APPLE CROP PROTECTION RESEARCH REVIEW
Thursday, January 25, 2018

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8:30		Hanrahan	Introduction	
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9:00	12	Sibayan	Cold tolerance, diapause, and survival of Brown Marmorated Stink Bugs: ***2nd year report***	16-17
9:15	23	Crowder	Dynamics of WAA on organic and conventional orchards	14-15
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FINAL PROJECT REPORT **YEAR**: 2 of 2

Project Title: Improved late- and post-bloom sanitation of fire blight pathogen

PI: Ken Johnson
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Budget: **Year 1: $18,100 (apple) *** **Year 2: $18,462 (apple) ***

Other funding sources

Agency Name: USDA NIFA ORG
Amt. awarded: $495K to Johnson, Elkins, Granatstein and Smith 10/14 - 9/17
Notes: Objectives of this proposal are supplemental to objectives for the above project.

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	**2016-17**	**2017-18**
Salaries Faculty Res. Assist. 2 mo.	9,200	9384
Benefits OPE 58%	5,336	5443
Undergraduate labor (&OPE 12%)	1064	1085
Equipment		
Supplies	1,250	1275
Local Travel	250	255
Miscellaneous		
Plot Fees	1,000	1,020
Total	**$18,100**	**$18,462**

***Footnotes: Total Budget** **Year 1**: $36,200 **Year 2**: $36,924 (2% inflation)
50% by WTFRC Apple Crop Protection, 50% by FPC/WTFRC Pear.

OBJECTIVES

1) Evaluate EPA-registered materials for their ability to reduce epiphytic populations of the fire blight pathogen, *Erwinia amylovora*, on pear and apple trees, and to kill *E. amylovora* in laboratory-based dose-response experiments.

2) Evaluate the mineral material, alum ($KAl(SO_4)_2$), for fire blight control, to reduce epiphytic populations of *E. amylovora* on pear and apple trees, and to kill *E. amylovora* in laboratory-based dose-response experiments.

3) Evaluate and characterize the abilities of near-commercial preparations of *E. amylovora*-specific phage and protective amendments (sunscreens and carrier strains) for fire blight control, to reduce epiphytic populations of *E. amylovora* on pear and apple trees, and to kill *E. amylovora* in laboratory-based experiments dose-response experiments.

SIGNIFICANT FINDINGS

- In each of eight pear and apple trails conducted in 2016 and 2017, epiphytic populations of the fire blight pathogen on flowers increased after full bloom and reached a maximum <u>at one week after petal fall</u>.
- In general, materials that suppress infection also reduce pathogen inoculum on flowers. In 2016, under weather conditions highly conducive for fire blight, numerous materials were only fair at inoculum suppression including Bacillus-based biorationals (e.g., Serenade Opti), a three-quart rate of Cueva soluble copper, and experimental phage-based materials.
- Blossom Protect (*Aureobasidium pullulans*) provided very good fire blight control, but this material does not effectively suppress pathogen populations of flowers.
- Integrated programs that began with Blossom Protect and ended with a non-antibiotic chemical were more effective at suppressing pathogen populations than programs based on a single non-antibiotic material.
- Alum (1%, 8 lbs/100 gal) provided intermediate inoculum sanitation and excellent fire blight control.
- Among EPA-registered materials for non-antibiotic fire blight control, Previsto soluble copper stood out as an effective material for both infection suppression and inoculum sanitation.
- Late bloom (petal fall) treatments of lime sulfur (2 to 4 %) provided good inoculum sanitation, fire blight control and improved fruit finish.
- Acidifying oxytetracycline with buffer protect (pH 4.5) improved the level of inoculum sanitation and fire blight control from this antibiotic.

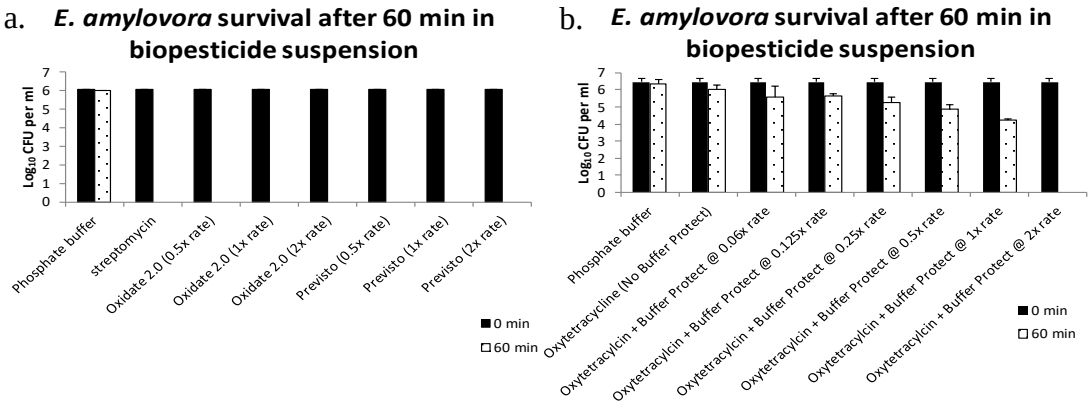
RESULTS & DISCUSSION

Obj. 1.a. Laboratory-based dose-response experiments to evaluate effect of EPA-registered materials on killing *E. amylovora* in vitro.

The purpose of this sub-objective was to develop laboratory-based assays to measure and compare the effects of fire blight-control materials on survival of *E. amylovora*. The assay exposed suspensions of pathogen cells (1×10^6 FU/ml) to a dose of a material for a period of time (e.g., 60 min). Pathogen cells were recovered from suspension by filtration, washed in phosphate buffer, then dilution plated on nutrient agar to determine survivorship relative to a non-treated control.

In conducting these assays, we obtained results with some materials that correlated positively with what we observe in the field and results with other materials that were contradictory to (not predictive of) what we see in the field. After numerous assays, we concluded that this approach is not a useful expenditure of time and effort. For example in **Fig. 1a**, labeled rates of streptomycin, Previsto and OxiDate 2.0 were highly effective at killing *E. amylovora* in the lab-based assay, but in field trials we observed that while strep and Previsto are effective at suppressing the pathogen on apple and pear flowers, OxiDate has only a slight effect these same floral populations. Another example shows that oxytetracycline was relatively poor at killing *E. amylovora* after a 60 min exposure in the laboratory suspension (**Fig. 1b**) even when buffered at a lower pH with citrate (Buffer Protect). In contrast, in the field, oxytetracycline by itself shows intermediate suppression of *E. amylovora* populations on flowers, which was significantly enhanced in the field by the addition of Buffer Protect (data below). Potential reasons for lack of correlation between lab assay and field performance of a material likely include the disparities in length of effective residuals in the different environments, rates of material uptake by bacterial cells, and potential interactions of a material with the host surface.

Fig. 1. Laboratory-assay results that were not fully consistent with field observations.



Obj. 1.b. Effect of EPA-registered materials on late- and post-bloom sanitation of fire blight pathogen on flowers in apple and pear orchards.

In contrast to laboratory assays, the measurement of epiphytic pathogen populations on apple and pear flowers during the bloom period was insightful for understanding the efficacy of the various materials for fire blight control. As in 2016 (see previous report), the highest epiphytic populations were usually measured on the water-treated control. Also as in 2016, the highest pathogen populations were observed in samples taken at 'petal fall + one week' (when compared to samples taken at full bloom or petal fall). This latter observation suggests that extending spray programs into petal fall could have beneficial effects on late bloom sanitation and infection suppression.

For the most part, measured epiphytic populations of *E. amylovora* correlated positively with incidence of infection but there were exceptions. The figures that follow (**Figs. 2-5**) depict effects of control materials on pathogen populations of flowers in four 2017 orchard trials (see previous report for 2016 data). Materials that suppressed final pathogens populations to less than 10^5 cfu/flower (100,000 cells/flower) provided excellent infection suppression. In this regard, antibiotics (streptomycin, kasugamycin, and oxytetracycline) and soluble coppers (Previsto 3 qt and Cueva 4 qt) showed most consistent suppression of the pathogen. Materials that did not cause a large reduction in pathogen populations included Bacillus-based materials (e.g., Serenade Opti), which also gave relatively poor disease control. Blossom Protect plus Buffer Protect is an example of a treatment that had only slight effects of epiphytic *E. amylovora* populations but was effective for disease control. The addition of a half rate of Buffer Protect to oxytetracycline improved the ability of this antibiotic to suppress floral pathogen populations.

Fig. 2. Effect of treatments applied to Bartlett pear trees to suppress fire blight on populations of *E. amylovora* strain 153N on flowers during the bloom period of April 2017. The 58-yr-old orchard was located near Corvallis, OR with each treatment applied to four replicate trees. Pathogen populations were determined by bulk sampling five flower clusters (~25 flowers) from each replicate tree with each sample washed in 25-ml of sterile phosphate buffer followed by dilution plating on to nutrient agar plus nalidixic acid. Owing to cold weather in early bloom, the pathogen was detected only in the 'petal fall plus one week' sample; results of 'full bloom' sample not shown. Panel A: Antibiotics and Serenade Opti with and without a 75 oz. rate of Buffer Protect. Panel B: biologicals and alum. Horizontal dashed line in Panel A indicates the detection limit of the assay. Data depict mean and standard error of each treatment program on each sampling date.

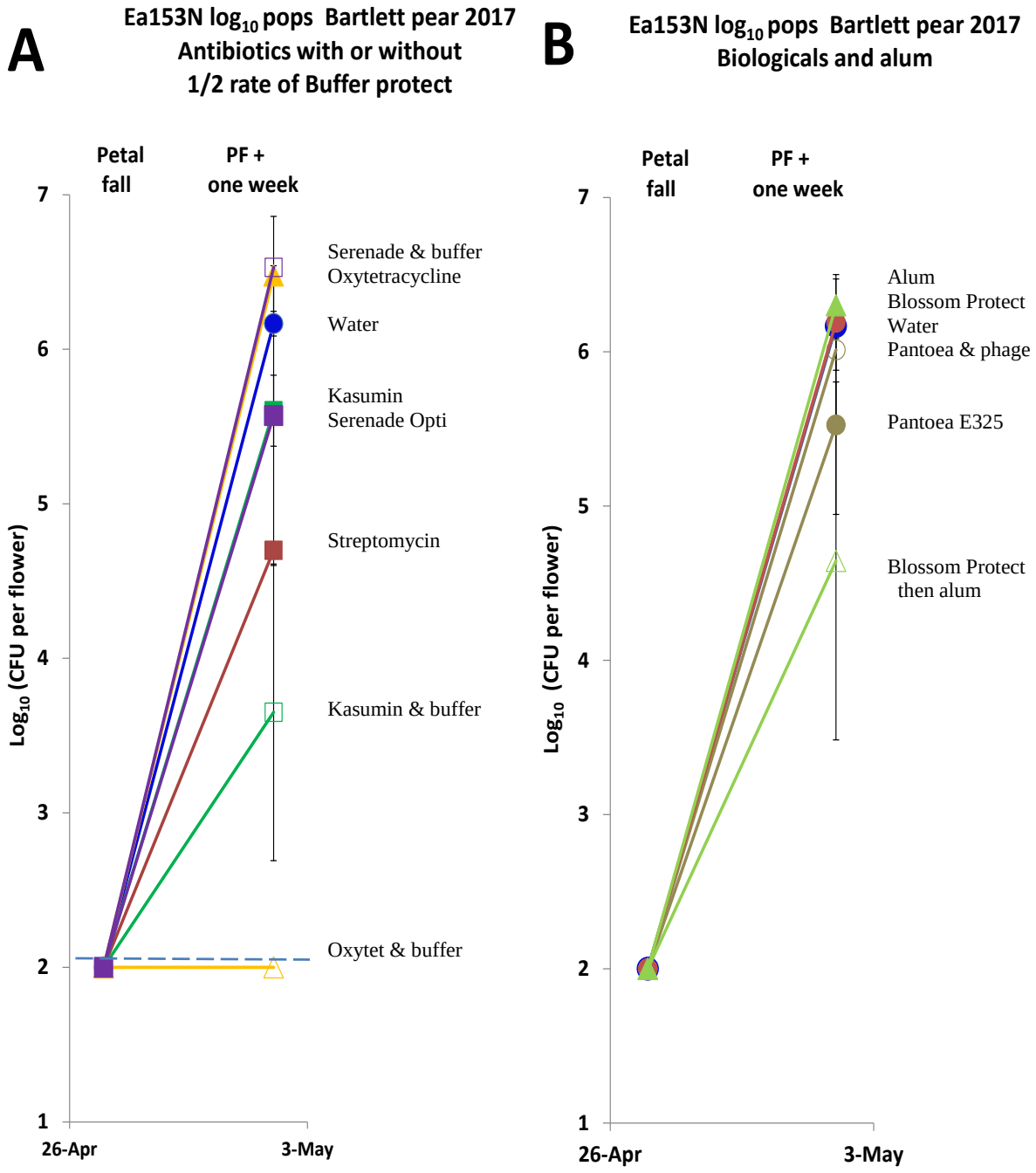


Fig. 3. Effect of treatments applied to Bartlett pear trees to suppress fire blight on populations of *E. amylovora* strain 153N on flowers during the bloom period of April 2017. The 17-yr-old orchard was located near Corvallis, OR with each treatment applied to four replicate trees. Pathogen populations were determined by bulk sampling five flower clusters (~25 flowers) from each replicate tree with each sample washed in 25-ml of sterile phosphate buffer followed by dilution plating on to nutrient agar plus nalidixic acid. Panel A: Integrated control programs. Panel B: streptomycin and selected soluble copper materials. Horizontal dashed line in Panel A indicates the detection limit of the assay. Data depict mean and standard error of each treatment program on each sampling date.

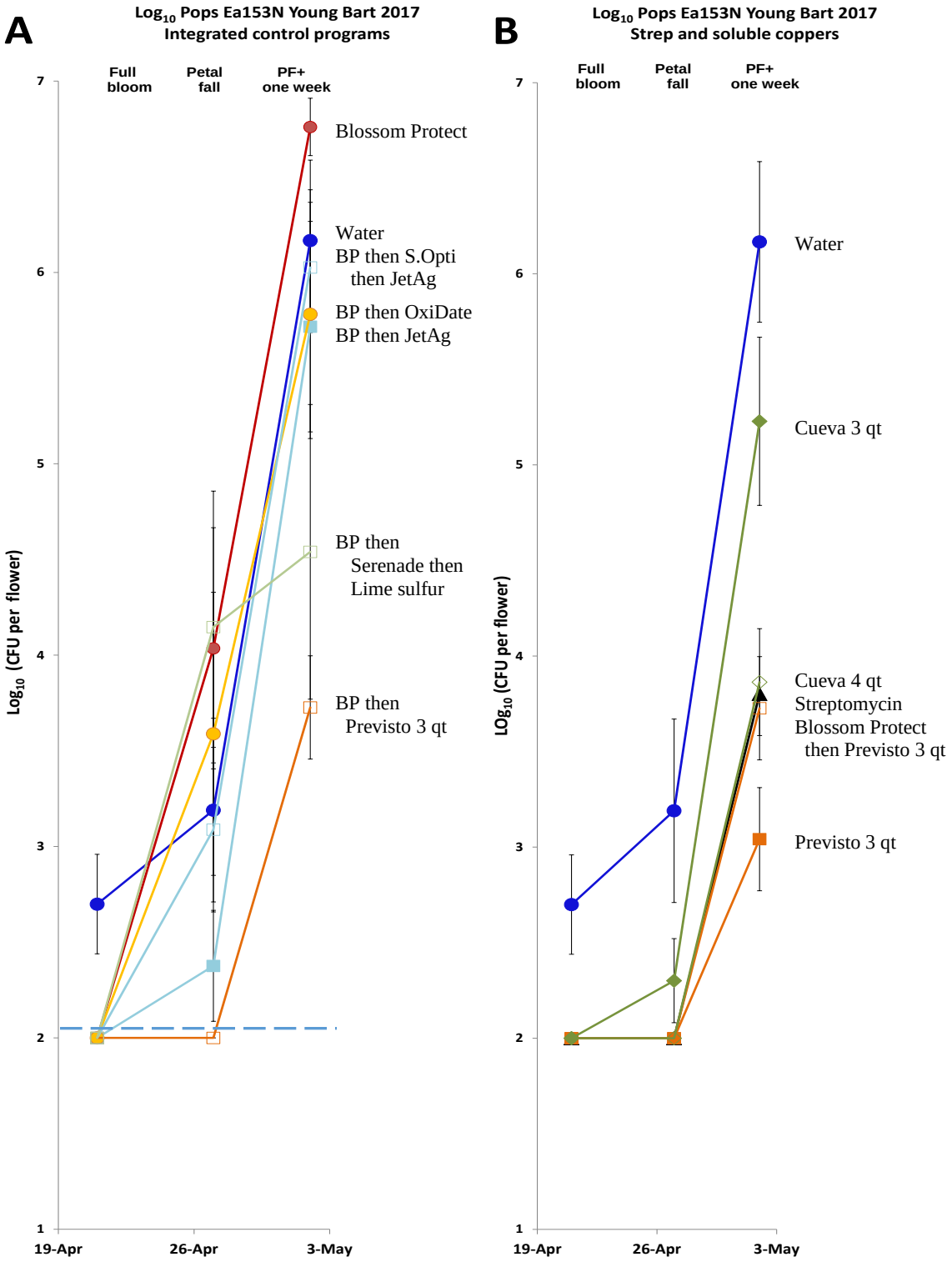


Fig. 4. Effect of treatments applied to Golden Delicious apple trees to suppress fire blight on populations of *E. amylovora* strain 153N on flowers during the bloom period of April and May 2017. The 37-yr-old orchard was located near Corvallis, OR with each treatment applied to four replicate trees. Pathogen populations were determined by bulk sampling five flower clusters (~25 flowers) from each replicate tree with each sample washed in 25-ml of sterile phosphate buffer followed by dilution plating onto nutrient agar amended with nalidixic acid. Panel A: FireLine (oxytetracycline) and Serenade Opti with and without a half label-rate of Buffer Protect. Panel B: Blossom Protect and Buffer Protect followed by alum, VP20, Previsto, or Serenade Opti then Rex Lime Sulfur; and solitary material treatments of Blossom Protect and Buffer Protect, alum, or VP20. Horizontal dashed line in Panel A indicates the detection limit of the assay. Data depict mean and standard error of each treatment program on each sampling date.

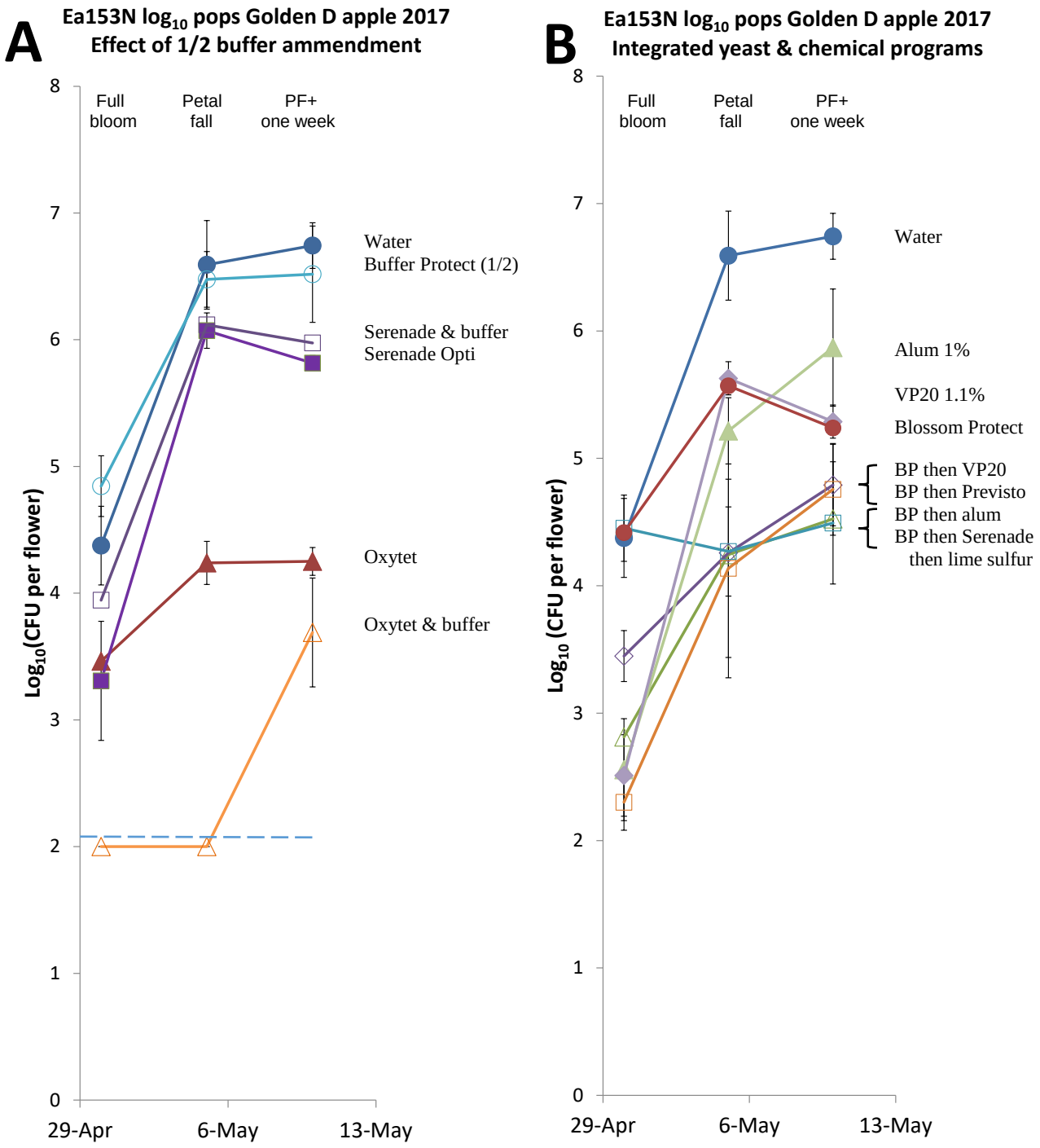
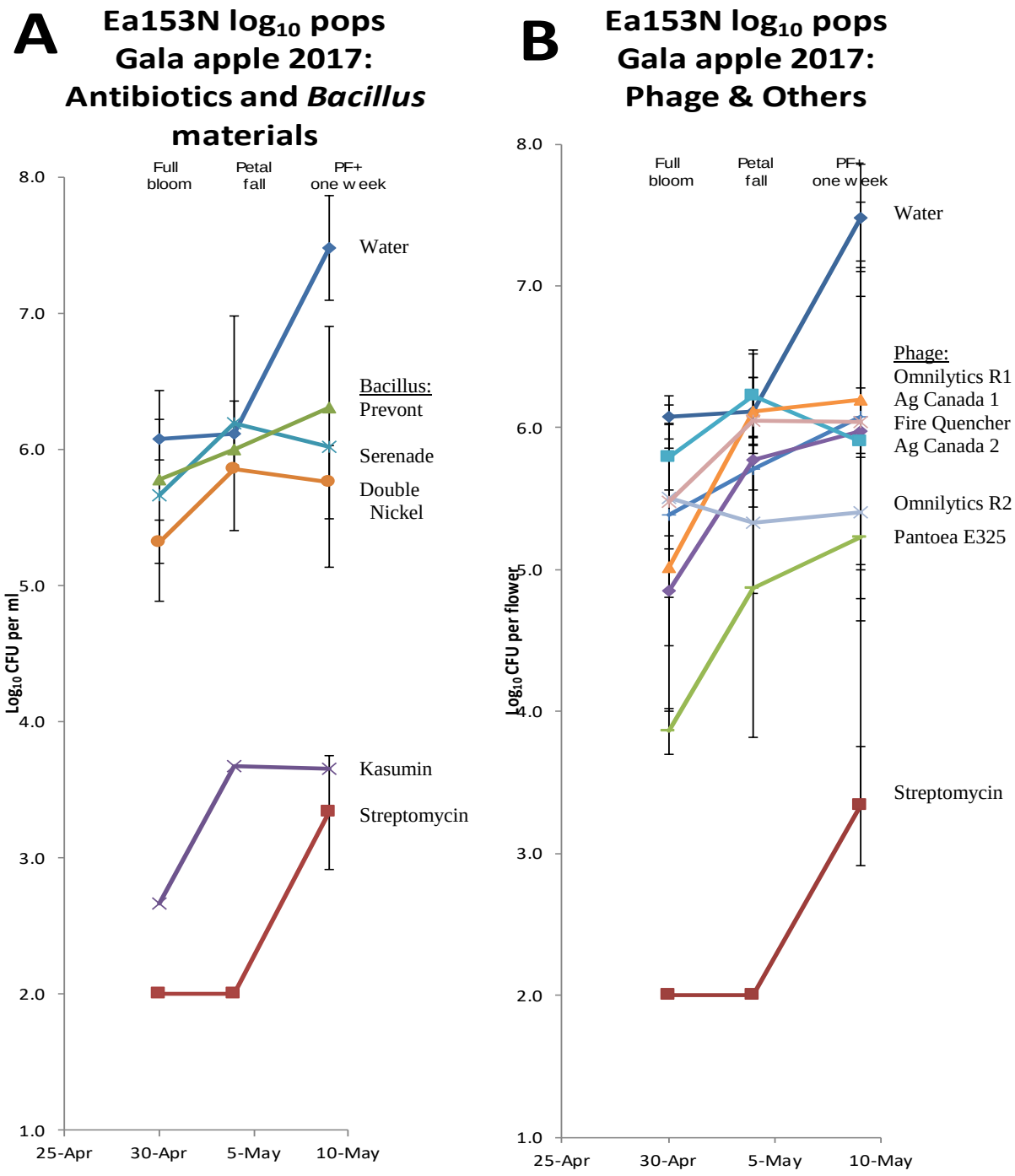


Fig. 5. Effect of treatments applied to Gala apple trees to suppress fire blight on populations of *E. amylovora* strain 153N on flowers during the bloom period (late-April to early-May 2017). The 17-yr-old orchard was located near Corvallis, OR with each treatment applied to four replicate trees. Pathogen populations were determined by bulk sampling five flower clusters (~25 flowers) from each replicate tree on each sample date with the sample washed in 25-ml of sterile phosphate buffer followed by dilution plating onto nutrient agar amended with nalidixic-acid (25 µg/L). Flowers were sampled on the days following the full bloom and petal fall sprays, and at 1-week after petal fall. Panel A: antibiotics and *Bacillus*-based materials; and Panel B: phage materials and *P. agglomerans* E325. Data depict mean and standard error of each treatment program on each sampling date.

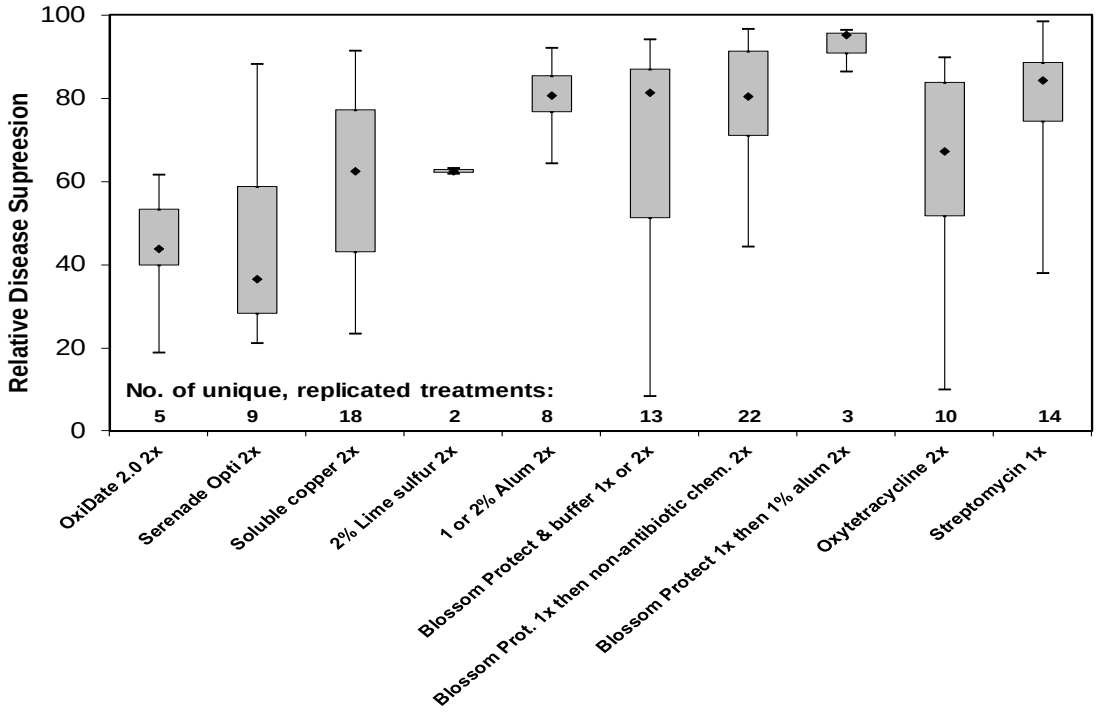


Obj. 1.c. Effect of EPA-registered materials on fire blight control in in apple and pear orchards.

Rather than show only 2017 data, a summary of important fire blight control treatments from 2013 to 2017 orchard trials is depicted below. The amount of fire blight in control trees of individual trials ranged from 7 infections per tree in Bartlett pear in 2017 to 673 infection per tree in Bartlett pear in 2014. Over all trials, the water-treated control averaged 147 infections per tree, which represented a mean of 26% of total flower clusters on the trees. As measured by the CougarBlight fire blight risk model, the conduciveness of the temperatures for epiphytic growth of *E. amylovora* varied by season with 2016 trials experiencing the most favorable conditions (extreme risk), and the 2017 season experiencing the least favorable (low risk in pear to moderate in apple) conditions. For other years, the conduciveness of temperatures for epiphytic growth was intermediate (moderate to high infection risk conditions). Primary conclusion is that non-antibiotic materials when applied as solitary treatments are less effective than antibiotics, but that integrated programs that begin with Blossom Protect (yeast) followed by a non-antibiotic chemical material can achieve a level of control on par with antiobiotics.

Fig. 6. Box and whiskers plot of relative fire blight suppression from 17 pathogen-inoculated pear and apple orchard trials conducted near Corvallis, Oregon from 2013 to 2017. Relative disease incidence was calculated for each treatment program by dividing mean number of infected flowers clusters observed on treated trees by the mean number of infected clusters on corresponding water-treated control. For each treatment, based on the trials in which it was present, the diamond is median relative disease suppression, the box is the range of the two quartiles of observations nearest the median, and the whiskers are the minimum and maximum observations. Each treatment consisted of one to three spray applications during the bloom period as indicated by the number preceding the 'x' in the axis label.

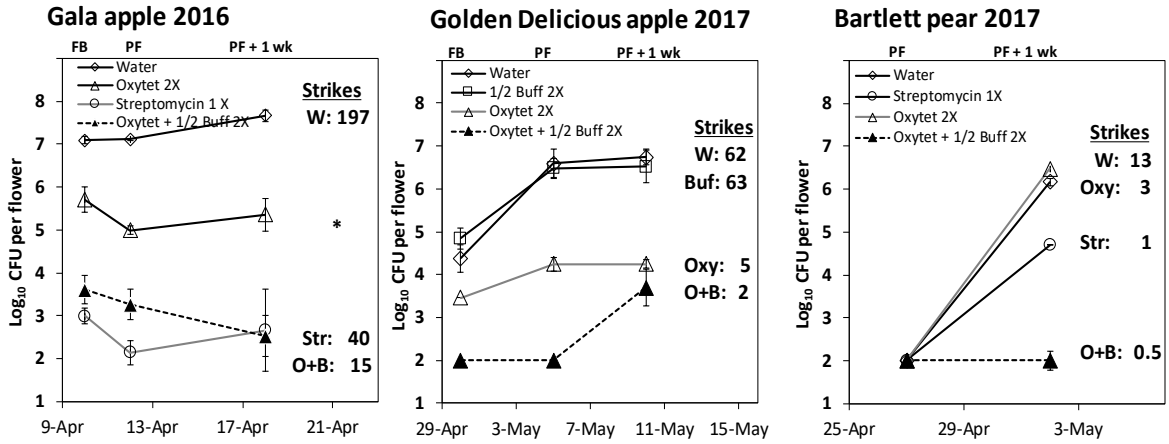
For the treatment 'Blossom Protect then non-antibiotic chemical', those chemicals were either Serenade Opti, a soluble copper (Previsto or Cueva), or OxiDate.



In the above chart (**Fig. 6**), fire blight was suppressed significantly ($P \leq 0.05$) by non-antibiotic or antibiotic treatments most of the time. The mean 'percent disease suppression relative to the control' (S_{rc}) for all evaluated non-antibiotic treatment programs was 65% $\pm$ (s.d.) 24 (n = 88). In contrast, for the antibiotic controls (streptomycin and oxytetracycline), mean S_{rc} for all treatments 72% $\pm$ (s.d.) 22 (n = 24). Box and whisker plots showed that specific NOP-approved, non-antibiotic materials (OxiDate, Serenade Opti, lime sulfur or the soluble coppers, Cueva and Previsto) tended to be only partially effective for fire blight suppression (median S_{rc}–values ranging from 35 to 62%) when sprayed as the only material in the program. For Blossom Protect and its companion buffer, a median level of control was 81%, which was intermediate to streptomycin and oxytetracycline (median S_{rc}-values of 84 and 65%, respectively). The experimental material, alum, and integrated programs that began with Blossom Protect followed by a NOP-approved non-antibiotic material also provided high levels of suppression with median S_{rc}-values of 81%. Integrated programs that consisted of Blossom Protect followed by another material showed less variability in suppression when compared to treatment programs comprised of a single material (**Fig. 6**).

A final consistent and potentially significant result from 2016-17 trials was enhanced late-bloom sanitation and disease suppression with pH-buffered oxytetracycline (i.e., the addition of a half label rate of Blossom Protect to the oxytet suspension) (**Fig. 7**). This result requires additional research to determine the optimal rate of a buffer amendment.

Figure 7. Effect of pH-buffering of oxytetracycline on fire blight-pathogen populations on apple and pear flowers sampled from orchards near Corvallis, OR. Each treatment was applied to four replicate trees. Y-axis is log scale: a value of '2.0' is 100 pathogen cells/flower (the detection limit) and a value of '6.0' is one million cells per flower.



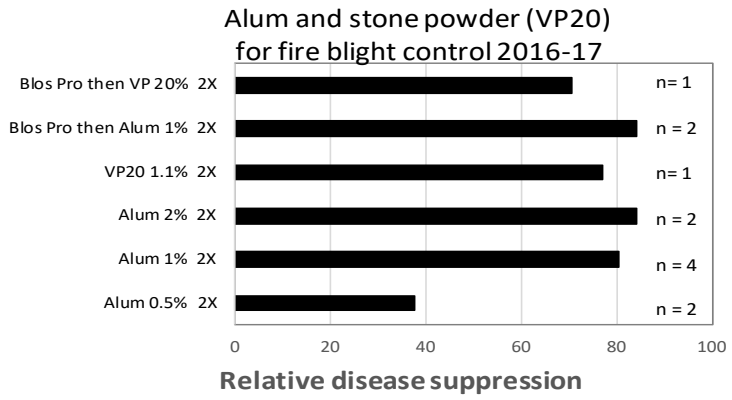
Obj. 1.d. Effect of EPA-registered materials on fruit russeting.

Because of Corvallis' wet spring environment, fruit russeting is typically moderate to severe regardless of treatment, especially for pears. For the last few seasons, we have collected fruit russeting data from selected fire blight control treatments. The results generally confirm the materials that have an enhanced risk of inducing fruit russeting (e.g., soluble coppers, and to a lesser extent, Blossom Protect). Most other materials (alum, Bacillus-based materials, biologicals, oxidizing agents) have not shown levels of russeting that are different from the water treated control. For two seasons, lime sulfur has shown a consistent reduction in fruit russeting, which we attribute to suppression of natural yeast populations (including *Aureobasidium pullulans*) (data available by request).

Obj. 2. Effect of mineral material, alum on late-bloom sanitation of fire blight pathogen on flowers and on fire blight control in apple and pear orchards.

Alum ($KAl(SO_4)_2$) provided only an intermediate level of pathogen-population suppression (**Figs. 2B and 4B**) but an outstanding level of disease control (**Fig. 6**). Alum's best fit in organic spray programs would be as a full bloom to petal fall treatment(s) after Blossom Protect. Effective rate is ~1% (8 lbs/100 gal). Alum is not currently approved for use in organic agriculture but a preliminary (and positive) OMRI-assessment was completed to utilize it as an manure amendment: http://www.ams.usda.gov/sites/default/files/media/Aluminum%20Sulfate%20Petition.pdf , and http://www.ams.usda.gov/sites/default/files/media/Aluminum%20Sulfate%20TR.pdf .
The alum containing stone powder we evaluated is an organic crop protection product sold in Europe under the name Mycosin (BIOFA AG, Münsingen, Germany, http://www.biofa-profi.de/en/about-us.html). We brought the material into the U.S. with help from Michael Braverman of IR-4 (Rutgers), who it was gave it the code name, 'VP20'.

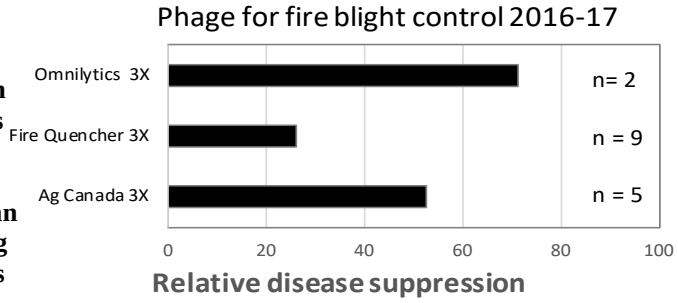
Fig. 8. Relative fire blight suppression from pathogen-inoculated pear and apple orchard trials conducted near Corvallis, Oregon from 2016 to 2017. Relative disease incidence was calculated for each treatment program by dividing mean number of infected flowers clusters observed on treated trees by the mean number of infected clusters on corresponding water-treated control. 'n' is number of times the treatment was trialed.



Obj. 3. Effect of *E. amylovora*-specific phage on late-bloom sanitation of fire blight pathogen on flowers and on fire blight control in apple and pear orchards.

Phage are viruses that attack bacteria, with which several groups are attempting to develop commercial products for fire blight management. In our hands, phage treatments have provided only intermediate levels of control (even after three applications) (**Fig. 8**), and generally poor levels of late-bloom sanitation (**Fig. 5B**). Disease suppression from phage treatments was better in 2017 than 2016, perhaps because we changed the protocol to apply the first treatment <u>within an hour</u> of the pathogen inoculation (how does a grower do this?). The primary drawback of phage is that they are very short-lived (µV sensitive) if their host (the fire blight pathogen) is not present at the time of treatment**.** Every season the formulations of evaluated phage materials have been modified from the previous season. Therefore, there is still a chance that one of the groups developing a product will hit on a formulation with improved efficacy.

Fig. 8. Relative fire blight suppression from pathogen-inoculated pear and apple orchard trials conducted near Corvallis, Oregon from 2016 to 2017. Relative disease incidence was calculated for each treatment program by dividing mean number of infected flowers clusters observed on treated trees by the mean number of infected clusters on corresponding water-treated control. 'n' is number of times the treatment was trialed.



EXECUTIVE SUMMARY

Project Title: Improved late- and post-bloom sanitation of fire blight pathogen
Investigator: Ken Johnson, Oregon State University

SIGNIFICANT FINDINGS

- In each of eight pear and apple trails conducted in 2016 and 2017, epiphytic populations of the fire blight pathogen on flowers increased after full bloom and reached a maximum at one week after petal fall.
- In general, materials that suppress infection also reduce pathogen inoculum on flowers. In 2016, under weather conditions highly conducive for fire blight, numerous materials were only fair at inoculum suppression including Bacillus-based biorationals (e.g., Serenade Opti), a three-quart rate of Cueva soluble copper, and experimental phage-based materials.
- Blossom Protect (*Aureobasidium pullulans*) provided very good fire blight control, but this material does not effectively suppress pathogen populations of flowers.
- Integrated programs that began with Blossom Protect and ended with a non-antibiotic chemical were more effective at suppressing pathogen populations than programs based on a single non-antibiotic material.
- Alum (1%, 8 lbs/100 gal) provided intermediate inoculum sanitation and excellent fire blight control.
- Among EPA-registered materials for non-antibiotic fire blight control, Previsto soluble copper stood out as an effective material for both infection suppression and inoculum sanitation.
- Late bloom (petal fall) treatments of lime sulfur (2 to 4 %) provided good inoculum sanitation, fire blight control and improved fruit finish.
- Acidifying oxytetracycline with buffer protect (pH 4.5) improved the level of inoculum sanitation and fire blight control from this antibiotic.

Industry implications: In the non-antibiotic era that began in 2015, the materials now used for organic fire blight control possess diverse modes of action, which are not completely understood. In particular, while it is possible to assign a ranking to material effectiveness for infection suppression during primary bloom, little has been known about how well these materials reduce (kill) floral pathogen populations that can carry over into the post-bloom period. The reason this distinction is significant relates to the fact that PNW apple and pear orchards frequently escape primary bloom infection, but develop fire blight in late and secondary flowers, and in rapidly growing shoots in warmer, unsettled weather of late spring. The late- and post-bloom period is also the period of high sensitivity to chemical-induced fruit russeting, which restricts choice of materials available for late- and post-bloom sanitation.

Results of this project showed that a) antibiotics are better at for inoculum sanitation than non-antibiotic materials, b) integrated non-antibiotic programs that begin with Blossom Protect followed by a non-antibiotic chemical is a valid strategy for fire blight control and offers an intermediate level of inoculum sanitation, and c) acidifying spray suspensions by buffering can potentially improve inoculum sanitation; this result needs further research. The material, alum (/organic stone powder) should be considered for commercial implementation. Materials based on bacteriophages (viruses that infect and kill the fire blight pathogen) were not particularly effective at reducing pathogen inoculum and likely are not commercially useful at this time.

FINAL PROJECT REPORT **Second year report**

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.

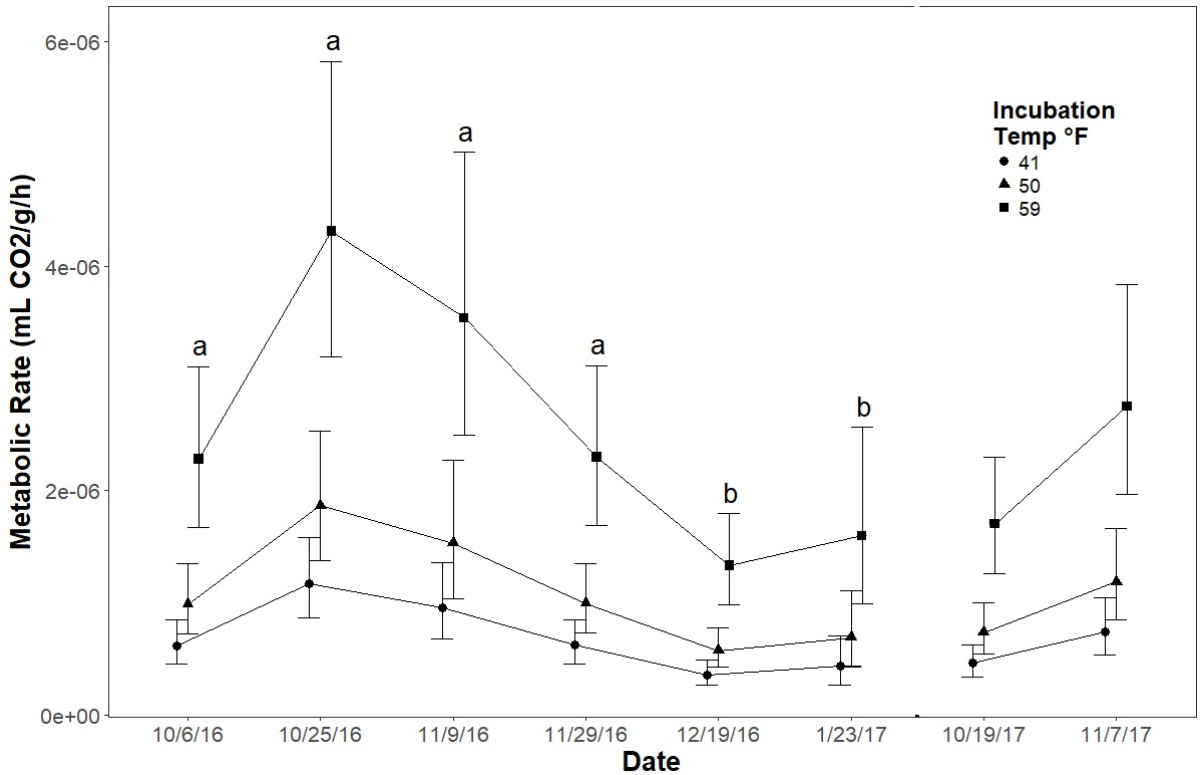

Figure 1. Mean metabolic rates including upper and lower confidence intervals, measured at 41, 50, and 59°F of overwintering adult brown-marmorated stink bugs (*Halyomorpha halys*). Metabolic rates of stink bugs, from the naturally-acclimated outdoor population, significantly differed across months in 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.

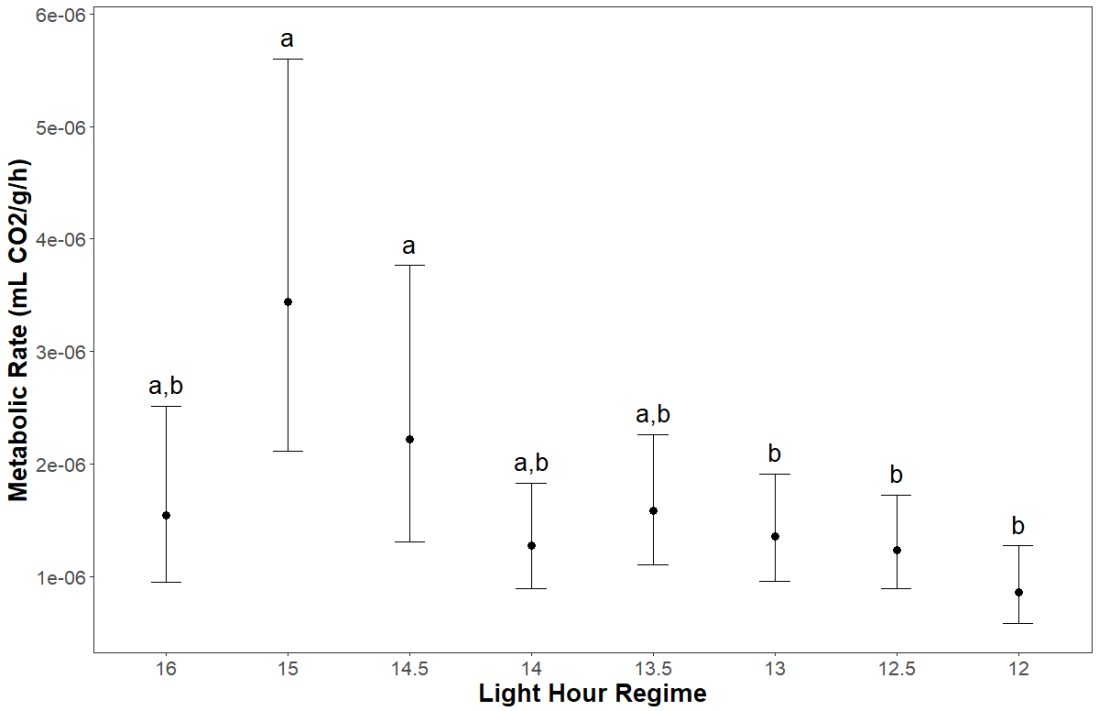

Figure 2. Mean metabolic rate including upper and lower confidence intervals, measured at 59°F of adult brown-marmorated stink bugs (*Halyomorpha halys*). Metabolic rates differed significantly across light hour availability ($F_{(7)} = 4.35$, $P < 0.001$). Metabolic rate response to incubation temperature 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.

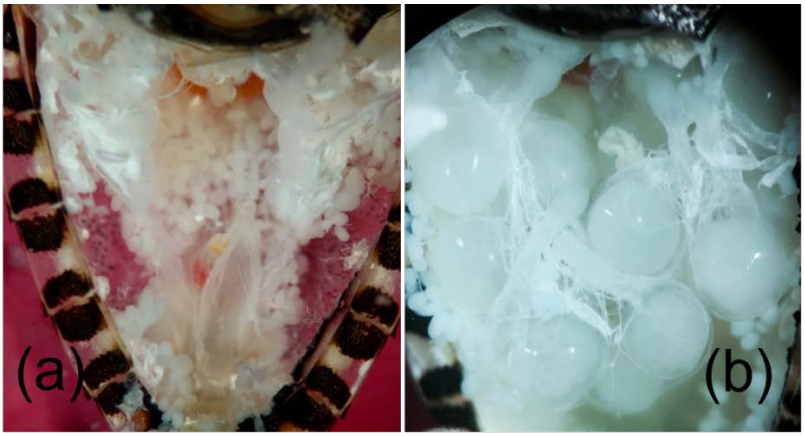

Figure 4. Female BMSB dissections demonstrating (a) lack of mature oocytes indicating a non-reproductive female (reproductive suppression during diapause) and (b) presence of mature oocytes indicating a reproductive female.

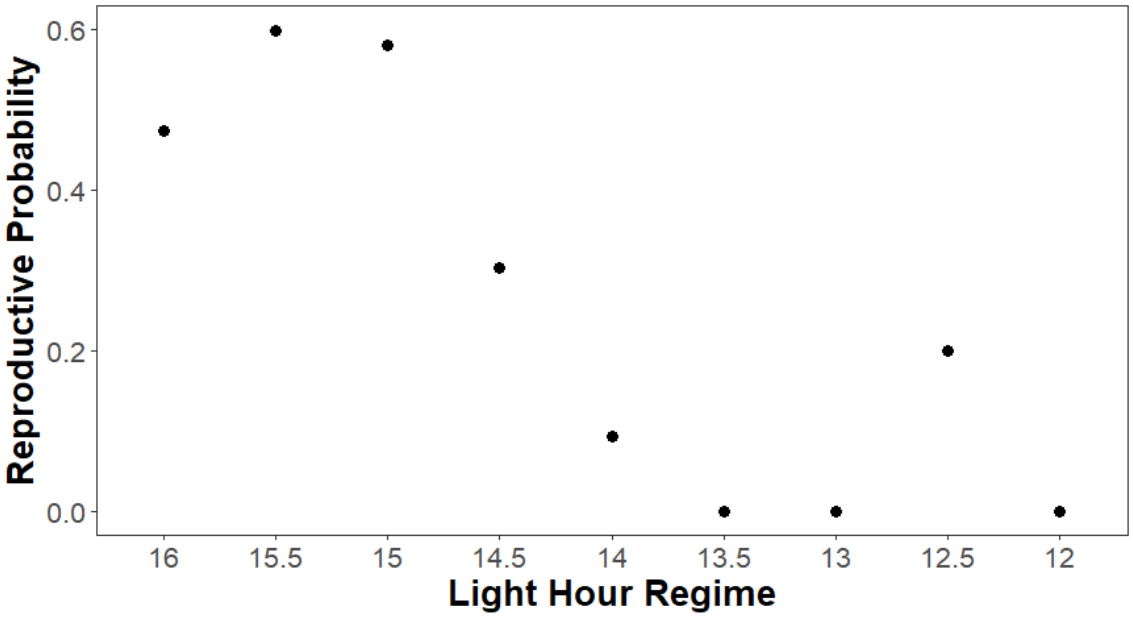

Figure 3. Probability of female Brown-marmorated stink bugs (*Halyommorpha halys*) being in a reproductive state at different day-lengths. The probability of reproductive BMSB differed across LD regime ($X^2_{(8)} = 15.53$, P-value = 0.049).

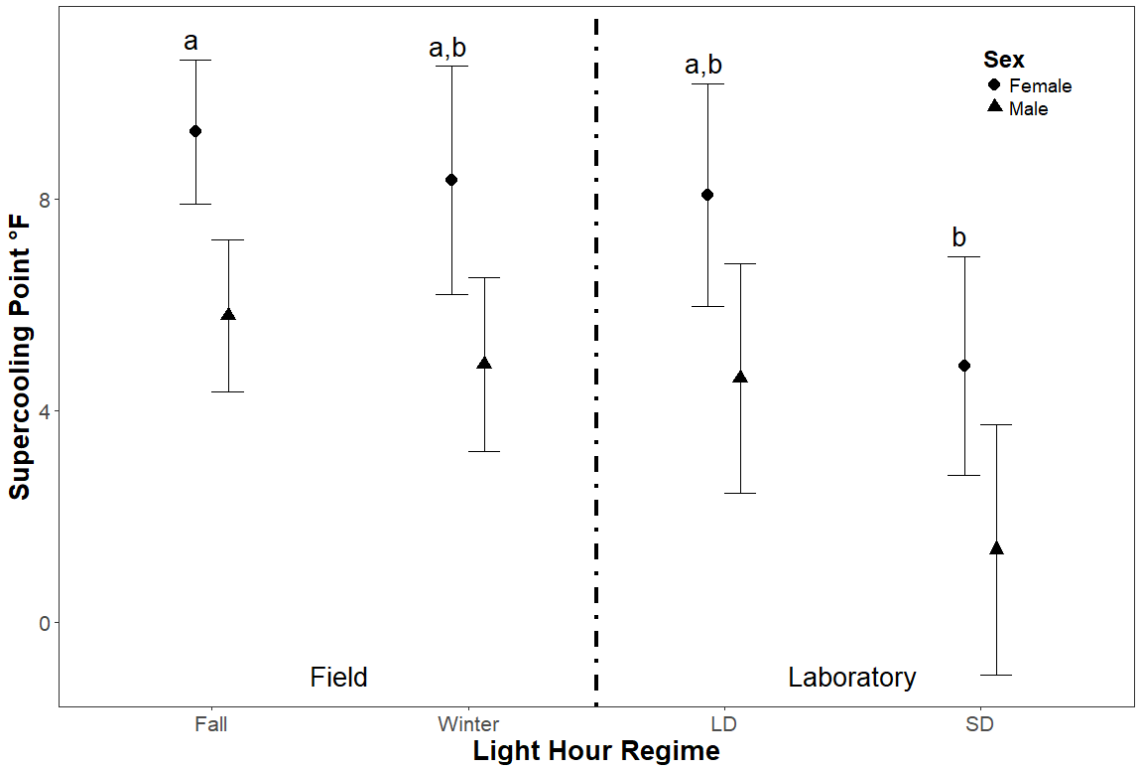

Figure. 5 Mean supercooling points of adult brown-marmorated stink bugs (*Halyomorpha halys*) including upper and lower confidence intervals, that differed significantly across day length (LD = long day, SD = short day), sex and mass ($F_{(3)} = 4.55$, $P = 0.0048$; $F_{(1)} = 9.49$, $P = 0.0026$; $F_{(2)}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

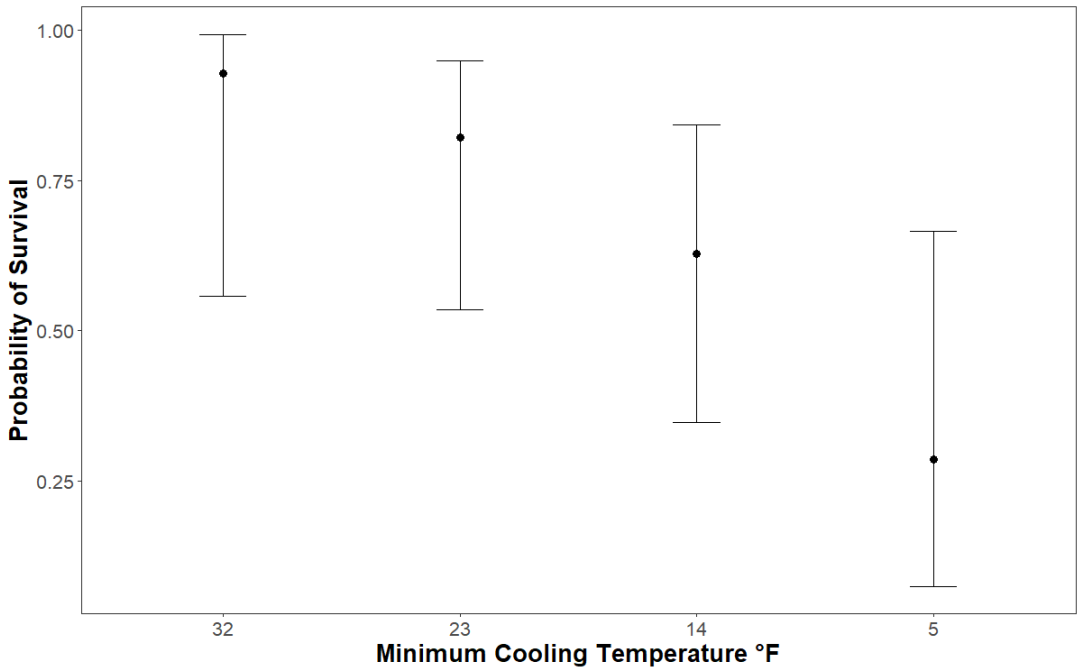

Fig. 6 Naturally cold-acclimated adult Brown-marmorated stink bugs (*Halyomorpha halys*) exposed to a series of minimum cooling temperatures, show significant differences in the proportion survived across temperature treatments ($X^2_{(3)} = 9.02$, p= 0.028), sex ($X^2_{(1)} = 6.22$, P-value = 0.012), and mass ($X^2_{(1)} = 5.09$, P-value = 0.024).

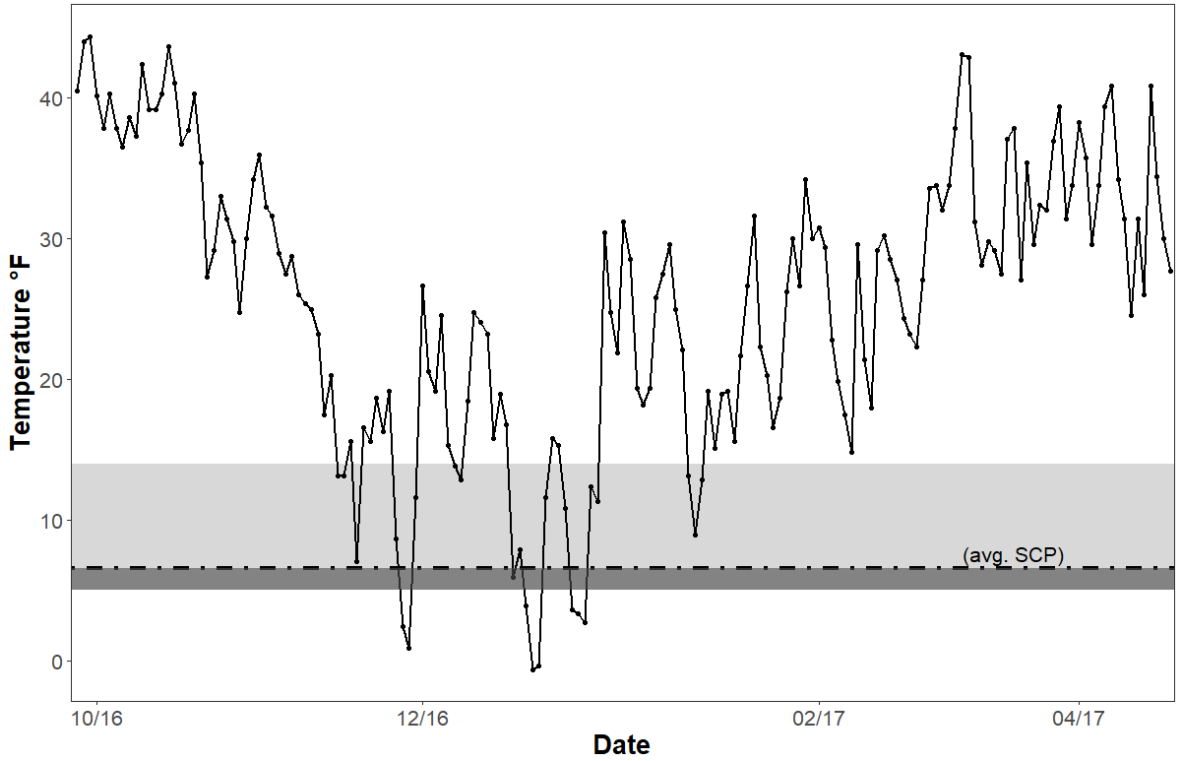

Figure 7. Daily minimum temperature (°F) recorded at an outdoor field enclosure in Yakima, WA from 10/28/16 to 4/15/17, with an observed minimum temperature of -0.65 °F on 1/6/17. The dashed line indicates the observed mean supercooling point of 6.6°F for BMSB, and the shaded area indicates the temperature 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.

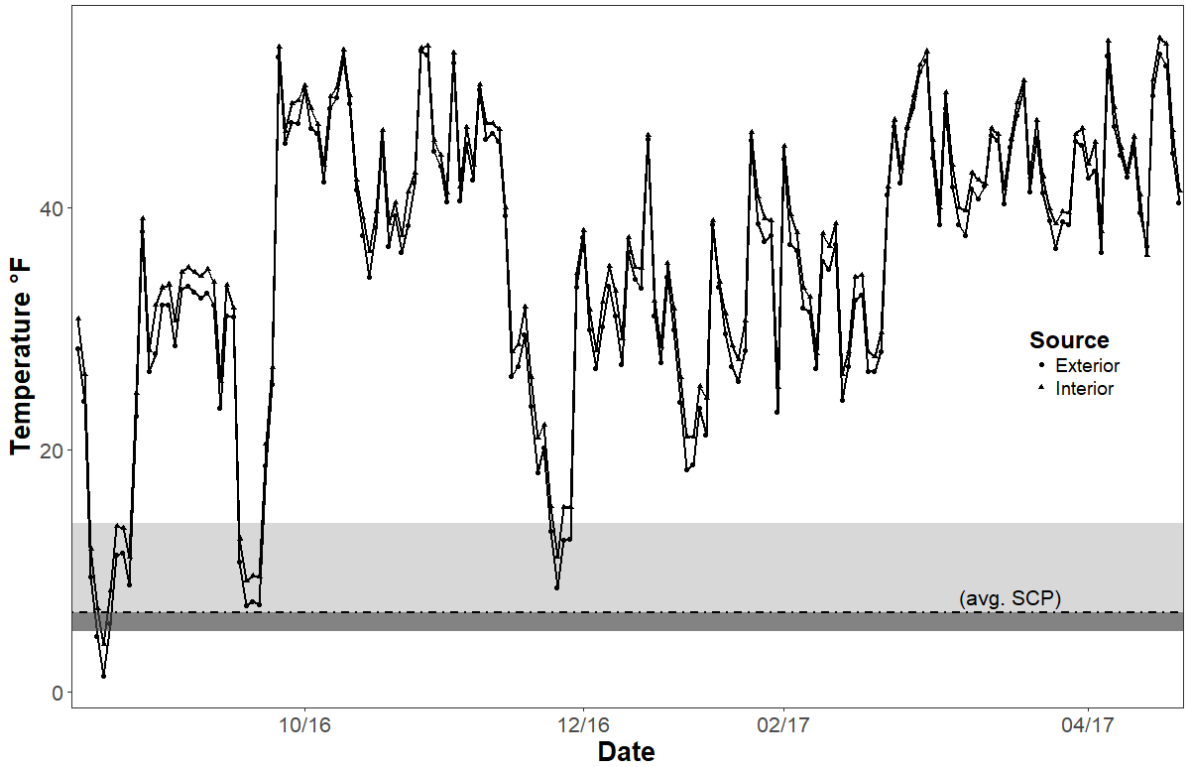

Fig. 8 Daily minimum temperature observed in Walla Walla, WA, at a naturally-selected overwintering site of Brown-marmorated stink bug (*Halyomorpha halys*), from 10/27/16 to 4/15/17. BMSB selected the tight spaces beneath cedar-shingle siding as refuge, with observed minimum temperatures from the interior 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. We used metabolic suppression and cessation of egg development as indicators of diapause metabolic rates of field-maintained BMSB were reduced 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 is this 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 to always 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 cold tolerance well 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 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 naturally-selected 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. Full mortality of BMSB was observed in the population maintained outdoors in Yakima, and 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.

Our research further emphasizes the importance of human structures and the potential role they play as a thermal buffer, preventing BMSB from encountering naturally occurring lethal temperatures. Individuals within this overwintering population who survive the adverse conditions of the winter months will be the source population that goes on to produce the next generation of BMSB throughout the upcoming growing season. These variables only increase the potential for BMSB to persist within this region, and increase management issues. If management of these structured were mitigated and the ability for BMSB to enter structures limited, our research strongly supports the notion that BMSB will not gain the ability to naturally support viable populations within eastern Washington.

FINAL PROJECT REPORT

WTFRC Project Number: 3043-3815

Project Title: Dynamics of woolly apple aphids on organic and conventional orchards

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Cooperators: Apple growers throughout Washington State

Total Project Request: **Year 1:** $56,279 **Year 2:** $57,669

Other funding sources

Agency Name: Washington State Department of Agriculture, Specialty Crop Block Grant Program

Amount Awarded: $194,910

Notes: This award expanded our project to include an analysis of how apple growers make management decisions for woolly apple aphid, other pests, and soils using in-depth interviews.

WTFRC Collaborative expenses: None

Item	2016	2017
Salaries[1]	$27,099	$28,183
Benefits[2]	$2,453	$2,552
Wages[3]	$11,133	$11,322
Benefits[4]	$594	$612
Supplies[5]	$7,800	$7,800
Travel[6]	$6,000	$6,000
Total	$56,279	$57,669

Footnotes:

[1] Project Assistant (1.0 FTE for 9 months)
[2] Project Assistant (9.055%)
[3] Summer Wages: Time-slip employee ($10 hr×40 hrs/wk×16 wks/yr); Project Assistant (1.0 FTE for 3 months)
[4] Time Slip (2.1%), Project Assistant (9.7%)
[5] Soil and leaf testing ($75/orchard for soil and $50 for leaf nitrogen×24 orchards = $3000/yr); Aphid and natural enemy and canker sampling supplies ($250/orchard ×24 orchards = $6,000/yr)
[6] Rental vehicle + gasoline + mileage + per diem + travel to research review ($6,000/yr)

Objectives:
(1) Sample populations of woolly apple aphids and natural enemies in organic and conventional apple orchards.
(2) Collect information on soil quality, plant nitrogen content, and perennial canker on these same orchards.
(3) Analyze linkages between soil quality, plant nitrogen content, pesticide-use intensity, natural enemies, and WAA populations in organic and conventional orchards.

Significant findings

- Woolly apple aphid counts in organic and conventional orchards tended to be similar on average
- Soil quality factors had no clear correlation to woolly apple aphid counts.
- Soil texture, however, was found to be associated with variation in woolly apple aphid density in orchards. In particular, densities were lower in sandier soils, perhaps because sandy soil is more difficult for woolly apple aphids to move through (aphids feeding on roots can migrate up to recolonize canopies).
- Leaf nitrogen had no clear correlation to woolly apple aphid counts.
- Perennial cankers were infested with woolly apple aphids more often than other possible feeding sites (such as burr knots), but incidence of cankers did not correlate to woolly apple aphid counts across orchards. However, this observation suggests that woolly apple aphids do prefer to feed at canker sites, which is perhaps why they have assumed to be a pathogen vector.
- Relationships between woolly apple aphid counts and green lacewings, *A. mali* wasps, and earwigs were difficult to interpret likely due to the limited resolution of our sampling strategy (only 3-5 data points per orchard per year). However, data suggest that predators (including earwigs) do play a critical role in suppressing aphid populations.
- Seasonal patterns of woolly apple aphid abundance appeared to be strongly related to temperature, with the hottest period of the summer always corresponding to crashes in woolly apple aphid populations. Insecticide applications during the hottest period of the summer are thus likely to be wasteful.
- In a greenhouse study, sandier soil reduced movement of woolly apple aphids down to roots, as did wood chip and paper slurry mulches, but migration was not completely blocked.
- In a field study, sticky bands designed to completely block woolly apple aphid movement up and down apple tree trunks did not significantly impact aboveground population dynamics. Aphids migrating up from roots were blocked, but this did not result in fewer aerial colonies.
- We expanded our study to include an investigation of the role of earwigs in woolly apple aphid control. Earwigs were released in sections of an experimental orchard. Compared to these sections, control areas with no earwigs added averaged about 400% greater woolly apple aphids at peak seasonal density, suggesting that earwigs are valuable predators of woolly apple aphids.
- The Fuji variety appeared especially susceptible to woolly apple aphid infestation in mixed plantings of Fujis, Galas, Goldens, and Jonagolds at WSU Sunrise Research Orchard.

Results and discussion

Soil quality and texture

Against expectations, no soil quality measurements differed significantly between organically and conventionally managed orchards, and neither did overall soil quality when all measurements were summarized into a normalized soil quality score from zero to one (one being the best). Though organic orchards are expected to have higher soil quality, our results are not consistent with this prediction. In our orchards, these findings might be explained by greater similarity in commercial organic vs. conventional management than in certain relatively controlled scientific studies.

There was no clear relationship between soil quality and woolly apple aphid abundance in either year of study (Figure 1). However, three of the orchards had especially high sand content in their soil (>60%) and these orchards also had low woolly apple aphid counts in both years of study (Figure 2). The low woolly apple aphid counts in these sandy orchards may have occurred because previous studies suggest that woolly apple aphids cannot easily move through sandy soil, thus preventing recolonization of canopies from root-feeding aphids.

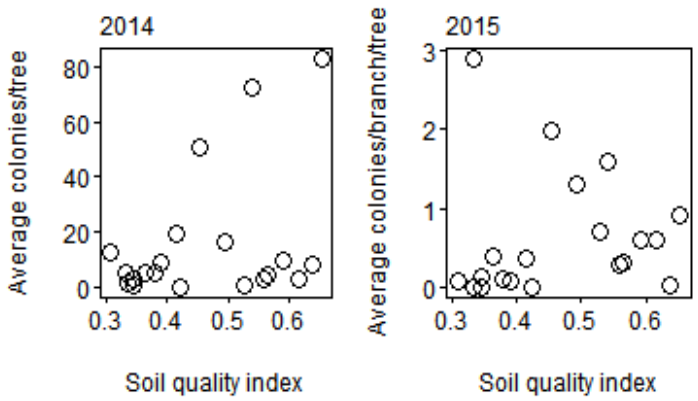

Figure 1. Woolly apple aphids and soil quality. Woolly apple aphid colonies were counted on entire trees (2014) or on each of 10 approx. 1' long branches on 16 trees per orchard (2015) on each of 3 to 5 visits throughout the season. Counts averaged to one number per orchard per year. The soil quality index is a score from 0-1 (0 = worst, 1 = best score) representing overall soil quality.

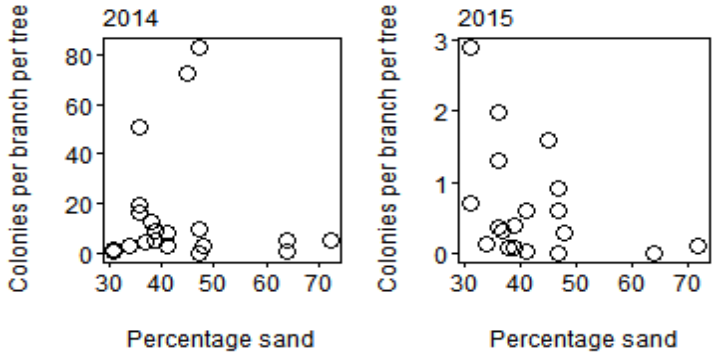

Figure 2. Woolly apple aphids and soil texture. Woolly apple aphids were counted as in Figure 1. Soil sand content was determined for each orchard by SoilTest Farm Consultants (Moses Lake, WA)

To follow-up on this, a greenhouse experiment was conducted to test a sandy potting mix, wood chip mulch, and a paper slurry mulch for blocking woolly apple aphid access to roots. Sandy soil and both mulches reduced woolly apple aphid infestation of roots (Figure 3). Similarly, sandy soil may reduce woolly apple aphid risk. ***Therefore, use of mulches on an orchard could be expected to reduce woolly apple aphid infestations***. This experiment was conducted in a greenhouse on small trees, and thus we should be careful with the inference from these results. It remains to be seen whether these results could be replicated under more realistic field conditions – this is potentially an area of future study that would be beneficial for improving management of woolly apple aphid.

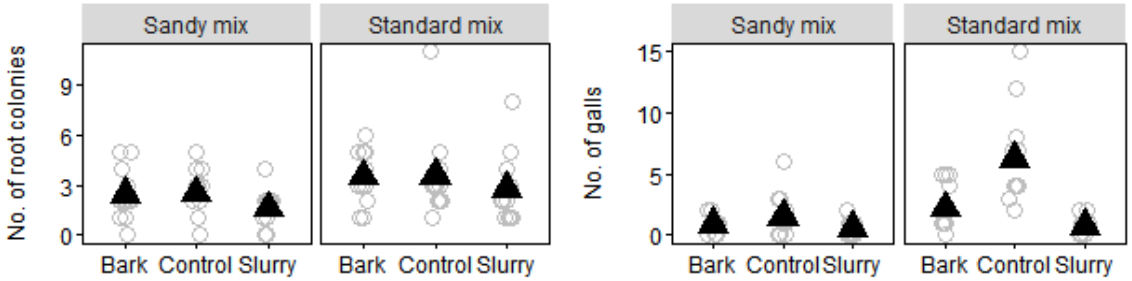

Figure 3. Soil and mulch greenhouse experiment. Woolly apple aphids were introduced to canopies of potted trees with different soil and mulch combinations. After two months, trees were pulled for root inspection and colonies and galls were counted. Circles represent counts on individual trees and triangles are the means for each group. The standard soil mix was equal parts perlite, vermiculite, and peat, while the sandy soil mix was 60% sand and 40% standard mix. The bark mulch was a 10 cm layer of chipped apple trees and the slurry is paper pulp poured wet and allowed to dry into resemblance of egg carton material.

Effect of blocking upward woolly apple aphid migration on aerial population dynamics in the field
To complement our mulch study, in 2017 we tested the hypothesis that upward movement of woolly apple aphids from roots to aerial parts of the tree significantly boosts aerial population abundance. In our experiments, even though thousands of migrating aphids were blocked from moving up tree trunks (data not shown), the trees with tanglefoot blocks did not have significantly lower aerial colony counts at any time during the season compared to the 'open' trees (Figure 4).

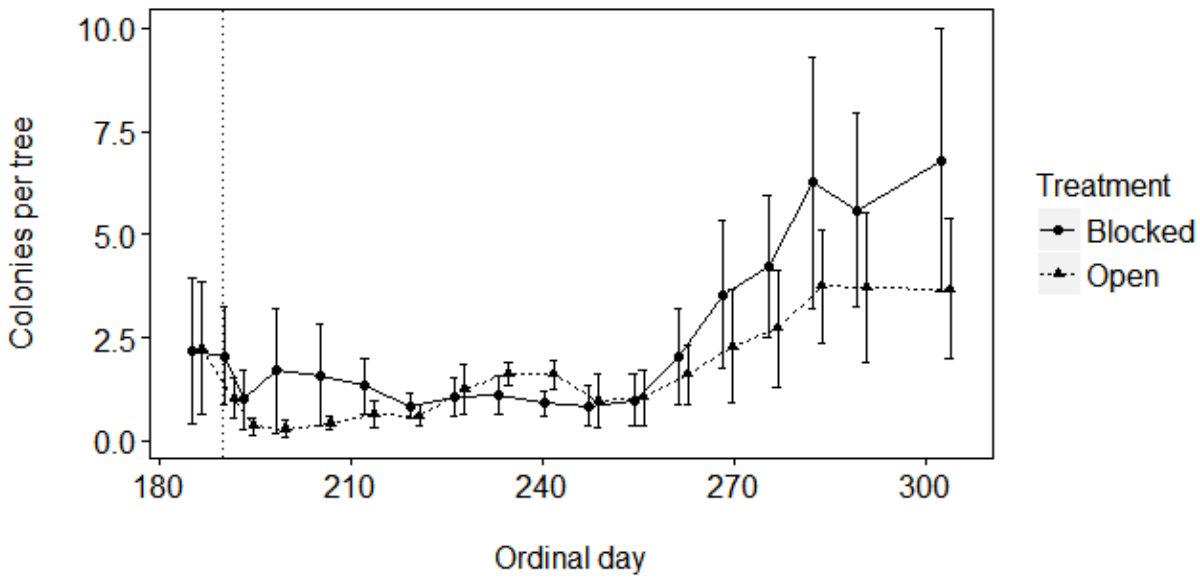

Figure 4. Woolly apple aphid colony counts on 'blocked' trees with tanglefoot bands and control 'open' trees. At Washington State University Sunrise Research Orchard, 12 sections of 12 trees were selected for study. Half of the sections received tanglefoot bands at the base of their trunk. The border trees in these sections were separated from neighbor trees by pruning and the addition of tanglefoot bands to trellis wires. The other 6 sections received no manipulation. All sections were monitored once a week from July until October. The total number of woolly apple aphid colonies found on the west side of each tree was counted. Each point shows the average number of aphid colonies per tree of the 6 sections, and error bars show 95% confidence intervals of the mean of aphid colonies per tree within each treatment. The vertical dotted line shows when the sticky tanglefoot bands were applied.

Our results do not support the hypothesis that blocking upward woolly apple aphid movement will ease aerial outbreaks. Apparently, existing aphid colonies in the canopy are a more important source of new aphid colonies in this situation. Because woolly apple aphids can overwinter aboveground, and are unlikely to be completely locally eradicated by pesticides or natural enemies, it seems unlikely that cutting off woolly apple aphid movement in and out of the soil will be an effective management tactic for growers. However, reduction of populations in the soil could still provide other benefits, despite the need for management of aerial colonies as well.

Plant nitrogen content

There was no clear relationship between leaf nitrogen and woolly apple aphid abundance in either year of our study (Figure 5). It was expected that new growth flushes, which can be spurred in part from nitrogen fertilization, would be associated with increased woolly apple aphid populations. Nitrogen can be a limiting factor for aphid growth, and woolly apple aphids often are found on new growth of apple trees. We did not observe this, however, perhaps because of the low resolution of sampling (3-5 visits throughout the whole season) or because leaf nitrogen is not a reliable proxy for phloem nitrogen, which is where aphids feed from.

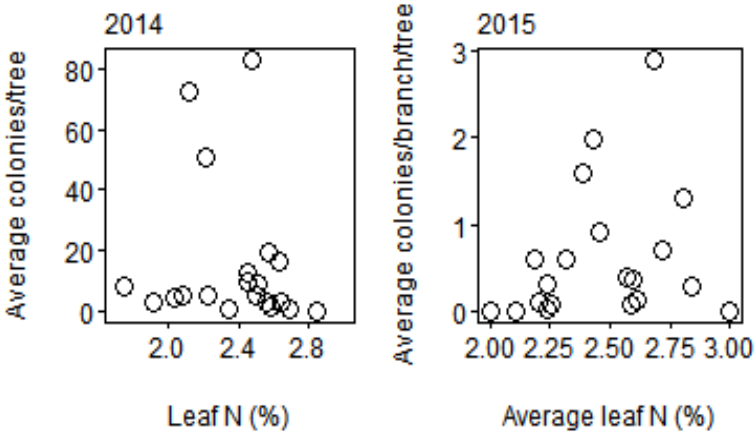

Figure 5. Woolly apple aphids and leaf nitrogen. Woolly apple aphids were counted as in Figure 1. Leaf nitrogen measurements were obtained from samples of mid-terminal fully expanded leaves sent to SoilTest Farm Consultants (Moses Lake, WA). Leaf samples were collected on one visit (2014) or three separate visits to each orchard throughout the growing season and (2015).

Pesticide use intensity

There was no clear correlation between pesticide use intensity and woolly apple aphids (Figure 6). We predicted that heavy use of broad-spectrum insecticides would induce woolly apple aphid outbreaks because of disrupted biological control, but this was not the case.

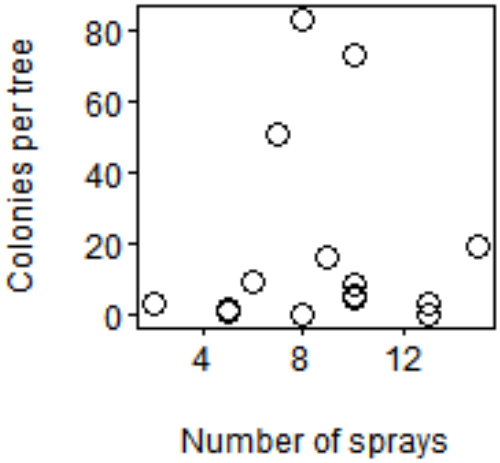

Figure 6. Woolly apple aphids and management intensity (broad spectrum insecticide sprays). Woolly apple aphid colonies were counted as in Figure 1. Oil and broad spectrum pesticides were included in this analysis, while more specific insecticides such as *Bt* and codling moth granulosis virus were not included.

Perennial cankers
Cankers were found at only one of the twenty study orchards, but they were rare (32 perennial cankers out of 4,500 trees inspected). While 90% of the perennial cankers were found to be infested with woolly apple aphids, there was not a clear connection between woolly apple aphid and perennial canker across all study locations because the 19 other orchards lacked perennial cankers. The high infestation rate of perennial cankers suggests that they are highly suitable feeding sites, but woolly apple aphids do not spread or cause first-year cankers. Furthermore, it was previously demonstrated that woolly apple aphids do not transmit the perennial canker fungus.

Natural enemies
Orchards with higher abundance of natural enemies (green lacewings, *Aphelinus mali* wasps, earwigs) tended to have lower woolly apple aphid counts, although results were highly variable, likely due to the other conditions on orchards. To follow-up on these results, a controlled experiment was conducted with earwigs, which appeared to be an important predator in our field surveys. The experiment clearly shows that the addition of this generalist predator resulted in fewer woolly apple aphids (Figure 7). Therefore, earwig conservation through timing and selection of pesticide sprays, and timing of tillage (to avoid destroying underground earwig overwintering nests) is suggested as a new integrated management tactic for woolly apple aphids. In addition, apples were inspected in the field for damage. Earwigs were sometimes found feeding in stem bowl cracks, but there was no evidence of any damage initially *caused* by earwigs because the occurrence of stem bowl cracks and overall damage was not higher in the earwig sections.

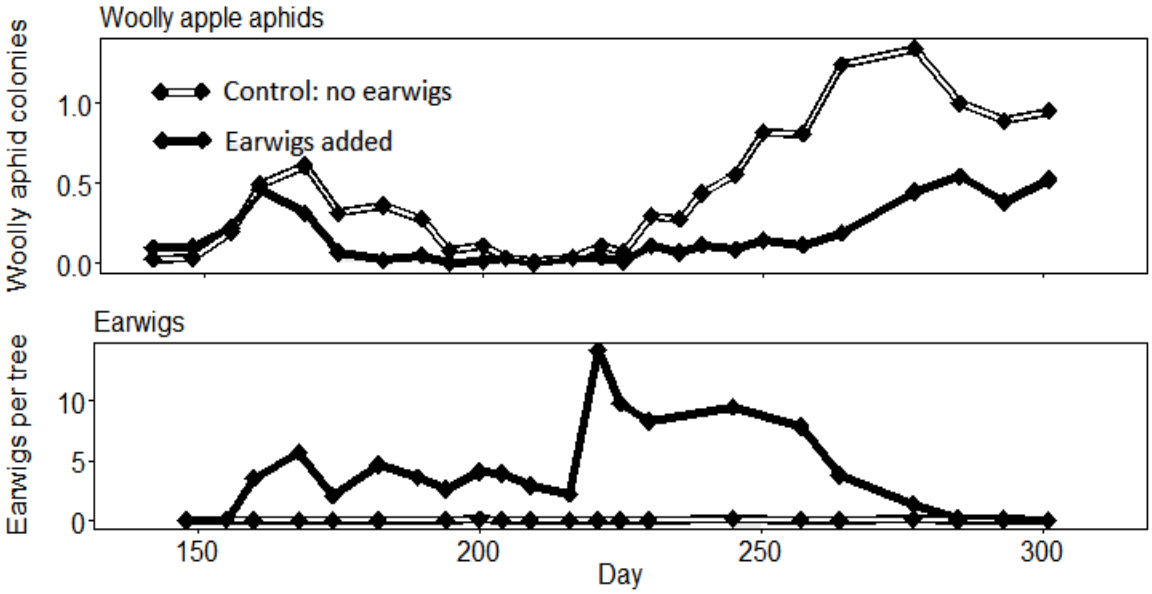

Figure 7. Earwig biocontrol experiment. Two years of observations indicated that no earwigs were initially present at the study block. Earwigs were first introduced to five 10 X 10 meter sections of the block on Ordinal Day 155 (see bottom chart) and were monitored each visit with counts in rolls of corrugated cardboard. A total of 675 earwigs per section was released between Ordinal Day 155 and 176. On Ordinal Day 217, 1,000 earwigs per section were released. The number of woolly apple aphid colonies (top chart) were counted on each of 10 branches of 21 trees in each of five earwig release sections and five unmanipulated control sections. Counts in the different section types were averaged to one number per visit for presentation here. The drop-off in earwig numbers at the end of the season is due to earwigs moving underground to nest over winter.

Woolly apple aphids and temperature

Temperature appeared to be an important predictor of woolly apple aphid population dynamics. Previous laboratory experiments showed that woolly apple aphids die at temperatures over 90 F. Consistently, when summer temperatures reached over 90 F for summer days, woolly apple aphid populations declined (Figure 8). We expect that in future years woolly apple aphids will also decline during extreme summer heat and management actions meant to control woolly apple aphids during such periods would be superfluous.

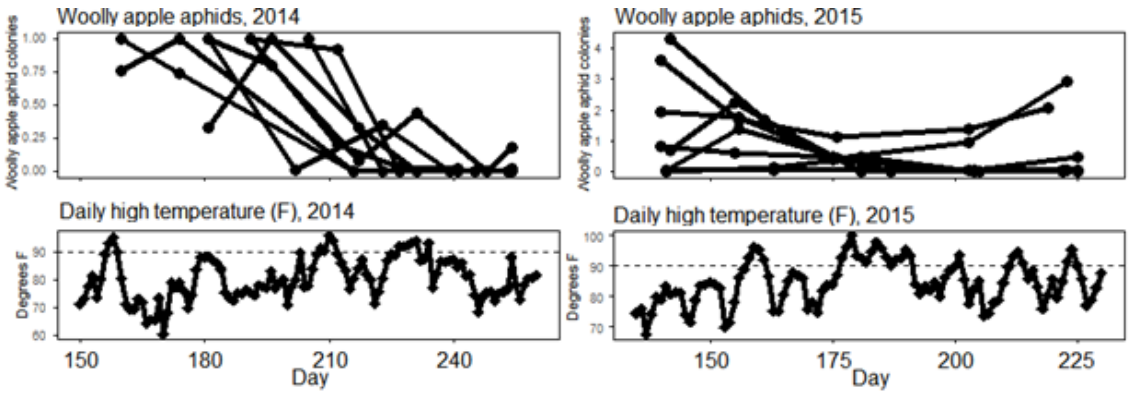

Figure 8. Temperature and season-wide woolly apple aphid counts. Woolly apple aphid counts (as in Figure 1) at orchards near Quincy, WA over time are presented along with daily high temperatures in degrees F. The dotted lines in temperature graphs are at 90F, above which woolly apple aphids do not grow well. Because of wide variation in woolly apple aphid counts in 2014, counts within each orchard were standardized values between 0 and 1 for purposes of visualization, while the 2015 chart shows average colonies per tree.

Woolly apple aphids and apple varieties

In our main study, only Fuji and Gala orchards were studied. In both years, woolly apple aphid counts averaged about twofold higher in Fuji orchards compared to Galas. Because of this, and because growers sometimes mentioned that they thought Fujis are more susceptible, we counted woolly apple aphid colonies in mixed plantings in the WSU Sunrise Research Orchard. The results suggest that Fujis are indeed more susceptible to woolly apple aphid infestation (Figure 9).

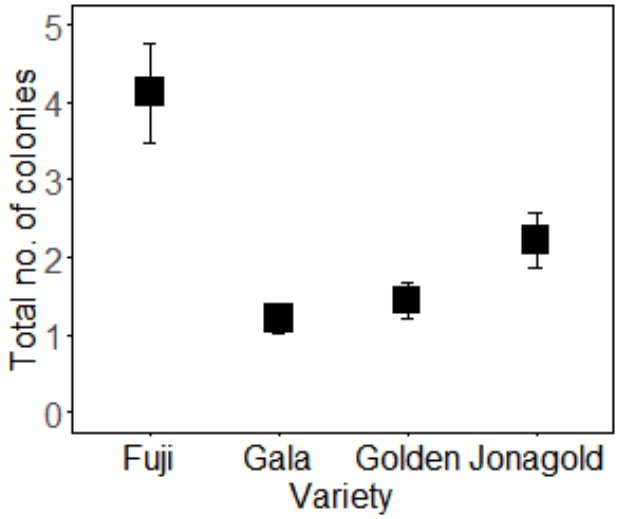

Figure 9. Woolly apple aphid counts on different apple varieties. In 3 mixed-planted blocks at the WSU Sunrise Research Orchard (containing alternating rows of Fujis, Galas, Goldens, and Jonagolds, the number of woolly apple aphids on October 6, 2015 were counted on ten ~1' long twigs on each of 312 total trees. Average counts per tree for each variety are shown with standard errors.

Executive summary

Summary of findings

- Our two-year observational study suggested that both temperature, natural enemies, and soil texture were important factors affecting variation in woolly apple aphid densities. Aphids were less common at high temperatures, in orchards with sandy soils, and in orchards with high natural enemy populations (earwigs in particular were key woolly apple aphid predators)
- In our two-year observational study, there was not clear evidence showing how soil quality, leaf nitrogen, organic management, and pesticide use influence woolly apple aphid incidence.
- Higher soil sand content may hinder woolly apple aphid movement into and out of soil, resulting in lower prevalence of aboveground colonies. Wood chip mulch and paper slurry mulch also reduce movement through soil, but our field study suggests that even complete blockage of woolly apple aphid movement up from roots may not help management efforts against aerial colonies. However, mulch or sand amendments might help prevent issues associated with colonies in the soil of orchards.
- Woolly apple aphids are often found feeding in perennial cankers, suggesting these are preferred wound sites for apples. However, a high abundance of woolly aphids does not induce canker outbreaks because woolly apple aphids do not transmit the fungus that causes perennial canker.
- Hot summer temperatures above 90°F are associated with woolly apple aphids die-offs, but woolly apple aphids resurge when temperatures cool off. Pesticide sprays at temperatures above 90°F are likely to be superflous
- Earwigs are important and underappreciated woolly apple aphid predators. Conserving earwigs has been shown to directly reduce aphid populations
- The Fuji variety is more susceptible to woolly apple aphid infestation than Galas.

Significance to industry

- Insecticide sprays for woolly apple aphids during peak summer heat appear to be unwarranted because the aphids will die anyway from the heat.
- Orchards planted on sandy soil may at lower risk of woolly apple aphid infestation.
- Fuji orchards may be at elevated risk for woolly apple aphids.
- Earwig conservation could reduce woolly apple aphid problems.
- Better IPM of woolly apple aphids that moves away from chemical strategies

Future directions

- New projects in our lab in 2017 expanded upon our ideas about earwig biological control. We have now produced strong evidence that earwigs are important aphid predators based on field manipulation studies and video recording studies. We will continue these investigations.
- PhD student Robert Orpet plans to compile and publish guidelines on earwig conservation for biological control of woolly apple aphids.
- PhD student Robert Orpet will compile current best recommendations for presentation at meetings.
- Continue to educate growers on strategies to improve aphid management
- Evaluation of survey data to better understand how growers make decisions concerning management of woolly apple aphid and other pests
- Evaluation of mulches in larger field settings as a method to reduce belowground aphid colonies

FINAL PROJECT REPORT
WTFRC Project Number: CP-16-104

Project Title: Phenotyping resistance traits of apple rootstock to replant pathogens

PI:	Yanmin Zhu	**Co-PI:**	Mark Mazzola
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Cooperators: Gennaro Fazio, apple rootstock breeder, USDA ARS

Agency Name: USDA ARS Tree Fruit Research Lab
Amount awarded: **Year 1: $**55,000 **Year 2: $**55,000

Budget history:

Contract Administrator: Charles Myers, Extramural Agreements Specialist		
Email:cwmyers@pw.ars.usda.gov		
Item	**Year 1**	**Year 2**
Salaries *	38,790	38,790
Benefits	13,577	13,577
Wages		
Benefits		
Equipment		
Supplies	1,733	1,733
Travel		
Miscellaneous		
Total	54,000	55,000

*The **salaries and benefits** are budgeted for a GS-7 technician dedicated to this project.

OBJECTIVES

1. Resistance response evaluation to multiple components of the ARD pathogen complex. Using established protocols for phenotyping the resistance response to *Pythium ultimum* infection, characterization of resistance responses will be expanded to other key components within the ARD pathogen complex including *Rhizoctonia solani* and *Pratylenchus penetrans*.

2. Field performance validation. The greenhouse based phenotype data will be evaluated at a replant site at the Columbia View (CV) experimental orchard. Field performance trials will be carried out using selected genotypes of both susceptible and resistant genotypes based on greenhouse data.

3. Phenotypic data will be used to improve the localization of previously mapped QTLs associated with ARD resistance. The resulting plant materials possessing reproducible and reliable phenotypes will be used in future gene-trait association studies.

SIGNIFICANT FINDINGS

- Individual genotypes from 'Ottawa 3' x 'Robusta 5' (O3R5) progeny were identified with a wide spectrum of resistance levels. More than sixty genotypes were assayed at least twice for response to *P. ultimum* infection. Forty-three genotypes were evaluated for their response to *R. solani* infection and more than thirty genotypes have been assayed for their ability to repel/attract nematodes *P. penetrans*.

- In assessment of the resistance response to *P. ultimum* infection, a primary focus of this work, the survival rates among individual genotypes ranged from 92% to 8% among genotypes. Reductions in root biomass resulting from *P. ultimum* inoculation varied from 5% to 60% and shoot biomass reduction varied from 10% to 60% between the most resistant and the most susceptible genotypes, respectively.

- Contrasting patterns of necrosis progression along the infected roots were associated with the resistant or susceptible phenotype, respectively. Rapid and undeterred necrosis progression was often observed along the roots of susceptible genotypes, and impeded necrosis progression was commonly associated with the roots of resistant genotypes.

- Substantial overlap was observed between resistance to *P. ultimum* and *R. solani* AG-5 but no correlation was found between resistance to *P. ultimum* and *P. penetrans* (nematode).

- Results from preliminary field evaluation at the replant site in Columbia View orchard suggests that the susceptible genotypes benefit more from soil fumigation than resistant genotypes do.

- Current phenotyping dataset of resistance to *P. ultimum* infection was used for detecting resistance QTL in collaborator's lab. However, no major QTLs were identified.

RESULTS AND DISCUSSION

By implementing an in-house plant micropropagation procedure and standardized infection protocol, more than sixty genotypes from O3R5 progeny were repeatedly assayed for their resistance responses towards infection by *P. ultimum.* Forty-three genotypes were also assayed for their response to infection by *R. solani*, and thirty-five genotypes were assayed for their resistance to infestation by *P. penetrans*. For pathogen of *P. ultimum*, the primary focus of this study, overall resistance levels were initially assessed based on overall survival rate of infected plants at 14 dpi (day post inoculation). Plant biomass reductions were determined at 28 dpi by comparison of root or shoot fresh weights between control and infected plants. Root necrosis patterns were examined under dissecting microscope at the early stage of infection, from one to ten dpi. Response to infection by *R. solani*, root infestation by the lesion nematode *P. penetrans*, as well as differential responses to chemical fumigation at a replant site, were also evaluated for selected O3R5 genotypes. The implemented tissue culture procedure enabled the first systematic and detailed analyses on apple root responses to individual components of ARD pathogen complex under controlled experimental conditions. The high-quality resistance phenotypes data of O3R5 population represents an important step for maximized exploitation of host resistance from apple root for managing ARD in future.

1. Wide-range and repeatable resistance responses to *P. ultimum* infection among O3R5 genotypes

Using a standardized phenotyping protocol and repeated infection assays, our data demonstrated a wide-spectrum of plant survival rates due to infection by *P. ultimum* among O3R5 genotypes (**Figure 1**). The top-10 most resistant and susceptible genotypes are listed in **Table 1**. In this study, "resistant" genotypes were assigned to those O3R5 progeny with average survival rate greater than 80%; those progeny with average survival rate lower than 30% were designated as "susceptible. The germplasm genotypes with intermediate values of survival rates were not included in this report.

Figure 1. Distinctive responses among O3R5 progeny to infection by *P. ultimum*. A. Three genotypes showing various responses to *P. ultimum* infection. **B.** Two genotypes, #161 and G.935, exhibiting highly resistant responses. **C.** Two genotypes, #115 and #132, exhibiting more susceptible phenotypes. Plants were inoculated by root dipping method; and inoculum solution contains 2 x 10^3 per mL oospores. All genotypes in Figure 1A were assayed at the same time; all genotypes in B and C were assayed at the same time. Most of these genotypes were assayed for 3-5 times with comparable results. The plants delineated by an orange-colored frame at the left side of the trays were mock inoculated control plants, which remained healthy throughout the assay. Images were taken at 14 dpi. Control and *P. ultimum* infected plants were maintained under identical greenhouse conditions.

Survival rates for a specific plant genotype were generally consistent or repeatable between infection events. Occasionally, an aberrant survival rate value was observed for a given genotype. Although the infection assay was conducted under "controlled" experimental conditions and efforts were made to maintain the consistency for each step of the experimental procedure, minor variations in plant materials and/or pathogen preparations could contribute to the variable survival rates observed between infection events. Environmental factors, such as fluctuating temperature and/or relative humidity can also present unexpected abiotic stresses which may influence the outcomes of plant-pathogen interactions. Synergistic effects between certain abiotic stress and *P. ultimum* infection may interfere with expression of resistance traits, but the details of their influence are largely unknown. Overall, repeatable and highly consistent results were observed for most of the assayed O3R5 progeny, particularly for the more resistant and susceptible groups as listed in Table 1. The survival rates of all tested genotypes were also analyzed for detecting potential resistance QTLs in collaborator's lab, though no major QTLs were identified.

Table 1. Top ten genotypes from both susceptible and resistant groups

O3R5 genotypes	Total plants assayed (survived)	Times assayed	Range of observed survival rates (%)	average survival rate (%)
#115	122 (24)	10	44-0	**19.5**
#132	57 (18)	4	31-33	**32**
#125	66 (16)	5	36-7	**21.5**
#106	22 (5)	3	33-12	**20**
#121	12 (1)	2	13-0	**6.5**
#47	19 (2)	3	25-0	**11.6**
#80	38 (8)	2	33-10	**21.5**
#34	15 (3)	3	33-0	**19.3**
#4814	25 (7)	3	38-20	**27.6**
#141	38 (9)	3	26-16	**17.3**
#58	49 (43)	5	71-100	**92.4**
#161	57 (51)	5	67-100	**91.6**
#164	91 (77)	6	83-100	**90.5**
G.935	124 (115)	7	86-100	**93**
#172	78 (68)	4	83-100	**87.3**
#173	48 (43)	5	83-100	**89**
#78	67 (61)	6	67-100	**89.4**
#63	38 (35)	2	85-100	**92.5**
#134	19 (17)	2	85-92	**88.5**
#142	42 (32)	3	66-100	**82.3**

Survival rates were scored at 14 dpi. Six plants from the same batch of micropropagation procedure were used as mock-inoculation control which were maintained under the identical conditions to plants inoculated with *P. ultimum*. Numbers of inoculated plants for different O3R5 genotypes varied from 10 to 30 depending on the available plants from tissue culture procedures at the time. At the later stage of the phenotyping study, effort was made to include at least one individual genotype from opposite group to monitor the inoculum preparation, the infection process and/or the presence of other abiotic factors. Average percentage is based on survival rate from each assay.

2. Measured reduction of root and shoot biomasses

In addition to genotype-specific plant survival rates, root and shoot biomass reductions were also measured at 28 dpi to examine the differential impacts of *P. ultimum* root infection on plant growth and

development. By comparing the values of root or shoot fresh weights between mock inoculated control plants and *P. ultimum* inoculated plants, greater reductions in biomass were generally observed for the susceptible genotypes (**Table 2)**. This observation is expected as more severe growth inhibition was often observed for surviving plants from susceptible genotypes as compared to infected plants from resistant genotypes (as shown in Figure 1). On the other hand, substantial reductions in biomass were observed for some resistant genotypes, such as #164 and #172. This observation indicated that although a high percentage of plants manage to be alive at 28 dpi, the growth of these surviving plants can be substantially inhibited due to the continuing influence of *P. ultimum*. In other words, even some of the resistant genotypes that possessed high rates of survival exhibited significant levels of growth inhibition. Therefore, survival rate and biomass reduction are two different aspects of resistance responses, and both parameters should be considered in the evaluation of overall resistance responses for a genotype.

Table 2. Root and shoot biomass reduction due to *P. ultimum* infection

O3R5 genotypes	**Root** biomass (average fresh weight, g)			**Shoot** biomass (average fresh weight, g)		
	Mock inoculation	*P. ultimum* inoculation	Biomass reduction (%)	Mock inoculation	*P. ultimum* inoculation	Biomass reduction (%)
#115 (S)	1.43 ± 0.39	0.71 ± 0.32	**50.3**	0.87 ± 0.05	0.56 ± 0.07	**35.6**
#132 (S)	1.68 ± 0.56	0.85 ± 0.54	**49.4**	1.31 ± 0.36	0.76 ± 0.31	**41.9**
#106 (S)	1.42 ± 0.28	0.42 ± 0.35	**40.8**	1.27 ± 0.27	0.45 ± 0.21	**64.6**
#122 (S)	1.65 ± 0.29	0.99 ± 0.23	**40.0**	1.47 ± 0.34	0.98 ± 0.38	**33.3**
#125 (S)	1.38 ± 0.24	0.86 ± 0.27	**37.7**	1.21 ± 0.02	0.50 ± 0.37	**59.2**
#58 (R)	1.11 ± 0.24	1.05 ± 0.17	**5.4**	0.94 ± 0.08	0.80 ± 0.11	**14.9**
#161 (R)	1.44 ± 0.32	1.35 ± 0.32	**6.2**	1.02 ± 0.12	0.92 ± 0.31	**9.8**
#173 (R)	1.14 ± 0.31	1.03 ± 0.20	**9.6**	0.71 ± 0.10	0.59 ± 0.17	**16.9**
#164 (R)	1.87 ± 0.39	1.35 ± 0.13	**27.8**	1.09 ± 0.07	0.77 ± 0.16	**29.4**
#172 (R)	1.40 ± 0.7	1.11 ± 0.53	**20.7**	1.18 ± 0.11	0.83 ± 0.06	**29.7**

The percentage biomass reduction was calculated by comparing the values of root and shoot fresh weight between the survived plants from *P. ultimum* infection and those of control plants at 28 dpi. Conceivably, the numbers of survived plants varied between resistant and susceptible genotypes from *P. ultimum* infection. R: denotes resistant genotype; S: denotes susceptible genotype.

3. Contrasting patterns of necrosis progression in roots between resistant and susceptible genotypes

The potential mechanisms that function in host resistance at the tissue and cellular levels, which may contribute to the distinct responses to *P. ultimum* infection, were investigated between resistant and susceptible O3R5 genotypes. Using a custom-made small glass box, necrosis progression was monitored continuously along inoculated apple roots under microscope. Based on analysis of serial time-lapsed images, distinctive patterns of necrosis progression were routinely observed along the roots between resistant and susceptible genotypes. As shown in **Figure 2A**, along a section of root system of the susceptible #115 plant, no identifiable infection was observed until 120 hours post inoculation (hpi); then rapid progression of root necrosis, indicated by the yellow or brown coloration from *P. ultimum* infection, spread through entire sections of the root system within 12 hours (120 to 132 hpi). It appeared that no restriction or deterrence existed in root tissues of the susceptible #115, which lead to the swift necrosis progression.

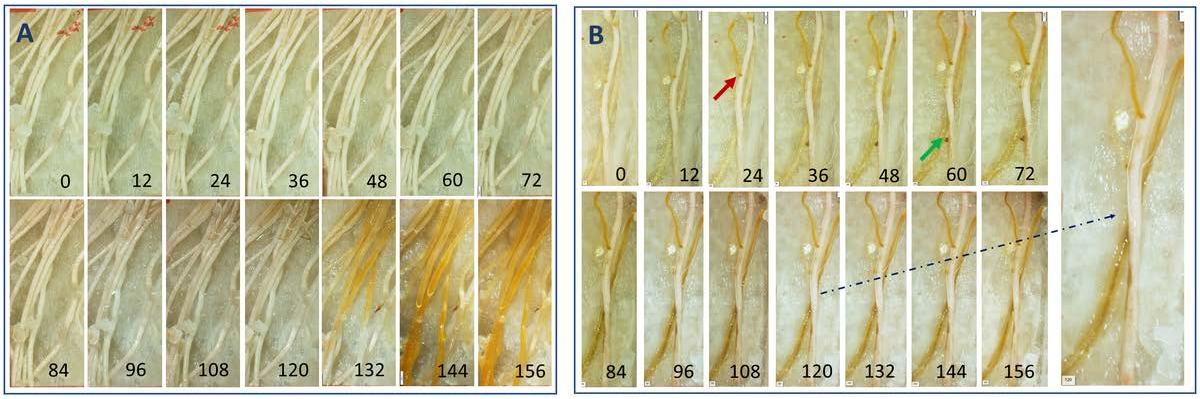

Figure 2. Progression patterns of root necrosis from *P. ultimum* infection. A. Time-lapse images of root necrosis development for the susceptible #115 in response to infection by *P. ultimum*. **B.** Time-lapse images of necrosis progression along the roots of the #161 resistant genotype in response to infection by *P. ultimum*. The number at the bottom of each image denotes the hour post inoculation (hpi).

In contrast, a very different pattern of necrosis progression was frequently associated with the resistant genotypes. As shown in **Figure 2B**, though the necrosis was detected as early as 12 hpi on a newly-emerged lateral root (red arrow on the image for 24 hpi), necrosis initiated from this lateral root seemed to be localized at the junction. A separate necrotic section was observed at 60 dpi from the low section of the roots, as indicated by green arrow, but healthy (white-colored) root tissues were still visible for an extended period of almost 100 hours, from 60 to 156 hpi. Such deterred or delayed necrosis progression was presumably due to the effective resistance responses operating inside the root tissues along roots of the resistant #161 genotype. A close-up image at 120 hpi at the right end of Figure 2B shows a clear "line" or "zone" appear to separate the white-colored healthy section from yellow-brownish necrotic section.

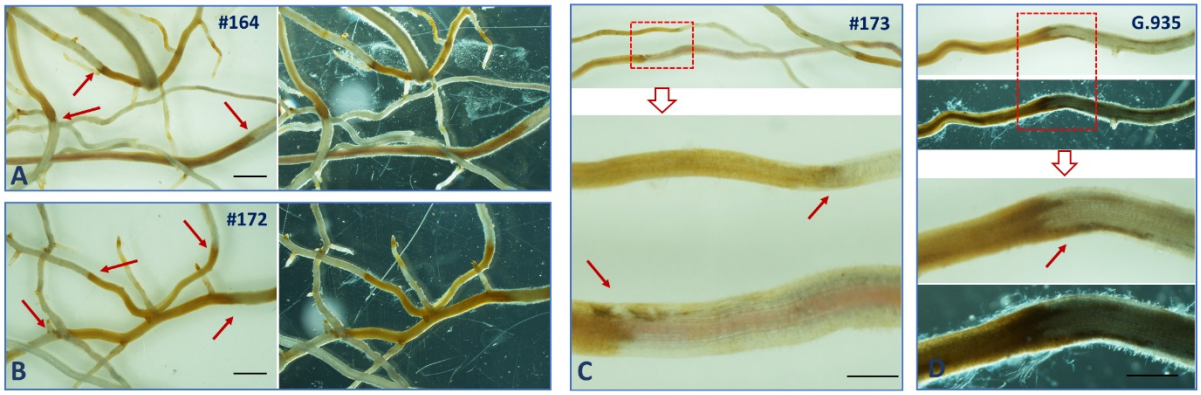

Figure 3. The defined lines separating healthy and necrotic tissues along the infected roots of selected resistant genotypes. The clear and defined "lines" or "zones" can often be observed between sections of necrotic and healthy tissues along the roots of the resistant O3R5 lines. A. Images from infected roots of #164; B. images from infected roots of #172. The same roots sections were documented against both white (left panel) and dark backgrounds (right panel). C. images from infected roots of #173; bottom image is the enlarged section of the top image. D. images from infected roots of G.935; bottom image is the enlarged section from the top image. The bars at the bottom of the image represent 500 µm for A and B; 200 µm for C and D.

Extensive microscopic examination indicated that such a defined "line" or "zone" along the infected roots was more widely associated with other O3R5 resistant genotypes such as #164, #172, #173 and G.935 (Figure 3). The existence of such a "clearly defined line" between healthy and necrotic root tissues strongly suggests that roots of resistant genotypes are capable of deterring the fast-growing *P. ultimum* from inflicting wide-spread necrosis throughout the entire root system. Such delayed necrosis progression could be one of the major factors contributing to the high survival rates of the resistant genotypes. It can be speculated that an active "chemical warfare" operates in the roots of these resistant genotypes towards an invading pathogen. The efficient generation of antimicrobial compounds from effective defense activation could lead to the delayed necrosis progression in the root of resistant genotypes, which in turn provides critical time for regenerating new root branches to compensate the loss of functional root tissue. These defined "zones" were very rarely observed among susceptible genotypes, and never observed from mock inoculated roots.

4. Overlapping resistance response to *R. solani* and that to *P. ultimum*

This part of the experiment was designed to address the question: do apple roots share similar or overlapping mechanisms of resistance to infection by *P. ultimum* and *R. solani*. As shown in **Figure 4**, plant survival rates suggested that considerable comparability exists between the resistance responses to infection by these two pathogens. In other words, those genotypes which were classified as *P. ultimum*-resistant showed a corresponding higher percentage of survival rate from inoculation with *R. solani* AG-5; and all *P. ultimum*-susceptible genotypes except one demonstrated a correspondingly lower survival rate in response to *R. solani* AG-5 inoculation.

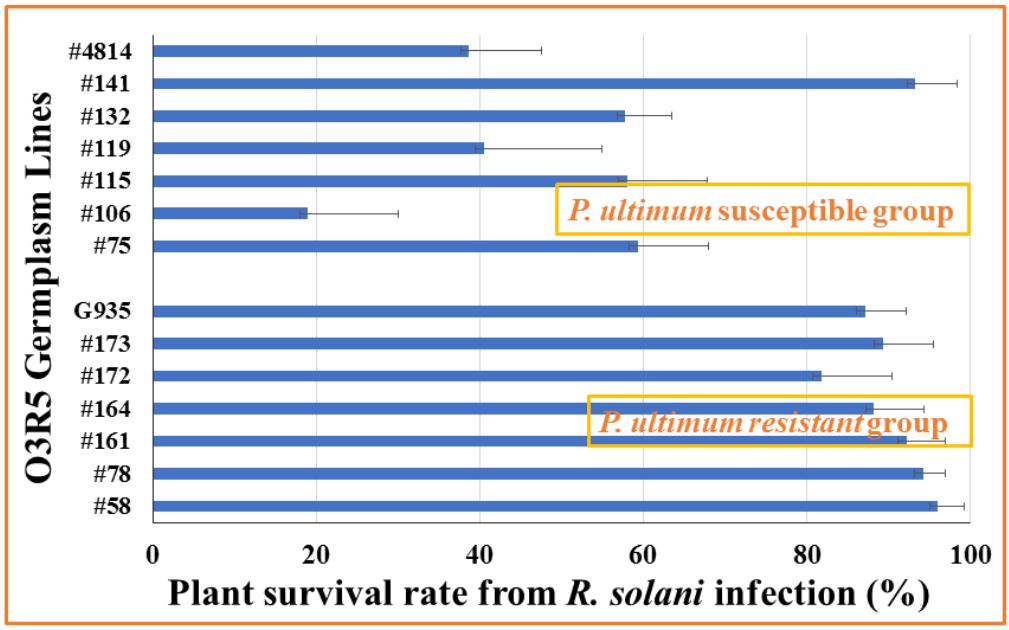

Figure 4. Overlapping resistance responses toward infection by *P. ultimum* or *R. solani*. Results represent the average values from at least two inoculation assays by *R. solani* AG-5. Plant survival rates were scored at 28 dpi. Data were grouped based on the resistance level to *P. ultimum* infection. Individual O3R5 genotypes in the upper group were more susceptible to *P. ultimum* infection, similarly, individual genotypes at lower group were more resistant to *P. ultimum* infection.

It is probably not surprising that comparable resistance responses may exist toward infection from either *P. ultimum* or *R. solani* AG-5 as both are necrotrophic pathogens. Although these two pathogens belong to very different categories (oomycete and fungus), they might exploit some comparable "attacking"

tactics towards apple roots. This observation seems to support the optimistic notion that a certain level of shared resistance mechanisms may exist towards infection by various components within the ARD pathogen complex. Then the level of difficulty could be alleviated in identifying a small set of apple genes to distinguish resistance or susceptibility to ARD. Unlike observations from *P. ultimum* infection, none of the germplasm lines demonstrated single-digit survival rates, this could mean that the *R. solani* AG-5 strain used in this study is less virulent, or less lethal, than the *P. ultimum* strain. However, abundant mycelia were routinely observed from R. *solani* AG-5 inoculated plant roots, suggesting adequate amount of inoculum was applied.

5. Relationship between resistance to *P. ultimum* and extracted *P. penetrans*

The question being asked from this part of experiment was: will those genotypes which showed either resistance or susceptibility to *P. ultimum* infection exhibit a differential effect on populations of the lesion nematode *P. penetrans*. Nematodes were extracted from 5-gram soil samples or 0.5-gram root tissue after plants from selected O3R5 genotypes have grown in the nematode-infested soils for 45 days. As shown in **Figure 5**, it appeared that no identifiable consistency was observed between the resistance to *P. ultimum* and recovered *P. penetrans* numbers. However, disregarding the resistance responses to *P. ultimum* there were considerable variations at recovered nematodes between individual genotypes. For example, #164 demonstrated consistently lower nematode root densities from repeated assays. Another example is obvious differences in term of recovered numbers of nematodes between roots and shoots of G.41. It seems that although resistance to *P. ultimum* infection does not share the trend of nematode density in the soil and root, the genotype-specific variations existed among O3R5 progeny. The detailed mechanisms behind such variation of extracted nematodes numbers are unclear based on the limited data from this pilot experiment. It can be speculated that the number of nematodes may be passively dependent on the genotype-specific availability of leaked nutrients or certain unique chemicals from root system.

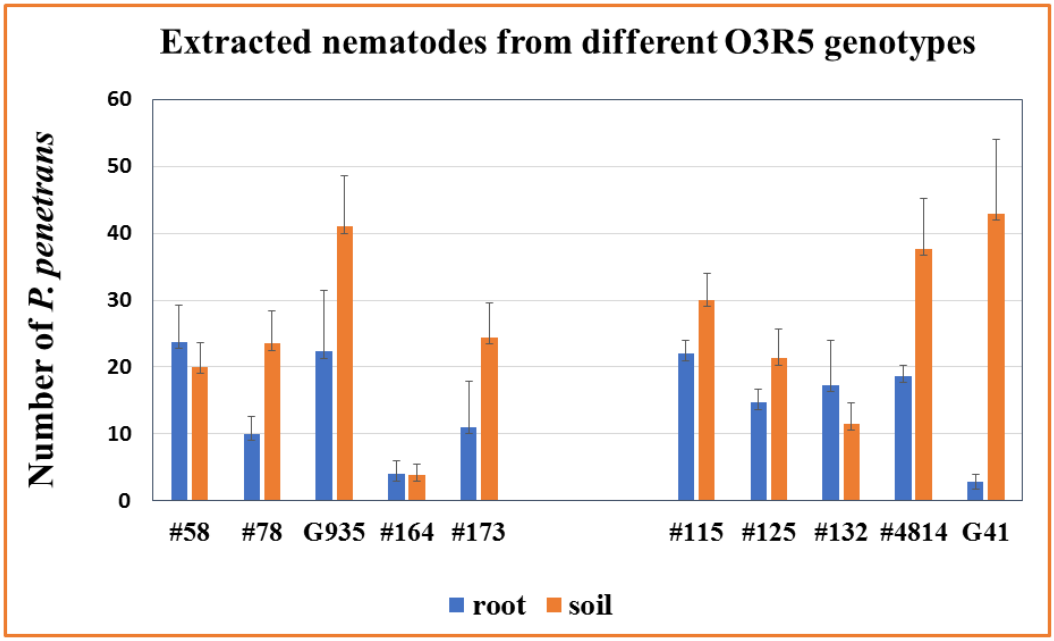

Figure 5. Extracted numbers of *Pratylenchus penetrans* from root and soil per genotypes. Selected O3R5 genotypes with contrasting resistance or susceptibility to *P. ultimum* infection were used to grow in nematode infested soil for 6 weeks. The values represent the average of multiple assays (2-4 times depending on genotypes). Nematodes were extracted from 0.5 g of root tissues and 5 g of soils.

6. Field evaluation of selected genotypes in both fumigated and non-fumigated rows

Field growth responses for selected *P. ultimum*-resistant and *P. ultimum*-susceptible genotypes was carried out at a replant plot at the Columbia View experimental orchard. The values of total plant biomass, were compared between plants growing in fumigated and non-fumigated rows for three months. Based on the data of a short-term (three months) growth responses, those *P. ultimum*-susceptible genotypes showed increased values of biomass in fumigated soil, but only half of *P. ultimum*-resistant genotypes show increased biomass values **(Table 3)**. In other word, those susceptible genotypes suffer more growth inhibition in the non-fumigated row. This preliminary observation seems to support the notion that susceptible genotypes benefit more from soil chemical fumigation, as indicated by the larger increased values of biomass. On the other hand, half of the tested resistant genotypes showed decreased biomass in fumigated soil as indicated by the negative net increase of total biomass values. The mechanism behind this different growth response based on the short-term observation is unknown from this pilot experiment. The possible contributing factors include genotype-specific growth habit or root regeneration patterns under field condition, nutrient utilization efficiency, the ability to overcome heat stress. The age variations between the tested genotypes at the time of being transplanted into soil were considerable for some of them, because of the time needed for generating these plants from tissue culture procedures. Therefore, this part of experiment was certainly a preliminary trial, and a more extensive field evaluation will be needed for more conclusive evidence so that the consistency between greenhouse assay of controlled infection from individual pathogens and overall field performance can be more reliably validated.

Table 3. Growth response of resistant or susceptible O3R5 genotypes to fumigation at replant site

O3R5 genotypes	Phenotype (*P. ultimum*)	total biomass (g); non-fumigated row	total biomass (g); fumigated row	% Net increase (non-F/F)
164#	R	55.7 ±7.4	37.9 ±5.8	-47
173#	R	47.8 ±6.8	43.9 ±7.5	-9
62#	R	189.5 ±19.6	174.3 ±12.8	-9
58#	R	142.0 ±17.3	134.5 ±11.4	-6
B9	S	129.1 ±15.6	127.5 ±13.7	-1
135#	R	89.6 ±11.2	109.2 ±9.9	18
142#	R	67.0 ±8.8	89.7 ±8.1	25
M9	S	259.7 ±22.1	357.9 ±28.3	27
G935	R	84.6 ±9.9	131.4 ±17.4	36
75#	S	79.4 ±11.4	123.6 ±8.4	36
161#	R	82.6 ±7.7	148.3 ±13.2	44
125#	S	31.7 ±5.8	112.2 ±10.1	72

Values represented the average of measured total biomass from all survived plants (up to 5) for each genotype; F: plants grown in fumigated row, non-F, plant grown in non-fumigated row. Designation of R (resistant) or S (susceptible) phenotypes was based on the result of greenhouse infection assay *by P. ultimum*. Fumigant (Telone C-17) was applied by deep (18 inches) untarp broadcast at the rate of 30 gallon per acre on May 25, 2016 by Custom Orchard Fumigation. Plants were planted on late June 2016 and harvested in early Oct for a growing period of more than three months at Columbia View replant site.

EXECUTIVE SUMMARY

Reliable phenotypes are a prerequisite for the conduct of careful molecular and genetic studies concerning the biology of interest. Apple rootstock germplasm with stable resistance traits (and susceptibility) are essential for elucidating the underlying molecular mechanisms. Until this study, a standardized phenotyping protocol for systematic and quantified analysis of apple root resistance responses had been lacking. Using our established phenotyping protocols, more than 60 individuals from O3R5 progeny have been evaluated for their detailed resistance responses to three representative ARD pathogens, i.e. *Pythium ultimum* (oomycete), *Rhizoctonia solani* (fungus) and *Pratylenchus penetrans* (nematode). For the primarily focused pathogen of *P. ultimum*, multiple genotypes with either highly resistant or highly susceptible resistance responses have been identified based on repeated infection assays. Their differential resistance responses were demonstrated by plant survival rates, reduction of root and shoot biomasses, as well as the microscopic features of root tissue necrosis patterns. While substantial overlap was observed between the resistance responses to *P. ultimum* and *R. solani* AG-5, no relationship can be derived between resistance to *P. ultimum* and the recovered nematode *P. penetrans* for a given O3R5 genotype. Preliminary field evaluation at the Columbia View orchard replant site seemed to suggest that the susceptible genotypes (which were based on *P. ultimum* infection assay in greenhouse) benefit more from fumigation than those resistant genotypes do. However, more extensive test with long-term field evaluation is needed. The available resistance phenotyping dataset was also analyzed for detecting potential resistance QTLs in collaborator's lab, though no major QTLs were identified suggesting complex genetics behind the observed resistance responses. This phenotype dataset will be converged with the results of our recently identified candidate apple genes from two transcriptome analyses on apple root defense responses to *P. ultimum* infection. These carefully phenotyped apple rootstock germplasms are pivotal for associating specific apple genes with observed resistance traits. It is worthy to note that the implementation of an in-house micropropagation procedure enabled us to overcome the unique obstacle for studying apple root resistance responses. Though it is a tedious and time-consuming process, the constant supply of uniform apple plants of defined genetic background, equivalent age, and non-contaminated root tissues is fundamental for the high-quality apple root resistance phenotypes. In summary, this study is the first careful and systematic effort to dissect genotype-specific apple root resistance responses to multiple ARD pathogens under controlled experimental conditions. This dataset of apple root resistance phenotype is the necessary step towards maximized exploitation of host resistance in managing ARD in the future. Progress in defining apple root resistance phenotypes from the current study were aligned closely with sustainability and profitability of Washington State apple industry.

FINAL PROJECT REPORT
WTFRC Project Number: CP-13-102A

Project Title: Season-long protection of apples from codling moth using kairomonal mass trapping.

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Cooperators: **Total Project Request:** **Year 1:** $29,000 **Year 2:**

Other funding sources: **2018None**

Budget 1
Organization Name: USDA, ARS **Contract Administrator: Chuck Meyers**
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Item	2017	**2018**
Salaries		
Benefits		
Wages	$22,400	
Benefits	1,800	
Equipment		
Supplies	4,300	
Travel	500	
Plot Fees		
Miscellaneous		
Total	$29,000	**0**

Note: Original proposal was for 2 years. Withdrawal of the second year of funding was requested by the PI.

OBJECTIVES

Objectives. The principal objective of this project was to demonstrate control of damage to apples by the codling moth in commercial apple orchard blocks using a kairomone lure in a Delta trap. The approach or research goal was to trap out enough female moths to prevent most oviposition and thus to prevent most attacks to the fruit.

SIGNIFICANT FINDINGS (for 2017 field season).

1. Mass trapping of codling moths using a kairomone attractant in the traps resulted in less damage/infestation of apples in treated plots compared to control plots in apple orchards with modest infestation levels.
2. Relatively large numbers of female and male codling moths were removed from treated plots, captured on the adhesive-coated liners of the Delta traps.

RESULTS AND DISCUSSION

Background: Work prior to this project showed the superior attractiveness to female codling moth of the combination of acetic acid, pear ester and N-butyl sulfide (Landolt et al. 2014). Additionally, we concluded that using an adhesive-coated surface (sticky trap) in place of a pesticide-treated surface for an attract-and-kill station was possible in commercial orchard settings where overloading of the trap liner surface is not severe. This approach is what has been called "mass-trapping" or "trapping out", and maintains the primary advantages of the attract-and-kill concept of using an attractant to bring pests to a discrete killing station. This approach has potential over cover sprays to reduce or replace insecticide use, and greatly reduce impacts on beneficial and other non-target insects. The mass-trapping approach has the additional benefit over pesticide baits of providing direct information on the insects removed, both pest and non-target.

Under the prior WTFRC project CP-13-102A "Codling moth attract-and-kill with kairomonal lures", experiments using a Delta trap in one-acre and 4-acre blocks of heavily-infested apple orchards, at a density of 50 traps per acre for ca 30 days, greatly reduced numbers of adult codling moths in monitoring traps, which we referred to as "knockdown". More importantly, percentages of apples that were damaged by codling moth were significantly less in the 4-acre plots with the 50 traps per acre, compared to the untreated plots (Jaffe et al. in preparation, WTFRC final report January 2017).

2017 Field Season. Methods and Approach. In the 2017 field season, under the current WTFRC project, the same methods generally were used as in the previous WTFRC project. Three aspects of this study differed from previous work.

First, rather than conduct tests for ca 30 days, which was intended to cover the flight period of a generation of codling moths, we conducted the test continuously from first moth flight until harvest. This involved setting up about 50 traps per acre in the 4-acre plots in early May and maintaining those traps until early September. Weekly trap maintenance involved counting and recording male and female codling moths in traps, removing moths and other insects if few in number, replacing liners that were dirty or with many insects captured, and occasionally replacing downed or damaged traps.

Second, rather than use apple orchards with codling moth populations that were uncontrolled as in previous tests, we used apple orchards with a history of damage and trap catch, but more modest codling moth populations.

Third, we deployed no traps in control plots. In earlier studies, we used kairomone, pheromone, and blacklight-traps to monitor numbers of moths in both treated and control plots. However, there was evidence that we were removing significant numbers of female codling moths with the monitoring traps in control plots. So, rather than compare trap catches (to determine knockdown) and damage to apples in treated vs control plots, we relied solely on assessing codling moth damage to apples.

2017 Field Season Results. In mid-season, following the end of the first moth flight, the percentages of apples that were damaged by codling moth were much less in plots with 50 traps/acre, compared to plots with no traps (Figure 1). The difference between damage to apples in treated vs control plots was stronger for the Parker site (ca 75 % less in the treated plot, and less dramatic for the Higgens site (ca a 55 % reduction). The Higgens site also had much less damage overall (<1 %) than the Parker site (up to 5+ %). Although there were individual apples that had more than one sting or hole (referred to below as damages), the percent of fruits damaged was not much less than the numbers of damages per fruit. That is, most damaged fruit had been attacked by one larva.

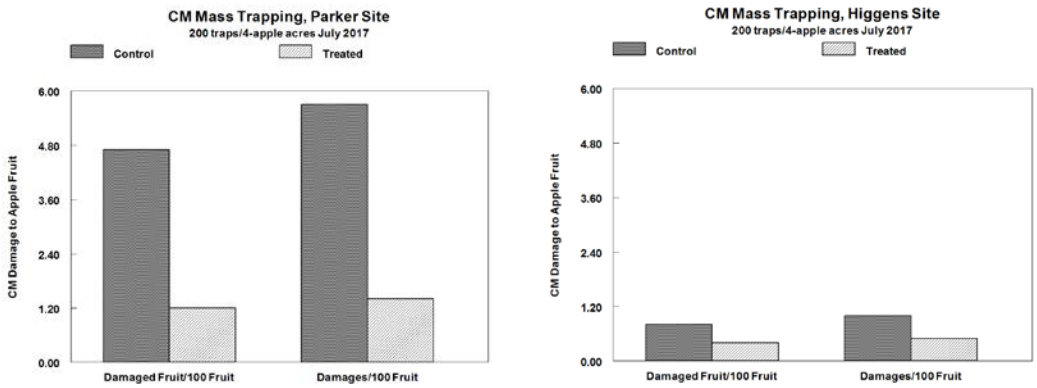

Figure 1. Amounts of damage to apple fruit by codling moths in orchard plots treated with 50 kairomone-baited traps per acre (Treated), or receiving no traps (Control). Observations of fruit were made in early July, between the end of the first adult generation and beginning of the second adult generation (flight).

At the end of the season, the percentages of apples that were damaged by codling moth again were much less in plots with 50 traps/acre (treated), compared to plots with no traps (control). This difference between treated and control plots held for both orchards. Treated plots had 75 % (Higgens) to 90 % (Parker) less codling moth-damaged fruit compared to control plots. At the Parker site, the percentages of apple fruit that were damaged were less at the end of the season compared to mid-season because of fruit thinning of both treated and control plots that took place in early August. No such thinning took place at the Higgens site.

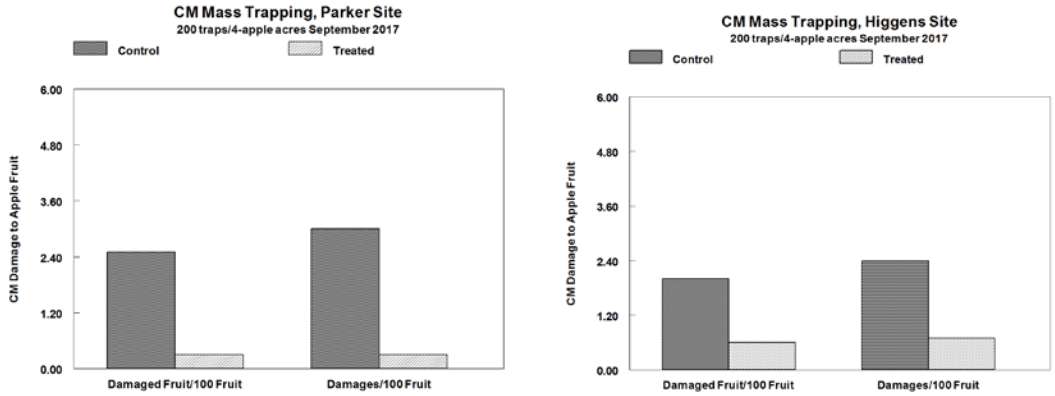

Figure 2. Amounts of damage to apple fruit by codling moth in orchard plots treated with 50 kairomone-baited traps per acre (Treated), or receiving no traps (Control). Fruit observations were made in late August into early September, near the end of the adult flight.

As in the prior 4-acre plot tests conducted for period of ca 30 days, the trap catch data shows that the lures were functioning well. Overall catch was somewhat male biased. Totals of 1314 females and 1721 males were removed from the Parker plot, and 294 females and 425 males were removed from the Higgens site. Captures of codling moths at the Parker site were much higher that at the Higgens site, in line with the much higher fruit damage at the Parker site.

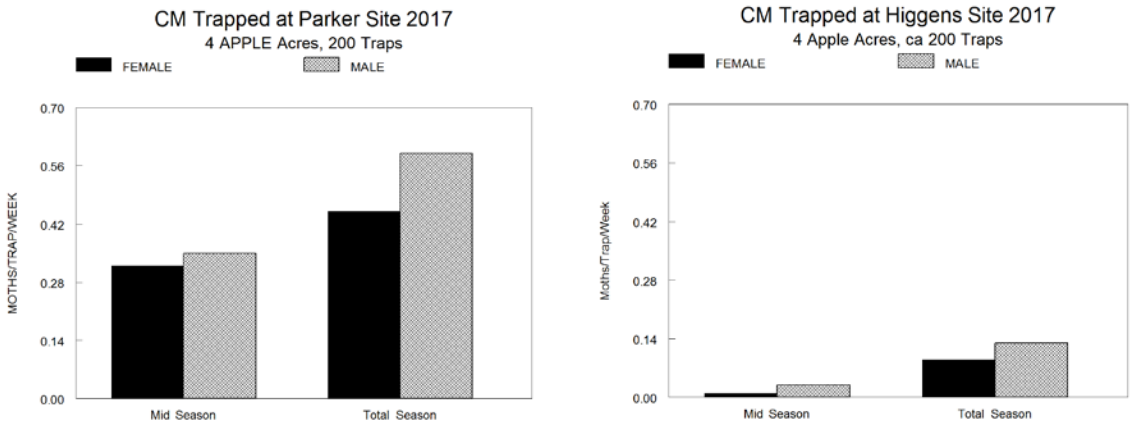

Figure 3. Average weekly numbers of codling moth males and females captured in Delta traps baited with a kairomone lure, for ca 200 traps per site and maintenance of traps for 16 weeks.

Discussion

The killing and removal of female codling moth from apple orchards has been a research goal, with prior trials indicating consistent suppression of populations of adult codling moth with these lures and traps in both one-acre and 4-acre plots. Success has been shown by the strong reductions in numbers of codling moth in kairomone- and pheromone-baited monitoring traps in plots with 50 kairomone-treated traps per acre. However, the ultimate objective of the work and a more relevant measure of success is the prevention of damage to the fruit. These trials, along with prior trials using very similar methods but for only 30 days at a time, show a good degree of protection of the crop with this mass-trapping approach, and over a broad range of codling moth populations.

Prior studies showed that females of all reproductive states/ages are attracted to our 3-chemical lure, and with the greatest value to the capture of females that have not yet oviposited or still possess much of their egg potential. These are represented in the trap catches as females that are unmated (no spermatophore) with abundant fat and no eggs, or females that are mated and have mature eggs. We expect then that the trapping of each of those females potentially prevents some oviposition and subsequent apple infestation. The additional trapping of males with the same lure may, or may not, have an impact on subsequent reproduction and damage to apples because a high percentage of males needs to be removed before female mating is impacted. This is why the research, and this discussion, focuses on the attractiveness of lures and effectiveness of traps for females.

These results indicate potential for this approach (mass trapping using lures for females) as one of the tools to use for management of codling moth. While the costs for labor and materials might be prohibitive for use of mass trapping as a long term stand-alone technique, it might be useful as a short term approach to reducing codling moth field populations to levels that can be managed with mating disruption. Additionally, such an approach may be helpful for localized problem areas,

sometimes called “hot spots”. Codling moth populations can become elevated on orchard borders, particularly where adjacent to or near uncontrolled populations such as urban/orchard interfaces, natural areas such as along creeks that may harbor “escaped” apple trees, or abandoned or poorly maintained orchards. A mass-trapping approach may be useful for such limited areas to keep larger orchard acreages under good codling moth control.

These studies and results do not suggest that the methods and materials used are either the best, or most cost-effective. Research conducted to date suggests that there is additional potential for alternative chemical blends in place of acetic acid plus pear ester and N-butyl sulfide. We have been working with alternative and much less expensive controlled release dispensers for the attractants by putting the chemicals in plastic sachets with specifications (type of materials, dimensions, film thickness, etc) to provide good release rates for long periods of time. Research results have indicated that other trap designs may be as effective in capturing attracted moths, while reducing the catch of certain non-target insects, and reducing the costs of traps. Most importantly, the approach worked (controlled codling moth) using these methods and this approach. Further improvements to the lure, the dispenser, and the trap will only further improve the effectiveness of the mass-trapping approach.

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Landolt, P. J., T. S. Davis, B. Oehler, D. Cha, and J. Brunner. 2014. N-butyl sulfide as a co-attractant with kairomones for male and female codling moths, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae). Environ. Entomol. 43: 291-297.

Executive Summary

Significant Outcomes
This study demonstrated control of damage to apples by codling moths with the use of 50 traps per acre, all baited with a kairomone attractant for female as well as male moths.

Summary of Findings.
Important specifications to the treatment were 50 traps per acre, in 4-acre blocks, an attractant comprised of acetic acid, N-butyl sulfide, and pear ester, an optimized controlled release dispenser system, Delta type traps with a sticky liner, and maintenance of traps and lures from first moth flight to just before harvest. These specifications were worked out or determined in earlier related WTFRC projects.
Reductions in damage to fruit by codling moth larvae were determined at mid season and end of season, by comparing treated and control blocks of apple orchards. End of season differences between treatment and control blocks indicated 75 to 90% reduction in damage to fruit by codling moth.
Direct comparisons of the kairomone attractant (acetic acid, N-butyl sulfide, and pear ester) dispensed from polypropylene vials and septa versus plastic sachets (bags) and septa, repeatedly showed a comparable performance of both controlled release approaches.

Future Directions.
Future directions could involve additional research, as well as technology transfer activities to make the technology and approach available to growers.
Research: There is reason to speculate that apple odor chemistry might suffice to replace the pear ester of the lure, for example, and if that is deemed advantageous. Additional field studies should be conducted to evaluate the technology and approach under a range of codling moth pest circumstances, such as “hot spots” caused by immigration from external sources, populations that have escaped control by mating disruption. Mass trapping with the kairomone lure should also be evaluated in combination with other soft approaches such as mating disruption, banding, virus, nematodes, and mass trapping of males with sex pheromone.
Technology transfer: Collaborative research and development will be needed with one or more companies. Such R & D will likely involve the pursuit of less expensive technologies (trap and lure), while maintaining effectiveness. Our demonstrations of efficacy of dispensing the attractant from inexpensive sachets to replace the polypropylene bottles, is one example of how that might work. Another possibility is to replace the Delta trap with a one-piece disposable cylinder trap. Additional field studies will be needed to proof the efficacy of any controlled release dispenser or trap replacement technology. Products and their application to pest control are regulated and costs and efforts will be needed for licensing.

FINAL PROJECT REPORT

Project Title: Economic Impact of Apple Maggot Infestation in Washington

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Total Project Funding:

Budget History:

Item	**2016**
Salaries	$ 18,722
Benefits	$ 5,834
Wages	
Benefits	
Equipment	
Supplies	$ 2,226
Travel	$ 4,105
Plot Fees	
Miscellaneous	
Total	$ 30,887

Recap original objectives

(1) To calculate the current direct short-term costs associated with apple maggot (*Rhagoletis pomonella*), -hereafter AM- in quarantine and non-quarantine areas for conventional and organic apple production.

(2) To estimate the indirect and induced costs of a potential AM spread in non-quarantine areas for the Washington State's economy.

Significant findings

- Direct costs of a potential AM infestation include additional chemical costs, storage cost and price effects.
- Higher losses in profits are observed for,
  - Conventional apple orchards with low CM pressure compared to moderate and high CM pressure in AM affected areas 1 and 2 (see Table 2).
  - Fuji compared to other apple varieties included in this study in all three AM affected areas.
- If there is a 100% loss of AM-free areas, the total output value of the Washington apple industry decreases by $547 million due to AM control requirements in an area of low CM pressure; $557 million due to AM control requirements in an area of moderate CM pressure; and $510 million due to AM control requirements in an area of high CM pressure. These losses are derived in comparison to their respective initial total output values (i.e., status quo).

Results & discussion

Data and assumptions

1. Representation of apple production costs and returns
   - Enterprise budget of Red Delicious – low value apples
   - Enterprise budgets of Fuji and Gala – mid-value apples
   - Enterprise budget of Honeycrisp – high value apples
2. Four areas were identified according to AM status: (1) AM threatened and quarantine, (2) threatened and non-quarantine, (3) non-threatened and quarantine, and (4) non-threatened and non-quarantine. Table 1 shows the information on the commercial apple orchards acreage located in each four areas identified, as of 2015 (WSDA Natural Resource Assessment Section, NRAS). "Threatened" means that the orchard boundary is within half a mile of an apple maggot detection that has occurred within the past 3 years (2012-2015). "Non-threatened" means that apple maggot has not been detected within a half mile of the orchard boundaries in the past 3 years (2012-2015). "Quarantine" refers to areas where the pest is established; "established" means present in an area, multiplying and expected to continue (Klaus, 2016). 0.83% of conventional and 0.24% of organic orchard acreage are located in a threatened and quarantined area. 85% of the conventional and 97% of organic apple orchard acreage are located in a non-threatened and non-quarantine area (Table 1).

Table 1. Apple orchard acreage by threat and quarantine status due to apple maggot.

Threat status of orchards (WSDA)	Quarantine status (WSDA)	Conventional apple orchards		Organic apple acreage orchards	
		Acres	% of total	Acres	% of total
AM Threatened [1]	Quarantine	1,351	0.83%	42	0.24%
AM Threatened	Non-quarantine	184	0.11%	0	0%
Non-threatened	Quarantine	23,224	14.21%	471	2.69%
Non-threatened	Non-quarantine	138,683	84.85%	17,007	97.08%
	Total	**163,442**	**100%**	**17,519**	**100%**

Source: WSDA NRAS, WSDA Pest Program (Personal communication, 2016).

For the study, we classify the areas (i.e., Area 1 through Area 4) according to the threat and quarantine status because the direct costs of AM infestation will not be the same across these four areas in terms of additional chemicals to control for AM and additional days in cold storage (see Table 2).

Table 2. Checklist of additional costs due to AM infestation by area.

Type of area	Status	Additional chemical costs	Additional storage costs[1]
Area 1	Threatened and quarantine	✓	✓
Area 2	Threatened and non-quarantine	✓	-
Area 3	Non-threatened and quarantine	-	✓
Area 4	Non-threatened and non-quarantine	-	-

[1] Includes the charge for 40 days of cold storage and the subsequent decline in the price of apples.

3. Direct costs of AM infestation in commercial Washington orchards are represented by two categories: additional chemical costs associated with AM threatened areas, and storage costs associated with AM quarantined areas.
   - WSU apple enterprise budgets were used as baseline. The baseline included control costs for CM but not AM. Therefore, we treat chemicals to control AM as additional cost to the growers. Entomologist Dr. Jay Brunner designed pest control scenarios for AM. The control window for AM overlaps with the control window for second and third generation codling moth (CM), in case second and third generation happens.

   - As the CM pressure increases, the growers spray more times to control for CM, leading to fewer sprays to control AM. The additional AM spray costs should be small when CM pressure is high, and high when CM pressure is low. Because we cannot assert with certainty the type of CM pressure an apple orchard faces, three different management scenarios to control CM were used considering a low, moderate, and high CM pressure. For each level of CM pressure, the costs of chemicals (materials and application) for additional sprays to control AM are estimated (Table 3). Also, the chemical control costs vary depending on the different harvest dates across varieties included in this study. We estimated the chemical costs for early season harvest varieties (Gala), mid-season harvest (Red Delicious, Honeycrisp) and late season harvest (Fuji). Under high CM pressure there are no costs associated with organic apples, regardless of the variety, because it is assumed that growers will not produce organic apples if CM pressure is high.

   - When estimating impact of AM to the WA economy, the costs depicted in Table 3 are considered for apples produced in AM threatened Area 1 and Area 2.

Table 3. Additional chemical (materials and application) cost for controlling AM under different degrees of CM pressure, $/acre

CM Pressure	Red Delicious	Gala	Fuji	Honeycrisp	Organic Red*	Organic Gala*	Organic Honeycrisp*
Low	$370.46	$257.50	$396.53	$370.46	$111.19	$111.19	$111.19
Moderate	$199.91	$86.92	$225.97	$199.91	$111.19	$111.19	$111.19
High	$0.00	$0.00	$26.07	$0.00	-	-	-

*All organic apples (both low and moderate CM pressure) have two applications of Entrust to control for potential AM infestation. Both applications are applied on or before the first week of August. Entrust is the product applied and has a 4 hour re-entry interval.
Source: Authors' estimates from AM pest control strategies provided by J. Brunner (Personal communication, 2016).

4. Besides the chemical costs, AM quarantine implies additional storage costs for apples shipped to Alberta (AB)/British Columbia (BC) in Canada, and China. These export destinations require apples coming from AM free zone, or having additional 40 days in storage at 1C, if they are grown in an AM quarantined area. Note that Alberta is included in the analysis to account for additional transportation costs as apples being shipped from Washington to Alberta are assumed to go through British Columbia.

   The storage cost for extra 40 days at 1C is $11 per bin. Exports to AB/BC and China represent 1.74% of total WA apple exports (based on 5-year average from 2012/13-2015/16 marketing seasons) (WSTFA, 2016). We take these percentages into account when estimating the total revenues and storage costs for apples produced in AM quarantined areas (Area 1 and Area 3).

5. The additional days in storage also cause a delay in exporting apples to AB/BC and China markets. It is assumed that if apples are not exported, importers will find other sources of apples (e.g., California) or other products that might replace apples to maintain their customer base in those destinations. As a result, WA apples will lose shelf space in these locations and exporters of apples will lower their price in order to entice the importers to purchase the WA apples when they come out of storage (F. Scarlett, personal communication, 2016). This study assumes a decrease in price of apples due to delay in shipment, and the estimation of the price decrease is further discussed below.

   WA apples that come out of storage will likely add to those quantities that are to be shipped in a given schedule (see shipment schedule in Table 4). For instance Gala quantities coming out of storage will be added to the Gala quantities actually scheduled for shipment in October, which means that the total export supply of Gala for that month will be in excess.

Table 4. Timing of harvest and export shipment of apples after 40 days in storage.

Apple varieties	Harvest schedule	End of 40-day storage	Shipment schedule
Gala	August 15	September 24	October
Red Delicious	September 15	October 15	November
Fuji	October 15	November 24	December

We estimated the elasticity of apple supply by month for Gala, Red Delicious, and Fuji to see how sensitive prices would be if there is excess supply. The supply elasticity estimates are -1.04% in October for Gala, -1.02% in November for Red Delicious, and -1.81% in December for Fuji. The supply elasticity estimate is interpreted as follows: every 1% change in the quantity of fresh shipments of Gala, leads to a -1.04% change in the price of Gala in October. The supply elasticity estimates for Red Delicious and Fuji are interpreted in the same manner.
The price discounts are applied on the month that the apples are shipped to AB/BC and China. The supply elasticity estimate is multiplied by the percentage of exports to AB/BC and China to derive the price discounts; for instance, the price discount for Gala apples shipped in October is -1.81% (i.e., -1.04% times 1.74%). The estimated price discounts after 40 days of storage for other apple varieties are shown in Table 5.

Table 5. Percentage of price reduction (5-year average from 2011/12-2015/16 marketing seasons) 40-days after harvest for each apple variety.

Red Delicious	Gala	Fuji	Organic Red Delicious*	Organic Gala*
1.78%	1.81%	3.16%	1.78%	1.81%

*Assumed to be the same as the conventional apple variety.

6. Growers who export, pay for a phytosanitary certificate regardless of where their operation is located (e.g., AM threatened or quarantined area). The cost of the phytosanitary certificate is included in the baseline costs.

7. In an alternative scenario where the entire apple production region is AM threatened and quarantined, the same additional costs will be incurred on AM chemicals, and/or storage cost, and price discounts as described above. However, there will be an additional cost for the Apple Pest Certification, which is $0.01875 per cwt net yield (M. Klaus, personal communication, 2017).

Partial budget results

AM control cost for individual WA apple operations
We compared the profits of apple operations growing in the three AM affected areas described in Table 1 with the best-case scenario, that is, when apples are produced in an area that is neither threatened nor quarantined (Area 4). Higher losses in profits due to a potential AM infestation are observed for apple orchards with low CM pressure, compared to moderate and high CM pressure, for both conventional and organic apples, in AM affected areas 1 and 2. This is mainly due to the higher costs incurred in controlling AM given a low CM pressure, compared to the moderate and high levels of pest pressure (see Table 3).

Higher losses in profits are observed for Fuji compared to the other apple varieties included in this study — when quarantined costs (storage cost and price decline) are considered (Area 1 and 3) due to Fuji's higher net yields relative to Red Delicious and Gala; and when AM threatening costs (additional chemical sprays for AM) are included (Area 1 and Area 2) since Fuji gets more chemical sprays for AM compared to other apple varieties.

Losses in profit for organic apples is the same in low and moderate CM pressure because in both degrees of pest pressure, there are two applications of Entrust to control for potential AM infestation. The detailed partial budgets are presented in Appendix A. The summary of partial budgets is presented in Table 6.

Table 6. Profit loss per year of full production ($/acre) for a representative block of different apple varieties due to AM infestation that involves additional chemical costs, storage costs and price decline for three areas.

	AM threatened, quarantined (Area 1)			*AM threatened, non-quarantined (Area 2)*			*AM quarantined, non-threatened (Area 3)*
	Low CM	Moderate CM	High CM	Low CM	Moderate CM	High CM	
Red Delicious	-$497	-$269	-$2	-$495	-$267	$0	-$2
Gala	-$370	-$127	-$3	-$367	-$124	$0	-$3
Fuji	-$568	-$326	-$41	-$565	-$322	-$37	-$4
Honeycrisp*				-$404	-$218	$0	
Org. Red Del.	-$150	-$150		-$149	-$149		-$2
Org. Gala	-$161	-$161		-$158	-$158		-$3
Org. Honeycrisp*				-$171	-$171		

*Not included in the analysis for Area 1 and Area 3 because Honeycrisp is not among apple varieties exported to AB/BC Canada and China; hence it does not require storage.

IMPLAN analysis results – AM spread cost for WA economy

Economic contributions of the Washington apple industry

To estimate the economic impact of an increased risk of an expansion of AM threatened and quarantined areas, we calculate the economic contribution of the apple industry to the WA economy. We used data from the WSU enterprise budgets. To estimate the aggregate effects of losses in profit depicted in Table 6, we considered the acreage of the 4 areas affected by AM as depicted in Table 1. For each AM area, we assume a variety mix acreage distribution similar to the WA acreage distribution. We also used the data from Globalwise, Inc. and Belrose, Inc. (2014) study as basis for the economic contributions of the fresh apple packing and processing industries, and the 2015 IMPLAN input-output (I/O) data. Second, we estimate the contributions of the apple industry to the WA economy under each degree of CM pressure: low, moderate and high. To estimate the economic contribution for 2017, we used the built-in GDP deflator in IMPLAN for 2017.

The estimated contributions of the apple industry to the WA economy go beyond the $2 billion value of fresh apple sales figure. Because we take into consideration the 3 sectors of the apple industry: apple production at the field level, fresh apple packing, and apple processing. Moreover, the contributions include direct effects, indirect effects, and induced effects. The direct effects are the immediate effects related to production, packing, and processing of apples. Indirect effects include changes arising from inter-industry transactions as supplying industries respond to the demand from the directly affected industry. Induced effects include the effects due to the household consumption expenditures by employees in the directly and indirectly affected industry sectors. IMPLAN reports the on employment, labor income, value added, and total output. Table 7 shows the Washington apple industry's estimated economic contributions. IMPLAN employment is the number of jobs, including full time, part time and temporary jobs, created after the apple industry.

Table 7. Estimated contributions of the apple industry* to the Washington State economy, 2017.

Variables	Impact type			
	Direct effects	Indirect effects	Induced effects	Total effects
Low CM Pressure				
Employment (number of jobs)[1]	37,357	25,487	13,588	76,431
Labor income ($ billion)[2]	$1.29	$1.30	$0.67	$3.27
Total Value Added ($ billion)[3]	$1.51	$1.84	$1.23	$4.58
Total Output ($ billion)[4]	$3.95	$3.42	$2.10	$9.47
Moderate CM Pressure				
Employment (number of jobs)[1]	38,273	25,609	13,536	77,418
Labor income ($ billion)[2]	$1.28	$1.31	$0.67	$3.26
Total Value Added ($ billion)[3]	$1.49	$1.86	$1.23	$4.57
Total Output ($ billion)[4]	$3.95	$3.44	$2.09	$9.48
High CM Pressure				
Employment (number of jobs)[1]	35,957	24,821	12,922	73,700
Labor income ($ billion)[2]	$1.19	$1.27	$0.64	$3.11
Total Value Added ($ billion)[3]	$1.41	$1.81	$1.17	$4.39
Total Output ($ billion)[4]	$3.81	$3.38	$2.00	$9.19

*Sum of economic contributions by the apple farming, fresh apple packing, and processed apple production sectors.
Definition (Source: IMPLAN):
[1] Employment = number of jobs.
[2] Labor income = employee compensation + proprietor income.
[3] Value added = labor income + proprietor income + other property income + indirect business taxes.
[4] Output = intermediate expenditures + value added.

Labor income is comprised of employee compensation (wages, salaries and benefits) and proprietor income (i.e., payments received by self-employed individuals and unincorporated business owners; includes capital consumption allowance and is recorded on Federal Tax form 1040C). Labor income totaled $3.27 billion of personal income to the WA economy at a low CM pressure in the commercial apple orchards; $3.26 billion at moderate CM pressure; and $3.11 billion at a high CM pressure (Table 6). The total value added is the sum of employee compensation, proprietor income, other property-type income and taxes. There are $4.58 billion in value added contribution to the WA economy at a low CM pressure; $4.57 billion at moderate CM pressure; and $4.39 billion at high CM pressure.

The direct output values of the WA apple industry are estimated in the range of $3.81 billion to $3.95 billion depending on the degree of pest pressure in the apple orchards. Due to inter-industry linkages, the total economic contribution of the apple industry, for example given moderate CM pressure, is $9.48 billion — comprised of 42% direct output, and 58% of output from other sectors within the State (the sum of indirect and induced effects). The total output multiplier for the apple industry is about 2.40 (i.e., total effects ÷ direct effects). This estimate implies that for every dollar of fresh apple packing and apple processing, about $2.40 is generated in the local economy; that is for every apple industry dollar, an additional $0.67 is generated in sectors providing inputs to the apple industry (indirect effects), and an additional $0.73 is earned by businesses providing goods and services to employees of the apple industry and indirectly affected sectors (induced effects). Note that the values reported in Table 7 are aligned with Globalwise, Inc. and Belrose, Inc. (2014) reports at $3.35 billion in direct effects, $3.67 billion in indirect and induced effects, and $7.03 billion in total effects.

Impacts of a further AM infestation on the WA economy

We examine the economic impacts considering a 100% loss of the AM free areas — that is all apples are grown in AM threatened and quarantined area, and compare to the status quo situation (distribution of apple acreage according to Table 1). This means that instead of only 0.8% of the conventional and 0.2% of the organic apple acreage incurring additional chemical costs for AM, storage costs, and price discounts, 100% of all apple acreage will incur all these additional costs. Furthermore, there is an additional apple pest certification fee per hundredweight of net yield.

Results indicate that if a complete loss of the AM free areas happens, the losses in total output value (direct, indirect, and induced) due to AM are approximately $547 million when there is low CM pressure; $557 million when there is moderate CM pressure; and $510 million when CM pressure is high, relative to their respective initial apple industry values (Table 8).

Table 8. Reduced output value[1] of the WA apple industry, considering 100% of the WA apple-producing areas are AM threatened and quarantined (100% loss of AM free area).

Degrees of pest pressure	Output value ($ billion)				Losses in total output value if 100% loss of AM free area ($ million)*	Percent reduction compared to initial apple industry value*
	Direct	Indirect	Induced	Total		
Low CM pressure	$3.69	$3.34	$1.90	$8.92	-$546.93	-5.78%
Moderate CM pressure	$3.69	$3.34	$1.90	$8.92	-$557.23	-5.88%
High CM pressure	$3.58	$3.27	$1.83	$8.68	-$509.78	-5.55%

*Change with respect to the total output value in Table 7 given different degrees of pest pressure.

Assuming a progressive loss of AM free areas, for example if 20%, 40%, 60% and 80% (instead of 100%) of the apple production region are both AM threatened and quarantined, the losses faced are listed in Table 9 and illustrated in Figure 1.

Table 9. Progressive loss of AM free areas — 20%, 40%, 60% and 80% of the apple production region are both AM threatened and quarantined.

Scenario	Low CM pressure			Moderate CM pressure			High CM pressure		
	Output value ($ billion)	Output value reduction relative to baseline ($/billion)	Percent reduction relative to baseline	Output value ($ billion)	Output value reduction relative to baseline ($/billion)	Percent reduction relative to baseline	Output value ($ billion)	Output value reduction relative to baseline ($/billion)	Percent reduction relative to baseline
Baseline	$9.47			$9.48			$9.19		
20% TQ	$9.36	-$0.11	-1.12%	$9.37	-$0.11	-1.14%	$9.09	-$0.10	-1.07%
40% TQ	$9.25	-$0.22	-2.28%	$9.26	-$0.22	-2.33%	$8.99	-$0.20	-2.19%
60% TQ	$9.14	-$0.33	-3.45%	$9.15	-$0.33	-3.51%	$8.88	-$0.30	-3.31%
80% TQ	$9.03	-$0.44	-4.61%	$9.03	-$0.45	-4.70%	$8.78	-$0.41	-4.43%
100% TQ	$8.92	-$0.55	-5.78%	$8.92	-$0.56	-5.88%	$8.68	-$0.51	-5.55%

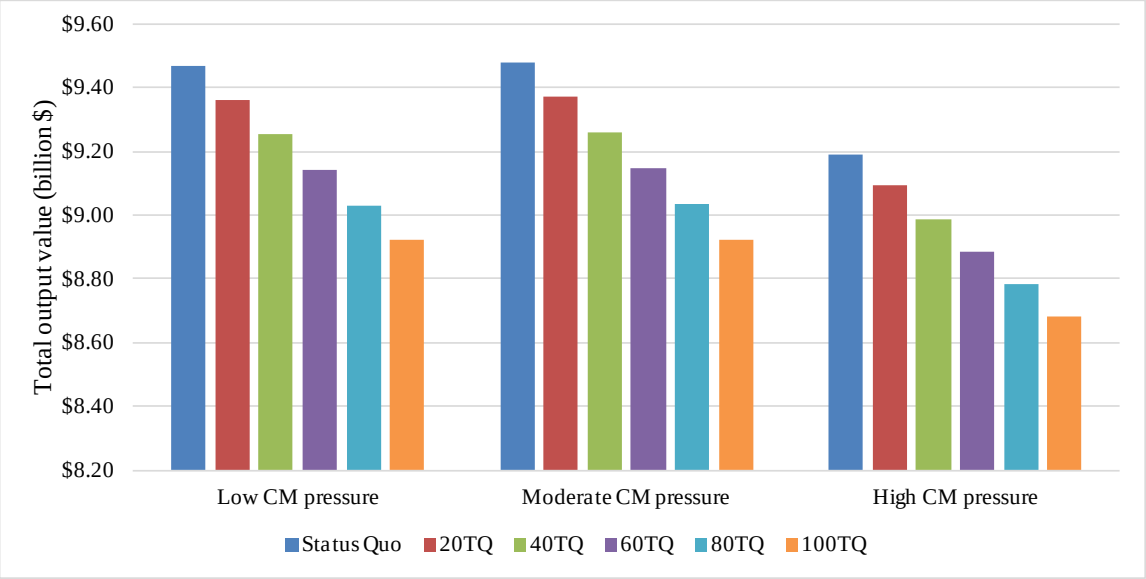

Figure 1. Changes in total output value of the apple industry given different percentages of apple production region that are threatened and quarantined due to AM and under different degrees of CM pressures. (Note: Status quo refers to current production areas affected by AM. TQ refers to threatened and quarantined areas.)

These analyses lead us to conclude the importance of an efficient control of codling moth and apple maggot due to the differences to the WA state economy for increases in CM pressure. Also findings revealed how revenues for the apple industry and thus its contribution to the overall WA economy are negatively impacted when apples are produced in quarantine areas. Given the increasing importance of export markets in special destinations such as China and Canada (BC/AB) this study illustrates the potential economic impact when quarantine areas are in risk of increasing.

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Executive summary

The conclusions of the Pest Risk Analysis* for AM moving on municipal green waste into the WA pest free area confirmed the high risk of spreading AM on the commercial fruit production areas as a result of this moving. In this report we estimated the potential costs to the WA economy of a potential spread of AM to the apple commercial production areas. We estimated three major drivers for the additional costs and consequent economic impacts: additional chemical sprays, additional time in storage, and reduction on output prices due to extended storage period required for AB/BC Canada and China.

We identified four areas according to AM status: (1) AM threatened and quarantine, (2) threatened and non-quarantine, (3) non-threatened and quarantine, and (4) non-threatened and non-quarantine. Less than 1% of conventional and organic orchard acreage is located in a threatened and quarantined area. Eighty five percent of the conventional and 97% of organic apple orchard acreage are located in a non-threatened and non-quarantine area. To estimate the additional chemical costs due to a potential AM infestation, we assume that the control window for AM overlaps with the control window for second and third generation codling moth (CM). As the CM pressure increases, already the growers are spraying more and the need for additional spray for AM declines. Thus, the additional cost of AM should be small when CM pressure is high, and relatively higher when CM pressure is low.

Because we cannot assert with certainty the type of CM pressure an apple orchard would face, three different management scenarios to control CM were used: low, moderate, and high CM pressure. Therefore, for each scenario, the costs of chemicals (materials and application) to manage CM are estimated, plus appropriate AM costs. Costs vary depending on the apple variety because of the harvest dates and the different timing of the chemical applications. We estimated the chemical costs for early season harvest varieties (Gala), mid-season harvest (Red Delicious, Honeycrisp), and late season harvest (Fuji). Additional storage costs for apples produced in AM quarantine areas and to be exported to AB/BC Canada and China are considered: (1) cost of additional 40 days in cold storage at $11/bin; and (2) decline in average price received by WA apple growers due to the required extra days in storage. This study assumes that exporters of apples will lower their price in order to entice the importers to purchase the WA apples when they come out of storage.

When comparing the profits of apple operations growing in the three AM affected areas with the best case scenario, Area 4 that is neither threatened nor quarantined, we found that: (1) Higher losses in profits are observed for conventional apple orchards with low CM pressure relative to moderate and high CM pressure in AM affected areas 1 and 2; (2) Losses are the same for organic apple orchards regardless of the degree of pest pressure because the additional chemicals costs for AM and storage costs are the same under low and moderate CM pest pressure; and (3) Higher losses in profits are observed for Fuji compared to other varieties included in the study in all affected areas.

The losses in the WA apple industry's output value due to a further spread of AM infestation to the entire apple production region are estimated at $547 million when there is low CM pressure, $557 million given moderate CM pressure, and $510 million given high CM pressure when compared to their respective initial total output values (i.e., status quo). The magnitude of the impacts of AM infestation on the WA economy depends on the proportion of the apple production area affected. The changes in the WA apple industry's total output value considering 20%, 40%, 60% and 80% of the production region that are both AM threatened and quarantined at different degrees of pest pressure are significantly lower than the changes in the output value if 100% of all apple production region is threatened and quarantined. The main reason is that instead of only some portions of conventional and organic apple acreages incurring additional chemical costs for AM, storage costs, apple pest certification fee, and price discounts, 100% of all apple acreage will incur all these additional costs in order to meet the requirements of exporting to AB/BC Canada and China.

*Sansford, C.E., Mastro, V. Reynolds, J.R., 2016. Pest Risk Analysis (PRA) for Apple Maggot (Rhagoletis pomonella) Moving on Municipal Green Waste into the Pest-free Area (PFA) of the State of Washington USA.

FINAL PROJECT REPORT

Project Title: Importation of the honey bee subspecies that coevolved with apples

PI:	Walter S. Sheppard	**Co-PI**:	Brandon Hopkins
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Total Project Funding:

Budget 1

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

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Item	**2014**	**2015** (No Cost Extension)	**2016**	**2017**
Salaries				
Benefits				
Wages				
Benefits				
Equipment				
Supplies	2,000			3,000
Travel	8,000			
Plot Fees				
Miscellaneous				
Total	$10,000	$0	$0	**3,000**

Notes:

Due to delay in project funding - only one additional year of funding beyond the start year was provided by WSTFRC over the 3-year period – total of $13,000

Objectives

1) Collect, cryopreserve and import semen from a diverse selection of *A.m pomonella* honey bee colonies in the apple forests of Kazakhstan and Kyrgyzstan. Significant findings - completed collection of honey bee germplasm from *A. m. pomonella* as planned from Kazakhstan. Some difficulty to retrieve cryopreserved germplasm though Kazakh customs and air shipment but managed to retrieve all materials. Entered USDA-quarantine protocol and were released as virus acceptable.
2) Following USDA-APHIS quarantine procedures, *pomonella* stocks will be recovered through backcrossing, undergo selection in Washington conditions and distributed to California queen producers for propagation. Significant findings- Inseminated queen bees containing imported genetic material were released from quarantine and one year of backcrosses to cryopreserved semen have been performed. Instrumentally inseminated *pomonella* stocks are being overwintered in 2017-2018 and will be further propagated in 2018 for distribution to producers. In 2018, *pomonella* stocks will also be used in research to evaluate mating behavior in inclement (colder) weather compared to currently available commercial US stocks.

Results and Discussion

The objectives outlined in the proposal were completed fully. WSU now has both a working stock of *A m pomonella* honey bees to use for further evaluation and field testing and a resource of cryopreserved honey bee semen from this subspecies in the WSU honey bee germplasm repository. This project represents an initial step in fundamental research on the honey bee subspecies that co-evolved with apples. The work by WSU in this area represents the single largest effort in adding genetic diversity to US honey bee populations, given the restrictions placed on importation of honey bee genetics that accompanied the Honey Bee Act of 1922. The main importance of this work is to assist in the stabilization and development of a sustainable beekeeping industry and tree fruit pollination system.

Executive Summary: No executive summary submitted

CONTINUING PROJECT REPORT **YEAR**: 1 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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Cooperators: Multiple growers and packers in Washington

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: WSU-TFREC **Contact Administrator:** Katy Roberts; Joni Cartwright
Telephone: 509-335-2885; 509-663-8181 x221 **Email:** arcgrants@wsu.edu;joni.cartwright@wsu.edu

Item	2017	**2018**	2019
Salaries[1]	12,635	9,371	0
Benefits	4,449	3,358	0
Wages	0	0	0
Benefits	0	0	0
Equipment[2]	13,735	0	0
Supplies[3]	4,000	2,000	3,000
Travel[4]	392	700	800
Miscellaneous	0	0	0
Plot Fees	0	0	0
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.

- Fortunately, enough genetic variability was found in the β-tubulin gene to sign specific primers for all 4 bull's eye rot species, i.e. *Neofabraea perennans*, *N. malicorticis*, *N. alba*, and N. *kienholzii* and for the speck rot pathogen-*Phacidiopycnis washingtonensi*.

- These primers are being tested for specificity in pathogen detection using pure DNA of each pathogen in laboratory conditions.

- 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.

- While field detection on cankers and twig dieback will be started in spring and summer of 2018 (objective 2a), detection of the 5 fungi on inoculated apples is being planned and should be conducted as soon as the primers developed in Objective 1.

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.

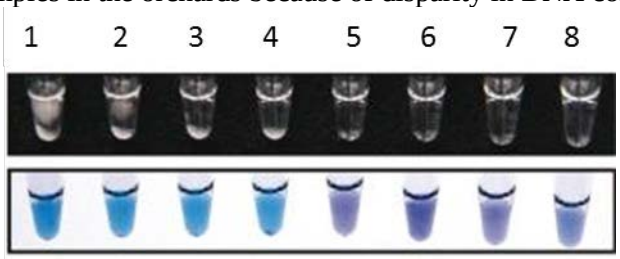

Figure 1. Example of detection sensitivity using LAMP assay based on tubidity (top) (note differences between samples 1 to 4) and fluorescent dye (bottom).

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

Primer design and LAMP assay optimization

The most critical point for the LAMP assay is primer design. Because its requires 6 primers compared to only 2 primers for regular Polymerase Chain Reaction (PCR) assay, this step can be challenging is the DNA sequence is not polymorphic enough to design the primers especially for species that are genetically so close such as *Neofabraea* species.

Now that our primers have been designed and obtained for all the 5-species included in this study, DNA of these fungi has been extracted and the primers are currently being tested for detection.

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 warehouse detection of pathogens.

CONTINUING PROJECT REPORT **YEAR**: 1 of 3
WTFRC Project Number: 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

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

Budget 1
Organization Name: WSU **Contract Administrator:** Katy Roberts/Joni Cartwright
Telephone: 509-335-2885/509 -663-8181 **Email**: arcgrants@wsu.edu/joni.cartwright@wsu.edu

Item	2017	**2018**	2019
Supplies[1]	23,609	2,000	2,000
Travel[2]	1,037	1,037	1,037
Plot Fees	0	0	0
Miscellaneous	0	0	0
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]	0	0	0
Miscellaneous	0	0	0
Plot Fees	0	0	0
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 a field scale experiment on 12 acres to test the efficacy of bio-renovation and anaerobic disinfestation as alternatives to soil fumigation for the control of apple replant disease. At the 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

- In the post-treatment bioassay, Gala seedlings grown in *Brassica* seed meal treatment soils performed comparable to pasteurized (simulating fumigation) control and superior to the no treatment control. Anaerobic soil disinfestation resulted in seedling growth that was comparable to the pasteurized control, but was less than seedling growth attained in *Brassica* seed meal amended soil (Figs. 1-3).
- Lesion nematode (*Pratylenchus penetrans*) root densities were lower in seedlings planted in *Brassica* seed meal treated soils compared to the control and were comparable to the nematode densities recovered from seedlings planted in the pasteurized control. Lesion nematode root densities for seedlings grown in in the anaerobic soil disinfestation treated soils were lower but not significantly different than the control (Fig. 4).
- Root infection by additional replant pathogens, including *Rhizoctonia* and *Ilyonectria* spp., were significantly reduced by all soil treatments relative to the no treatment control (Fig. 5)
- The cumulative anaerobicity (cumulative mV hours with reduction potential (*Eh*) below 200 mV) in anaerobic soil disinfestation plots did not reach the target level of 50,000 mV hr with the exception of one plot where the target rate was reached for only 20,764 mV hr.
- Analysis of bacterial and fungal populations showed good separation for *Brassica* seed meal treated soils and fumigated soils, but anaerobic soil disinfestation soils had variable populations (figure 6).

METHODS

Experimental site and design: A 12-acre field trial was established at BMR Orchard in Othello WA (46.933876, -119.392096). Soil treatments included mustard (*Brassica*) seed meal biorenovation (BSM), anaerobic soil disinfestation (ASD), pre-plant soil fumigation (Telone-C35), and a no treatment control. The field was divided into four experimental blocks and each treatment, with exception of the control, was implemented in an 0.8 acre plot (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. Soils at the site are an Adkins very fine sandy loam, 0-5 percent slope.

Site preparation: Soils 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.

Triticale biomass measurements: Biomass for use in the application of ASD treatments was produced on-site in the form of triticale. Total biomass resulting from growing tritcale was estimated by cutting and harvesting total plant production from three six-square-foot subsamples per plot. Samples were dried at 80˚ C (176 ºF) and weighed to calculate biomass per acre.

Soil moisture: Volumetric water content (VWC) attained after application of irrigation for ASD treatments was determined by measuring the dielectric constant of the media using capacitance/frequency (EC-5 Decagon Devices). Soil moisture was measured at the commencement of laying plastic for blocks A/B and C/D and as plastic laying finished. Moisture was measured again two hours after blocks were re-wetted.

ORP: ORP measurements were taken with oxidation reduction potential sensors with platinum polished rod double junction (SC500C-ORP, Sensorex, Garden Grove, CA) with measurements recorded every ten minutes for days 1 to 7 and 9 to 20 after tarping. Three probes were placed 10 feet apart in reps B and C, with 2 probes placed in rep D.

ASD treatment: 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 Masschio 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.

Table 1. Field Operations Anaerobic Soil Disinfestation.

Operation	Tractor	Implement/Equipment	Setting	Hrs/A
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
Incorporation	John Deer 7200	Celli spader	all the way down (8" depth)	2
Tarping	Kubota M8540	Mulch later (Mechanical Transplant Co Model 90)	N/A	2

Mustard seed meal treatment: 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 spader 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 2. Field Operations *Brassica* Mustard Meal Disinfestation.

Operation	Tractor	Implement/Equipment	Setting	Hrs/A
Irrigation		Hand lines (R33 sprinklers)	6 gal/min, .28"/hr	12
mustardmeal 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

RESULTS & DISCUSSION

Soil samples were obtained post-treatment application from ASD, BSM and control plots on August 30, 2017 (3 weeks after tarp removal). Plant bioassays were conducted in these orchard soils using 'Gala' seedlings in order to provide preliminary findings as to whether treatments effectively controlled replant disease pathogens. A pasteurized soil control was used as a surrogate for soil fumigation. Seedlings cultivated in *Brassica* seed meal treated soils performed better than the pasteurized control and the no treatment control. While plants cultivated in soils receiving the anaerobic soil disinfestation treatment had significantly higher plant weight and root weight compared to the no-treatment control, plant performance was inferior to that attained in response to *Brassica* seed meal treatment (Figs. 1-3). Lesion nematode root densities were lower in seedlings cultivated in *Brassica* seed meal treated soils compared to the control and statistically similar to that attained in response to soil pasteurization. Lesion nematode root densities for seedlings grown in the anaerobic soil disinfestation treated soil were lower but not significantly different than the control (Fig. 4). All soil treatments reduced seedling root infection by *Rhizoctonia* and *Ilyonectria* spp., replant complex fungi (Fig. 5). There was no significant difference among treatments in recovery of non-pathogenic *Fusarium* spp. from seedling roots. The soil cumulative anaerobicity measured in anaerobic soil disinfestation plots did not reach the target level of 50,000 mV hr with the exception of in one plot where the target rate was reached for only 20,764 mV hr. 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 soil fumigation treatments relative to the no treatment control. However, microbial communities in anaerobic soil disinfestation soils exhibited significant variation and demonstrated greater relation to the control than corresponding communities from either of the other soil treatments (Fig. 6).

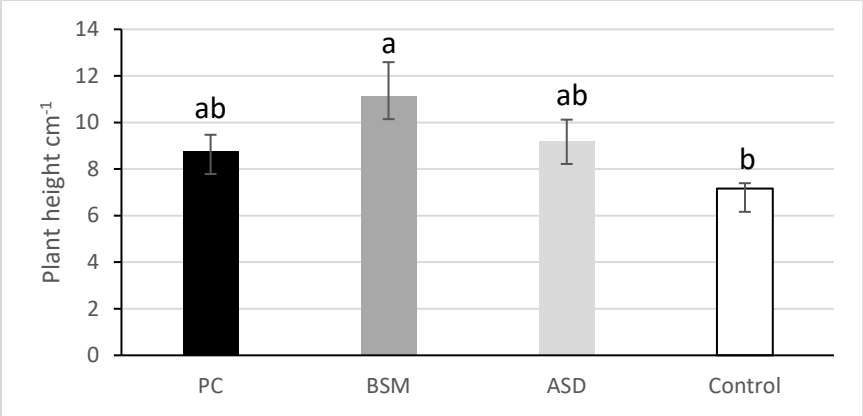

Fig. 1. Growth of Gala seedlings in a plant bioassay conducted in soils treated at the experimental field site. Treatments: Pasteurized control (PC), *Brassica* Seed Meal (BSM), Anaerobic Soil Disinfestation (ASD).

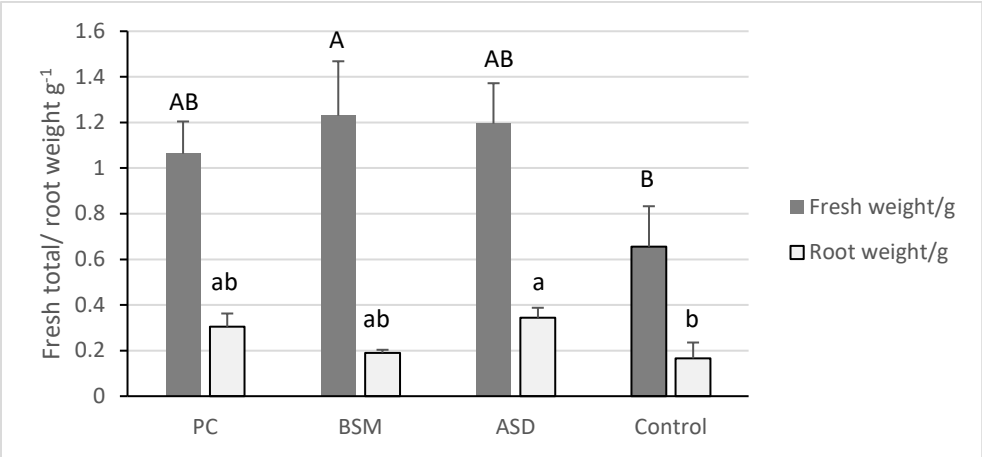

Fig. 2. Effect of field treatments on growth of Gala seedlings grown post-treatment soils. Treatments: Pasteurized control (PC), Brassica Seed Meal (BSM), Anaerobic Soil Disinfestation (ASD).

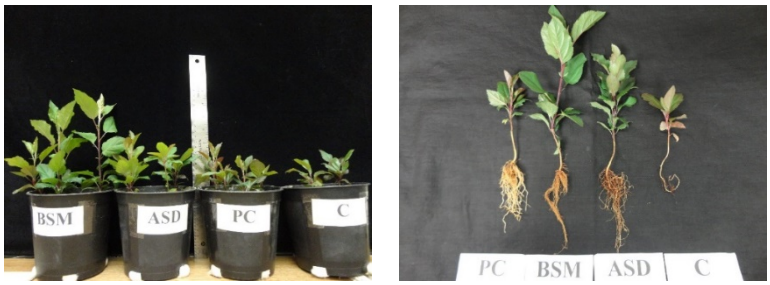

Fig. 3. Example of plant growth attained in seedling bioassays. Treatments: Pasteurized control (PC), Brassica Seed Meal (BSM), Anaerobic Soil Disinfestation (ASD).

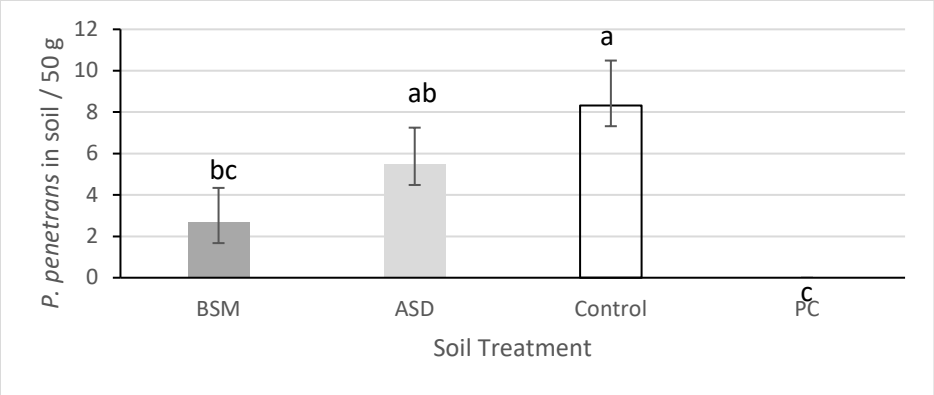

Fig. 4. Effect of soil treatments on lesion nematode root densities. Treatments: Pasteurized control (PC), *Brassica* Seed Meal (BSM), Anaerobic Soil Disinfestation (ASD).

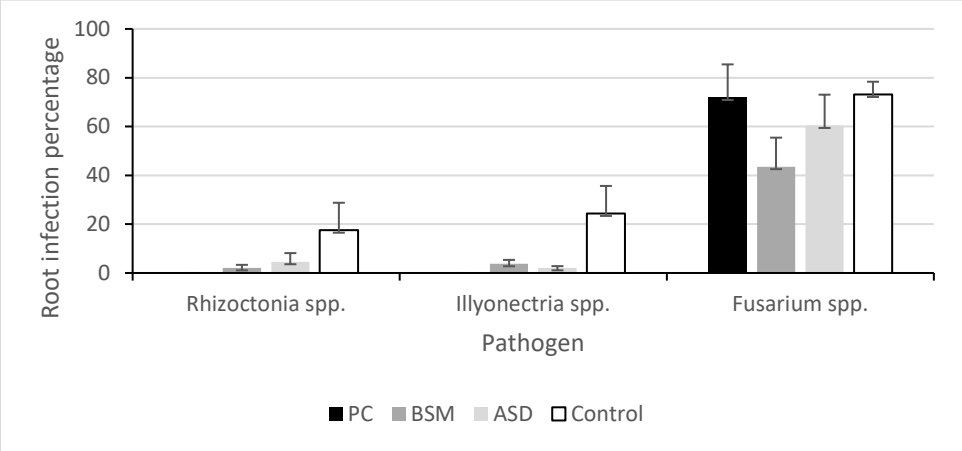

Fig. 5. Relative recovery (%) of fungal genera from Gala apple seedlings in plant bioassay. Treatments: Pasteurized control (PC), Brassica Seed Meal (BSM), Anaerobic Soil Disinfestation (ASD).

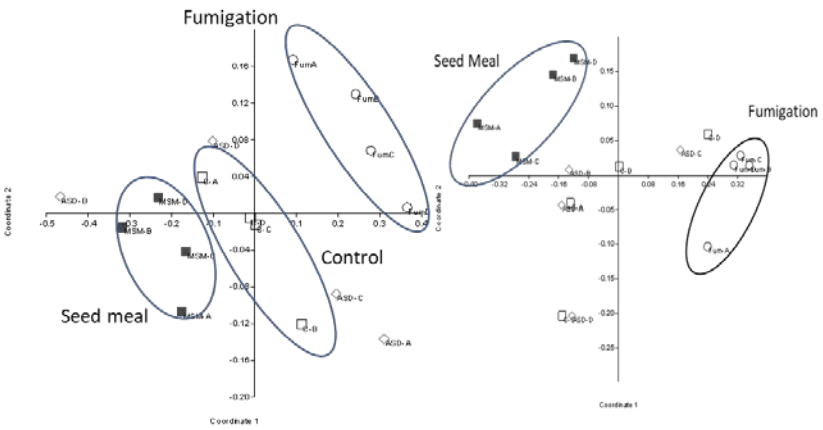

Fig. 6. Effect of soil treatment on relative similarity of bacterial (left panel) and fungal (right panel) communities recovered from orchard field soils. Communities were characterized by terminal restriction fragment length polymorphism analysis of amplicons generated targeting the bacterial 16S rRNA gene and the internal transcribed spacer region of fungal rRNA genes. Treatments: Control (C), *Brassica* Seed Meal (MSM), Anaerobic Soil Disinfestation (ASD), Telone-C35 fumigation (FUM).

CONTINUING PROJECT REPORT **YEAR**: 1 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:** $84,826

Other funding sources: None

Budget 1
Organization: WSU-TFREC **Contract Administrator:** Katy Roberts/Joni Cartwright
Telephone: 509-335-2885/509-663-8181 x221 Email: arcgrants@wsu.edu/joni_cartwright@wsu.edu

Item	2017	**2018**	2019
Salaries[1]	45,000	46,800	48,672
Benefits[2]	17,069	17,751	18,461
Wages	9,600	9,984	10,383
Benefits[3]	259	270	280
Equipment	0	0	0
Supplies[4]	2,500	2,600	2,704
Travel	4,000	4,160	4,326
Miscellaneous	0	0	0
Plot Fees	0	0	0
Total	78,428	**81,565**	84,826

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:
- Results of our spray timing trial for improved obliquebanded leafroller (OBLR) management did not yield useful data for updating WSU's DAS OBLR spray recommendations in 2017. OBLR did not develop in our test site.
- A partial DD model had been validated for WTLM. A similar model for its major parasitoid, *Pnagalio flavipes*, could not be assessed because too few parasitoids were captured on traps.
- Monitoring for western tentiform leafminer (WTLM) in adjacent commercial and organic orchards showed similar numbers of male WTLM captured in pheromone traps. However, WTLM damage to leaves was only observed in organic apple orchard treated with the high label rate of Entrust SC.
- Evaluating spray records from several organic orchards showed that Entrust SC is commonly used for early OBLR management. These orchards often have populations of WTLM develop in the orchard late-season.
- Use of Entrust in organic orchards does not always lead to outbreaks of secondary pests. However, we suspect that ill-timed applications of Entrust SC in organic orchards is responsible for inducing outbreaks of other pests including WTLM, white apple leafhopper and brown mite, because it is toxic to their natural enemies.

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 OBLR using conventional and organic insecticides. The treatments for both objectives were combined into one experiment because there was some overlap of treatments and the research site was large enough to accommodate a combined study.

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* 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.

For conventional OBLR pest management, our data from a past study showed that leaves treated with a broad range of larvicides during the summer are extremely long (>50 days at >85% mortality). This makes the window starting at 900 DD relatively easy to cover with a single application. However, the shoot growth during this time essentially causes the plant to "dilute" the residue as new terminal leaves are formed which can allow leafrollers tend to move to the newer (and hence, untreated) foliage. Our treatments in conventional orchards will be similar to those above (for treatment 1) because this is a critical period for natural enemy development and use of a conventional material may greater reduction in pest mortality from natural enemies.

The study was conducted in a 10 A 3-yr old orchard consisting of cider apple cultivars 'Dabinette' and 'Yarlington Mill' located in Quincy, WA. The site was chosen because it was a relatively new planting that was vigorous and had not received insecticides the previous year. The trees were planted on a 5' x11' row spacing. Each treatment was replicated four times. Each single treatment replicate consisted of two rows of 12 trees (24 trees per replicate) and was approx. 0.2 A in size. Insecticide sprays were applied at 100 GPA using a tractor mounted Rears PacBlast sprayer driven

through the site at 2 MPH. Both sides of test trees were treated. Most of the replicates were separated from each other by four rows of buffer trees and 35' within rows. The site received sprays for diseases but no additional insecticides were applied.

Sprays were timed for either 90 DD_{43}, 900 DD_{43}, or both (Table 1). Some treatments had additional applications to extend coverage during periods of expanding foliage. The 90 DD sprays targeted larvae that overwintered in the orchard. The 900 DD sprays targeted summer generation larvae.

Two pheromone traps baited with OBLR pheromone were placed in this block on 1 May and shecked weekly through the experiment. Lures were replaced weekly.

Results: The treatments were assessed on 15 May, 16 June and 12 July. Assessments consisted of visually scanning each tree within a rep for signs of OBLR (larvae, webbing and/or feeding damage to foliage). No OBLR or damage was observed on any of the sample dates. No adult male OBLR were captured in the traps. The lack of OBLR in this plot caused us to forgo the final applications in treatments 2, 3, 7 and 9.

Work next year: We will continue to work with fieldmen to find sites apple blocks with OBLR so we can test our hypotheses about optimal timing for OBLR management.

Table 1. Insecticides applications targeting obliquebanded leafroller on apple, 2017.

			Application timing DD_{43} [Target DD_{43}] (Date)				
Trmt	Insecticide	Rate/A	Application 1	Application 2	Application 3	Application 4	Application 5
1	*Bt*[1]	1 lb	92 [90] (24 Apr)	+1wk (1 May)	+1wk (8 May)		
2	*Bt* + oil	1 lb + 1 gal	874 [900DD] (30 Jun)	+1wk (7 Jul)			
	Bt	1 lb			+1wk (NA[2])		
3	*Bt* + oil	1 lb + 1 gal	874 [900DD] (30 Jun)	+1wk (7 Jul)			
	Bt	1 lb			+1wk (NA)	+1wk (NA)	
4	Trmt 1 + Trmt 2		92 [90] (24 Apr)	+1wk (1 May)	+1wk (8 May)	874 [900DD] (30 Jun)	
5	Trmt 1 + Trmt 3		92 [90] (24 Apr)	+1wk (1 May)	+1wk (8 May)	874 [900DD] (30 Jun)	+1wk (7 Jul)
6	Delegate + oil (field rate)	7 oz + 1 gal	874 [900DD] (30 Jun)				
7	Delegate + oil (25% field rate)	1.75 oz + 1 gal	874 [900DD] (30 Jun)	+3wk (NA)			
8	Trmt 1 + Trmt 6		92 [90] (24 Apr)	+1wk (1 May)	+1wk (8 May)	874 [900DD] (30 Jun)	
9	Trmt 1 + Trmt 7		92 [90] (24 Apr)	+1wk (1 May)	+1wk (8 May)	874 [900DD] (30 Jun)	+3wk (NA)
10	Control						

[1]Bt = Deliver Biological Insecticide

[2]NA = planned timing but not applied because no OBLR

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

Methods: WTLM was monitored in one organic orchard and an adjacent conventional orchard located in Quincy, WA using pheromone traps baited with the WTLM lure and from weekly leaf samples. The study site was selected because the organic orchard had a WTLM outbreak the year before while the adjacent conventional orchard did not have WTLM problems.

Three pheromone traps were deployed in each orchard before the start of the second WTLM generation and checked weekly. Lures were replaced every four weeks. The pheromone traps are highly active and attract extremely high numbers of males. Unfortunately, the start of the first generation of moths was missed because monitoring did not start until 1 May in the organic block; this was most of the way through the first summer generation. However, we had data from 2013 in an organic block near Brays Landing where WTLM were found in reasonable numbers on panel traps that were monitoring natural enemies, and that data was used to estimate the flight curve for the first generation and confirm the flight curves for the subsequent generations.

Leaf samples consisting of 450 leaves per orchard were collected weekly starting on 24 Apr and examined for WTLM mines in the field until after harvest (leaf sampling ended 24 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.

Three yellow sticky card traps were also placed in the organic block on 24 Apr and in the conventional block on 30 May. Traps were changed weekly until 24 Oct.. They were assessed in the laboratory under a dissection scope for adult parasitoids of WTLM, primarily *Pnigalio flavipes*. Later, we went back and counted the number of adult white apple leafhoppers, *Typhlocyba pomaria* McAtee (WALH), on each yellow sticky card trap when it became apparent that there was an outbreak of this pest in the organic block.

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. There were comparable numbers of adult male WTLM captured in pheromone traps in both the organic and conventional orchard (Fig. 1). However, mines caused by WTLM larvae feeding within the leaves were only found in leaves collected from the organic site from the end of July through harvest while no mines were observed in the conventional site. The preliminary model (see next session) shows that these larvae came from second generation WTLM, while later flights added to the numbers. The number of sap-feeder (SF) mines (seen only on leaf bottoms) and tissue-feeder (TF) mines (seen from the tops of leaves) peaked in early October (Fig. 2); which came from the fourth adult generation. However, at no point in time did the infestation reach treatment threshold of 5 SF mines per leaf nor did the percent parasitism of mines reach levels high enough to regulate the current or overwintering WTLM population in the organic orchard (Beers et al. 2007).

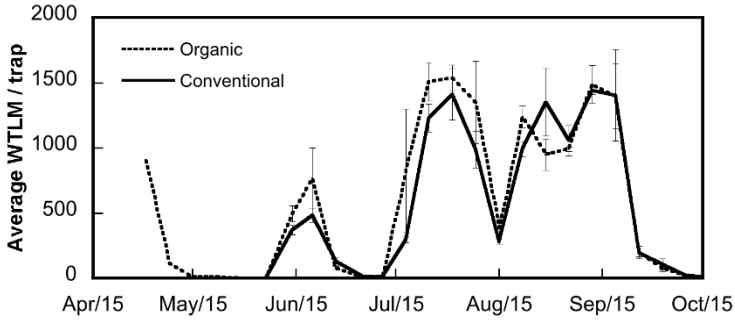

Fig. 1. Average number of adult male WTLM per pheromone trap: 2017.

Preliminary model for WTLM. Evaluation of the data on WTLM development from the work of Barrett (1988) showed the lower threshold for development was 40.6°F and the upper threshold was 82.4°F. The lab studies showed a complete generation should take 1037 DDF. We were also able to digitize the data in the dissertation that showed trap catch from two years data (1985 and 1987) and pair that with weather data from the same locations.

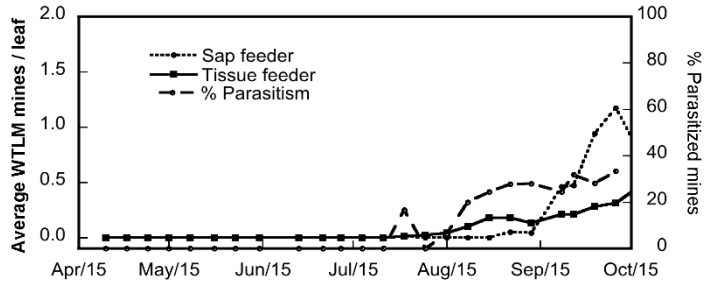

Fig. 2. Average number of sap-feeding and tissue-feeding mines per leaf and associated % parasitism.

Evaluation of the trap catch data showed four (+) generations occurred in the summer at approximately the 1050 DDF intervals predicted by the laboratory data (Fig. 3). The predictions were generated based on data from 2013 & 2017, and then plotted with the data taken from Barrett's dissertation. The flights were similar for all sites and years and generations, except for the first summer generation from Barrett's data which started at the same time, but showed a slower increase after about 80% of the adults had emerged. As we don't know any spray records from Barrett's data, it is not clear why this occurred, but the subsequent generations track the model developed using only the 2013-2017 data. We are strongly encouraged that this model using data from different orchards and widely different years/treatment background seems to predict the flight patterns. The field data showed that a horizontal upper threshold matched the lab data better than a vertical upper threshold.

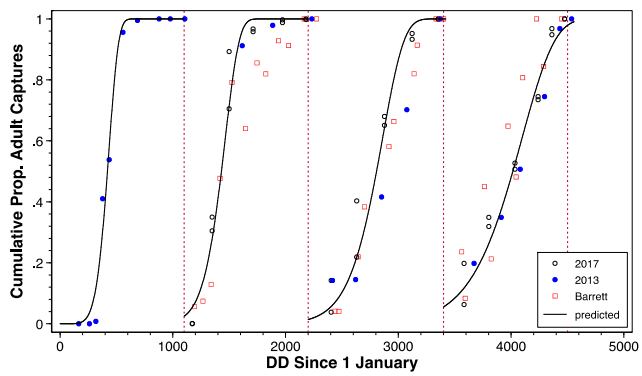

Fig. 3. Cumulative proportion trap catch versus degree days for four generations. Data from 2013 (solid circles) and 2017 (open circles), and from Barrett 1985 & 1987 (open squares).

Preliminary model for Pnigalio flavipes. The data from Barrett (1988) also showed the lower threshold for development for the parasitoid *Pnigalio flavipes* was much higher that of WTLM at 47.8°F with and upper threshold of 83.4°F but with a much shorter total duration of 331 $DD_{47.8°F}$. Attempts at field collections of *P. flavipes* to develop the model were not successful; only 59 individuals were found on yellow panels over the course of the summer in three different locations. We were able to digitize the data from Barrett's dissertation, but have not been able to evaluate the data as a potential basis for the model.

Review of spray records. The organic site used in this study had one spray of Entrust SC applied at 10 oz/A during the end of May. OBLR was the target pest and WTLM was in-between adult generations thus likely were not directly exposed to this spray. Using Barrett's developmental parameters for *P. flavipes* puts the application timing of Entrust SC at approx. 500 $DD_{47.8°F}$ from Jan 1, which is about halfway through the 2nd *P. flavipes* generation.

Three other spray records from organic apple orchards in 2017 were assessed. All three had applications of Entrust SC applied during the mid-later part of May. The application rate at two of the sites was 10 oz/A and these sites had WTLM mines increase in numbers towards the end of the season. The third site's spray record shows entrust SC was applied at 0.25 oz/A on 20 May. This is

either a typo in the records or an error by the applicator. Regardless, no WTLM were apparent during the growing season at this site.

Additional observations: Levels of WALH captured on yellow sticky traps increased rapidly in the organic block starting in mid-Aug (Fig. 4). Levels of WALH were high enough in the organic site that frass from adult and immature WALH accumulated on the fruit. We also observed high numbers of the WALH parasitoid *Anagrus sp.* on the yellow sticky traps towards the end of the season (data not presented). We speculate that the late-May application of Entrust disrupted WALH by killing the parasitoid. It is possible that the overwintering levels of *Anagrus sp*. are high enough to regulate next year's WALH population, as long as they are not killed off next year. Additionally, we observed, but did not monitor, an outbreak of the brown mite, *Bryobia rubrioculus* (Scheuten), in the organic block. We suspect this was another result of disruption caused by an application of Entrust. The black lady beetle, *Stethorus* punctum Casey (Coleoptera: Coccinellidae), appeared in this orchard and built up to high levels on leaves and the yellow sticky traps. This mite predator does not usually respond to and regulate low levels of prey.

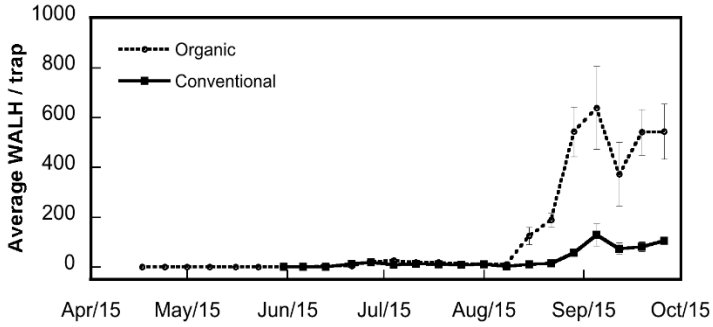

Fig. 4. Average number of white apple leaf hopper captured on yellow sticky traps.

Next year. We need to find a much larger number of orchards with WTLM present. The high quality of lures should allow us to monitor even low population levels. We will be canvassing organic growers that use Entrust, as that is the only material in our organic orchard that should have disrupted the high levels of parasitism that is common in Washington orchards. When we are able to validate the P. *flavipes* model, we will be able to more accurately assess the impacts of spray timings on the disruption of this key natural enemy as well as potential negative impacts on natural enemies of other pests, including those that help regulate WALH and brown mites.

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.

Beers, Elizabeth H., Jay F. Brunner and Bruce A. Barrett. Western tentiform leafminer. WSU TFREC Orchard Pest Management Online. http://jenny.tfrec.wsu.edu/opm/displaySpecies.php?pn=520.

CONTINUING PROJECT REPORT **YEAR**: **3rd year report**
WTFRC Project Number: CP-15-100 (A, B and C)

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

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Cooperators: none

Total Project Request: **Year 1**: $32,425 **Year 2**: $31,974 **Year 3**: $41,711 **Year 4:** $0

Other funding sources

Agency Name: USDA-NIFA-Specialty Crop Research Initiative
Amt. requested/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

WTFRC Collaborative expenses: None

Budget 1
Organization Name: USDA-ARS-NEA **Contract Administrator:** Rebekah Huson
Telephone: (760) 546-3171 **Email address:** rebekah.huson@ars.usda.gov

Item	2015	2016	2017	2018
Salaries	6,132[1]	6,255[1]	6,380[1]	
Benefits	491	500	510	
Wages	0	0	0	
Benefits	0	0	0	
Equipment	0	0	0	
Supplies	3,550[2]	3,550[2]	2,650[2]	
Travel	0	0	0	
Miscellaneous	2,800[3]	0	0	
Plot Fees	1,700	1,700	800	
Total	**14,673**	**12,005**	**10,340**	**0**

Footnotes: 1: summer student to assist with fire blight inoculation, recording data and plant maintenance, **2:** inoculation ($500), greenhouse ($1,250) and orchard ($1,800 yr1-2, $900 yr3), **3:** genotyping of Splendour population (96 individuals).

Budget 2

Organization Name: WSU-TFREC **Contract Administrator:** Katy Roberts & Joni Cartwright

Telephone: 509 335 7667,509 663 8181 **Email address:** ARCgrants@wsu.edu; joni.cartwright@wsu.edu

Item	2015	2016	2017	2018
Wages[1]	4000	9152	9518	
Benefits	392	897	933	
Orchard maintenance supplies	2000	1500	1500	
Fire blight testing consumables	500	2000	2000	
Travel[2]	560	1120	1120	
Plot Fees	2800	2300	2300	
Total	**10,252**	**16,969**	**17,371**	**0**

Footnotes:

[1]Wages for time-slip labor for orchard management and trait phenotyping

[2]Travel to research plots.

Budget 3

Organization Name: Washington State University **Contract Administrator:** Katy Roberts

Telephone: (509) 335 4564 **Email address:** ARCgrants@wsu.edu

Item	2015	2016	2017	2018
Salaries				
Benefits				
Wages				
Benefits				
Equipment				
Supplies				
Travel				
Plot Fees				
Miscellaneous	$7,500[1]	$3,000[1]	$14,000[1]	
Total	**$7,500**	**$3,000**	**$14,000**	**0**

Footnotes:1: genotyping

OBJECTIVES:

The overall goal of this project is to enable selection of new Washington apple varieties that are fire blight resistant and have superior fruit quality, as soon as possible. The three objectives below are sequentially of long, mid and short term time frames.

1. Determine the best sources of fire blight resistance in *Malus sieversii* to incorporate strong resistance into the **W**SU **a**pple **b**reeding **p**rogram (**WABP**).
    - Goal is to incorporate strong fire blight resistance from *M. sieversii* into the WABP
    - Previous WTFRC project identified 21 *M. sieversii* accessions that are highly resistant to fire blight shoot infection
    - Based on evaluation of fruit quality (WSU-TFREC) and resistance to blossom blight infection (ARS-AFRS), 4 *M. sieversii* accessions were selected for use in the WABP
    - To start incorporating this resistance into WABP crosses were made with 3 of these *M. sieversii* accessions in 2016 and a cross was made with the 4th accession in 2017
    - Seedlings from these crosses are currently being evaluated for their resistance to fire blight

2. Determine fire blight resistance levels in RosBREED reference germplasm for current use in the WABP.
    - The goal is to identify potential sources of resistance and unknown **f**ire **b**light resistance influencing **l**oci (**FBL**) among individuals in the RosBREED reference set
    - The RosBREED apple reference germplasm set was propagated and planted in a replicated planting at the WSU Columbia View Orchard in Wenatchee, WA in 2015 (WSU-TFREC)
    - In order to determine levels of resistance, germplasm was challenged with *Erwinia amylovora* (fire blight pathogen) in 2016 and 2017
    - Results of 2016 and 2017 trials are currently being analyzed to identify FBL

3. Develop DNA tests to enable the fire blight resistance of select WABP parents to be efficiently evaluated in seedlings and to evaluate genetic resistance in current WABP elite selections.
    - Goal is to develop and evaluate DNA tests for FBL present in 'Enterprise' and 'Splendour' which have been used as parents in WABP
    - DNA tests are to be developed based upon research conducted in the RosBREED project to identify the genetic loci responsible for resistance
    - Due to unforeseen delays in completing this research a no cost extension has been granted to complete the development of the DNA tests in 2018

This research addresses the Washington apple industry's critical need for improved fire blight management options (i.e. fire blight resistant apple varieties) and improved scion genetics (i.e. DNA tests for resistance and identification of fire blight resistant elite lines in WABP).

SIGNIFICANT FINDINGS:

- In order to facilitate the identification of sources of fire blight resistance and the associated loci within current apple cultivars and breeding material, 1650 trees of 556 elite cultivars and their seedlings were challenged with fire blight.
- Although 'WA38' (Cosmic Crisp® brand apple) had a fairly tolerant response to fire blight in 2016, it appeared slightly more susceptible in the 2017 trial.

METHODS:

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

Evaluation of fruit quality (completed 2015/2016/2017):

- Best horticultural, insect pest and disease control practices will be followed to produce high quality fruit.
- Fruit will be evaluated at WSU-TFREC by Evans following WABP standardized protocols.
- Fruit produced in WV will be express shipped to WSU-TFREC for evaluation.

Evaluation of resistance to fire blight blossom infection (completed 2015/2016):

- Two-4 branches containing 75-100 blossom clusters will be identified on replicate trees and flagged prior to bloom.
- When at least 50 clusters contain at least 1 freshly opened blossom, the branches will be spray inoculated with a suspension of the fire blight bacteria using a back-pack sprayer.
- After symptom development in susceptible control (Gala), test trees will be qualitatively evaluated for blossom infection (severe, moderate, light, none).

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

- Due to the sporadic nature of fire blight, reliable evaluation of fire blight infection in the field requires controlled challenging (inoculation) of individuals with the fire blight pathogen.
- In 2015, a trial orchard of the RosBREED apple germplasm reference set was established at the WSU Columbia View orchard in Wenatchee, WA.
- In 2017, vigorously growing shoots were inoculated with a suspension of *Erwinia amylovora* by dipping a pair of scissors into the inoculum solution and clipping the tip of the two youngest leaves.
- This was the second year of inoculation (2016 and 2017).
- Disease severity was quantified by measuring the shoot length and lesion length of each shoot.
- The 2016 and 2017 data is currently being analyzed using FlexQTL™ software to identify fire blight resistance influencing loci. FlexQTL™ was previously used by RosBREED and our analysis will leverage the genotypic data and expertise from the RosBREED project.

Objective 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.

- DNA tests for 'Enterprise' FBL and related varieties 'GoldRush' and 'CrimsonCrisp' will be designed (2016/2017) based upon FBL recently described in the literature.
- DNA tests for 'Splendour' FBL will be developed in the current RosBREED2 project.
- Test populations (ca. 300 individuals/ populations) of 'Enterprise', 'GoldRush', CrimsonCrisp' and 'Splendour' crossed with 'Pinata' have been developed (2015-2016).
- To validate the DNA tests, test populations will be screened with the DNA tests and evaluated for their resistance to fire blight by controlled pathogen challenge.
- Resistant progeny will be evaluated with DNA markers for fruit quality loci and resistant progeny containing desirable fruit quality alleles will be turned over to the WABP following the establishment of appropriate material transfer agreements between USDA-ARS and WSU.

RESULTS & DISCUSSSION:

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

A previous WTFRC project identified several *M. sieversii* (wild apple) accessions that are highly resistant to fire blight shoot infection and could serve as sources of strong fire blight resistance for breeding. To identify the best 1-5 accessions to use in the WABP we evaluated these shoot blight resistant accessions for their fruit quality and resistance to blossom blight.

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: In 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 will be used to select among the accessions still under consideration for use in the WABP. In 2016 crosses were made with 3 accessions, including GMAL4002.k. In 2017 a additional cross was made with GMAL3688.c. Some of the seedlings from the 2016 crosses are currently being evaluated for their resistance to fire blight. Seed from the 2017 cross has been sown and should be ready for fire blight evaluation in spring 2018.

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

While Objective 1 focuses on identifying the best sources of fire blight resistance for future use as parents in the WABP, the purpose of Objective 2 is 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 'Cripps Pink' 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. In this project, we will identify fire blight resistance-influencing loci (**FBL**) 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. As part of RosBREED 1, extensive genetic analysis and evaluation of fruit quality was carried out on this germplasm. However, since RosBREED 1 was focused on fruit quality, none of the existing orchards of the reference germplasm set were conducive to fire blight inoculation. As a result, a new planting of the RosBREED reference germplasm set was established at the WSU Columbia View Orchard in 2015 for evaluation of fire blight resistance/susceptibility.

In May 2017 (18th), 1650 trees of 556 individuals of the RosBREED apple germplasm reference set were challenged with fire blight at the WSU Columbia View research orchard. 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.

In many cases, individuals responded similarly to being challenged with fire blight in 2017 as in 2016 (Table 1 attached). 'Enterprise', which has been used extensively as a parent in the WABP, is a potential source of tolerance. In both the 2016 and 2017 trials, 'Enterprise' had a resistant response with a very high average proportion of healthy tissue (Table 1). Although 'Splendour', which has been used as a parent in the WABP, has previously shown tolerance to fire blight, it was more susceptible than expected in the 2017 trial (Table 1). 'Aurora Golden Gala', an offspring of 'Splendour', demonstrated tolerance to fire blight in both trials (Table 1). As expected, 'Delicious' demonstrated moderate tolerance to fire blight infection (Table 1). 'WA38', a recent release from the WABP, had a lower average proportion of healthy tissue in 2017 than in 2016 (Table 1). Due to the variability of fire blight, individual tests can lead to spurious results for a cultivar because of variable environmental conditions or vegetative vigor of the individual at the time of inoculation. For example, 'Fuji' is known to be susceptible to fire blight but in the 2017 Columbia View trial appeared to be more tolerant than expected (Table 1).

Currently, the results of 2016 and 2017 trials are being analyzed using FlexQTL™, previously used in the RosBREED project, to identify fire blight resistance-influencing loci (FBL). This is expected to be completed in 2018. FBL identified will allow for the development of DNA tests in a future research project.

Objective 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 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 is 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 that were derived from these parents.

Before a DNA test can be developed for a specific genetic locus, the loci need to be genetically characterized and identified. Identifying the genetic 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 ('Splendour') and the European FruitBreedomics ('Enterprise') projects and we expected results from these projects would allow us to develop the DNA tests within the third year of this project. Unfortunately, the loci have taken longer than anticipated to identify due to unforeseen complexities in the inheritance of fire blight resistance and we have therefore requested a no cost extension to complete the development of the DNA in 2018. The loci controlling fire blight resistance in 'Enterprise' have now been identified (van de Weg *et al.* 2018) and the development of DNA tests

is currently underway. Using new genomics methods candidate loci for fire blight resistance have been identifiedin 'Splendour' and are currently being characterized and evaluate within the RosBREED project. Furthermore, the results of objective 2 above are facilitating a FlexQTL™ analysis similar to that used in the European project which successfully identified the resistance loci in 'Enterprise' (van de Weg *et al.* 2018). Due to these recent advances we are confident we will be able to develop DNA tests and complete our goal for Objective 3 in Year 4 of the project.

DNA tests will be validated by comparing DNA test results of seedlings with results from direct challenge of seedlings with the fire blight pathogen. Over 300 seedlings of 'Enterprise' and 'Splendour' have been challenged with the fire blight pathogen to determine their resistance to fire blight. In general, 25-50% of these seedlings were found to be fire blight tolerant, depending upon parents used in specific crosses. DNA was isolated from the seedling prior to being challenged with fire blight and will be used for DNA testing in 2018. The DNA tests will then be evaluated based upon the association between the observed resistance of the seedling after pathogen challenge and predicted resistance from the DNA test. We anticipate running these DNA test validation in 2017 contingent upon successful development of DNA tests.

Incorporating fire blight resistance into the WABP 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.

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/s11032-017-0755-0

Table 1: 2016 and 2017 resistance and susceptibility levels observed in select cultivars and sources of fire blight tolerance inoculated in the WSU Columbia View fire blight trials.

	2016				2017		
Individual	**Avg. Proportion of current shoot healthy**	**Avg. age of wood infected**	**Max. age of wood infected**		**Avg. Proportion of current shoot healthy**	**Avg. age of wood infected**	**Max. age of wood infected**
'Enterprise'	1.00	0.0	0.0		1.00	0.0	0.0
Malus floribunda 821	0.97	0.1	1.0		1.00	0.0	0.0
'Aurora Golden Gala'	0.80	0.5	2.0		0.93	0.5	1.0
'COOP17' (‘Goldrush’ parent)	0.78	0.6	1.0		0.90	0.4	1.0
'Delicious'	0.73	0.9	2.0		0.82	0.4	1.0
'Honeycrisp'	0.39	1.5	3.0		0.71	0.7	2.0
'WA38'	0.71	0.9	3.0		0.67	0.5	2.0
'Fuji'	0.68	0.6	1.0		0.66	1.0	2.0
'Splendour'	0.80	0.5	2.0		0.54	0.9	1.0
'Zestar'	0.29	1.3	2.0		0.54	0.9	1.0
'Cripps Pink'	0.49	1.2	3.0		0.39	1.2	2.0
'Gala'	0.25	1.5	2.0		0.34	1.5	2.0
'Jonathan'	0.11	2.4	3.0		0.12	1.5	2.0

CONTINUING PROJECT REPORT **3rd Year Report**
WTFRC Project Number:

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

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City/State/Zip: Summerland, British Columbia, Canada V0H 1Z0

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

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: Grant was awarded to Dr. Judd on 1 April 2016. Dr. Judd's WTFRC request was reduced to $4,000 for 2016 and to $6,000 for 2017.These changes are reflected in the revised CRADA with AAFC signed 20 October 2016. We request transfer of this reduction from Dr. Judd to Dr. Knight for the remaining two years of the project.

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[d]
Salaries			18,000[a]	0
Benefits				
Wages	17,500	20,000	22,000	0
Benefits	1,500	1,500	2,000	0
Equipment				
Supplies	6,000	7,500	8,000	0
Travel (local to research plots)	1,000	3,000	3,000	0
Miscellaneous			3,000[b]	0
Plot Fees	3,000	3,000	3,000[c]	0
Total	29,000	35,000	59,000	0

Footnotes: [a] $20,000 that was approved for 2016 ($59,000) was not requested for current use. Our intention would be to use these funds in 2017 to support a visiting Italian scientist ($38,000 total for salaries).
[b] These funds would support the visits of two foreign scientists working on this project.
[c] Funds are now required to support the operations of the Research Farm in Moxee.
[d] No additional funds are requested to complete the research.

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**[b]
Salaries	0	0	0	0
Benefits	0	0	0	0
Wages[1]	18,000	0	0	0
Benefits	2,500	0	0	0
Equipment	0	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[a]	6,000[a]	0

Footnotes: [a] The drop in requests to WTFRC for 2016-17 was due to the awarding of Canadian funds to support this project.
[b] No additional funds are requested to complete the research.

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

- The project-developed high emission rate acetic acid lure (TRE1468) produced by Trécé Inc (Adair, OK) was found to last only six weeks in the field, and a new longer-lasting version (TRE1531) was formulated and tested. This lure lasts the entire season covering the two flight periods of leafrollers.
- A new combination host-plant volatile (HPV) lure (TRE1379) was developed that contains equal amounts of 2-phenylethanol and phenylacetonitrile 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 do elicit a feeding response by both sexes of leafroller adults. This suggests they may signal the presence of a food source for the moths.
- Preliminary results from culture-independent characterization of leafroller damage has revealed that feeding can increase the total abundance of both bacteria and fungi in the apple leaf phyllosphere. Unique isolates of each are currently being identified to species and stored for future experimentation; efforts of which are being complimented with a full culture-independent characterization of the apple leaf microbiome.
- Volatile captures of cherry, peach, and apple trees were conducted to address several specific research interests in combination with the analytical laboratory of Dr. Jim Mattheis (ARS,

Wenatchee, WA). This work has revealed several interesting interactions of insects with fruit trees that could have future importance in biological control studies with several aphid species in tree fruits (black cherry aphid, rosy apple aphid, and green peach aphid). Results in 2017 germane to our project include validation of the absence of the key tortricid attractants being released due to drought stress, overwatering, aphid feeding, and powdery mildew, thus demonstrating they are not unique to leafroller feeding.

- Characterization of apple volatiles and testing of their potential for leafroller attraction has led to a discovery of a new attractant for codling moth. Spin-off studies are in place in South America to validate our initial results and to refine the development of a new bisexual lure for codling moth that could be useful in both monitoring and mass trapping efforts.
- 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.

Goals and activities for next year: We have requested an extension of our project to allow the completion of the various objectives we initially laid out for the project. The remaining funds will primarily support the continued work of my technician Bill Stewart throughout the 2018 field season. Our work will target a final assessment of the potential of these new lures to achieve effective mass trapping of leafrollers. Studies will continue to be done in combination with mass trapping of codling moth as the lures are compatible and controlling two pests with one set of traps is clearly cost effective. The second focus of our final year will be to make the optimized lures available to pest managers in the Industry for testing. Lures can be used to track seasonal population dynamics of leafrollers in orchards treated with sex pheromones for mating disruption. We also believe these lures can be used to provide additional information on the within-field presence of leafrollers and allow users to gain experience to establish action thresholds of moth catch with these lures. The third focus of our work continues to be developing a basic understanding of why the volatiles are attractive and whether we can develop approaches to identify additional volatiles that might further improve the management of these tortricid pests. For example, do these new attractants signal to the moth the presence of a food source or a host plant suitable for oviposition or a rendezvous site for copulation? Answering this question is important both to refine their uses but also their possible limitations in future management of these key pests, and to develop new lures for these and other pests. Dr. Judd will expand his collaborations with Dr. Gerhard Gries at Simon Frazier University in finishing EAG studies to characterize antennal responses of both sexes of oblique-banded and *Pandemis* leafrollers to apple volatiles. He will also complete his proboscis-extension studies with these species to ascertain the feeding stimulus of the various plant volatiles to adults. Following the identification of the major microbial species that benefit from leafroller feeding by Dr. Schaffer we will conduct further volatile collections of shoots sprayed with populations of these microbes to ascertain what influence they have on the volatile profile released from leafroller-infested shoots. We are looking to identify whether release of one of the attractants, phenylacetonitrile is due to the microbes alone, by the plant but is stimulated by the microbial growth that occurs in the wound created by the leafroller, by the plant only due to the leafroller's feeding, or increased by the interaction of the two events. The other attractant, 2-phenylethanol is a known yeast volatile and likely signals a food source to the moth. Studies addressing the role of microbes as a food source on adult longevity and fecundity will be continued.

Schedule of Activities:

1. Flight tunnel tests to study the adult behavior of virgin and mated male and female OBLR and PLR with the key volatiles are finishing this spring at YARL and Ag Canada. Flight

tunnel tests with the insecticide-impregnated netting will also be conducted to assess its potential effectiveness prior to possible field testing.

2. Microbial identification via whole-genome sequencing should be completed in January. Key microbes will be cultured from our samples and maintained for further testing.
3. Volatile analyses will begin in June with Drs. Giacomuzzi and Mattheis and will continue through the summer.
4. Laboratory studies addressing the role of microbe-feeding on adult moth fitness will continue beginning early in the year.
5. Field testing of HPV lures (TRE1379 plus TRE1531) and comparison with sex pheromone lures in both conventional and sex pheromone-treated orchards will begin in May and will continue through the summer.
6. Replicated mass trapping evaluations will be established in the spring and continue all season.

METHODS

Flight tunnel studies. Flight tunnel bioassays are being conducted at both the Agriculture and Agri-Food Canada (2 research tunnels) and USDA laboratories (3 research tunnels) to study moth behaviors to the key compounds previously identified. Mated and virgin males and females of *Pandemis* and OBLR are being tested for their response to key volatiles alone and in combination with sex pheromone.

Volatile collection. Headspace samples from host plants (apple, peach, and cherry) will be sampled from clean shoots, shoots sprayed with key microbial species, shoots with feeding larvae, shoots with both microbes and larvae under controlled laboratory conditions using our established protocols. Samples are collected on Tenax and thermally desorbed and analyzed in Dr. Mattheis laboratory. Final analysis of the chromatograms will be conducted by Dr. Valentino Giacomuzzi at the Free Bolzen University in Bolzano, Italy.

Microbial identifications. Samples of the total DNA from samples washed from apple shoots either with or without associated OBLR larval feeding were collected in 2017 and are now being analyzed in a laboratory in California. Previously, samples were plated out on standard medium and individual colonies from all the major morphological types were isolated, re-plated to purify isolates, and are now being maintained in Dr. Crowder's laboratory in Pullman. Following the identification of the most abundant culturable species of bacteria and yeasts these will be transferred to our laboratory. Microbial populations will be maintained and replicated apple shoots will be sprayed with individual species, including both intact and leafroller-injured shoots. Volatile samples will be collected from these shoots and characterized.

Role of microorganisms in feeding: The proboscis-extension assay will be used to evaluate the preference of adult leafrollers to feed on solutions of the major species of microbes found from the larval-infested apple shoots. Additional studies will be conducted to examine moth longevity, mating success, and fecundity when presented with microbial solutions.

Monitoring populations. Final versions of the HPV lures have been developed in close collaboration with Trécé Inc. and no further modifications of lures are anticipated during 2018. Lures will be free for testing during 2018 to all interested field managers. Data will be collected on the phenology of both male and female catches in traps with these lures, and these data will be compared with the current phenology models for OBLR and PLR in DAS. Traps with pheromone lures for both codling moth and leafrollers will also be distributed for a similar seasonal evaluation. We will attempt to correlate seasonal cumulative and peak moth catches in these traps with spring and summer larval

populations and the close proximity of other sources of leafrollers, such as cherry or infested orchards and/or unmanaged backyard trees. Our goal is to have at least 20 orchards monitored.

Evaluation of mass trapping. At present, five sites have been selected for these studies including an organic block in southern Oregon infested with codling moth, oblique-banded leafroller, and eye-spotted budmoth. The second site is the USDA Experimental Farm which is infested with codling moth and both *Pandemis* and oblique-banded leafrollers. The third site is an organic orchard situated south of Kennewick and is infested with European leafroller (*Archips rosanus*) and codling moth. A fourth site is situated south of Wapato and is infested with codling moth and oblique-banded leafroller. A fifth site is organic and is situated in British Columbia and is heavily infested with eye-spotted budmoth and apple clearwing moth (a pest which is also attracted to acetic acid and pear ester). This site is currently treated with sterile codling moths, but trapping has found a low level of wild moths each year. We anticipate that several additional sites will be selected based on certain criteria: we prefer to work in orchards with both codling moth and leafrollers and are interested in the role of mass trapping within certified-organic orchards. We will use the binary TRE1379 lures with the newly improved acetic acid lure (TRE1531) to insure we have season-long trap effectiveness (16 weeks). Both mass-trapped and untrapped plots (3 – 5 acres) will be included at each site and levels of fruit injury will be assessed.

Development of 'Lure and Kill'. Flight tunnel bioassays will be conducted to evaluate male and female contact with an insecticide-treated netting that has recently been registered by the EPA. Both direct mortality and sublethal (subsequent mating success and resulting fecundity) effects will be measured. We do not anticipate doing field trials with the netting this year.

RESULTS & DISCUSSION

Lure development. Weight loss of membrane lures in the laboratory at 25°C varied widely among the various acetic acid (AA) and HPV lures used in our trials (Table 1). Unexpectedly, the current high-emission AA lure (TRE1468) used in the first generation in all our mass trapping trials was only effective for five weeks when aged in the laboratory. Previously, this lure was thought to be effective for 8 wks. These new results suggested that this lure might have been even shorter-lived in the field during the 2017 trials due to higher temperatures (late-season) and possible unknown wind effects. Unfortunately, these lures were replaced after 8 weeks in the mass trapping studies and because the HPV lures are not attractive without the acetic acid this suggests the traps likely loss their effectiveness for catching leafrollers, particularly in the second generation. The new AA lure, TRE1531 was as attractive to leafrollers as TRE1468 in comparative field trials, had a three-fold higher emission rate, and lasted >16 weeks in our laboratory aging test. In the field, these lures were used for the second generation in the three G80 plots but were not used at the Moxee Farm or in the Lateral A blocks. The three HPV lures had relatively consistent and long-lasting emission rates. These lures are at present being aged to measure weight loss out to 20 weeks.

Table 1 Weight loss measurements of the membrane lures loaded with acetic acid (AA) or host plant volatiles (HPV) including 2-phenethanol (PET) and phenylacetonitrile (PAN) used in our trials under laboratory conditions at 25 ºC.

			Mean (SE) weight loss (mg) per day		
Lure	Target pest	Content	First week	Last week tested	Effective emission (wks)
TRE3321-AA	CM	AA	5.5 (0.1)	2.1 (0.1)	>20
TRE1468-AA	LR-old	AA	39.2 (0.5)	2.2 (0.7)	5
TRE1256-HPV	LR-old	PET	13.5 (0.1)	11.2 (0.1)	>12
TRE1381-HPV	LR-old	PAN	32.8 (0.2)	26.8 (0.1)	>12
TRE1531-AA	LR-new	AA	73.7 (1.1)	5.4 (0.5)	>20
TRE1379-HPV	LR-new	PET+PAN	26.0 (0.3)	19.0 (0.1)	>12

Lure comparisons: Studies were run to compare the effectiveness of three acetic lures which had been evaluated for their weight loss over time (see Table 1). Data were collected for both codling moth (CM) and leafrollers (OBLR) using traps baited with pear ester for CM and TRE1256 for OBLR. No significant differences were found among the AA lures used in this study for either species nor for total or female moth catches (Table 2). The TRE3321 lure is the commercial lure available for use with the Combo lure (pheromone plus pear ester) used extensively for CM. Previous studies had shown that increasing the emission rate of acetic acid could increase catches of leafrollers. The current study included a new acetic lure with an even higher emission rate to develop a season-long lure for use in mass trapping. Moth catches were numerically lower with this new lure but across many other studies conducted during the summer there was no indication that leafroller catches with either of the two lures TRE1468 and TRE1531 were different. However, due to the clear greater longevity of the TRE1531 lure it will be used in 2018.

Table 2. Comparison of moth catch in traps baited with a membrane lure loaded with 2-phenylethanol (PET, TRE1256) and a grey septa (TRE3460) loaded with pear ester (PE) plus one of several types of acetic acid (AA) lures which varied in their emission rate (see Table 1).

		Mean (SE) moth catch			
HPV lure	AA lure	CM-total	CM-females	OBLR-total	OBLR-Females
PET+PE	TRE3321	18.6 (2.0)	4.2 (1.2)	8.8 (2.3)	3.4 (1.2)
	TRE1468	19.4 (3.2)	5.0 (1.4)	15.4 (2.6)	7.2 (2.2)
	TRE1531	26.4 (6.3)	6.8 (1.6)	11.4 (1.6)	5.6 (1.6)
ANOVA		$F_{2, 12} = 0.78$	$F_{2, 12} = 0.84$	$F_{2, 12} = 2.33$	$F_{2, 12} = 1.20$
		$P = 0.48$	$P = 0.46$	$P = 0.14$	$P = 0.34$

Studies were also conducted to compare moth catches with the three HPV lures in combination with the TRE1468 AA lure for several species of leafrollers. (Table 3). In general, traps baited with the HPV plus AA lures caught significantly more total and female moths than traps baited with only AA lures. The one exception was for the total and female-only ESBM study in Oregon where traps baited with PET pus AA failed to outperform AA alone. Only with ESBM did PAN+AA outperform PET+AA and only in Canada. Interestingly, with this species in Canada the ternary lure of PET/PAN+AA outperformed both binary lures. Only with ELR did the PET plus AA lures outperform the PAN+AA lures. However, there was a clear trend (2X) with PET+AA catching higher mean numbers of moths than PAN+AA for both the key leafrollers in Washington, OBLR and PLR.

Similar studies have been conducted with collaborators in Europe with a number of other tortricid species. In general, only the light brown apple moth, (LBAM) *Ephyidas postvittana*, and the eye-spotted budmoth are more attractive to PAN+AA than PET+AA. Thus, using the TRE1379 combo lure instead of the PET lure TRE1256 would have only marginal effect in monitoring leafrollers globally. Yet, LBAM is an important quarantine pest affecting California, and ESBM is the major leafroller pest in British Columbia. ESBM is also present in some organic orchards in Washington and is a key pest in an organic orchard in Southern Oregon. Thus, studies planned for 2018 will use only the TRE1379 combo HPV lure.

Table 3. Summary of field trials comparing the attractiveness of membrane lures loaded with acetic acid alone (AA), the binary combination of AA and either 2-phenylethanol (PET) or phenylacetonitrile (PAN), or a ternary blend including the AA lure and a second lure loaded with PET and PAN.

	PET + AA		PAN + AA		PET/PAN+AA	
Species[a]	Total	Female	Total	Female	Total	Female
ESBM[b]	7.2 (1.4)c	4.4 (1.0)C	13.0 (4.4)b	9.4 (3.5)B	26.6 (4.1)a	16.2 (3.9)A
TLLR[b]	26.9 (4.2)a	12.4 (2.3)A	15.3 (2.8)b	7.6 (1.2)B	21.1 (3.4)a	10.0 (1.7)A
OBLR[c]	3.9 (0.8)a	3.4 (0.60A	1.7 (0.5)ab	1.5 (0.5)AB	4.9 (1.3)a	3.9 (0.9)A
ESBM[c]	2.6 (0.9)ab	1.6 (0.50AB	6.9 (2.50a	5.0 (1.7)A	5.2 (2.7)ab	3.1 (1.5)AB
ELR[d]	12.8 (0.9)a	6.2 (0.8)A	6.6 (0.5)b	3.4 (0.7)A	8.4 (1.2)ab	5.4 (0.7)A
PLR[d]	12.6 (2.8)a	2.4 (0.5)AB	6.2 (3.1)ab	2.0 (1.0)BC	11.0 (0.9)a	5.8 (1.0)A
OBLR[d]	5.8 (1.6)a	3.8 (1.2)A	3.8 (0.7)a	2.2 (0.7)AB	7.8 (1.0)a	5.4 (1.1)a

Row means followed by a different letter (lower-case for total and Uppercase for females) were significantly different, $P < 0.05$.

[a] ESBM, eye-spotted budmoth, *Spilonota ocellana*; TLLR, three-lined leafroller, *Pandemis limitata*; OBLR, obliquebanded leafroller,
Choristoneura rosaceana; ELR, European leafroller, *Archips rosanus*; and PLR, Pandemis leafroller, *Pandemis pyrusana*.

[b] Studies conducted in British Columbia.

[c] Studies conducted in Oregon.

[d] Studies conducted in Washington.

The HPV plus AA lures always catch fewer moths than a sex pheromone lure when used in a conventional orchard. However, we showed that the HPV lure was not affected by the use of mating disruption and thus is a superior lure compared with the sex pheromone lure in these orchards. Combining a sex pheromone lure with either PET or PAN in the same trap reduces the catch of male moths (Table 4). In addition, with *Pandemis* it also reduced the catch of female moths relative to the HPV lure alone.

Table 4. Comparison of moth catch in traps baited with a species sex pheromone (PH) alone and in combination with either PET or PAN alone or a ternary blend with acetic acid

Species	Lure	Males	Females	Total
Pandemis limitata	PH	9.3 (1.1)a	-	9.3 (1.1)a
	PH+PET+AA	4.6 (0.8)b	2.3 (0.4)b	6.9 (1.2)ab
	PET+AA	1.1 (0.5)c	4.9 (0.9)a	6.0 (1.4)b
Spilonota ocellana	PH	42.3 (5.4)a	-	42.3 (5.4)a
	PH+PAN+AA	28.8 (4.3)b	12.1 (1.8)a	41.2 (6.9)a
	PAN+AA	8.0 (1.7)c	12.2 (2.9)a	20.2 (4.4)b

Putative attractiveness of apple seedling infested with leafrollers. Studies conducted with potted apple trees in a screened cage demonstrated that adult OBLR were less likely to be caught on oil-coated clear vertical traps surrounding trees and for females to lay fewer eggs on trees with conspecific larval feeding than on clean trees (Fig. 1). This contradicts an initial report from New Zealand. However, we feel it is not surprising because we found in subsequent that at least two of the plant volatiles (from among the list in Table 6) stimulated by this larval feeding are repellant to adult OBLR when tested alone. These studies also showed that neither PET or PAN are either attractive or repellant to moths. We continue to study why when presented in combination with acetic acid they are attractive to both sexes of these moths.

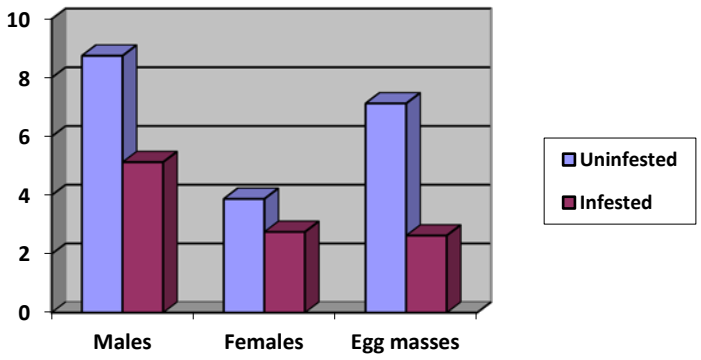


Figure 1

Attractiveness of female moths caught in traps to male. One question that has been asked is whether male attraction to the HPV lures is only because traps have calling females already on the liners. This question was asked previously with codling moth and pear ester and we demonstrated that both sexes were attractive to pear ester. Recently, new liners have become commercially available that have a less messy adhesive. Thus, studies compared moth catches of codling moth and oblique-banded leafrollers in delta traps using two different types of sticky liners coated with either a sticky gel (SG, Tangletrap, Trece Inc.) or a hot-melt pressure sensitive (HMPS, No-Mess, AlphaScents Inc.) adhesive. Laboratory and field studies demonstrated that delta traps with either liner type and baited with a pair of 2 to 3-d-old virgin females can catch males, but often at significantly different levels (Table 5). Male moth catches in traps baited with two virgin females placed on the HMPS liner were significantly greater than on SG liners in the field for OBLR but not for CM. Similar results were observed in a laboratory flight tunnel. This difference in moth catch between liners was not due to levels of female mortality, but instead was correlated with the occurrence of the female's ventral abdomen becoming stuck in the adhesive. The observed difference between species was due to the size of the moth and the proclivity of becoming stuck on the liner. Across all the species studied female leafrollers have generally been mated at high levels in traps baited with these HPV lures, 70-100%. Because, mated females generally do not begin to call again for several days after their first mating we looked at this factor and found that mated females were significantly less likely to lure males into traps. Thus, it is probably unlikely that substantial numbers of males are caught with the HPV lures because of the presence of female moths in the trap. In general, the sex ratio with the leafroller HPV lures has been ca. 1:1. We feel this is more proof that the lures are not signals for mating rendezvous sites but are more likely signals for food.

Table 5. Male moth catches of *C. pomonella* (CM), and *C. rosaceana* (OBLR) in field trials in traps baited with two virgin female moths in traps with either SG or HMPS liners or a screened cylinder with a HMPS liner.

	Mean (SE) male catch in traps when Virgin females placed		
Species	On HMPS liner	On SG liner	In Screened cylinder
CM	0.80 (0.29)b	0.64 (0.41)b	7.64 (2.80)a
OBLR	1.90 (0.64)b	0.82 (0.70)c	6.86 (1.59)a

Importance of adult feeding on fitness Previous studies with several tortricid species have all shown that adult feeding is not required for females to mate and lay a full complement of eggs. However, moths were paired on Day 1 in all of these studies. We found similar results in that situation but if the two sexes were kept apart for $\geq$ 1 day and then allowed to mate we found that a provision of water or honey water significantly increased mating, longevity, and female fecundity. Thus, we suppose that there would be a selective advantage for moths to locate food or water sources in natural environments where host trees with potential mates may be widely-spaced. We also feel that the presence of acetic acid may be a volatile signature for the presence of water and sugary sources produced by microbial fermentation of fruit and other plant parts. Yeasts which produce PET can be a rich nutritional source for insects and are typically included in the artificial diets used to rear insects. At present, he ecological importance of PAN is unknown.

Microbial identifications. This study was conducted to characterize the potential role of apple's microbiome on the plant volatile profile we have previously reported. We are interested in whether phenylacetonitrile might be of a microbial origin as this is unclear from the literature. Previous work has shown that the other attractant 2-phenylethanol is produced by most yeasts and is reported from flowers of many plants, including apple. There is a possibility that phenylacetonitrile is produced by one or more species of bacteria. Unfortunately, it is never clear from reports of volatile captures from plants how important the microflora covering the plant can be on this phenotypic expression. Our initial data shows that the population densities of both bacteria and yeast are increased on apple shoots following leafroller feeding. Our focus is to characterize the impact of leafroller foliage feeding on both the volatiles released and the populations of microbes from apple shoots. Subsequent studies planned for 2018 will then spray key microbes on the shoots and evaluate volatile profiles to ascertain the origin of the new attractants. These data are also critical in understanding the ecological roles of these volatiles and our ability to develop new attractants for use in pest management.

Volatile collections. Twenty volatile compounds were significantly increased with larval feeding by OBLR on apple shoots (Table 6). These data were similar to our previous report with PLR larvae.

Table 6. Mean emission rate (pmol dm^{-2} h^{-1}) of volatile compounds detected in the headspace of apple shoots that were either uninfested (U) or infested (I) with *C. rosaceana* larvae, N = 7. The three shaded rows are the key attractants.

		Mean (SE) emission rate		Rate increase
Compounds	L.R.I.	Uninfested (U)	Infested (I)	I / U
(*Z*)-3-Hexenyl acetate	1323	440.9 (152.1)	1814.8 (126.9)	4.1
(*E*)-2-Hexenal	1228	9.3 (4.7)	532.8 (47.7)	57.3
Acetic acid (AA)	1457	154.8 (43.7)	525.8 (109.9)	3.4
(*Z*)-3-Hexen-1-ol	1393	22.5 (6.3)	311.3 (67.7)	13.8
Acetaldehyde	851	41.7 (10.5)	108.1 (15.9)	2.6
(*E*)-2-Hexenyl acetate	1344	15.4 (5.2)	52.6 (13.5)	3.4
Acetone	963	13.1 (2.6)	50.5 (7.8)	3.9
Phenylacetonitrile (PAN)	1937	0.4 (0.2)	48.8 (8.1)	122.0
Methyl salicylate	1788	11.9 (2.8)	48.5 (6.5)	4.1
(*E*,*E*)-α-Farnesene	1748	1.2 (0.3)	41.7 (10.1)	34.8
DMNT	1306	6.6 (2.1)	40.1 (7.9)	6.1
2-Phenylethanol (PET)	1922	2.3 (0.5)	23.6 (3.9)	10.3
(*E*)-β-Ocimene	1238	1.4 (0.6)	23.4 (5.1)	16.7
Benzyl alcohol	1884	3.0 (0.9)	16.3 (2.0)	5.4
Phenylacetaldehyde	1648	1.7 (0.4)	14.4 (2.0)	8.5
Linalool	1548	0.9 (0.7)	7.1 (1.3)	7.9
Indole	2426	0.1 (0.03)	4.4 (1.3)	44.0
(*Z*)-3-Hexenyl benzoate	2130	0.5 (0.2)	2.7 (0.2)	5.4
2-Phenylethyl acetate	1823	0.3 (0.1)	1.8 (0.2)	6.0
(*Z*)-Jasmone	1960	0.5 (0.2)	1.5 (0.3)	3.0

In addition, new data from sampling in the field during 2017 showed that both 2-phenylethanol and phenylacetonitrile were not significantly increased by water relations in the orchard, phloem feeding by aphids, or the growth of powdery mildew (Table 7). Only emissions of acetic acid among the three attractants was significantly higher with GAA feeding and over-irrigation compared with the control apple trees.

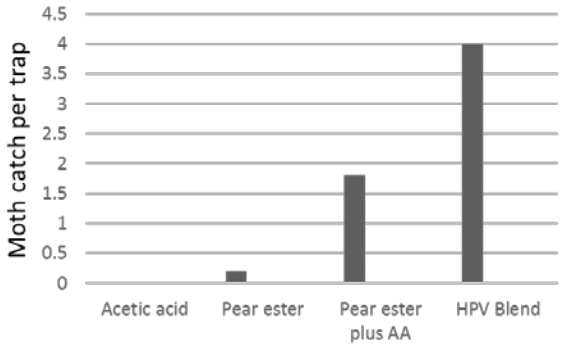
Table 6. Mean emission rate (pmol dm^{-2} h^{-1}) of the three attractants detected in the headspace of shoots from apple trees that were either uninfested (control), non-irrigated (drought), over-irrigated, infested with green apple or rosy apple aphids, or powdery mildew (PM), N = 3-4.

Volatile	Mean (SE) emission rate					
	Control	Drought	Over-irrigated	GAA	RAA	PM
AA	60.6 (7.7)b	175.5 (12.7)ab	377.0 (45.0)a	465.9 (161.8)a	50.7 23.8)b	26.0 (25.8)b
PAN	0.1 (0.1)	0.3 (0.02)	0.4 (0.1)	0.3 (0.1)	0.1 (0.1)	0.2 (0.03)
PET	0.5 (0.04)	3.6 (1.5)	1.6 (0.9)	2.8 (1.7)	0.4 (0.3)	1.5 (1.4)

New attractant for codling moth. Based from our collaborative work in Europe on the leafroller attractants a new discovery was made of a volatile blend that appears to be more attractive for codling moth than the use of pear ester plus acetic acid. Our test was conducted at the end of the season in Washington and confirmed the results of this European study (Fig. 2). Lures have been prepared and are currently being evaluated in three countries in South America. The identity of the new volatile blend has not been made public due to pending patent concerns. If results continue to be positive studies outside of this project will continue and this new lure will be tested in combination with the

leafroller HPV lures to examine the joint use of mass trapping for CM and leafrollers beginning in 2019.

Figure 2. Catches of codling moth in end-of-season trial with a new HPV blend in comparison with pear ester, acetic acid, and the two together.



Attract and Kill. Limited studies have been conducted with a recently EPA-registered fabric (ZeroFly screen, Vestergaard-Frandsen) impregnated with 0.4% deltamethrin. These nets have recently been tested with the brown marmorated stink bug, *Halyomorpha halys*, in the eastern U.S. Only a preliminary 10-sec exposure assay has been conducted to date with codling moth and OBLR, and results have been similar to our previous testing with experimental deltamethrin-impregnated cloth and a PVC material. During 2018, sublethal tests following exposure and field aging studies are planned. Flight tunnel tests will also be conducted with the HPV and AA lures for both sexes to compare moth mortality on the netting with moth catch with a trap.

Mass trapping. Studies were conducted in seven blocks situated in three areas of Washington State (Table 8). Our initial trials to assess the potential of these new lures to mass trap leafrollers experienced a few hiccups, including the lack of comparative non-trapped plots, the unexpected short residual activity of the acetic acid lure, the relatively low population density of leafrollers in most plots, and the unexpected management of these plots. All mass trapping trials conducted in 2017 used the TRE1256 PET lure plus an acetic acid lure. Traps were also baited with the Combo lure for codling moth. Multipher bucket traps were used and these were loaded with a blend of organic mineral oil and organic vinegar to retain moths for use in the organic orchards or with propylene glycol in the non-organic sites. Traps were serviced mid-season and lures and liquids were replaced. The three organic blocks all had very high codling moth populations the previous year and during 2017 an intensive program was used, including mating disruption and sprays of spinosad and virus. By mid-season, a large number of codling moths were trapped and catches in the second half of the season were reduced > 90% which was a very good result and suggests the intensive program was effective. An attempt to sample levels of fruit injury was circumvented by the small plot size and the resulting strong gradient of fruit injury along the borders of all the plots. Populations of leafrollers in these organic blocks were relatively low and consisted primarily of *Archips rosanus* even though this species was not being monitored by the grower. The two Moxee blocks were isolated and had high levels of codling moth and no management program other than mass trapping was used to control this population. Thus, the level of reduction witnessed during the season was much lower in these blocks (36 and 54%). Also, one of the two blocks had no fruit due to a spring frost. Leafroller populations were much higher here than in the organic site and consisted of both *Pandemis pyrusana* and *Choristoneura rosaceana*. Reductions in these populations was much higher, but unfortunately the use of the TRE1468 acetic acid lure may have impacted the catch of leafrollers in the second half of the season and these results cannot be strongly supported. The two small plots in the center of the Lateral A orchard had extremely high catches of codling moth and these catches increased from early

to late in the season. This likely reflects the two acres being surrounded by 78 acres of unmanaged and completely infested orchard. At the start of the season we anticipated that the owner would implement a full management program but the ownership was lost and no actions were taken to manage the orchard. Levels of leafrollers were low in these two plots and populations also increased between generations, perhaps due to the immigration of moths from outside the treated plots and the lack of any surrounding management program.

Table 8. Summary of mass trapping studies conducted during 2017.

	Cumulative male/female moth catch and levels of mating			
	Codling Moth			
	Mid-season		End-of-season	
Block	M/F	% unmated F	M/F [% change]	% unmated F
G80C-4	489/162	59	7/26 [-95]	22
G80B-7N	550/188	43	10/37 [-94]	19
G80B-11S	400/124	48	11/29 [-92]	41
Mox G	326/156	17	95/125 [-54]	14
Mox R	193/179	18	103/135 [-36]	16
LatA-2	1373/2609	18	1780/4358 [+154]	25
LatA-3	1417/2874	27	1319/4176 [+128]	31

	Leafrollers [a]			
Block	Mid-season		End-of-season	
G80C-4	M/F	% unmated F	M/F [% change]	% unmated F
G80B-7N	42/26	8	0/2 [-97%]	0
G80B-11S	43/24	11	2/2 [-94%]	0
Mox G	49/30	7	0/1 [-99%]	0
Mox R	379/299	18	23/33 [-92%]	24
LatA-2	267/200	14	44/57 [-78%]	21
LatA-3	7/12	33	43/13 [+295]	15
Block	5/12	42	30/17 [+276]	6

[a] Leafrollers were primarily *Archips rosanus* in the organic G80 orchards, *Pandemis pyrusana* in the Mox orchards, and *Choristoneura rosaceana* in the LatA blocks.

CONTINUING PROJECT REPORT **YEAR**: 1 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:** $82,207

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: WSU-TFREC **Contract Administrator:** Katy Roberts/Joni Cartwright
Telephone: 509-335-2885/509-663-8181 x221 Email: arcgrants@wsu.edu/joni_cartwright@wsu.edu

Item	2017	**2018**	2019
Salaries[1]	31,146	51,561	48,041
Benefits[2]	7,439	17,143	17,167
Wages	8,000	8,320	8,653
Benefits[3]	216	225	234
Equipment	0	0	0
Supplies[4]	3,500	3,640	3,786
Travel[5]	4,000	4,160	4,326
Miscellaneous	0	0	0
Plot Fees	0	0	0
Total	54,301	**85,049**	82,207

Footnotes:
[1] Project Assistant (Y1 -12 months, Y2 – 3 months); Y2-3 Ute Chambers 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

- We found clear evidence that earwigs suppress woolly apple aphids at our four study orchards.
- There was no evidence that earwigs initiated fruit damage in any of our four study orchards (one Gala orchard and three Fuji)
- Molecular gut content analysis shows that earwigs eat a variety of foods in apple orchards including fungi, plants, and numerous arthropods, not just aphid pests.
- 1,680 hours of woolly apple aphid colony video footage were collected show direct evidence that earwigs frequently eat woolly apple aphids

Objective 1. Effects of earwig manipulation on woolly apple aphids and apple fruits.

Methods

Study sites. We worked at four orchard blocks described in Table 1. In 2017, sites M, T, and O were each divided into 12 sections consisting of two adjacent rows of 8 trees. All sections were at least 30 meters apart from each other and the edges of the block. Each of the 12 sections was assigned to an earwig treatment, either 'control', 'augmentation', or 'removal' (explained below). Site W was set up in 2016 and differed in that there were only 10 sections and two treatments ('augmentation' and 'control') and each section consisted of three adjacent rows of 14 trees. This was because two years of previous monitoring data suggested that there was no (or a very small) natural earwig population, so removal and control treatments would be redundant.

Insect monitoring and earwig manipulations. Approximately once a week, from April to November, all trees in study sections of sites M, T, and O, and every other tree at site W were monitored for woolly apple aphid colonies and earwigs. Woolly apple aphid colonies were recorded as the number of infested axils on a survey of ten ~1' long twigs plus all colonies on pruning cuts and trunks. Earwigs were monitored by counting the number found in rolled tubes of corrugated cardboard placed in each tree.

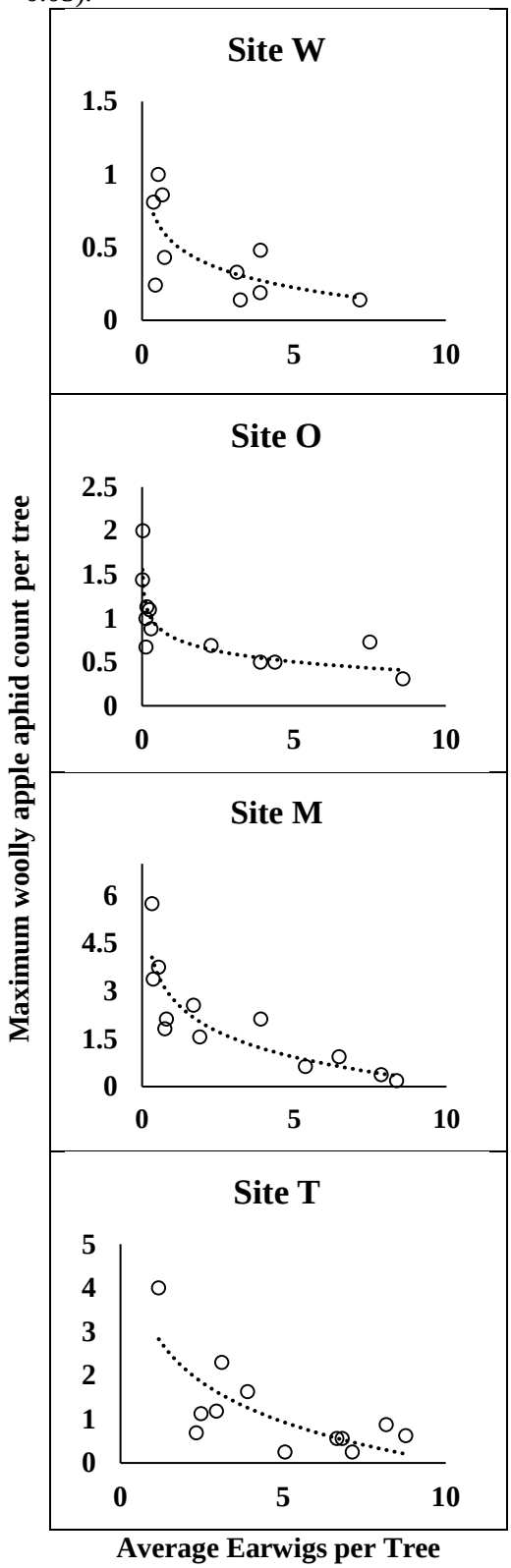
Table 1. Information about study sites

Block name	Nearest town	Variety	Spacing (trees x rows)
M	Quincy	Fuji	3.5' x 12'
T	Quincy	Fuji	7' x 15'
O	Orondo	Fuji	5' x 13'
W	Winchester	Gala	3' x 10'

In control and augmentation treatment areas, all earwigs found were counted and released. In the removal areas, earwigs were counted and collected into a plastic bag. In addition, earwigs were collected by the thousands at an orchard near Quincy, counted, and released into augmentation areas from May to July. In total, 120 earwigs per tree were released in augmentation areas of site M, 350 per tree at site T, and 175 per tree at site O. At site W, 38 earwigs were released per tree in 2016 in augmentation areas. There was no further manipulation of site W in 2017 because earwigs established at the augmentation areas and were more abundant there than in the control areas.

The number of earwigs released per tree varied between sites depending on the amount we found during monitoring. If less than ten earwigs were found per tree during monitoring, or if there was

Fig. 1. Correlation between woolly apple aphids and earwigs at four sites (linear regression after Log transformation, all P < 0.05).



no significant difference between earwig counts in augmentation vs. control areas, we released more earwigs on the next visit. The number of earwigs released per tree may seem like a very large amount, but our trap counts were not extremely high compared to monitoring data collected from commercial orchards in 2014 and 2015.

Fruit damage survey. Within 5 days of harvest, we inspected up to 30 apples on each study tree in earwig augmentation and removal areas at sites M, O, and W. Site T was not evaluated because earwigs remained prevalent in the removal treatment areas. Each inspected apple was scored as 'good' (having no visible defects) or categorized by defects.

Data analysis. To quantify the relationship between earwig and woolly apple aphid abundance, we summarized each study section of each site into two numbers: 1) the maximum count of woolly apple aphid colonies per tree (to represent 'how bad the problem got') and 2) the average earwig count during the observation period. To correct for variation in the number of days between observations, the average earwig count was calculated as $\sum (T_{i+1} - T_i)[(Y_i + Y_{i+1})/2]$ divided by total days of observation, where T is the day of an observation, and Y is the number of earwigs found per tree during an observation.

Results and discussion

Woolly apple aphid suppression. At all four orchard blocks, locations with fewer earwigs had elevated risk of woolly apple aphid outbreaks, while at higher levels of earwigs, woolly apple aphid populations remained consistently low (Fig. 1). This strongly suggests that earwigs suppress woolly apple aphid populations.

At sections with over 5 earwigs found per tree averaged across the season, woolly apple aphid counts never reached more than 1 colony per tree. At below an average of 5 earwigs per tree, the maximum woolly apple aphid colonies per tree ranged from approximately 1–6 fold greater, depending on the site.

Earwig damage to fruit. We found no evidence that earwigs caused increased fruit damage. There was evidence that rounded and expanded stem splits were more common in earwig augmentation areas at site W, and when all sites were pooled, but the overall occurrence of any type stem bowl splitting was not significantly greater in earwig augmentation areas at any site or overall (Table 2). Strangely, stem bowl splitting was more prevalent at Site M in earwig removal areas, but when all sites were pooled,

augmentation and removal areas were not significantly different in total stem bowl split occurrence. Overall, the results suggest that while earwigs can attack damaged apples, they do not initiate damage frequently enough to be detectable. In addition, the frequency of apples with putative earwig exacerbation of stem bowl damage was very low: 0.3% in augmentation areas and 0.1% in removal areas.

Table 2. Apple damage survey. Chi squared tests were conducted to assess the chance of finding apples belonging to each category of damage in earwig augmentation vs. removal areas. Tests were conducted within each study site and for total apples pooled across sites.

Site	Trt.	Number of apples								
		Total	Good	Round hole	'Bird' hole	Depression	Side crack	Stem bowl split		
								Normal	Expanded	Total
W	Aug.	2692	2585	2	0	14	1	81	8	89
	Rem.	2632	2536	0	0	9	2	81	0	81
	Chi-square P:		*0.53*	*0.16*	*NA*	*0.32*	*0.55*	*0.90*	***0.005***	*0.63*
O	Aug.	1478	1394	3	3	20	7	11	3	14
	Rem.	1482	1393	2	6	10	8	11	4	15
	Chi-square P:		*0.71*	*0.65*	*0.32*	*0.07*	*0.80*	*0.99*	*0.71*	*0.86*
M	Aug.	1835	1778	2	1	18	12	13	6	19
	Rem.	1831	1758	3	0	16	12	35	1	36
	Chi-square P:		*0.15*	*0.65*	*0.32*	*0.74*	*0.99*	***0.001***	*0.06*	***0.02***
Total	Aug.	6005	5757	7	4	52	20	105	17	122
	Rem.	5945	5687	5	6	35	22	127	5	132
	Chi-square P:		*0.57*	*0.58*	*0.52*	*0.07*	*0.73*	*0.12*	***0.01***	*0.47*

Objective 2. Molecular analysis of earwig diet in an apple orchard

Methods: During previous experimentation at site W in 2016, samples of 20 earwigs were collected on 6 visits between June 17 and September 21. Collections were made within an hour of sunrise into plastic Ziploc bags stored in a cooler with ice and transported to a -20C laboratory freezer. Later, each earwig's stomach was dissected the DNA extracted using QIAGEN's DNeasy spin column kit. Each set of extractions included a negative control with no earwig stomach to check for DNA contamination.

Extractions from the 20 earwigs from each day were pooled to yield one sample representing earwig diet for each of the 6 collection days. These samples, along with a pooled sample of negative control extractions were sent to RTL Genomics in Lubbock, TX, for sequencing on Illumina MiSeq platform. Sequencing involved different sets of 'universal primers' designed to amplify DNA from the COI region for arthropods, trnL for plants, and ITS for fungi. RTL Genomics also performs analysis and identification of DNA sequences.

Results
The RTL Genomics commercial laboratory analysis identified in total 441 'operational taxonomic units' from animals, 120 from fungi, and 16 from plants (Table 3).

Table 3. List of taxa found in earwig stomachs according to DNA analysis. OTUs represent genetic diversity, not abundance in the stomach.

Kingdom	Order	Species name, explanation	OTUs	Ecological relevance
Animal	Capitellida	Polychaete worm	4	
	Araneae	*Anames sp* spider endemic to Australia	1	
	Opiliones	*Phalangium opilio* harvestman	1	Predator
	Prostigmata	*Abacarus lolii* grass mite	1	
	Lithobiomorpha	*Lamyctes africanus* centipede	1	
	Entomobryomorpha	*Entomobrya unostrigata* 'slender springtail'	1	
	Coleoptera	*Stethoris punctillum* spider mite destroyer	1	Predator
		Carpophilus sp sap beetle	1	
	Dermaptera	*Forficula auricularia* European earwig	18	
		Unclassified or unknown	14	
	Diptera	*Pollenia rudis* calliphorid fly	1	
		Drosophila melanogaster vinegar fly	1	
		Symplecta sp crane fly	1	
	Hemiptera	*Macrosiphum euphorbiae* potato aphid	1	
		Dikrella californica Blackberry leafhopper	2	
		Zonocyba pomaria white apple leafhopper	2	Pest
		Campylomma verbasci campylomma bug	1	Pest and predator
		Daraeocoris brevis predatory bug	1	Predator
		Eriosoma lanigerum woolly apple aphid	1	Pest
		Pemphigus sp aphid	2	
	Hymenoptera	*Aphelinus varipes* wasp	1	Parasitoid
		Aphidius ervi wasp	1	Parasitoid
	Neuroptera	*Micromus sp* brown lacewing	1	Predator
	Thysanoptera	*Frankliniella occidentalis* Western flower thrips	1	Pest
	Passeriformes	*Cardellina pusilla* Wilson's warbler bird	1	
	Rhabdita	Unclassified roundworms	3	
	Tylenchida	*Bursaphelenchus mucronatus nematode*	1	
Fungi	31 Orders		142	
Plant	Bryales	*Bryum sp* moss	1	
	Dicranales	Unknown moss	1	
	Poales	Unknown grass	2	
	Brassicales	Unknown mustard	1	
	Caryophyllales	Unknown cactus	1	
		Polygonum sp buckwheat	1	
	Fabales	*Medicago sativa* alfalfa	1	

Oxalidales	Cunoniaceae, a family from the S. Hemisphere	1
Pinales	Conifer	3
Rosales	Oleaster	1
Solanales	Unknown potato family plant	1

'Operational taxonomic units' (OTUs) are groupings of very similar DNA sequences. A unique OTU usually corresponds to a unique species, but one species can also have multiple OTUs due to genetic variation in the species.

When databases do not contain sequence data for the species, or there was too much uncertainty in which species a sequence may belong to, OTUs cannot be identified. Some of the taxa identified were odd, such as a spider endemic to Australia and a cactus thought to occur only in the Southern Hemisphere, which may be indicative of some closely related taxa present in Washington, but not currently in the gene sequence databases. Many of the insects identified by the databases were expected in apple orchards. Notably, the earwig stomachs contained DNA sequences from both pest insects and beneficial insects. It is important to note that this analysis does not address the quantity of any food eaten and whether the food was killed by the earwig or already dead and scavenged. Nonetheless, the potential for earwigs to interfere with biological control by eating other predators would seem to be a worthwhile research question in the future.

Work next year: Earwigs were similarly collected at the four sites used in Objective 1 of this study in 2017 and will be analyzed similar to the 2016 samples explained above.

Objective 3. Woolly apple aphid video monitoring

Methods

Study site. Our video recording study was conducted at a 2-acre block at Washington State University Sunrise Research Orchard. The block receives minimal management inputs and received no insecticides during our study. The block has 3' x 10' spacing. Each row consists of 12 trees divided in to 4 sections of 12 trees, with each section separated by 2 yards. We selected one 12-tree section of Golden Delicious trees for this study.

Earwig releases. Initial monitoring revealed low abundance of earwigs in the study block. Because we were specifically interested in studying them, we released earwigs into the experiment section. Twenty earwigs collected from a different orchard were released per tree on June 9, 12, and 14, and sixty were released per tree on June 27.

Video monitoring. Each camera was manually focused on one or more woolly apple aphid colonies in the experiment section. The two edge trees of the section were never used. After downloading video from the server to a computer for viewing, we used digital zoon to standardize the viewing area to approximately 13 by 13 cm. All visible branch or trunk area where the woolly apple aphid colony was located was defined as the observation area of interest.

There were two periods of study: 1) from June 13 to July 4, we recorded woolly apple aphid colonies from 7PM to 7AM each night, and 2) from July 4 to July 11 we recorded continuously. Cameras were focused on new woolly apple aphid colonies each week or after colonies disappeared.

On the computer, the number of times and amount of time any natural enemy appeared in the viewing area was recorded, along with the time and duration of any interactions between creatures with each other or the aphid colony.

Results

Thus far, only data from the first week of our video observation has been analyzed due the heavy time burden in reviewing video (Table 4).

During the first week of observation, earwigs were clearly the dominant predator of woolly apple aphid colonies, appearing in nearly 15 hours of the recordings, with nearly half of that time spent feeding on the woolly apple aphid colonies. Each colony observed was attacked at least once by an earwig, and one colony was completely destroyed. In contrast, green lacewings appeared more rarely, but also attacked colonies. The only other predator observed was the common harvestman, which appeared rarely and never attacked aphids. Interestingly, ants frequently scared off earwigs. When contacted by an ant, earwigs consistently raised their forceps in alarm, increased their movement speed, and changed their direction; this occurred 36 times in our observations so far. These frequent interactions may limit sustained feeding or colony discovery by predators. Earwigs rarely encountered one another, but were also aggressive when they did (only 2 encounters observed).

Table 4. Video data from the first week of observation. Visits were defined as any time an insect spent time in the observation area, and attacks were defined as any contact with the woolly apple aphid colony. Ant attacks were defined as a change in earwig behavior after contacting an ant. These ant attacks were instantaneous events which took no appreciable time.

Colony	Dates	Earwig			Green lacewing adult	
		Visiting (minutes)	Attacking (minutes)	Attacked by ant (#)	Visiting (minutes)	Attacking (minutes)
Trunk 1	June 13-19	37	16	21	0	0
Trunk 2	June 13-19	175	81	6	0	0
Twig 1	June 15-19	310	72	8	60	41
Twig 2	June 13-14	38	26	0	0	0
Twig 3	June 14-19	331	220	1	30	7
	TOTAL:	890	414	36	90	48

Overall, this portion of the study provided direct observations that earwigs frequently eat woolly apple aphids in a field situation and can provide biological control when other natural enemies are not yet present.

Work next year: the work on this objective will finish up the current monitoring data, but will also focus on the effect of adding the lures (next year's planned objective 4). We will use similar tactics as this year, where we will focus on WAA colonies, but compare the rates of visits with natural enemy lures present and absent.

Objective 4. Natural enemy lures and woolly apple aphid biological control

Work on this objective was not scheduled for the first year and will be started this next field season. Briefly, our work will focus on a larger scale implementation in orchards where WAA populations are sporadically distributed. We will locate 10 such areas within each of 3 orchards/yr. The 10 areas/orchard will be randomly assigned to either a control (no lures) or lures attractive to *Chrysopa nigricornis* and *Chrysoperla plorabunda* and monitored at weekly intervals to evaluate WAA population levels and egg deposition by the lacewing species. The focus of the sampling will be to evaluate the long-term effects of lure placement on WAA population levels.

CONTINUING PROJECT REPORT **YEAR**: 2 of 3
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. Requested: SCRI grant: $9,164,909 (funded); WSCPR: $16,356 (#16PN25, funded); $18,733 (#17AN029; funded); new proposal December 2017 (#18AN011, $21,851 - pending and contingent on continued WTFRC funding).

WTFRC Collaborative Expenses: None

Organization Name: WSU-TFREC **Contract Administrator:** Katy Roberts/J. Cartwright
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Item	2016	2017	**2018**
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**

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, 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 found in 21 counties in Washington, and populations in both eastern and western Washington are expanding
- The samurai wasp, *T. japonicus* was found for the first time in eastern Washington, in a park in Walla Walla
- The samurai wasp attacked 8 out of 15 (53%) of the sentinel egg masses placed in a Vancouver site
- *T. japonicus* was released for the first time in eastern Washington in a park in Yakima, where surveys in 2017 indicated it was not present
- Net enclosures continue to be promising for exclusion of direct pests such as stink bugs and codling moth; however, woolly apple aphid and spider mites continue to be problematic inside the cages, likely due to an insufficiency in biological control
- Single wall net barriers may be a practical substitute where complete enclosures are not feasible
- Native stink bugs appear to migrate between native vegetation and the orchard throughout a prolonged period during the summer; the majority of stink bugs entering an orchard were between 4-6 feet above the ground

Obj. 1. Determine distribution of *Trissolcus japonicus* in Washington

Obj. 1 Methods. Trissolcus japonicus was found for the first time on the west coast in 2015 in Vancouver, WA, and its presence confirmed there in 2016. This prompted broader survey to determine if it had extended its range to eastern Washington, and if not, to release it as a classical biological control strategy. Four sites in eastern Washington were surveyed (2 in Walla Walla, 2 in Yakima). Additional egg masses were deployed in 2 sites in Vancouver, WA to check if this population was perennial, and to collect more *T. japonicus* for release.

Plate 1. *Trissolcus japonicus* ovipositing in a BMSB egg mass.

A sentinel BMSB egg mass survey was used to determine the presence of *T. japonicus*. Eggs 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 1 to 10 days later (avg. 3.9 days) after deployment, returned to the laboratory and held at 22 °C (72 °F) until host or parasitoid emergence was complete.

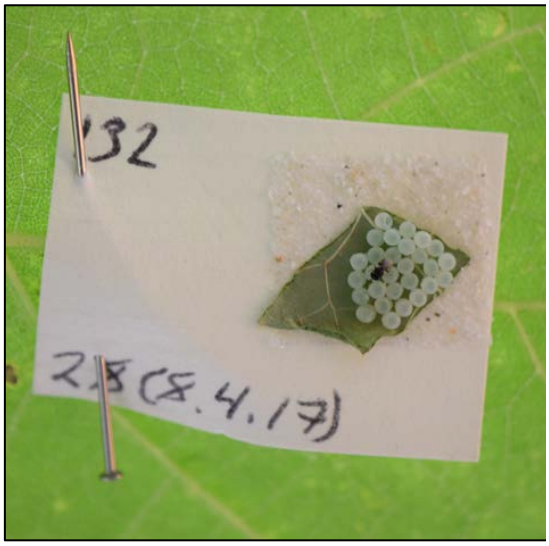

Plate 2. BMSB sentinel egg mass pinned to underside of a host plant leaf.

Obj. 1 Results. A total of 173 BMSB sentinel egg masses were deployed in six Washington sites in 2017. *T. japonicus* was found in 6 egg masses in the original site in Vancouver in June/July, yielding 120 females and 6 males. Eight BMSB egg masses were found parasitized in a second Vancouver site which had been positive the previous year, yielding 165 females and 16 males. In addition, a single wild BMSB egg mass was found at this site, also parasitized by *T. japonicus* (25 females, 0 males).

A total of 17 BMSB sentinel egg masses were deployed at a park in Yakima in July and August, all were negative for *T. japonicus.* Fourteen egg masses were deployed (June-August) at a second Yakima site, with similarly negative results. In one Walla Walla site, 24 egg masses (July-August) were deployed with no *T. japonicus* recovered. Importantly, two egg masses (out of 71 deployed) in **Pioneer Park, Walla Walla** yielded 36 female and 6 male *T. japonicus* in mid-August of 2017. **This is the first record of this parasitoid occurring in eastern Washington**, and thus represents a substantial range expansion within the state (ca. 210 miles from the Vancouver finds). Species identification was confirmed by Elijah Talamas, Florida Dept. of Agriculture and Consumer Services, Gainesville) on 27 September 2017), and were forwarded to the laboratory of Dr. Marie-Claude Bon (USDA-ARS-European Biological Control Laboratory, Montpellier, France) for haplotyping.

Obj. 2. Maintain a laboratory culture of *T. japonicus* in preparation for release

Obj. 2 Methods. The 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.
Obj. 2 Results: Based on the negative survey results in Yakima, we released parasitoids at one of the Yakima sites on 12 October 2017. **This is the first attempt at re-distribution of this parasitoid within the state of Washington.** Releases were made in the form of parasitized egg masses on card stock from the *T. japonicus* colony, and pinned to the underside of a host tree (the same method as the sentinel egg mass survey). A total of 21 masses were distributed on 3 host trees, for a total of 551 eggs parasitized by *T. japonicus*. When the egg masses were checked a few weeks later, 41 had exit holes, indicating some of the adults had emerged.

We elected not to release *T. japonicus* in Walla Walla in order to determine if natural spread and establishment occurs as determined by future surveys. A survey is planned for the summer of 2018, along with continued releases in Yakima.

Obj. 3. Evaluate IPM-friendly management strategies for BMSB

3a. Physical exclusion, large field cages.

Obj. 3a. Methods: To test the principle of physical exclusion, we used a native stink bug (the consperse stink bug, *Euschistus conspersus*), as a model for BMSB until such time as wild BMSB populations

start causing damage to commercial tree fruits. We also tested the ability to exclude other important direct pests (codling moth and leafrollers), and examined nontarget impacts on pests and natural enemies. The experiment was conducted in a 1.2-acre block of apples at the WSU Sunrise orchard near Rock Island, WA. The trees were planted in 2008 at a 3 x 10 ft spacing. Three treatments were tested: 1) cages made from shade netting (plus supplemental sprays), 2) conventional management (routine airblast sprays), and 3) an unsprayed check. The plots were 4 rows x 12 trees, with 4 replicates per treatment in a randomized complete block design. Each plot had one row of four different cultivars (Jonagold, Gala, Granny Smith, and Golden Delicious). For Treatment 1, cages were constructed around the 48-tree plot with trellis posts and dimensional lumber, and covered with commercial white shade net (Green-Tek pearl, 20% shade). Treatment 2 received routine airblast cover sprays for codling moth, but no other secondary pests; Treatment 3 received only herbicides and fungicides.

All of the assessments of pests and natural enemies in the block were from naturally occurring populations, with the sole exception of stink bugs. At the beginning of August 2017, consperse stink bugs from a field-collected colony were released near each plot to create artificial pest pressure. The ability of the insects to penetrate the netting of the cage and cause fruit damage were measured with aggregation pheromone traps and fruit damage samples.

Pests and natural enemies were sampled every two weeks from April through October, with the exception of mites, which were sampled every two weeks from June through September. Woolly apple aphid colonies and lady beetles were counted on all trees in the plot. *Aphelinus mali* was trapped on yellow sticky cards stapled to the lower trunk. Lacewing and syrphid adults were trapped using a GMP lure (plant volatiles; geraniol, methyl salicylate, and 2-phenylethanol) on a white sticky panel. Earwigs and spiders were trapped with a 4-inch roll of cardboard tied to the trunk with flagging tape. Mite samples consisted of 25 leaves per variety in each plot, mites were removed with a leaf-brushing machine, and counted with the aid of a microscope. Three tortricid pests (codling moth, obliquebanded leafroller, and pandemis leafroller) were sampled using their respective sex pheromones in a delta trap (1 trap/plot). Fruit damage was assessed after the first codling moth generation (July), and preharvest (September). In the latter assessments, direct pest damage and sunburn were recorded. A single index of the seasonal counts for each insect was calculated (cumulative insect days, or CID) and analyzed using analysis of variance (SAS 2016, PROC GLIMMIX). The CIDs are the average of two successive counts multiplied by the number of intervening days and summed over the season.

Obj. 3a. Results and Discussion. Stink bug fruit damage was significantly reduced in the caged (0.08%) vs the other two treatments (airblast 0.50%; check 0.36%) (Fig. 1). Codling moth pheromone trap captures were reduced 3-4-fold inside the cages compared to the other two treatments; obliquebanded and pandemis leafroller captures were negligible throughout the season. Codling moth damage averaged 5.0% inside the cages, compared to 13.5% (airblast) and 56.9% (untreated check) (Fig. 1). Surprisingly, thrips damage was higher in the cages (thrips data not shown). Spider mite densities were high in the mid-July sample (maximum of 2.64 mites/leaf; 100% brown mites), but not significantly different among treatments, although they tended to be higher inside the cages. Woolly apple aphid densities (CID) were 100-115-fold higher inside the cages than in the check and airblast treatments, respectively (Fig. 1). *Aphelinus mali* adults on traps were also higher inside the cages, indicating that either the netting did not represent a barrier to entrance of parasitoids, or the population present at the time of construction was able to perpetuate itself. The captures of lacewings and syrphids inside the cages were greatly reduced relative to the other two (uncaged) treatments. Earwigs tended to be higher in the checks, but with no significant differences. Sunburn was significantly reduced inside the cages (2.8%); surprisingly, the airblast treatment (10%) also reduced sunburn relative to the check (21%).

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.

Obj. 3b. Results and Discussion. 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) (Fig. 1). Thrips damage was significantly less inside the cages (0.21%). Woolly apple aphid densities were 400-500 fold higher inside the cages than in the airblast and check treatments, respectively (Fig. 1). 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. 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%).

Obj. 3c. Physical exclusion, single-wall barriers.

Obj. 3c. Methods. For the exclusion study, four commercial apple orchards in the Manson, WA area were chosen based on their history of stink bug damage. A border facing native habitat was divided into two 200-foot sections. A net barrier (200 ft long x 15 ft high; Fig. 2a) of commercial shade netting (20% pearl leno [white] net; Green-Tek West, Dinuba, CA) was constructed near the orchard border of one of the sections, and the other section served as a check. The grower was asked to treat the entire orchard for stink bugs as he/she normally would; thus, the barrier acted as a supplement to insecticidal controls. Stink bugs on the barrier were counted every four days and recorded based on which side of the barrier they were on (orchard vs vegetation). 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 July- September to determine the height at which stink bugs migrate into orchards. A sticky barrier (8 ft high x 6 ft wide; Fig. 2b) made of two trellis posts and horizontally attached dimensional lumber was constructed at the orchard boarder in five locations (2 in Stemilt Hill, 3 in Manson). Clear sticky panels, 1 x 6 ft (Alpha Scents, West Linn OR), were attached to the lumber from the ground up to eight feet high, with a two inch spacing between each trap. Stink bugs on the sticky panels were removed and their height of interception was recorded every four days.

3c. Results and Discussion. Net barriers. In all samples, stink bugs were present from the middle of June to the end of August. Beat tray samples of the surrounding vegetation resulted in substantially higher counts (559 stink bugs) than samples in the orchards (61 stink bugs). There was no significant difference in the amount of stink bugs found in the orchard between the barrier and check area. Stink bug damage levels were extremely low with 0.07% fruit damage in the barrier section, and 0.04% damage in the check. While differences were not statistically significant, the numbers of stink bugs found on the vegetation side of the net was twice that found on the orchard side.

3c. Results and Discussion. Sticky barriers. The numbers of stink bugs caught on the sticky barrier varied significantly by height. The highest percentage of stink bugs (21%) were trapped between 5 and 6 ft, while the least amount were trapped between 0 and 2 ft (5%) with none trapped on the ground (data not shown).

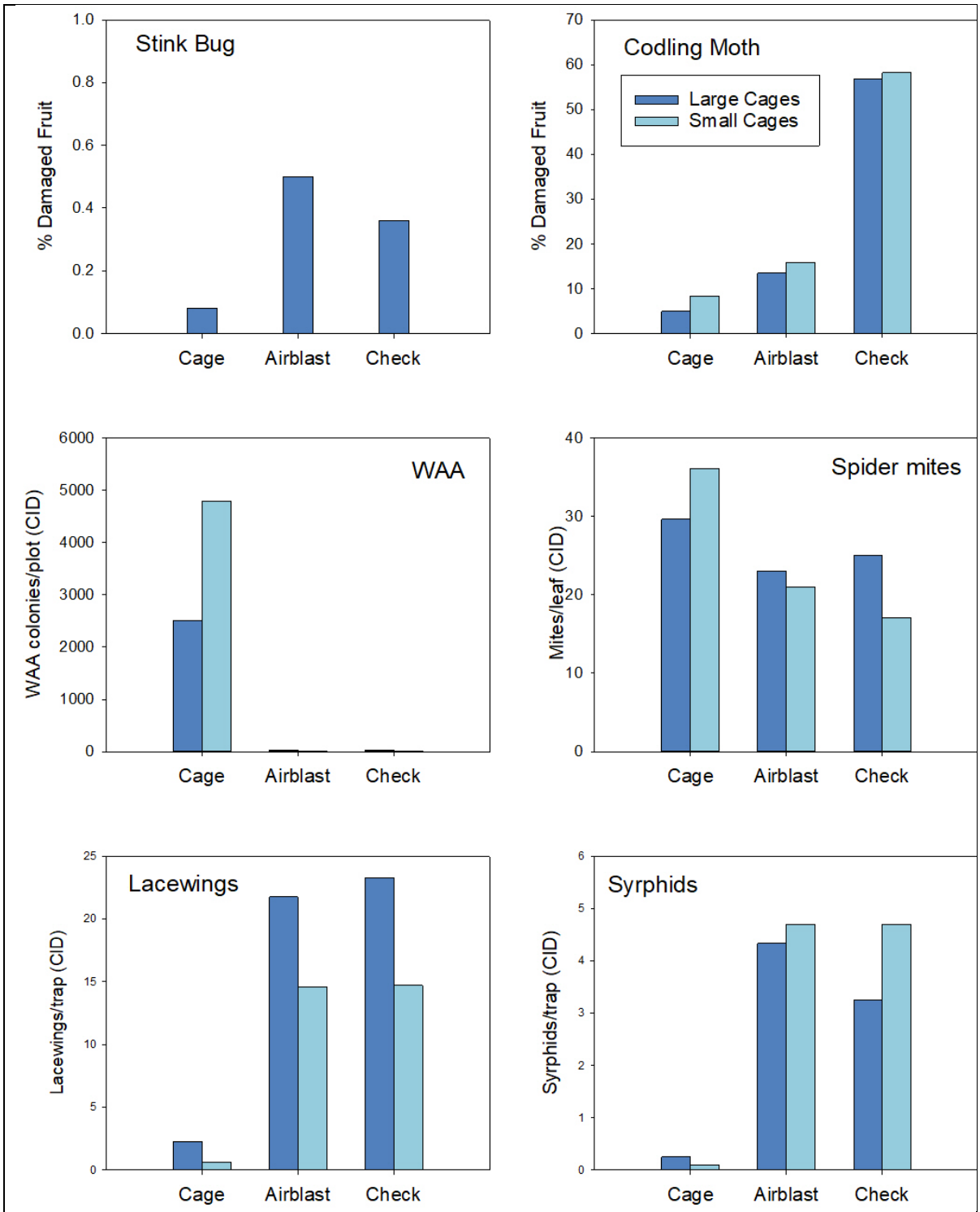

Fig. 1. Fruit damage or pest/natural enemy densities in caged, airblast, and check treatments, 2017.

Fig. 2a. Single wall net barrier between sagebrush habitat and orchard.

Fig. 2b. Barrier with clear sticky panels between sagebrush habitat and orchard.

Obj. 3 Conclusions. The preliminary information indicates there is potential for complete enclosure stink bug exclusion, but low orchard pressure in these tests makes it difficult to evaluate this completely. On the other hand, codling moth pressure in the research blocks was high, and both pheromone trap captures and fruit damage indicate a high degree of success in excluding this pest. The small cages, which have been in place for several years, only had a capture rate of 15 moths/cage, with a correspondingly low level of fruit damage (8%). The large cages, which were constructed in 2015, also had a low level of codling moth damage (5%) inside the cages. A mark/recapture study with codling moths in 2018 should provide evidence if the net is completely excluding external populations.

It is also clear that woolly apple aphids, and mites are increased by caging. In the case of woolly apple aphid, *A. mali* densities were actually higher in the cages, indicating netting is likely not a barrier to this tiny parasitoid. Conversely, two of the generalist predators, lacewings and syrphids, were almost absent inside the cages. Their absence is the most likely explanation of aphid increase, given that earwigs were either higher or unaffected by the cages. The consistently higher mite levels inside the cages is puzzling, given the lack of effect on predatory mites. Other factors, such as changes in microclimate caused by the cages, may be responsible.

Although the natural vegetation, orchard, and net barrier sampling in Manson did not result in any significant differences, it did give insight into stink bug seasonal migration habits. Stink bugs were abundant in both the natural vegetation tap samples and the net barrier visual counts throughout the season. This provides evidence that there may not be a single, large migration into orchards as previously thought, rather they may frequently move between the natural vegetation and the orchard throughout the season. The sticky barrier trial complements this finding in that stink bugs were caught on both sides of the panels continually from July through August. This trial also found that adult stink bugs fly into orchards, as few to none were caught on the ground trap. These results imply that mechanical barriers, such as netting, may not need to extend into the ground.

CONTINUING PROJECT REPORT **YEAR**: 2 of 3
WTFRC Project Number: CP-16-103

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

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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

Dovetail Genomics LLC and the UCR Office of Research and Economic Development provided funds for sequencing the WAA genome: $13,550

Budget 1
Organization Name: University of California-Riverside
Contract Administrator: Teeny Ellis
Telephone: (951) 827-2205 **Email address: teeny.ellis@ucr.edu**

Item	**2016 (WSU)**	**2017**	**2018*** **
Salaries*	$32,836	$20,646	**$32654**
Benefits*	$3,424	$16,463	**$22044**
Wages			
Benefits			
Equipment			
Supplies**	$22,450	$11,970	**$2,000**
Travel			**$500**
Plot Fees			
Miscellaneous			
Total	$58,710	$49,079	**$57,198**

Footnotes: *Salaries and Benefits are to support one PhD student for a year, a research scientist (Zhao) for two months.
**RNA sequencing services, lab supplies for tissue assays or extractions, and high-performance computer server access.
***2018 has an adjusted budget to reflect costs to travel within CA and WA for insect collection, and personnel time needed for a collaborator to complete objectives within the proposed project.

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 and proteins active in aphids. Comparing across cultivars that vary in resistant phenotypes will identify the processes in common and the different networks that define resistance. Linking insects to plants will help identify how aphids overcome resistance, identify how insect populations vary across growing regions, and aid in screening for more genes involved in resistance networks. Completion of this project will substantially increase our understanding of how apple responds to biotic stress, with a definitive list of aphid proteins that challenge apple immunity. Overall, we will increase the number of known plant traits that deter aphids and other organisms, and link these traits to genotypes to improve apple growth and production over the long term. Our project also aims to train one PhD student in molecular techniques with a focus on wooly apple aphid (WAA) biology.

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. Josh Wemmer is leading this as part of his dissertation and will be comparing population differences in aphid saliva across a latitudinal gradient. To date, the whole body transcriptome and salivary gland assemblies are complete, and proteome samples are being collected. In late 2017 we secured funding for the WAA genome to increase the ability to detect genes and their products related to colonization. Once completed, these data will represent the most comprehensive database of WAA genetics. The salivary secretion assessments will be completed by mid 2018, with an anticipated genome wrap-up by winter 2019. To expedite this process we have adjusted the original budget (proposed in 2016) to include personnel time devoted solely to genome, transcriptome, and proteome analysis.

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. Data analyses will commence in Winter 2018.

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 can phenotype the underlying biochemistry related to resistance. These assays will be informed by the final analysis of the plant transcriptome (Objective 2). This work will begin by summer 2018.

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. Once we conclude our network analysis, (Objective 2) we can increase our interaction with RosBreed scientists to aid in marker development.

Timeline. Our expected timeline is outlined below. We collected and assessed WAA during year 1, and are combining these data with a new genome assembly this winter. We are currently assessing plant immune function winter 2018, and will link this information together in a final summary during year 3.

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

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. Genes found only in salivary glands will be matched to the proteins secreted. 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. This year we will complete the transcriptome assay of resistant and susceptible *Malus* rootstocks under WAA attack to identify the genes mediating a successful immune response. Functional plant traits such as immune/defense pathways, or their metabolites will then be assessed this summer to link immune response to the gene networks and resolve the discrete traits that provide resistance. 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 1st-4th 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. To resolve this we are increasing the number of aphids used to provide saliva for each replicate, using a salivary stimulant, resorcinol, to increase saliva production, and will compare to the genome once assembly is complete. The use of a genome for this analysis is paramount because it will list all possible protein products found with an aphid, thereby improving our ability to screen substances found in saliva against known targets.

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. We will leverage this approach to determine 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 36h. RNA was extracted as described above. Samples are currently in the queue to be sequenced at the UC-Davis Genome Center. After processing, read alignment will be done by using free software (TopHat2; Kim et al. 2013) and the apple reference genome (Velasco et al. 2010).

Given what we know about other aphid-plant interactions, and insects that gall plants, we predict reactive oxygen signaling (ROS) and defenses to be active in addition to stimulation of the structural antiherbivore defense compound: callose. We will verify these pathways in the WAA transcriptome using the gene visualization software Mapman that syncs transcriptome data from the

latest genome assembly to metabolic pathways (Thimm et al. 2004), and through a gene co-expression network analysis. We will then fulfill **Objective 3** by assaying tissues to confirm what immune compounds are active during WAA feeding. Reactive oxygen compounds can be assessed via colorimetry where enzymatic-driven color changes of tissue extracts correlate with ROS (e.g., peroxidase, polyphenol oxidase; Nabity et al. 2006). Callose accumulation at feeding sites will be visualized using cleared tissue sections and aniline blue stain (Casteel et al. 2014). Given WAA increases total phenolics and alters amino acid profiles depending on resistance (Zhou et al. 2013), we will also link these functional traits to their genetic pathways as part of our systems approach to identifying additional biological functions that define or underlie immunity. This work is feasible given the lab's experience (e.g., Nabity et al. 2013a, 2013b)

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

From the WAA whole body transcriptome, we predicted 184 effectors that comprise 61 families of proteins (Table 1). We tested our modified bioinformatics pipeline on other insects, including the Hessian fly, and found that 97% of predicted effectors match the effectors identified previously. Therefore, these 184 putative WAA effectors are very likely the most important protein molecules that WAA secretes and injects into plant tissues during feeding to conquer host defense and/or interrupt plant physiological processes. Generating the WAA genome assembly will provide a definitive list to compare against and identify genes that may be lowly expressed (thus not detected but important) or vary among populations in different growing regions.

Among the 184 putative WAA effectors, 140 (76%) show little or no sequence similarity to proteins of any other organisms. This novelty suggests that the WAA evolved a specific suite of proteins for its interaction with apple. Once the functions of these proteins are validated, they could serve as important insecticide targets whose breakdown would paralyze the insect during initial infestation. It should be noted that because these proteins are specifically evolved in WAA, insecticides designed to target at them would be highly specific without harming beneficial species in nature. An example of such insecticides is the double-stranded RNA molecules whose gene is introduced into plant cells through genetic modification or transiently through topical spray/powder applications, and during WAA feeding, the double-stranded RNA silences only its corresponding insect effector gene, leading to the reduction of insect infestation.

Interestingly and importantly, we discovered one WAA effector is an aphid transmission protein, which is found in various caulimoviruses and potyviruses whose natural hosts are plants. This protein is critical for virus transmission by aphids and will likely prove significant in understanding more about virus-apple interactions. It is likely that WAA acquired this virus gene inside the insect genome and uses it as an effector to transmit viruses for host manipulation. With the completion of the genome, we predict many more fundamental discoveries of WAA biology to greatly enhance management strategies.

From the aphid survival assays, we identified the same immune phenotype (50% survival) in genotypes that differ in resistance: 5087 is known as resistant whereas 16 is susceptible based on genetic markers and longer duration performance assays. This sets up an excellent comparison of immune function beyond the ER2 gene, and is the target of sequencing assays for **Objective 2**. In another survival assay, 10 genotypes were scored for performance over 6 days. Six genotypes containing the ER2 gene but with unknown resistance phenotypes were found to reduce survival below 40% in 4 days, with one genotype preventing colonization entirely, leading to aphid dispersal and eventual death (Fig 1). This provides an excellent candidate for further analysis to identify the traits underlying this response. We anticipate adding this genotype to functional trait assays once physiological processes have been identified at the gene expression level.

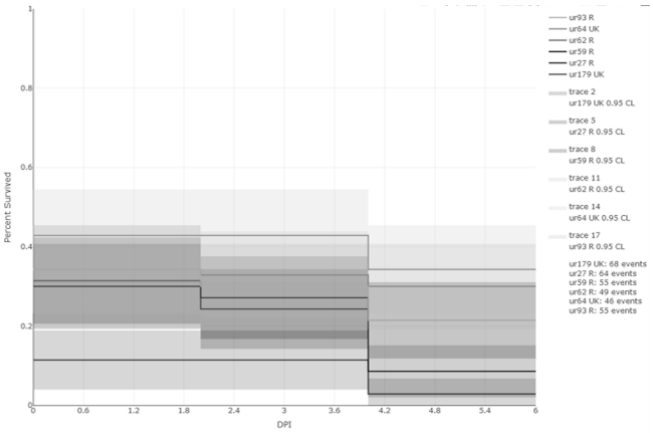

Fig 1. Unreleased genotypes with known resistance genes were screened for aphid survival over 6 days to assess immediate immune functions. Each line represents a genotype within a confidence interval. All lines indicate low colonization (<50%) and reduced survival through time.

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