiles to locate the host plants and then use contact evaluation of plants to detect less or non-volatile chemical compounds to determine suitability for egg laying (Honda 1995). For codling moth, oviposition is stimulated by apple odor (Wearing 2016), and several volatiles in apple odors including α-farnesene, β-farnesene and nonanal have been specifically identified as stimulants (Sutherland et al.

1977, Witzgall et al. 2005). ORs expressed in the female antennae are thought to important play roles in host plant finding, bringing the moth in proximity of a suitable oviposition site. The hypothesis to be tested in this project is specific ORs expressed in the abdomen tip are important for contact evaluation in determining plant suitability for egg laying.

To determine if an OR gene knocked out by CRISPR/Cas9 genome editing has an effect on codling moth oviposition, a bioassay will be used. The assay uses jars or cups in which 10 mated females are placed in the presence or absence of varying concentrations of a volatile compound (see below for compounds to be tested). After one hour, females are removed from their container and eggs are counted. It is expected that oviposition stimulants will result in a greater amount of eggs laid vs controls. To determine which ORs play key roles in detecting oviposition stimulants, CRISPR/Cas9 edited females will be placed in the cups in the presence or absence of varying concentrations of volatile compounds and a positive result would be fewer eggs laid compared to unedited females. To determine statistical significance, full factorial analysis with concentration, treatment and their interaction as the dependent variables. All statistical analyses will be done using SAS 9.4 with the GLIMIX procedure.

There are at least 64 volatile compounds found in headspace collections from apple at different phenological stages (Bengtsson et al 2001). Using the assay above, apple volatile compounds that produce antennal activity in females will be tested (Bengtsson et al 2001). These compounds are (Z)3-hexenol, butyl butanoate, propyl hexanoate, hexyl propanoate, butyl hexanoate, hexyl butanoate, hexyl 2-methyl-butanoate, hexyl hexanoate, methyl salicylate, benzyl alcohol, 4,8-dimethyl-1,3,(E)7-nonatriene, β-linalool, β-caryophyllene, (E)-β-farnesene, germacrene D, (Z,E)-α-farnesene and (E,E)-α-farnesene, all of which are commercially available. In addition, compounds found in apple leaves will be also be tested, including a mixture of Theaspirane, (2R, 5R) and (2S, 5R), and geraniol, which acts as a codling moth repellant (Wei et al 2004). Initially, volatiles previously identified as oviposition stimulants (α-farnesene, β-farnesene and nonanal) will be tested and candidate ORs for these compounds will be edited.

6) Expected Outcomes.

Completion of this project will identify ORs expressed in the abdomen tips of female codling moth that play key roles in oviposition site selection. This information, along with identification of ORs expressed in female antennae that have key roles in attracting females to oviposition sites, will provide targets that may be manipulated for codling moth control. Examples can include: 1) development of new compounds that are more potent attractants which can be used to trap females; 2) development of compounds that block receptor activity to prevent females from finding oviposition sites; and 3) development of a system that attracts females (using a more potent attractant from example 1) to a platform that contains oviposition stimulants enticing the females to lay eggs away from apple. In the long term and when socially acceptable, these targets can be knocked out in wild populations using CRISPR/Cas9 technology are currently being commercially developed (Bayer Crop Sciences and DuPont are working on this technology).

RESULTS AND DISCUSSION

The central role of olfaction (sense of smell) in the life of insects has been well documented. Olfactory cues from insects of the same species and environmental/ecological sources mediate most vital behaviors, including mate and host seeking, oviposition and predator avoidance. Extensive research efforts have characterized the role of odorant receptor (OR) proteins in the detection of odorants, where ORs provide the interface for insects with the environment and serve as the molecular gateway to olfactory centers in the brain and downstream behavior. Traditionally, ORs are thought to be localized to insect antennae. For codling moth, we have determined that ORs are also

expressed in the female abdomen tip. The function of these ORs is not known, but it is hypothesized that they are present to identify males of their species, or environmental odors to find oviposition sites.

Identification and cloning odorant receptors expressed in female codling moth

We have prepared a transcriptome (a compilation of all genes expressed in a particular tissue) from abdomen tips dissected from codling moth females. When examined, 38 transcripts encoding ORs were discovered. In year one of this project, 10 OR transcripts were cloned to verify expression and DNA sequence for the design of guide RNAs for use in future CRISPR genome editing experiments to determine function. When analyzed, three of the 10 cloned OR transcripts showed evidence that they were products of a phenomena know as alternative splicing. Alternative splicing allows for multiple proteins to be produced from a single gene. Because insects have a reduced number of odorant receptor genes compared to vertebrates, alternative splicing provides a mechanism that allows for the production of more functional proteins than the number of genes present in their genome. For insect odorant receptor genes, alternative splicing has not been fully explored and a manuscript has been accepted describing this phenomena in codling moth (Garczynski et al. accepted by Journal of Economic Entomology citing this project as the primary funding source).

In collaboration with Dr. William Walker, a female biased OR transcript was expressed in a functional assay system to determine the odorant that activates that protein. From a panel of plant volatiles and male produced pheromones, Dr. Walker has determined that this OR responds to a male produced pheromone. This pheromone is structurally related to one identified in the spruce budworm, *Choristoneura fumiferana*, which is in the same family as the codling moth. The pheromone produced by the spruce budworm has been shown to be important for mating courtship behaviors. Currently, codling moth male sex pheromones are being extracted so that we can identify the native compound that activates the female OR. This gene is also being targeted for CRISPR genome editing to determine its role in mating and if disruption of this gene provides a target for the development of new compounds that can be used to disrupt mating in codling moth control programs.

Additional receptors identified in the female abdomen tip transcriptome

The goal of this project is to identify proteins expressed in codling moth females that when disrupted cause females to stop laying eggs on apple. We proposed looking at the ORs expressed in the female abdomen tip, but in searching the transcripts in our transcriptome we have identified additional proteins that may also be valuable targets for control. The most interesting of the targets are neuropeptide receptors that are involved in letting the female know when she has been mated and play key roles in egg production. A collaboration has been initiated with Dr. Man-Yeon Choi at the ARS facility in Corvallis, OR. Dr. Choi will be cloning and expressing these receptors in his cell-based assay system to identify the native peptide hormones that activate these proteins. Dr. Choi holds a number of patents on compounds that disrupt neuropeptide receptor function in moths with the goal of developing new chemicals for insect control.

Conclusion

The progress made on this project is in line with the timeline provided in the initial proposal. In year 2 of this project we will complete the cloning of additional ORs expressed in the abdomen tip and design guide RNAs that target these receptors for CRISPR genome editing experiments. We have completed initial CRISPR genome editing experiments on four ORs and in year 2 we will be generating stable populations to use in behavior bioassays. We have received funding from Pacific West Area Office for a flight tunnel and a camera/software system that analyzes behavior in real time. This system will be used to identify codling moth behaviors modified by knocking out OR genes. The system is much more accurate than manual behavior monitoring in that it can detect variations not apparent to the naked eye. The success that we have had in the past year has attracted

collaboration of additional researchers whose expertise's will allow for the development and testing of novel semiochemicals and chemical compounds that may be useful to control codling moth in the orchard.

CONTINUING PROJECT REPORT **YEAR 1 (No-cost extension)**

Project Title: Improved monitoring and lure and kill for codling moth management

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City/State/Zip: Valdivia, Chile 5110000

Other funding sources: None

Total Project Funding: **Year 1:** $21,090 **Year 2**: $0

Budget 1
Organization Name: ARS, USDA
Contract Administrator: Chuck Myers
Telephone: (510) 559-5769
Email address: chuck.myers@ars.usda.gov

Item	2018	2019
Equipment	-	
Supplies	12,000	
Travel	3,090	
Miscellaneous	-	
Plot Fees	-	
Total	$15,090	$0

Budget 2
Organization Name: Universidad Austral de Chile
Contract Administrator: Ricardo Leal **Telephone:** +56 63 222 1778
Email address: rleal@uach.cl

Item	2018	2019
Travel (visit USDA lab June)	6,000	
Total	$6,000	$0

OBJECTIVES:

1. Develop two newly discovered attractant blends that can significantly increase catch of both sexes of codling moth to improve monitoring and increase the effectiveness of lure and kill technologies for this pest.
2. Field test and fine-tune the components of mass trapping for codling moth using a *hands-free approach* during the season.
3. Develop and evaluate *Zerofly* netting for use against codling moth.

SIGNIFICANT FINDINGS

- The ***4-way K*** lure was developed through extensive testing of host plant volatiles. This non-pheromone lure outperformed the standard pheromone lure used by the industry and caught 60-70% females
- The ***4-way K*** lure caught 4-fold more females than the previous best female attractant (pear ester plus acetic acid) and was effective in variable apple orchards with different cultivars, fruit loads, and throughout the season (green fruits, mature fruits, injured fruits, and fruits rotting on the ground).
- Lure development is continuing in South America.
- Mass trapping of codling moth using 24 combo plus acetic acid-baited bucket traps per acre were used to reduce levels of fruit injury 71% at harvest across four organic apple orchards. Traps were only serviced once at mid-season to replace the lures.
- The *Zero-fly* deltamethrin-impregnated netting was developed into a ***Grey Ghost*** and evaluated with moths in laboratory experiments. These tests demonstrated that the netting killed moths within 24 h with contacts as brief as 5 s. A strong sublethal effect on mating and egg laying occurred in moths that did not die within 24 h.
- The ***Grey Ghost*** remains toxic throughout the season and may last for more than one year.
- ***Grey ghost***-treated plots had reduced moth catch in monitoring traps and 50% less fruit injury at midseason.
- Lures used with the ***Grey Ghost*** were not replaced and both the lure attractiveness and fruit protection declined after mid-season.

METHODS

The remaining research supported by this project will be conducted up until 30 April 2019. Two types of research are ongoing. First, we are testing various lure combinations in Chile and Uruguay. These studies have replicated lure treatments in delta traps randomized in apple orchards. Studies are run for 3-7 days and new lure treatments are then tested. These studies will continue until February. Second, we are continuing to look at the effectiveness of the ***Grey Ghost*** in flight tunnel assays. This involves flying both males and females to lures placed on the netting and recording the length of the moth contact and subsequent mortality. Sublethal effects are examined by pairing treated and untreated male and females and recording mating success and the numbers and fertility of eggs laid. Funds are being used to support a technician who runs these studies and assists my permanent person in rearing insects. Funds are also used to purchase the various host plant volatiles and materials needed to produce lures. Funds will also be used to develop a detachable lure holder for use with the ***Grey Ghost***.

RESULTS AND DISCUSSION

A series of studies were conducted during the 2018 season in a heavily-infested set of apple orchards situated near Wapato, WA. Several host plant volatiles were evaluated in delta traps to see if they were attractive to adult codling moth. Individual compounds, binary, ternary, and quandary blends were compared. All of these compounds and blends were compared to the effectiveness of pear ester plus acetic acid lures as the industry best lure for female codling moth. Only the quandary blend

outperformed the PEAA lure and caught nearly 4-fold more total moths and females with levels of females comprising as much as 80% of all moths caught (Figs. 1 and 2). This blend was coined ***4-way K*** because there are four kairomone compounds. Late in the season various modifications of this blend were evaluated with various substitutions of compounds. Just prior to the end of the season several additional volatiles were identified that could also be used in various substitutions. However, this work was incomplete and ongoing studies have been established in Uruguay and Chile to continue these studies.

The significance to the WA tree fruit industry of this discovery is tremendous. Identification of an attractant that is more powerful than sex pheromones and catches a very high proportion of female moths can benefit growers in several ways. The lure is very effective in orchards treated with sex pheromones for mating disruption as the traps are not disrupted. Enhanced capability to track female moths can allow significant improvements in timing sprays to target egg hatch. The lure does not draw moths into orchards and effective thresholds based on moth catch could be established. Data from 2018 suggested the ***4-way K*** lure worked well in several cultivars, throughout the season, and in blocks with few fruit and heavy crop loads. Also, it worked whether the crop was injured, moderately injured, heavily injured and even when the orchard floor was littered with damage fruit. Trap height does not seem to be as important compared with sex pheromone lures, so traps could be placed at a lower and more convenient height in the orchard. The dogma in insect behavior research is that the best attractant makes the best disruptant of sexual behavior. Thus, it may be possible to develop improved mating disruption dispenser systems using one or more of these compounds. Experimental dispensers have already been formulated and will be tested in 2019. Finally, the tremendous increase in female catch afforded by the use of the ***4-way K*** lure could make mass trapping extremely effective. Our results from last season using just 24 bucket traps baited with the Combo plus acetic acid lures (71% less injury) for an organic grower was exciting to both him and us, and perhaps these good results could be greatly improved using the 4X more potent ***4-way K*** lure.

The ***4-way K*** lure may also facilitate the eventual use of an attract-and-kill concept we explored in 2018. The *Zero-fly* netting was developed for battling malaria in third world countries and has been used effectively in research to manage the brown marmorated stink bug in Pennsylvania orchards. We developed the ***Grey Ghost*** which is baited with codling moth lures and hung in the canopy (Fig. 3). Our laboratory studies demonstrated that moths are not repelled and land on the netting in response to the lures. Moths typically walk on the netting for 1-60 s and often rest on the netting for longer periods. Forced touch tests demonstrated that as little as a 5 sec contact with the netting kills moths within 2 h and most moths by 24 h after contact ended. Sublethal effects are also pronounced with survivors unable to mate or to lay a full complement of eggs. A small field research study was established in 2018 using replicated 1-acre plots. We had hoped that the lures would last all season so once the ***Grey Ghosts*** were applied we did nothing all season except check traps and sample fruit injury. Lures at mid-season were still effective, and moth counts and levels of fruit injury were about 50% lower. However, by the end of the season no difference in fruit injury between the treated and untreated plots were seen and no difference in moth catches occurred in the two treatments. Lures near the end of the season only caught 30% as many moths as new lures. We consider these results to be interesting and will develop a method to attach a lure holder to the ***Grey Ghost*** that will allow lures to be more easily replaced at mid-season. We have a 3D printer at the laboratory to develop this device. We demonstrate that the netting does not lose any toxicity over the course of the season and could perhaps last more than one year.

Figure 1. Comparison of the 4-way K lure with industry standard lures.

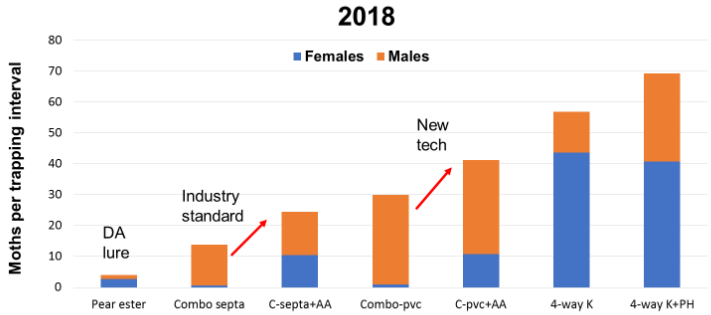
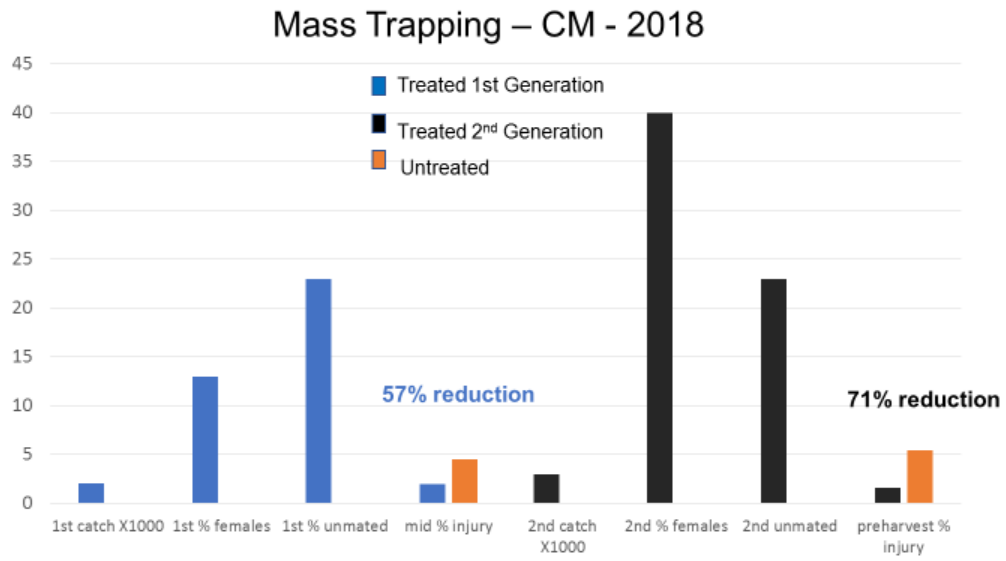


Figure 2. All the codling moths (males at top, females below) sorted and removed from the one liner on the right after only one night (21 July 2018) using the ***4-way K*** lure in combination with sex pheromone. *Note*: scale-less areas on liner are where the lures were placed.

Figure 3. Photograph of the ***Grey Ghost*** developed with *Zero-fly* netting for attract and kill of codling moth, 2018.

Figure 4. Summary of mass trapping experiments N = 4, using 24 bucket traps per acre baited with Combo plus acetic acid lures, Tieton, 2018.



CONTINUING PROJECT REPORT **YEAR**: 3 of 3 (No-cost extension)
WTFRC Project Number: CP-16-103

Project Title: Assessment of apple immune responses to wooly apple aphid saliva

PI: Dr. Paul D. Nabity
Organization: University of California-Riverside (1st year with WSU)
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Cooperators: Dr. Chaoyang Zhao, UCR; Dr. Gennaro Fazio, USDA-ARS/Cornell University

Total Project Request: $164,987 **Year 1**: 58,710 **Year 2**: 49,079 **Year 3**: **57,198**

Other funding sources

Agency Name: Dovetail Genomics LLC & University of California, Riverside Office of Research and Economic Development
Amount awarded: $13,550
Notes: Dovetail Genomics LLC and the UCR ORED provided funds for sequencing the WAA genome

Budget 1
Organization Name: University of California-Riverside
Contract Administrator: Cynthia Carr
Telephone: (951) 827-4372 **Email address:** cynthia.carr@ucr.edu

Item	2016 (WSU)	2017	2018	2019*
Salaries	$32,836	$20,646	**$32,654**	
Benefits	$3,424	$16,463	**$22,044**	
Wages				
Benefits				
Equipment				
Supplies	$22,450	$11,970	**$2,000**	
Travel			**$500**	
Plot Fees				
Miscellaneous				
Total	$58,710	$49,079	**$57,198**	**$0**

Footnotes: *No money is requested in 2019 as this is a no-cost extension to finalize data and reporting.

OBJECTIVES

All plants share networks of related genes and proteins that work together to generate immune responses to both insects and pathogens. The main goal of our project is to identify these networks in apple as they relate to aphid feeding, although resolving these immune networks will inform upon any biotic stress response imposed on apple in the future. A complementary goal is to examine how the aphids trigger these networks. Our approach combines transcriptomic information on the apple genes induced by aphid colonization with the genes active in aphids as a first hurdle in linking insects to plants to better understand resistance, how insect populations vary across growing regions, and identifying additional genes involved in resistance networks. Our specific objectives were to:

1. Identify the WAA salivary proteins that alter plant form and function in roots and shoots. When feeding, WAA discharges salivary constituents into plant tissues. These proteins play critical roles in reprogramming the physiological processes of infested plant tissues, i.e., roots and shoots. Because salivary proteins are secreted by salivary glands, we used a transcriptomic assessment of extracted salivary glands to identify all the genes that encode secretory proteins in WAA. We compared this to whole body extractions to rule out transcripts expressed in dissected tissue but not associated with salivary glands. To verify the gene products, we also collected salivary proteins for proteomic analysis. Initial proteome screens (2016-2017) revealed more material was necessary to increase replicates and detection. However, other recently published studies indicate the number of proteins found in insect saliva is much less than what should be produced from protein-encoding genes annotated in the transcriptome (Thorpe et al. 2016, Boulain et al. 2018,). Thus, transcriptome profiling of salivary glands is a better approach to identifying insect secretory proteins that antagonize plants compared to proteome collections. Because of this we did not pursue more proteome studies. To date, the whole-body transcriptome and salivary gland assemblies are complete. In late 2017 we secured funding for the WAA genome to increase the ability to detect genes and their products related to colonization. This genome is still under assembly, but we will soon open it up to the scientific community at large to improve annotated gene models. Once completed, these data will represent the most comprehensive database of WAA genetics.

2. Characterize the plant immune response in resistant and susceptible species and rootstocks. Apple resistance to aphids is known to depend on at least four genes. By assessing transcriptomes of apples that differ in susceptibility to aphid attack we can identify how networks of genes interact to protect against aphids. We may also identify aphid-specific genes not yet annotated in the draft genome of apple currently available to increase candidates for resistance breeding. Sample collection was completed in Fall 2017 using the susceptible genotype G935 and two commonly used genotypes with greater resistance G16 and G87 determined by previous performance trials. Sequencing data was returned Spring 2018. Data analyses commenced in Fall 2018. We also screened novel rootstocks from a resistance mapping population for performance with WAA in collaboration with G. Fazio during Summer and Fall 2017.

3. Identify functional plant traits that confer immunity to WAA.
Once gene networks are identified, we can infer the functional plant defense chemistry and signaling that result from aphid attack. Preliminary screens of commercial and unreleased rootstocks with known resistance genes showed variable colonization by WAA. With performance trials completed, we originally planned to phenotype the underlying biochemistry related to resistance. Given the gene network analyses (objective 2) indicate RNA signaling, transcription, and post translational processing such as ubiquitination (degradation) of proteins were strongly upregulated, we chose not to screen for biochemical changes in phenolics, callose, or reactive oxygen species. Rather it is likely that effector proteins from the aphid are targeting upstream genetic processes that regulate transcription and translation to ensure colonization. These pathways also regulate resistance in plants,

and were co-expressed with nucleotide-binding site leucine-rich repeats (NBS-LRR) proteins that act to monitor effector targeting of plant processes by pathogens (McHale et al. 2006).

4. Map these traits to genes in apple to facilitate marker-assisted breeding.
Breeding-program specific DNA tests for high impact attributes are required to streamline cultivar development. One way to advance the creation of these tests is to identify the genes and their nearby markers. Thus, a first step toward marker-assisted breeding for WAA is to identify the genes and their functional traits that enable immunity. Our collaboration with Dr. Gennaro Fazio provides access to unreleased genotypes of known heritage (genetic maps) to better understand the location on chromosomes of genes that are central to resistance networks. Combining this mapping population with our transcriptome analyses we can screen individual response to aphids to understand how effector recognition is inherited. We are currently working on the gene networks in both the insect and plant to better predict which changes in the plant are linked with known resistance genes.

Timeline. Our expected timeline is outlined below. We collected and assessed WAA during year 1, and assessed the plant during year 2-3. We are combining all these data currently with an anticipated completion of Summer 2019 (when the lead student Josh Wemmer graduates).

Objective	2016	2017	2018	2019
1 – insect transcriptome	x			
1 – insect proteome	x	x	x	NA
2 – plant transcriptome		x	x	
3 – plant functional traits			x	
4 – gene to trait linkage			x	x
Final summary			x	x

2016 SIGNIFICANT FINDINGS

- 184 proteins were identified as putative effectors from the transcriptome.
- 75% of these proteins are unique to the WAA and do not occur in other insects.
- At least one protein mimics a transmission protein necessary for successful infection of two families of plant viruses (the caulimoviruses and the potyviruses).

2017 SIGNIFICANT FINDINGS

- 10 unreleased genotypes were screened for resistance.
  - One genotype prevented colonization that led to aphid dispersal/death in 5 days (Fig 1).
  - 9 genotypes showed a range of survival between 15–40% (5 shown in Fig 1).
- Rescreening aphid performance on resistant (5087) and susceptible Geneva (16) rootstocks showed similar survival (50%). This indicates resistance is beyond genes on chromosome 17 and provides support for our hypothesis that immune systems are complex, and a transcriptomic approach will help resolve new resistance traits/genes.

2018 SIGNIFICANT FINDINGS

- Of the 349 genes differentially expressed when aphids feed, protein regulation is enriched in expression with 51 genes and includes genes recognizing biotic stress (specifically NBS-LRR proteins, Ca+ signaling, MYB domains, WRKY, and bHLH)
- In comparisons among plants along a resistance spectrum, uninfested plants showed more active pathogenesis response proteins, including those involved in ubiquitination (and predicted targets of aphid effectors).
- In comparisons between G16 and G87, G16 showed greater response to aphid colonization (274 vs 66 genes) with significant enrichment in hormone signaling and cell wall formation, but opposite expression patterns in several pathways. This links the induced/gall phenotype to

the first moments of feeding. When combined with survival data from 2017 this suggests success does not depend on gall formation per se, but rather subversion of pathogen recognition genes.

METHODS

Experimental Design - *Overall Strategy:* We will use a combination of genetic, transcriptomic, and functional trait assessments to resolve how WAA colonizes a plant. This will allow us to identify plant genetic targets (and immune functions) that deter or reduce WAA feeding. We first measured the genes expressed in WAA and compared these to those in salivary glands currently being sequenced. This will be guided by the draft genome funded through a cost share with Dovetail Genomics LLC and the UCR Office of Research and Economic Development. We are employing several statistical approaches to complete the transcriptome analyses of resistant and susceptible *Malus* rootstocks under WAA attack and identify the genes mediating a successful immune response. Lastly we will link the WAA salivary effector proteins (Objective 1) to their immune targets (Objective 2) to begin to identify the plant markers associated with genes underlying traits of WAA resistance (Objectives 3 and 4).

Identification of WAA salivary enzymes/proteins that promote parasitism in apple:

Salivary Gland Transcriptomics: As part of **Objective 1**, salivary glands from fourth instar larvae and wingless adult WAA were dissected under a Zeiss Stemi 508 stereoscope. Total RNA was extracted using a combination of a Trizol RNA isolation protocol and a commercially available RNA extraction kit (Qiagen). Extracted RNA was assessed for quality and quantity using an Advanced Analytics Fragment Analyzer and RiboGreen quantification kit, respectively. Libraries were built with the Illumina TruSeq RNA kit and assessed again for quality as above. RNA-Seq of the whole body insects was done at the WSU Genomics Core on an Illumina HiSeq2500. All expressed RNA (transcriptome) data were *de novo* assembled using Trinity software (Grabherr et al. 2011) with a minimum fragment overlap of 35 base pairs (bp) to create final contigs (>200 bp). To discover the salivary effector proteins that WAA uses to attack the host, we adapted a bioinformatics pipeline that has been successfully applied to identify spider mite effectors (Villarroel et al. 2016). Briefly, this pipeline was designed based on the four features of effectors: 1) secretory, 2) small sized, 3) fast-evolving, and 4) gene-duplicating. The putative WAA effector proteins were then used to search against the public protein and domain databases according to their sequence and structure similarities to understand how they may function in attacking host plant.

Salivary Proteomics: The final activity for **Objective 1** involved collecting salivary proteins from WAA using similar methods described by Vandermorten et. al.(2014). Feeding chambers were created using 40-mm diameter plastic cylinders with diet (15% sucrose solution and 100 mM each of the following amino acids: glutamine, serine, methionine, arginine, and asparagine) sealed between two layers of stretched Parafilm. 150-200 1^{st}-4^{th} instar larvae and adult WAA were placed into feeding chambers to feed for 48 hours. The diet after feeding (containing saliva) was collected under sterile conditions and concentrated using Vivaspin 20 centrifugal concentrators with a 3000 Da molecular weight cut-off . Protein concentration was quantified with a micro BCA assay and Nanodrop 2000 spectrophotometer. Protein samples were analyzed by the Tissue Imaging and Proteomics Lab at WSU by Drs. David Gang and Jing Wang. These samples showed few proteins because of low excretions by aphids and a lack of a protein library to screen against. Because new studies indicate proteomics approaches to aphid saliva can be limited, we are abandoning this approach and focusing on the transcriptome data.

Identification of apple immune responses to WAA:

Plant Assessment: A first step to defining complex traits, such as resistance in apple, is to use a systems-genetics approach. This approach uses transcriptome networks to assist in discovery of single genes underlying relevant biological function. Using genotypes that vary in resistance we show how apple immunity functions against WAA during both successful and unsuccessful colonization events.

To fulfill **Objective 2**, we sampled across 50 resistant (5087) and susceptible (16, 935) cultivars, which surprisingly showed similar survival after 6d. This indicates the traits imparted by the main resistance locus on chromosome 17 is not the only contributor to resistance. Plant and insect tissues were harvested after 50h. RNA was extracted as described above. Samples were deduplexed (BBtools; DOE/JGI), aligned to a reference genome (Hisat2), and assessed using statistical and visualization packages in R.

Given what we know about other aphid-plant interactions, and insects that gall plants, we predicted reactive oxygen signaling (ROS) and defenses to be active in addition to stimulation of the structural antiherbivore defense compound: callose. Initial screens did not support these hypotheses so we chose not to evaluate plant biochemistry. Rather we are applying several novel network analysis approaches to characterize co-expressed gene modules related to resistance. Combined with the gene visualization software Mapman that syncs transcriptome data from the latest genome assembly to metabolic pathways (Thimm et al. 2004), we will fulfill **Objective 3.**

Trait Mapping: Apple breeding is often slow and challenging because of long generation times and complex inheritance. Markers linked to biological functions expedite cultivar development, but this still remains an enduring process. One step toward resolving WAA markers more quickly is to use transcriptional profiling to identify gene expression during incompatible WAA-apple interactions. Expression values of genes when not challenged by WAA can be representative of baseline resistance or susceptibility, depending on the cultivar examined. However, using a comparative framework with both resistant and susceptible cultivars challenged by WAA will allow us to subtract out genes or pathways not directly linked to WAA resistance or genes that are induced upon colonization. Once genes are identified we can identify chromosome locations for each gene to link them to markers to assist cultivar screens for WAA-relevant traits to complete **Objective 4**. Follow-up studies can then be planned to assess cultivars along the resistance – susceptible continuum to resolve which genes and processes provide greater immunity. Our collaboration with the USDA in Geneva and RosBreed will greatly facilitate finding the WAA genes on apple genetic maps to advance future studies.

RESULTS & DISCUSSION

During 2018 we continued work on the WAA genome assembly and the transcriptome analyses. We generated two WAA assemblies, both with 95% of genes known to be conserved across insects, thus indicating high quality datasets. By combining the assemblies and overlaying the transcriptome data we are improving gene annotation. This is a computationally intensive process and will take additional time to complete. We expect the genome assembly to be drafted during 2019, and creep toward completion with aid from expert genome biologists when we open up this assembly to the scientific community. This is a critical step toward enhancing apple production across geographic boundaries.

The transcriptome analyses now include the training of another graduate student to understand how resistance to aphids is linked through novel plant pathways. Using standard differential expression analyses and a network coexpression analysis we found the following summary points. WAA induces 349 genes within the first 50h of feeding and a significant number of these genes (51) are enriched in the protein signaling/degradation pathways. This links transcription, and especially post translational modification of proteins to plant proteins in a manner to degrade these plant targets through ubiquitination, a process often targeted by galling insects to disrupt immune responses. We also found gene expression patterns differed between G16 and 87, but were less pronounced in G935. In comparisons between G16 and G87, G16 showed greater response to aphids (274 vs 66 genes) with enrichment in hormone signaling and cell wall formation. This links the induced/gall phenotype to the first moments of feeding. When combined with survival data from 2017 this suggests success does not depend on gall formation per se, but rather subversion of pathogen recognition genes.

Data analyses are ongoing, however some immediate patterns stand out among genotypes and relative to biotic stress genes in apple (Figure 1).

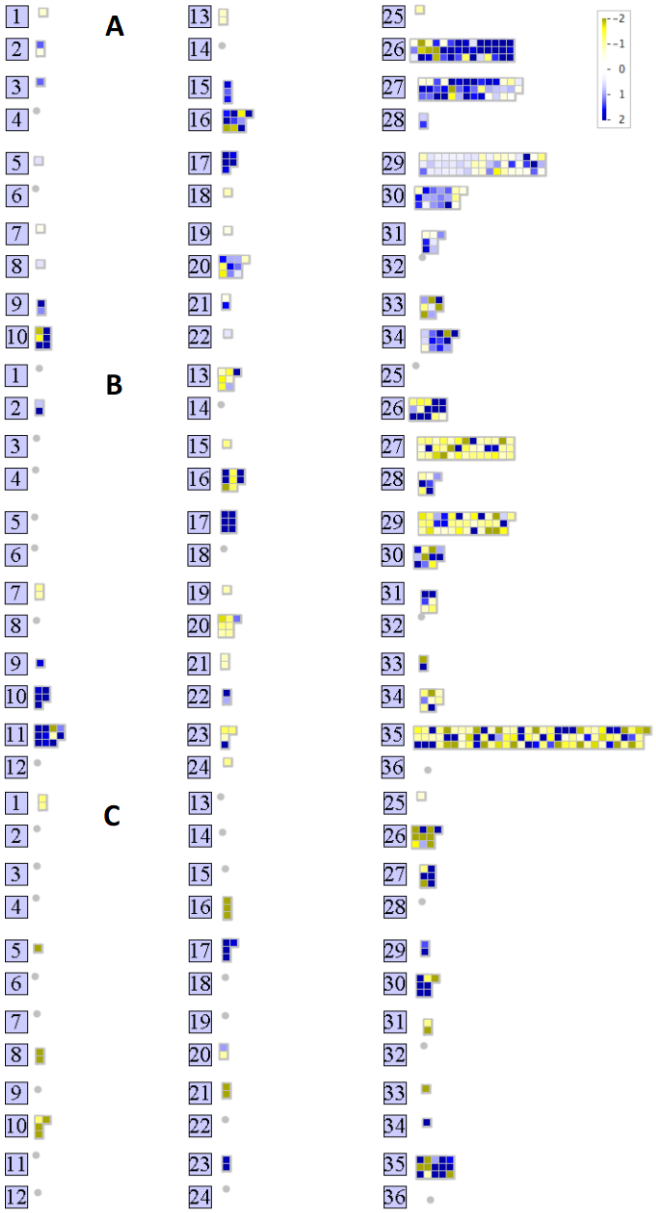

Figure 1. Gene expression profiles for **A)** aphid infested versus uninfested pooled across genotypes, **B)** aphid infested versus control for G16, and **C)** aphid infested versus control for G87/5087. The numbers in blue boxes represent metabolic processes in plants whereas the colors represent the strength of the gene regulation in aphid attacked plants (blue=up; yellow=down). The individual boxes that vary from yellow to blue represent unique genes linked to specific pathways. **One general summary across all genotypes is that aphids upregulate numerous processes in plants, with significant enrichment in protein manipulation (translation, transcription, degradation, #29), cytochrome P450 regulation (#26), and serine/esterase lipase (also in #26).** When looking specifically at two genotypes (G16 and 87) the **general summary is more gene expression in G16 than 87, but opposite patterns in pathways.** ***The degree that these patterns link to function of resistance genes is still under investigation.***

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