APPLE CROP PROTECTION RESEARCH REVIEW
Thursday, January 26, 2017

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FINAL PROJECT REPORT **YEAR**: 4 of 3
WTFRC Project Number: CP-13-102A

Project Title: Codling moth attract-and-kill with kairomonal lures

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Total Project Request: **Year 1:** $22,000 **Year 2:** $40,000 **Year 3:** $40,000

Other funding sources: None

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

Item	2013	2014	2015
Wages	$13,000	$13,000	$13,000
Benefits	3,000	3,000	3,000
Supplies	5,000	4,000	4,000
Travel	1,000	1,000	1,000
Total	$22,000	$21,000	$21,000

Footnotes: Supplies needed are the materials to construct the A & K stations, vials, sachets, and chemicals for the kairomone lures, and for additional and replacement BL traps and batteries. Travel costs are for trips to multiple field sites.

Budget 2
Organization Name: Plant and Food Research New Zealand
Contract Administrator: Claire Hall
Telephone 64 3 977 7340 **Email address:** claire.hall@plantandfood.co.nz

Item	2013	2014	2015
Salaries			
Benefits			
Wages		$16,000	$16,000
Benefits			
Equipment			
Supplies		$3,000	$3,000
Travel			
Plot Fees			
Miscellaneous			
Total	0	$19,000	$19,000

OBJECTIVES

The overall objective or goal of the project is to develop and demonstrate control of codling moths (CM) in orchard plots using the attract-and-kill approach (A & K). Our prior research has led to the use of a sticky trap as killing station and a recently developed 3-chemical kairomone attractant as the lure. The technical objectives of the work are to:

1. Determine a best A & K density (traps per acre) to use.
2. Determine interactions between deployment of A & K traps baited with kairomone and traps baited with pheromone lures.
3. Determine the interactions of mating disruption and A & K traps baited with kairomone lures.
4. Determine efficacy of A & K traps for reducing oviposition and for prevention of infestation of fruit in orchard blocks early season as well as at harvest.

SIGNIFICANT FINDINGS (for 2013, 2014, 2015. 2016 field seasons).

1. The adhesives used in Alpha Scents or Trece trap liners or with spreadable Tanglefoot were equally effective in holding captured moths. However, there was a problem with Tanglefoot spray, with reduced moth catch.
2. The synergy of acetic acid, pear ester, and N-butyl sulfide for male and female codling moths was confirmed in additional tests in New Zealand.
3. One-acre Washington field tests showed strong reductions in catches of female codling moth in kairomone traps but not males in pheromone traps. There is no indication of any incompatibility with mating disruption, as indicated by catches in kairomone traps. It is well known that it is more difficult to monitor the moth populations with the pheromone in orchards under mating disruption.
4. Field testing of a dispenser that combined all three chemicals (acetic acid, pear ester, and N-butyl sulfide) in one sachet indicated a weakness compared to the prior dispenser method of using polypropylene vials for dispensing acetic acid and N-butyl sulfide and a rubber septum for pear ester. The problem was determined to be the release of the pear ester from the mixture. Subsequent laboratory work produced a dual sachet system that provides adequate long term release of all three chemicals. The two sachets are attached back to back so as to employ a single device in the trap.
5. Four-acre Washington field tests were conducted in the summer CM flight of 2014, the spring and summer CM flight of 2015, and the spring and summer flights of 2016. In all five of these tests, there was much less infestation of apples in treated vs untreated plots in heavily infested orchards, following 30 days of kairomonal trapping with 50 traps per acre.

RESULTS AND DISCUSSION

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 in place of a pesticide-treated surface for an attract-and-kill station target was suitable in commercial orchard settings where overloading of the surface is not a concern. Note that this approach is the same as prior attempts at “mass trapping” or “trapping out”, and maintains the primary advantages of the attract-and-kill concept of reducing or replacing insecticide use, and greatly reducing impacts on non-target insects including beneficial insects.

Early work on this project led us to conclude that we can use a commercial white Delta trap with commercial adhesive as an A & K trap. In addition, we settled on a formulation for our lure comprised of acetic acid + pear ester + N-butyl sulfide, to be used in the A & K traps. In 2013, we obtained preliminary evidence that a density of 50 A & K traps per acre significantly reduces numbers of adult codling moths, which we referred to as “knockdown”.

Much of the effort in 2014 replicated and confirmed evidence of knockdown of female codling moths in orchard plots, and then late in the season tested the hypothesis that the knockdown of moths results in reduced infestation of apples. Field plot tests in 2015 and 21016 provided similar results, with greatly reduced increases in CM-damaged apples in treated plots compared to control plots, under heavy CM pressure.

One acre plot moth knockdown. Thirteen replicates of this paired test were conducted in the spring flight of 2014. Plot monitoring for the 3 days preceding attract and kill deployment indicated similar moth populations in treated vs control plots. A & K traps deployed in treated plots captured 28.8 ± 16.3 female and 35.2 ± 17.1 male codling moths during the 7 days of the test. Totals of 375 females and 458 males were removed from plots by these traps during the one week duration of the test. Both male and female codling moths captured in the kairomone-baited monitoring traps in treated plots were reduced compared to those in control plots. Numbers of male codling moths captured in pheromone-baited traps were similar between control and treated plots, and numbers of moths in light traps were numerically, but not statistically, reduced in treated plots.

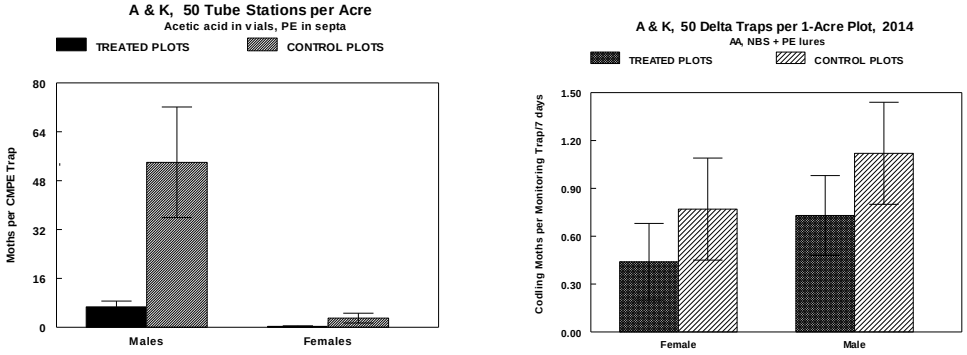


Figure 1. Mean numbers of codling moths captured in monitoring traps in A & K plots. On the left, in 2013, numbers of moths were greatly reduced in kairomone-baited monitoring traps in plots with tube shaped A & K traps. On the right, in 2014, numbers of moths in kairomone-baited monitoring traps were again reduced in plots with Delta-shaped A & K traps, but not so dramatically.

Four acre plot infestation reduction. Pairs of 4-acre plots were set up in August of 2014, June of 2015 and August of 2015, to compare CM infestation rates in apples with and without deployment of attract-and-kill traps. A & K traps baited with acetic acid + pear ester + N-butyl sulfide, were evenly spaced at ca 50 per acre, and these were maintained for 20 to 30 days. Treated and control plots were each monitored with four pheromone traps and four kairomone traps (AA + PE + NBS). Two thousand fruit were inspected in the field in each plot to determine codling moth damage rates. In the first and second tests, fruit were inspected at the end of the test. In the third test, fruit were inspected both at the beginning and at the end of the test. Fruit samples were 20 apples inspected per tree, for 10 trees per row, for 10 rows of trees per plot.

Numbers of CM in kairomone-baited monitoring traps were reduced in treated plots compared to control plots (Figure 2), while numbers of male moths in pheromone-baited monitoring traps were similar between the plots. Percentages of apples that were damaged by codling moth were less with the deployment of the A & K traps, compared to the untreated plots in all three tests (Figure 3). Over the four weeks of each test, from 352 to 397 female CM were captured in the A & K traps of the treated plot, and numbers of males captured were generally higher.

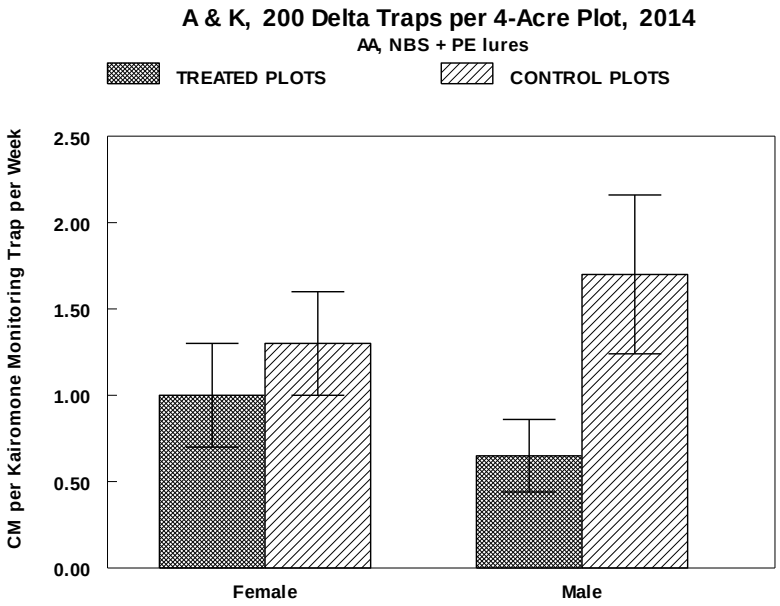
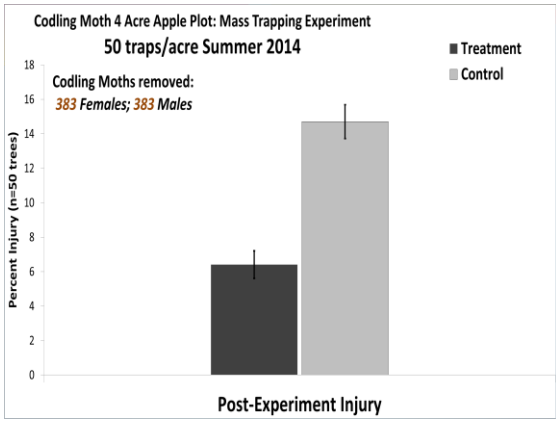


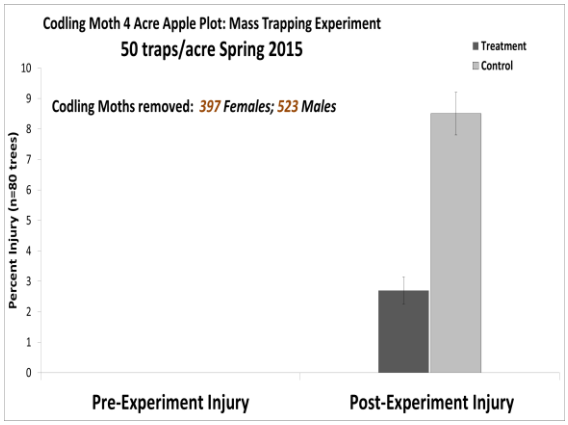
Figure 2. Mean numbers of male and female codling moths captured on monitoring traps baited with kairomone lures, in 4 acre plots treated with 200 A & K traps in untreated control plots.

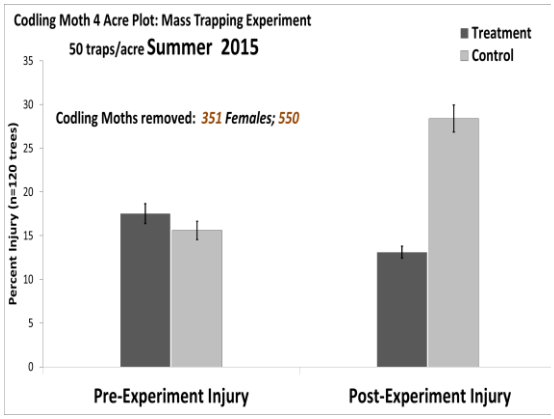
Results of the field plot tests consistently indicate an impact of the A & K traps on CM; both reducing the numbers of moths in plots, and reducing the codling moth damage to fruit. However, additional replicates of the larger scale plot tests need to be conducted before firm conclusions can be drawn.

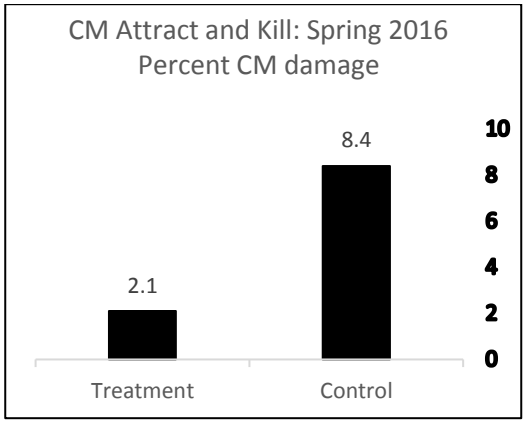
One might ask why the results with monitoring traps are not more dramatic, given the apparent strong prevention of infestation of apples. That is, with ca 50 A & K traps per acre, using a bisexual 3-component kairomone lure, why do we not see a complete elimination of codling moths in monitoring traps within the plot?. We suggest two possible factors; immigration and competition between stimuli. *Immigration*: In relatively small plots within larger orchards, moths can move freely from untreated to treated areas, confounding and obscuring results. With the use of chemical attractants, there is great risk of luring many moths into treated plots from untreated areas, again confounding and obscuring results. This was certainly seen in studies of mating disruption of CM, and the problem is diluted as an edge effect with an increase in the size of the treated area (such as in Areawide programs). *Competitive stimuli*: The moth response to the kairomone lures might be impacted by other sources of the same chemicals, by food sources, and by other types of attractants. For example, infested apples in the heavily infested orchard used for the 4 acre plot test could be a competing stimulus, and calling females and pheromone lures in monitoring traps could be competing stimuli that reduce male response to the kairomone lure. These are speculations, but call for the need for more research in what is a new area of exploration.

With the positive but varied trap catch results, the fruit infestation data from the 4 acre plots is most encouraging. Killing and removing female codling moths from the orchard is a reasonable goal, but the most important parameter to measure is the damage to the fruit. Although we will be conducting additional tests of lures, dispensers, and traps, we feel that the critical aspect of work to be done in 2016 is further replication of the 4 acre plot tests of the 50 kairomone A & K traps per acre, to provide rigor to conclusions regarding efficacy of this approach in protecting the fruit. Reducing the cost of the method is also an important goal, hence the continued focus on less expensive but effective dispensers and traps.









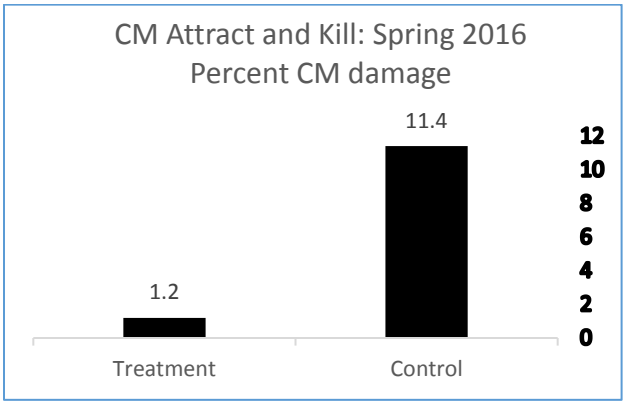


Figure 3. Mean percentages of apple fruit damaged by codling moth, in 4 acre apple blocks that were untreated controls, or were treated with 200 attract and kill traps at 50/acre.

REFERENCES CITED

Landolt, P. J., D. M. Suckling, and G. Judd. 2007. Synergism of a feeding attractant and a host kairomone for the codling moth (Lepidoptera: Tortricidae). Journal of Chemical Ecology. 33: 2236-2244

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

The codling moth remains the most important insect pest of apples. Control of the pest is usually obtained with applications of pesticides and pheromonal mating disruption, and other secondary measures. There remains a need for additional strategies and technologies (tools in the tool box) under some circumstances. For example, mating disruption loses its effectiveness where the moth population "escapes", and additional measures are then needed to bring that population level down to a point where MD works. Also, the measures used for control of CM in organic orchards are limited. The use of "mass trapping" to remove enough moths with traps to effect control could be a valuable tool to augment mating disruption. The method also has potential to meet organic certification requirements.

It is thought that attractants for females should be more effective for this type of approach, compared to sex pheromones that lure males, because the removal of females directly impacts oviposition and fruit infestation, while removal of males only indirectly and somewhat weakly impacts reproduction. Prior attempts in the 1970s to control CM using pheromone-baited traps gave mixed results that were mixed at best. Later work then has focused on the development of an increasingly stronger set of attractants for female CM, based on their need to locate oviposition sites and food. These efforts produced a lure comprised of acetic acid, pear ester, and N-butyl sulfide as a trap lure that provided a strong attractant for both male and female CM.

This lure has been evaluated for mass-trapping CM in apple orchards over a series of years. Earlier tests were conducted in one-acre plots and indicated strong reductions in the numbers of moths present in the block but not reductions in fruit damage. It was surmised that a problem existed with orchard edge effects that obscured the advantage obtained with the removal of those moths in traps, and perhaps the attraction of moths that were not captured in traps. Additional work on lure and trap optimization, and the expansion of the work to 4-acre apple orchard blocks resulted in consistent good evidence of fruit protection by the use of the new lure in Delta traps, at 50 traps per acre. All of these experiments were conducted in commercial orchards with strong CM pressure and high levels of damage. In total, the experiment was conducted 5 times, each time over a 30 day period at the peak of a flight. Two of these tests were conducted in the spring flight of the moth, and three were conducted during the second or summer flight. Apples were surveyed for CM damage either at the end of the 30 day test for spring flights (with a zero infestation at the start of the flight), or at the beginning and again at the end of the 30 days for the summer flight. This provided then estimates of damage incurred over the 30 day test period, with strong differences in damage incurred in control plots compared to trapped plots. These orchards with high populations of CM were purposely selected for the studies so that we could readily obtain the numbers needed (moths trapped, fruit infested) to determine the impact of the trap deployment.

Future Directions

These results demonstrate feasibility of this approach as a new means of controlling CM in apple orchards. We think it is necessary to pursue the approach further to 1) assess control over the entire field season rather than 30 day time periods, 2) reduce costs with less expensive lures and traps, and 3) continue to improve lure efficacy. Regarding #1 above, it is expected that the control of CM damage will only be more complete with maintenance of traps from first CM flight until harvest. Regarding #2 above, we have done much work to develop a sachet system as attractant dispensers for these chemicals, but additional field validation of lure efficacy needs to be done. Additionally, it appears that a simpler disposable trap that is a tubular shape would be cheaper to use than the Delta trap. And regarding #3 above, there always seem to be opportunities to change or improve the power or consistency of such lures.

FINAL PROJECT REPORT Year 4 of 3 (with a one year no cost extension)

WTFRC Project Number CP-13-100A

Project Title: Chemical mediation of aggregation by brown marmorated stink bug

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Cooperators: Tracey Leskey, USDA, ARS Kearneysville, WV
Helmuth Rogg and Todd Adams, Oregon State Dept. Agric., Salem Oregon

Other funding sources

Agency Name: USDA, CSRS, SCRI program.
Amount awarded: $16,500
Notes:

Total Project Funding: $120,000

Budget History: Combined Wapato and Riverside

Item	Year 1:	Year 2:	Year 3:
Salaries			
Benefits			
Wages	$24,535	$24,776	$25,022
Benefits	6,328	6,415	6,513
Equipment			
Supplies	8,137	7,809	7,465
Travel	1,000	1,000	1,000
Plot Fees			
Miscellaneous			
Total	$40,000	$40,000	$40,000

RECAP OF ORIGINAL OBJECTIVES

The overall objectives of the project were to discover and develop chemical attractants and attractant synergists for brown marmorated stink bug (BMSB) based on their host-and mate-location behavior. The experimental objectives were to:

5. Determine sex attraction responses of female BMSB, including physiological and environmental regulators of that behavior.
6. Determine host plant preferences, and female and male BMSB attraction to host plant odor.
7. Determine host plant effects on BMSB sexual pheromone behavior.
8. Isolate and identify plant kairomones that mediate or enhance BMSB attraction behavior.
9. Determine interactions between male BMSB pheromones and host plant kairomones, to develop superior attractants.

SIGNIFICANT FINDINGS

6. Both attraction and repulsion of bugs by plant odors was demonstrated. This provides initial target plants and a bioassay to use in isolating and identifying plant kairomones.
7. Strong female BMSB attraction to males was demonstrated, providing opportunities to isolate a male-produced attractant, and a bioassay method to use for that purpose. This behavior was subsequently shown to be related to male aggregation within shelters provided to them.
8. A pheromone was recovered from solvent washes of jars housing male BMSB that is very attractive to females and is repellent to males.
9. An alarm pheromone response was demonstrated for BMSB. This work is being pursued to determine the functions of alarm pheromone, and to understand the various roles of complex BMSB body odor and signal chemistry.
10. Thigmotaxis was demonstrated. This behavior is important to study and understand the conditions under which BMSB aggregates, and then the roles of pheromones in that aggregation.
11. An electro-antennal detector (EAD) was modified for the BMSB antenna and its effectiveness was demonstrated using BMSB and published pheromone chemicals. This system was then used to determine which compounds in defensive secretions are detected by BMSB, and which compounds in samples of male volatiles are detected by female BMSB.
12. The volatile chemistry of BMSB males and females was characterized and compared, to provide a baseline from which to detect and determine chemical signaling.
13. The volatile chemistry of BMSB defensive/alarm secretions was characterized. A set of these chemicals were found to be repellent to paper wasps that are potential predators, affirming the defensive roles of these compounds.
14. A new combination of pheromone lure and trap was found to be significantly more effective in capturing BMSB, compared to the most-used commercial trapping system.

RESULTS AND DISCUSSION.

We were able to demonstrate several behaviors in BMSB that relate to attraction and aggregation. Knowing that these behaviors exist provides the opportunities to pursue isolation and identification of active semiochemicals, and the development of bioassays that are necessary to isolate the active chemicals involved. These behaviors include attraction and repulsion by plant odors, sex attraction, alarm and defense, and thigmotaxis (arrest in response to contact with surfaces).

Attraction and repulsion of bugs by several plant odors. A Y-tube olfactometer system (Landolt et al. 2000), was used to determine BMSB responses to plant odor (Figure 1). For each plant species, we tested female BMSB response to a bouquet of foliage (often with fruits) versus an empty chamber. A minimum of 60 bugs were tested one at a time per plant species, with the bouquet replaced for each ten females. For most plant species, there was not a significant response. Of particular note was the repellency of wild *Clematis*, which is a preferred late season plant for native stink bugs, and

attractiveness of both potato and green beans to BMSB. We also determined that the bug responses are faster in a vertical orientation compared to a horizontal orientation. The strong difference between male and female stink bug response in this test (Figure 1) suggests that the behavior is host finding for purposes of oviposition site selection, rather than a search for adult food.

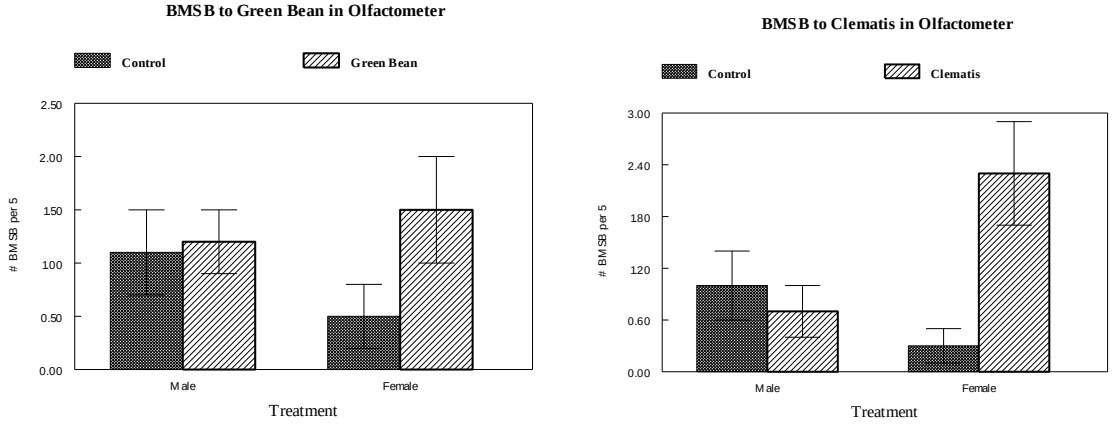

Figure 1. Examples of numbers of female BMSB responding to airflow from over plant material (green beans and clematis) in a choice olfactometer. N = 60, as 12 groups of 5. Control is airflow through an empty jar.

BMSB Thigmotaxis. BMSB appear to seek out and hide in tight places. We constructed 3D slatted shelters out of cardboard, and placed a 3 inch wide shelter in the corner of a 16 X 16 X 16 inch screened cage. Most stink bugs moved into these shelters and stayed in these shelters (Figure 2). This behavior may be an important aspect of one type of aggregation behavior and was the basis for our assay for sex attraction.

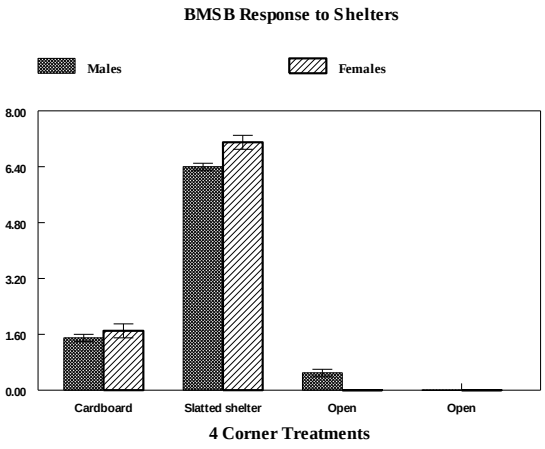

Figure 2 (left) Numbers of male and female BMSB moving into shelters made of cardboard and placed in upper corners of cages. Numbers are counts of those within or on shelters or in the remaining open upper corner of the cage.

Female BMSB attraction to males. Several experiments tested the hypotheses of female attraction to males and male attraction to females. Either male or female BMSB were placed in a cage with a shelter in which they entered and stayed. This shelter was in turn tested for attractiveness to other BMSB in an olfactometer assay. Each assay involved the testing of 60 stink bugs, one at a time, in serues of 6 batches of 10, with the treatments (stink bug shelters with residual odor) replaced for each set. Responses of females to males was strong, but not female response to females (Figure 3). A

much stronger response was seen when the assays were conducted in the scotophase (night) under red light versus the photophase (day), and when the olfactometer was oriented vertically compared to horizontally (Figure 3). A further breakthrough was the successful "capturing" of the sex pheromone in solvent used to rinse glass jars housing males in shelters. This solvent was then very attractive to females (Figure 4), indicating the presence of the pheromone in the sample, which was then analyzed by GC-MS. Interestingly, the same samples were repellent to males (Figure 4).

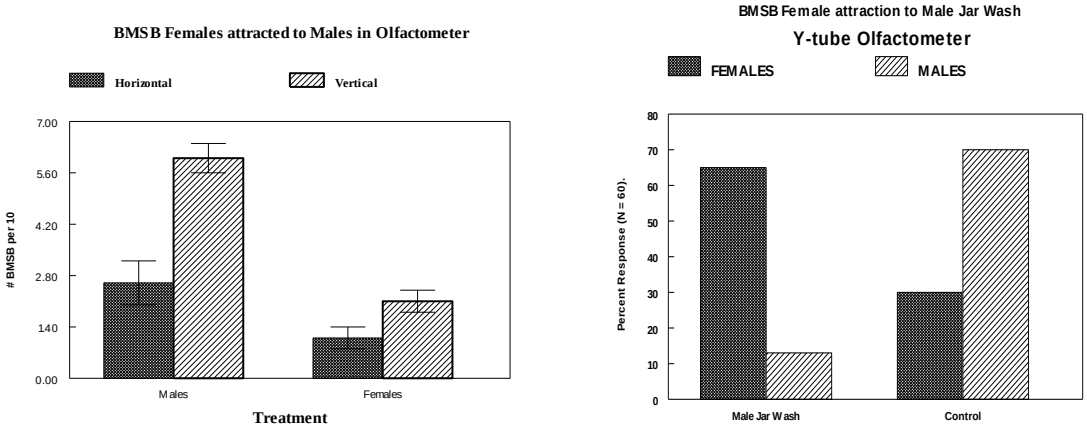

Figure 3 (left). Numbers of female BMSB orienting to airflow from over a male-occupied shelter versus a female-occupied shelter in a choice olfactometer. N = 60.
Figure 4. (right). Percentages of female and male BMSB responding to a solvent wash of a jar housing male BMSB, applied at a rate of one male-day equivalent. The control is a solvent wash of an empty jar.

BMSB Alarm Response. An alarm pheromone response was demonstrated for BMSB. In an arena type assay, stink bugs showed an escape reaction in a 20 second response to a puff of air from a chamber with a disturbed bug (Figure 5). This work was pursued to determine if there is a conspecific alarm-type response to BMSB defensive chemistry (an alarm pheromone), and to obtain overall a better understanding of the complex roles of BMSB body odor chemistry.

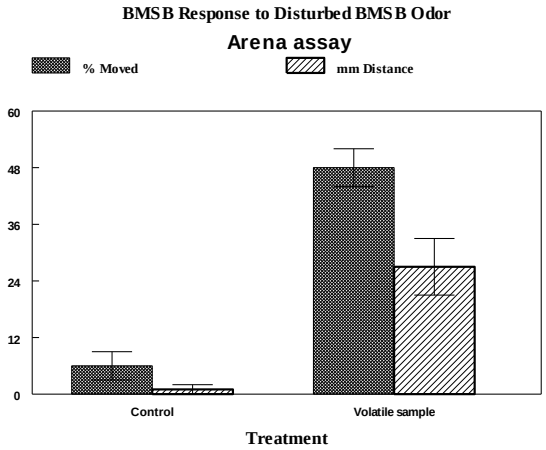

Figure 5. Numbers of male and female BMSB (combined) responding to airflow from a single disturbed BMSB. Movement was noted, as well as the distance moved in the 20 second long test. N = 100 as 20 groups of 5. Control is air from an empty jar.

Additionally, we showed repellency of *Polistes* paper wasps to the odor of disturbed BMSB and to individual chemicals that make up the defensive secretion of the bug.

Coupled Gas Chromatographic-Electroantennographic Detection (GC-EAD) Analysis. Coupled GC-EAD analysis was performed. The column effluent was split 1:1 in the oven to produce airstreams to both to the GC detector and into a humidified air stream directed toward the mounted antennae of brown marmorated stink bug.

One of the two antennae was separated from the head and it was positioned between two gold wire electrodes immersed in saline-filled (46mmol NaCl, 182mmol KCl, 3 mmol CaCl2, and 10mmol TrisHCl at pH 7.2) micropipettes in an acrylic holder. The output signal from the antenna was amplified (10×) by a customized high input impedance DC amplifier and converted to a digital signal (IDAC-232, Syntech) and recorded on a computer using a dedicated software (GC-EAD, Syntech). A total of ten antenna set-ups were prepared and each antennae preparation was tested on SPME headspace adsorption of a commercial stink bug lure (Sterling). Consistent and significant antennal responses were achieved for 5 different female pheromone chemicals, using male BMSB antennae. This development is important because there are no good precedents in the literature for the methods or even the ability to obtain electroantennal responses to semiochemicals from stink bugs. This accomplishment provides a powerful tool for us to isolate other semiochemicals such as plant kairomones or pheromones involved in BMSB aggregation behavior. This technique for example was critical to our rapid identification of a feeding attractant lure for spotted wing drosophila, using volatile chemicals from a wine/vinegar bait (Cha et al. 2012).
Consistent and measurable antennal responses were obtained to synthetic samples of pheromones reported in the literature. This GC-EAD system was then used to determine which chemicals in disturbed BMSB samples are detected by paper wasps, and which chemicals in male BMSB samples are detected by female BMSB, using behavioral assays developed.

The volatile chemistry of BMSB males and females was characterized and compared, to provide a baseline from which to detect and determine chemical signaling. Volatile collections from over female BMSB showed the presence of 6 compounds when the stink bugs were quiet, which increased to 20 compounds when they were disturbed. Undisturbed males released 4 compounds, while disturbed males released 20 chemicals. All of these chemicals are identified and a number of them have been evaluated in bioassays and field tests.

Field sampling of stink bugs. During 2015, a dozen traps were placed in residential properties, and about 60 additional field collections were made to assess the species makeup of stink bugs, to detect the presence and spread of BMSB, and to determine potential preferred host plants. Sampling was accomplished with a beating sheet and sweep net to sample foliage in non-agricultural habitats. These collections were principally in Yakima County, and yielded nearly 450 stink bugs, all which were identified to species. About 20 BMSB were collected in 2015 as a part of this study, in pheromone traps, on plants, on structures, and through WSU Extension of Yakima and the Master Gardener Program, in the cities of Yakima and Sunnyside. BMSB were found over a broad area of the west side of the city, but not in agricultural or rural areas. During 2016, the use of 20 pheromone baited traps in addition to plant sampling, observations on structures, and the Master Gardeners, yielded over 250 BMSB collected in Yakima, with the largest numbers west of downtown and with increased numbers and sites in the West Valley area out to Ahtanum and the Apple Tree Gulf Course.

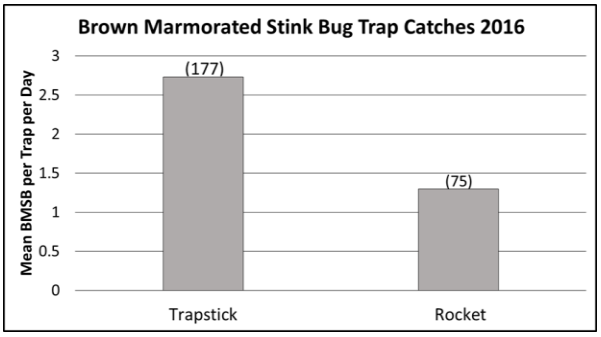

Figure 6. Mean numbers of brown marmorated stink bugs captured in Sterling International Inc. TrapStik versus Rescue (Rocket) traps. N = 18. Yakima County, WA.

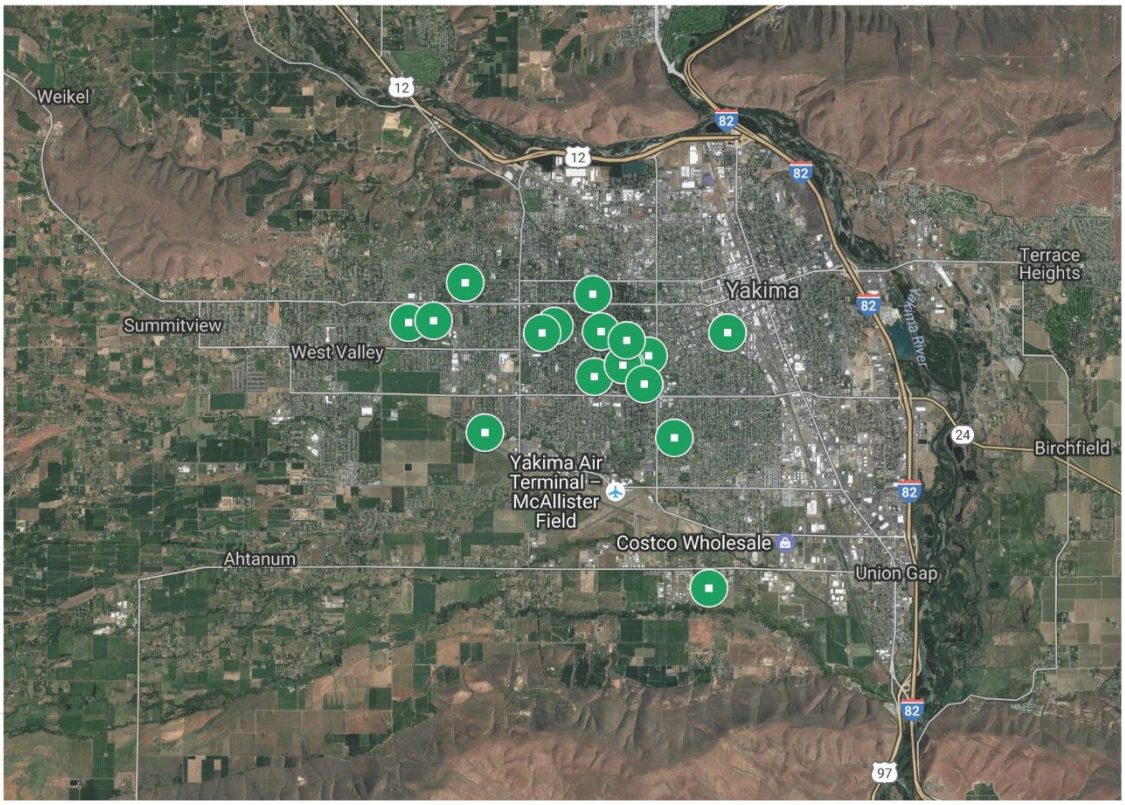

Figure 7. Positive sites in Yakima for BMSB, Sept./Oct 2016, using Sterling Inc. TrapStik and Rescue (Rocket) pheromone-baited traps.

EXECUTIVE SUMMARY

The adult brown marmorated stink bug is long-lived. The adult stage may persist for nearly a year and the bug displays a diversity of semiochemical-mediated behaviors over the course of that year that provide multiple opportunities for manipulation if they are determined and understood. By demonstrating different behaviors (aggregation, attraction, and alarm) and behavioral responses to odor signals in the laboratory (sex attraction, adult aggregation, alarm) we developed and provided assay methods needed for isolation and determination of the chemical signals that elicit these different behavioral responses.

We characterized the chemistry of BMSB secretions and odors, and related those to the behaviors studied. We determined BMSB volatile compounds that are specific to males and those specific to females. We determined the compounds emitted by BMSB when they are disturbed and showed that several of these chemicals repel predatory wasps, confirming the defensive nature of those compounds. We also showed that alarm pheromone stimulates BMSB dispersion. There is potential for these compounds to be used to disrupt BMSB aggregations and other activities. We characterized the chemistry of male-specific chemicals released when males are attractive to females and confirmed the attractiveness of those chemicals to BMSB.

At the end of the 2016 field season, some of this work came to a fruition with a significant improvement to a pheromone-based trapping system. A change in the combination of pheromone lure and trap design more than doubled trap catch compared to the commercial system most commonly in use. This result provides a simpler, cheaper, and more effective means of detection of BMSB in the field. We took advantage of this development to take a quick late season assessment of the prevalence of BMSB in Yakima. That assessment shows the widespread nature of the bug in the city.

Although the techniques that we used to assess local BMSB populations changed over the life of this project, the results of sampling and trapping show a clear and sharp upward trend in their numbers and distributions, although still largely restricted to urban and suburban landscapes. That trend is expected to problems where orchards and human habitations and neighborhoods are in proximity. This situation appears to differ from that of the native consperse stink bug which is problematic in apples where orchards border largely feral (old field, and Steppe) rather than urban/suburban landscapes.

Future directions. Our findings and experimental results, together with advances made by other laboratories, indicate 3 potential roles for applied chemical ecology to manage BMSB. 1) The determination of BMSB defensive chemistry having an alarm function suggests a possible application to disrupt aggregation behavior. 2) The movement of bugs to shelters and production of a female attractant while in those shelters suggests that females can be captured and removed by a suitable combination of male pheromone and shelter. Similar shelters have been evaluated for capture of overwintering bugs, and this might be expanded with chemical attractants, but also for use with non-overwintering bugs. 3). There are currently problems with stink bug pheromones attracting bugs that are not captured in traps; potentially worsening a situation in a crop. Strong advances in trap efficacy may be a solution to that problem, and improve the use of pheromone-baited trapping for monitoring in cropping systems.

FINAL PROJECT REPORT

Project Title: Evaluating plant volatiles for augmenting biological control

PI: Vincent P. Jones
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City/State/Zip: Wenatchee, WA 98801

Cooperators: Jay Brunner, TFREC

Other funding sources

Jones VP and C O'Leary. Spatial and temporal dynamics of attracting green lacewings to synthetic lures in apple orchards for pest suppression**.**
Agency Name: WSU-BioAg Grant
Amt. awarded: $37,866

WTFRC Collaborative Expenses: None

Total Project Funding: Year 1: 54,573 Year 2: 55,493

Budget History:

Item	2015	2016
Salaries[1]	26,826	29,030
Benefits[2]	2,492	2,406
Wages	18,720	18,720
Benefits[3]	1,835	449
Equipment	0	0
Supplies[4]	2,500	2,600
Travel[5]	2,200	2,288
Miscellaneous	0	0
Plot Fees	0	0
Total	54,573	55,493

Footnotes:
[1] New PhD student
[2] 4.8%
[3] 2.4%
[4] includes lab and field supplies
[5] w/in state travel

Objectives:

1. Determine the area of attraction of lures for the lacewings *C. nigricornis* and *C. carnea*.
2. Evaluate the effect of lure placement on population growth of woolly apple aphid.
3. Determine if increased lacewing egg deposition occurs within the active area of the lures and if this results in greater larval lacewing densities.

Significant Findings:

- Activity of lacewings is primarily concentrated around dusk and dawn.
- Distance of attraction from both the squalene and the AMP lures was <10' for both species of lacewings. This suggests large-scale disruption of natural enemy searching will not cause pest outbreaks in other areas of the orchard and that the lures can be used to manipulate lacewing adults on a small scale.
- Video observations showed lacewing activity around squalene lures was 18-fold higher than around control lures and 2.2 fold higher around AMP lures than control lures.
- Lacewings attracted to the squalene lures only spent an average of 8.8 min on lures, so that normal behaviors associated with mating, feeding, and oviposition would not likely be significantly affected and biological control would not be interfered with.
- Use of lures appears to reduce the buildup of WAA populations in the fall after the summer population crashes related to high temperatures.

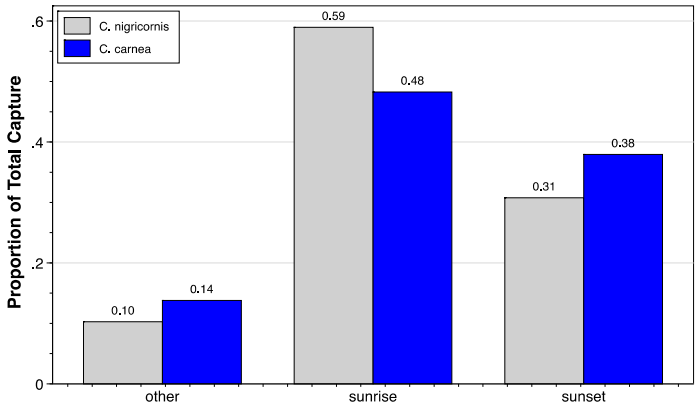
Results and Discussion

Objective 1. Determine the area of attraction of lures for the lacewings C. nigricornis and C. carnea.

Methods:

Determine the area of attraction of lures for the lacewings *C. nigricornis* and *C. carnea*.
We used high-resolution video cameras at distances of 0, 5, 10 and 150 feet from a tree where either a squalene or an AMP lure (Acetic Acid, Methyl Salicylate, and 2-Phenylethanol) were placed. The camera at the 150-feet distance was considered to be a control that indicated the general activity level in the orchard. Studies were performed from late June to late August 2015, with the lures being left in the field for a 4-day period and recording each day. PVC frames held the cameras 8 feet above ground level, pointing vertically down at an 8 in. x 8 in. platform held at 4 feet off the ground. The platform, made of wood, was covered in gray construction paper and a one-sided 8 in. x 8 in. clear sticky trap. Lures were attached on the top side of the platform using binder clips. The cameras were set to record for 10 hours total over each day; the hours were adjusted throughout the summer in order to record the hour immediately before and after sunrise and sunset. The other 6 hours of recording were evenly distributed throughout the rest of the day and night. During the non-recording days, lures were removed and the entire recording set up was shifted so that residual volatiles would not interfere with the next set of recordings.

Fig. 1. Proportion of total observations of C. nigricornis or C. carnea at different times of the day.



Results:

Daily Activity Patterns:
Video analysis of the time of day showed that peak activity of *both C.*

nigricornis and *C. carnea* occurred around sunrise, with the second peak around sunset. Flight activity at other times of the day or night was very limited, with only 10-15% of the total flights observed (Fig. 1).

Distance of attraction:
C. nigricornis was most abundant on the video on the lured tree, with the activity five or 10 feet way not significantly different from the activity recorded with the 150-foot control camera (Fig. 2). We were surprised to see *C. carnea* in the vicinity of the squalene lure (squalene traps almost exclusively catch *C. nigricornis*), but the distance drop-off was similar to *C. nigricornis* with the numbers significantly higher on the lured tree, but no significant difference between the longer distances. It is possible that squalene affects *C. carnea* much like it does female *C. nigricornis* which are attracted near squalene lures, but will not enter traps.

The AMP lure had a slightly slower drop-off with distance, with both *C. carnea* on trees five feet away from the lure still showing activity about half of that on the tree containing the lure (Fig. 3). However, trees 10 feet from the lure showed no more attraction than the general orchard seen 150 feet away. *C. nigricornis* is also attracted to the AMP lure (although not as strongly as to squalene), but there were no significant distance effects likely because only 17 lacewings total were captured.

These results show that the lure activity is very restricted spatially. These results are similar to work with other types of plant volatile lures that have been performed in corn and soybean and independent results performed by Dr. Tom Unruh at USDA-ARS in 2016. These results strongly support the idea that we can use the lures to aggregate populations of natural enemies in areas of high pest density without disrupting the overall spatial distribution of within the orchard.

Fig. 2. Percent of total activity observed at each distance from a squalene lure. Bars for each species with the same letter are not significantly different at p = 0.05.

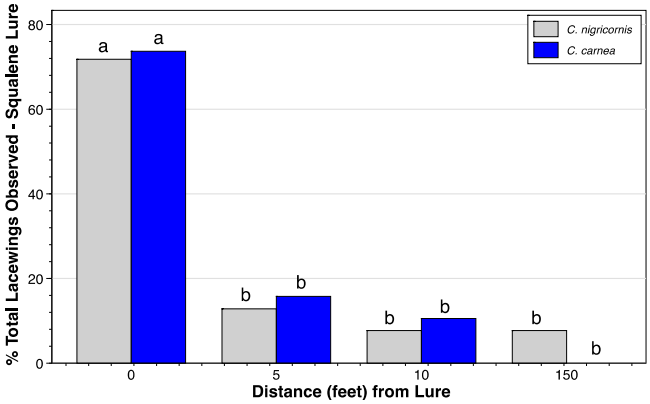
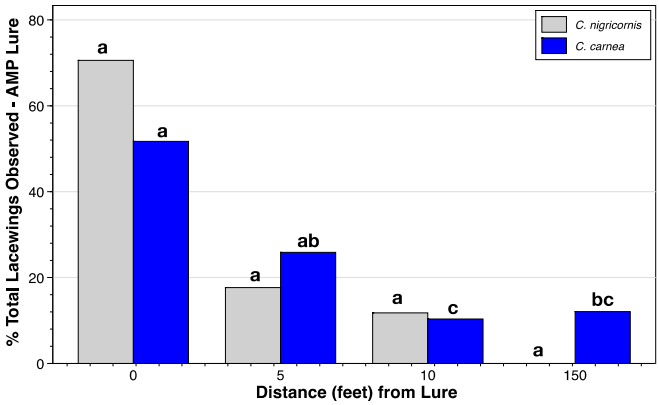


Fig. 3. Percent of total activity observed at each distance from an AMP lure. Bars for each species with the same letter are not significantly different at p = 0.05.



Objective 2. Evaluate the effect of lure placement on population growth of woolly apple aphid.

Methods:
In 2016, we used video recording to evaluate activity of lacewings in trees with either an AMP lure, a blank (water filled) control lure, or a squalene lure. We set up two replicates of trees spaced 4 rows apart; within a row, six consecutive trees were considered a treatment (AMP, Control, or Squalene) with a single lure put between tree 3 and 4 in the row; all treatments were separated by >60 feet. Because some lures are suspected of inducing the production of plant volatiles in trees that are attractive to natural enemies, we moved the lures each time cameras were moved between replicates; this kept the trees from being exposed continuously to the volatiles from the lures, which would

minimize plant induction of volatiles. Monitoring began 10 June and ran through 23 Sept. Video recordings started 28 June and ran through 23 Sept. During that time, we moved the cameras from replicate 1 to replicate 2 or vice-versa at weekly intervals.

Cameras were focused on a platform with a lure and recorded for 8 hours a day, with most of the coverage overnight. Cameras recorded from 6-8 pm, 12-1 am, 3-5 am, 12-1 pm, 4-5 pm, and 8-10 pm. When any lacewing came into the field of view, its behavior was classified into seven different categories: (1) crosses the video screen without stopping, (2) seen on a branch, leaf or trellis adjacent to the platform, (3) on the platform but not on the lure, (4) circles lure, (5) actually on the lure, or (6) interacts with another lacewing generally on the lure. For each category, the number of individuals and the length of time to complete the behavior was calculated. We summarized the data by plot as well as a grouped (both plot data).

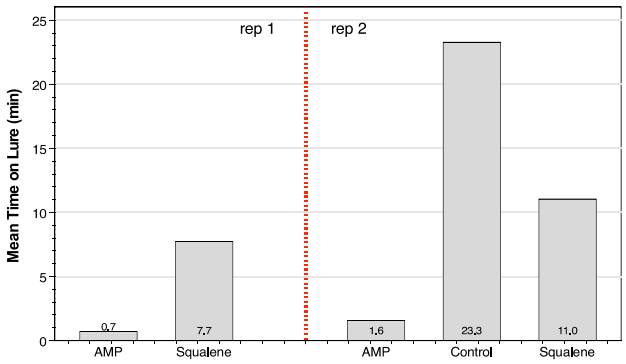
Results:
The data clearly showed that in both replicates, the squalene lure had the greatest activity of any kind adjacent to the lures (Table 1). Over both replicates, 85% of all activity was seen in the squalene treatment versus 10% and 5% for the AMP and Control lures, respectively. In the squalene treatments, 49% of the activity was a lacewing "landing on the lure" and 25% "landing on the platform adjacent to the lure." We also found some instances of interaction between different lacewing individuals, but only on the squalene lure; most of these were short-lived and resulted in one of the individuals leaving the lure.

Table 1. Number of times different behaviors were observed in field video observation in 2016.

	Treatment			
Behavior	**AMP**	**Control**	**Squalene**	**Total**
Crosses screen w/o stopping	96	52	145	293
On leaf, branch or trellis	26	9	106	141
On platform	24	5	336	365
Circles lure	5	0	42	47
On lure	4	3	620	627
GLW-GLW interaction	0	0	35	35
Total Observed	**155**	**69**	**1284**	**1508**

The data showed a completely different pattern for the AMP and Control treatments where the most common behavior was "moving across the screen without stopping" (63% and 72%, respectively), followed by "resting on a leaf, branch or trellis" and "resting on the platform" (Table 1). The relatively low lacewing activity associated with the AMP lure is likely caused by much lower population levels of *C. plorabunda* which is the primary lacewing attracted to AMP; trapping in areas close by (but not in the plots) showed the trap catch of *C. nigricornis* (highly attracted to squalene) was 4-fold higher than that of *C. plorabunda*.

Fig. 4. Mean time lacewings spent on lures for different lure types in the two different replicates.



This data confirms observations made in previous years using unbaited sticky traps,

where we found that trees with lures had a roughly 8-10-fold increase in lacewing activity over untreated areas.

A major concern of using the lures has always been whether the lacewings might spend too much time on the lure at the expense of mating, laying eggs or feeding in its vicinity. On average, video analysis showed that lacewings spent 7.7-11 minutes on the squalene lures (Fig. 4), with some spending as much as 2 hours; however, 75% of individuals spent less than 12.2 minutes and 90% spent less than 21 minutes on the lure (summarized over the two replicates). These data suggest that the lures are not so disruptive that biological control would be negatively affected.

Unfortunately, the experimental protocol in 2016 – designed to minimize the induction of plant volatiles – proved to be ineffective for evaluating biological control of WAA associated with the lures. The plots were too small and re-randomized several times, and the lures were never in place for more than 7-10 days in any plot. However, results in 2015 detailed below show that the longer term use of lures has the potential to slow population growth and provide additional control. We plan to perform additional studies this coming year on a larger scale in commercial orchards to evaluate suppression of WAA population levels and will provide a report on the results.

Suppression of WAA populations on lured trees (2015):

Methods:
We set up 24 trees in a modern fruiting wall orchard that were separated from each other by 200 feet and each randomly assigned one of the three treatments: a squalene lure placed on the tree, an AMP lure, or no lure at all. We evaluated WAA population levels at weekly intervals by evaluating 10 randomly selected shoots in each of the 24 trees and determining the average percentage infestation. The evaluations were made over a six-week period.

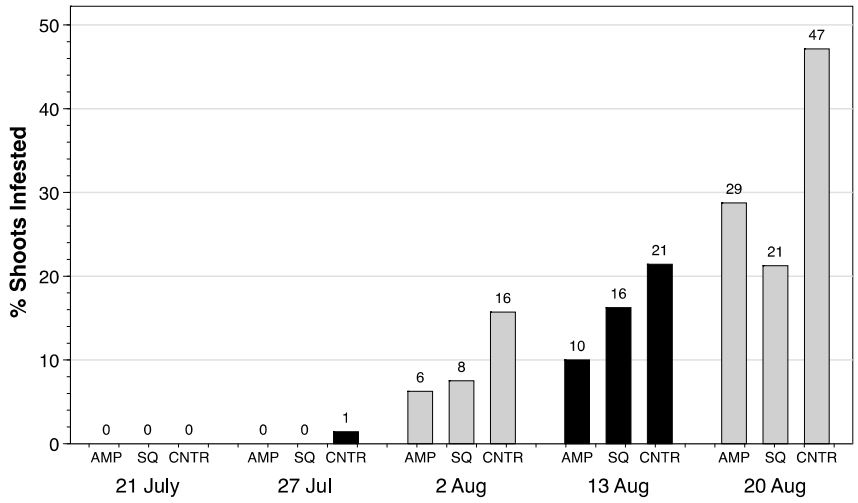
Results:
We found that the initial set up of the experiment did not account for the extraordinarily warm conditions. We had initially hoped to sample for a week or two, then put out the lures and see how the population changed after the lures were placed. However, the warm temperatures completely flat-lined the population so that from 6 July to 21 July 2015 no infested shoots were found. Fortuitously, this allowed us to follow the population rebounding after the temperatures started to drop over the period from 27 July to 20 August. Overall, the percentage infested shoots increased from 0% (21 July) to nearly 50% in the control by 20 August, with the lure-treated trees having significantly lower infestation levels compared to the control trees (Fig. 5).

Objective 3. Determine if increased lacewing egg deposition occurs within the active area of the lures and if this results in greater larval lacewing densities.

Methods:
Monitoring of lacewing egg deposition in 2015 was performed in the same plots as reported in Objective 2 (*Suppression of WAA populations on lured trees*). Each week, lacewing eggs were counted using a 3-minute visual count on the central tree and the two adjacent trees within the row. The evaluations were made over a six-week period.

Results:

Fig. 5. % Shoot infestation with WAA over time.



The number of eggs present on the trees was similar in all three treatments when averaged over the six-week period. ANOVA showed there were no significant differences between the different treatments, even though in 4 of 5 samples the AMP lure had higher numbers of egg masses found. Although there is an expectation that there would be more eggs laid on lured trees, the higher levels of infestation on control trees would tend to induce natural plant volatile emission which could result in higher egg-laying on those trees.

Executive Summary:

This project developed the basic information on lacewing behavior around the tested natural enemy lures needed to understand if the lures might interfere with biological control. The results clearly show that the range of attraction of the lures is short (<10 feet), they increase activity 8-18-fold in their immediate vicinity, and do not cause lacewings to spend inordinate amount of time just sitting on the lures. In addition, studies in 2015 showed that lures applied early in the summer helped reduce the population buildup of WAA after populations rebounded when temperatures dropped in mid-late August.

After testing the lures for enhancing biological control on a small scale in this grant, we intend to start some studies this next year on a larger scale in commercial orchards and will provide a new progress report that gives results next year. Overall, we were able to show that the use of the lures (especially squalene) could be a viable option to help reduce WAA in orchard hot spots. Our concerns about negatively affecting lacewing behavior were not confirmed. The results that suggest no practical interference with biological control, so that use of the lures in areas should be able to re-distribute natural enemies within an orchard block and reduce WAA population levels. That we did not find the expected increase in lacewing egg deposition around the lures was most likely due to the relatively small scale of our experiment, which makes it hard to see significant differences. The larger-scale studies we will start this next year should answer this question, while at the same time allowing us to get a better measure of biological control for WAA over a much larger scale.

Final Report

Project Title: Economic impact of apple maggot infestation

PI: R. Karina Gallardo
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Co-PI (4):
Organization:
Telephone:
Email:
Address:
Address 2:
City/State/Zip:

Cooperators: Jon DeVaney, Washington State Tree Fruit Association

Total Project Request: Year 1: $30,887

Other funding sources

Agency Name: WSU-CAHNRS-SES
Amt. requested/awarded: $15,158
Notes: The cost above indicated covers the salary of a PhD student for one semester. This salary would be covered by WSU-CAHNRS-SES through the ARC appointment.

Budget 1 (R.K. Gallardo, S. Galinato and D. Granatstein)
Organization Name: WSU-SES-PREC **Contract Administrator:** M. Stephens/K. Proudfoot
Telephone: 253-445-4508/253-445-4507 **Email address:** mstephens@wsu.edu/ karen.proudfoot@wsu.edu

Item	2016
Salaries	$18,722
Benefits	$5,834
Supplies	$2,226
Travel	$4,105
Total	$30,887

Footnotes: Salary for K. Gallardo for 4% FTE for 1 year (base salary $11,616/mo). Benefits $1,639 calculated at 29.4%. Salary for S. Galinato for 12.5% FTE or 1.5 months for 1 year (base salary $5,903.06/mo). Benefits $2,891 calculated at 32.65%. Salary for D. Granatstein for 5% FTE for 1 year (base salary $7,512.38/mo). Benefits $1,304, calculated at 30.4%. Travel for K. Gallardo to meet with growers in Pasco at $1,013 ($337.67 per trip x 3 trips) and to present study results at the Washington State Tree Fruit Association Annual Meeting in Wenatchee at $643 (airfare & taxi = $270, lodging = $110/day x 2 days, M&IE = $51/day x 3 days); Travel for S. Galinato to meet with growers in Yakima at $852 ($284 per trip x 3 trips) and to present study results at the Washington State Tree Fruit Association Annual Meeting at $447 (airfare = $235, lodging = $110/day x 1 day, M&IE = $51/day x 2 days); and Travel for D. Granatstein for meetings and presentations with organic growers statewide at $1,150 ($115/trip x 10 trips).Supplies include 2015 IMPLAN Data for Washington State ($1,110), poster ($116), and publication ($100 per page x 10 pages = $1,000)

NOTE: Gallardo will have handouts of her report available at the Review.

CONTINUING PROJECT REPORT **YEAR**: 2 of 3
WTFRC Project Number: CP-15-100

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

PI: Jay Norelli
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Co-PI (2): Kate Evans
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Co-PI(3): Cameron Peace
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Email: cpeace@wsu.edu
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Address 2: Department of Horticulture, Washington State University
City/State/Zip: Pullman, WA 99164-6414

Cooperators: none

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

Other funding sources

Agency Name: USDA-NIFA-Specialty Crop Research Initiative
Amt. 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
Salaries	6,132[1]	6,255[1]	6,380[1]
Benefits	491	500	510
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**

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
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**

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
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**

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 of Objective 1 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
- in 2015 and 2016 these 21 selected accessions were evaluated for their fruit quality (WSU-TFREC) and their resistance to blossom blight infection (ARS-AFRS)
- this objective is near completion and crosses were made with 4 *M. sieversii* accessions in 2016 to start incorporating their resistance into WABP (WSU-TFREC&ARS)
- additional crosses are planned for 2017 and a few *M. sieversii* accessions may be re-evaluated for fruit quality and/or resistance to blossom blight infection in 2017 (TFREC&ARS)

2. Determine fire blight resistance levels in RosBREED reference germplasm for current use in the WABP.

- goal of Objective 2 is to identify unknown **fi**re **b**light resistance **l**oci (**FBL**) among the current parents and seedling of the WABP by leveraging genomic resources developed in USDA-NIFA-SCRI RosBREED project
- to accomplish this the RosBREED apple reference germplasm (a collection of elite cultivars and their seedlings) was propagated and a replicated planting was established at the WSU Columbia View Orchard in Wenatchee, WA (2015) (WSU-TFREC)
- in 2016 this planting was challenged with the fire blight pathogen to determine the resistance of the elite cultivars and seedlings
- the collection of plant material will be challenged again with the fire blight pathogen in 2017 and results will be analyzed in 2017 to identify FBL within the WABP

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 of Objective 3 is to develop and evaluate DNA tests for known FBL among some of the fire blight tolerant parents that have been used in the WABP, specifically ‘Enterprise’ (PRI), ‘CrimsonCrisp’ (PRI), ‘GoldRush’ (PRI) and ‘Splendour’
- in 2015-2016 seedlings of the above cultivars were evaluated for their fire blight resistance (ARS), these seedlings will be used to validate DNA tests in 2017 (WSU-Pullman & ARS)
- in 2016 genetic analysis of ‘Splendour’ populations was conducted to identify FBL and will continue in 2017 (ARS)
- in 2016 genetic analysis in the PRI cultivars above was conducted to develop DNA test and will continue in 2017 (WSU-Pullman)
- these DNA tests will be employed in the WABP at the completion of the current project (2018) to identify elite selections of the WABP carrying fire blight resistance genes and for the selection of fire blight tolerant seedlings

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:

- Crosses were made with four fire blight resistant *Malus sieversii* accessions to incorporate higher levels of fire blight resistance into the WABP. Work was featured in Northwest Public Radio piece titled "The Future of Apple Breeding May Be Hidden in Ancient Species". See: http://nwpr.org/post/future-apple-breeding-may-be-hidden-ancient-species
- Approximately 1,600 trees of 552 elite cultivars and their seedlings were challenged with the fire blight pathogen to facilitate the discovery of fire blight tolerance genes among the existing seedlings and selections of the Washington apple breeding program. Trail was featured in a Good Fruit Grower article 'Tracking fire blight', December 2016 pages 71-73 (http://www.goodfruit.com/tracking-fire-blight/).
- WSU38, or Cosmic Crisp™, appeared to be fairly tolerant to fire blight in the 2016 trial, behaving similar to 'Delicious' following challenge with the fire blight pathogen (See Table 2). However, additional testing of WSU38 will be required before we can reliably determine its fire blight susceptibility.

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):

- 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) 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.

- Because fire blight is a sporadic disease from year to year and in its distribution within the orchard, reliable evaluation of fire blight resistance requires controlled challenge of test plants with the fire blight bacteria.
- A trial orchard of the RosBREED reference germplasm was established (2015) at the WSU Columbia View orchard for the evaluation of fire blight resistance.
- Vigorously growing shoots were challenged (2016) by dipping a pair of scissors in a suspension of the fire blight bacteria and then cutting the youngest leaves of the shoot tip.
- Above inoculations will be repeated in 2017.
- Results will be analyzed (2017) using FlexQTL software to identify and predict fire blight resistance loci (**FBL**). This software was developed and used in the previously funded RosBREED project utilizing high-resolution genome scans already completed by the project and our analysis will draw upon 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 testss 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 2015 and 2016, 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. One accession (PI657116), selected in 2015 for reasonable fruit quality, showed extensive internal breakdown after storage in 2016. Four accessions (including PI657116) were used as pollen parents in the WABP crossing program in spring 2016. Seeds were collected from all three crosses and have been sown ready for fire blight evaluation in spring 2017.

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

Although Objective 1 will identify the best sources of fire blight resistance to be used in future crosses, it will not facilitate selection of fire blight resistance among the existing seedlings and selections of the WABP. The goal of Objective 2 is to leverage resources developed in the first RosBREED project to identify genetic factors which are already present within the WABP that affect

the severity of fire blight. Although the material currently in the WABP may not be immune to fire blight, we know there is a large gradation of susceptibility from "tolerance" to high fire blight susceptibility. An example of a tolerant variety is 'Delicious', which may occasionally get some fire blight but devastating financial losses due to fire blight rarely occur in 'Delicious'. This type of resistance is very useful, however most of the genetic factors controlling this "tolerance" are currently not known. To identify these **f**ire **b**light resistance-influencing **l**oci (**FBL**) we will determine the fire blight resistance of the RosBREED reference germplasm, a collection of elite cultivars and their seedlings. In depth genetic analysis of these elite lines and their seedlings has already been conducted in the RosBREED project and software has been developed to identify genetic loci controlling traits within the germplasm by a process known as pedigree analysis. Because existing orchards of the RosBREED reference germplasm established for the evaluation of fruit quality could not be appropriately challenged with fire blight without risking the destruction of many lines, a planting of the reference germplasm was established at the WSU Columbia View Orchard (2015) for fire blight evaluation.

In 2016, approximately 1,600 trees of 552 cultivars of the RosBREED reference germplasm were challenged with the fire blight pathogen at the WSU Columbia View Orchard on April 28th and 29th. The number of shoots inoculated per tree ranged from 3-10. There was a wide range of fire blight resistance responses from highly susceptible to highly resistant. The mean proportion of the current season's shoot length that remained healthy following fire blight challenge ranged from 0.0 (all challenged shoots killed by fire blight) to 1.0 (no fire blight development on any of the challenged shoots). 26.3% of the cultivars evaluated had mean proportions of current shoot healthy ≤ 0.25 and should be considered susceptible, whereas 23.4% of genotypes had mean proportions ≥ 0.75 and should be considered fire blight tolerant. The remaining 50.3% of genotypes had means between 0.25 and 0.75 healthy tissue. The average age of wood infected ranged from 0 to 2.6. An average age of wood infected > 1.0 indicates that on average, fire blight infections on challenged shoots extended into the previous season's growth.

'Enterprise' is a potential source of fire blight tolerance that has been used widely in the WABP. Both 'Enterprise' and the original source of its resistance, *Malus floribunda* 821, had resistant responses in the 2016 trial (Table 1). 'Splendour' and its offspring 'Aurora Golden Gala' have both been used as parents in the WABP and both showed tolerance to fire blight (Table 1). The fire blight tolerance of 'GoldRush', which has also been used as a parent in the WABP, is believed to have originated from its COOP17 parent, which was fire blight tolerant in the trial. WSU38, also known as Cosmic Crisp™, appeared to be fairly tolerant to fire blight in the 2016 trial, with fire blight symptom development similar to that of 'Delicious' and intermediate between its parents 'Enterprise' and 'Honeycrisp' (Table 1). However, additional testing of WSU38 will be required before we can reliably determine its fire blight tolerance or susceptibility. Fire blight is a very variable disease and cultivars can have spurious results in individual tests due to either environmental conditions or the growth vigor of the cultivar in the trial. For example, 'Fuji', which we know to be susceptible to fire blight, appeared more resistant than expected in the 2016 Columbia View trial (Table 1).

In 2017 the WSU Columbia View test block will again be challenged with the fire blight pathogen. The combined results of the 2016 and 2017 trials will be analyzed (2017) using FlexQTL software developed in the RosBREED project to identify and predict fire blight resistance loci (**FBL**) within the current plant material of the WABP. This is expected to be completed by the end of the project. DNA test for newly identified FBL will be developed in subsequent research.

Table 1. Fire blight resistance and susceptibility observed in 2016 WSU Columbia View fire blight trial for select cultivars and sources of fire blight tolerance in WABP.

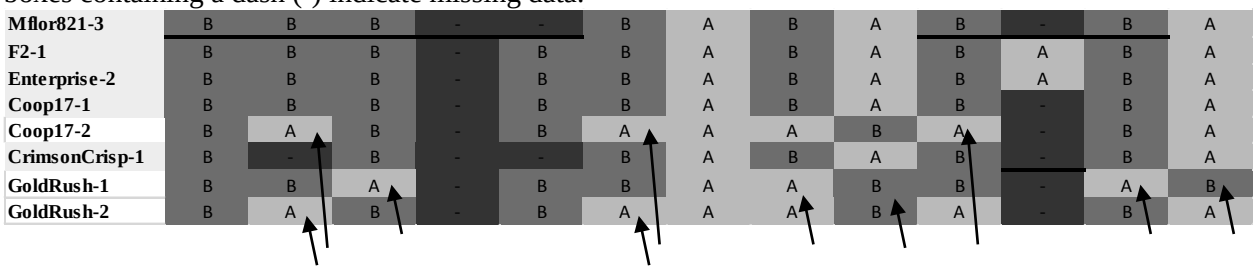
Cultivar	Proportion current shoot healthy	Average age of wood infected
Enterprise	1	0
Malus floribunda 821	0.97	0.1
Splendour	0.8	0.5
COOP17 (GoldRush parent)	0.78	0.6
Aurora Golden Gala	0.77	0.8
Delicious	0.73	0.9
WSU38	0.71	0.9
Fuji	0.68	0.6
Cripps Pink	0.49	1.2
Honeycrisp	0.39	1.5
Zestar	0.29	1.3
Gala	0.25	1.5
Jonathan	0.11	2.4

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', 'Crimson Crisp', 'GoldRush' 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.

Previous research in the USA and Europe has identified genomic regions, or "loci", in the ancestors of 'Enterprise', 'GoldRush' and 'CrimsonCrisp' that influence fire blight tolerance (Durel et al. 2009, Khan et al. 2013). In 2016, genetic analysis of these regions within these cultivars was conducted using genetic resource information developed in the RosBREED project. Durel et al. (2009) identified a genomic region at the end of chromosome 12 that was associated with the fire blight resistance of *M. floribunda* 821 which is an ancestor of all three of the above cultivars. That genomic region was found to be present in 'Enterprise' and 'Crimson Crisp' (Fig. 1) but not 'GoldRush'. Khan et al. (2013) identified genomic regions on chromosomes 6 and 15 of COOP17 associated with fire blight tolerance and similar analyses were conducted. In 2017, DNA tests will be developed for each of these three genomic regions based upon the analyses conducted in 2016 using available DNA sequence information for the regions.

Figure 1. Genetic similarity at bottom of chromosome 12 within a region associated with the fire blight resistance of *M. floribunda* 821 (Mflor821-3). 'Enterprise', 'Crimson Crisp', F2, and COOP17 (allele -1) share similarity with *M. floribunda* 821 in this region, whereas 'Goldrush' does not (arrows highlight some of the difference). F2 is a fire blight resistant seedling of *M. floribunda* 821. Dark boxes containing a dash (-) indicate missing data.



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', 'GoldRush' and 'CrimsonCrisp' were challenged with the fire blight pathogen in 2015 and 2016 to determine their resistance to fire blight. In general, 25-50% of these seedlings were found to be fire blight tolerant, depending upon parent. DNA was isolated from the seedling prior to being challenged with fire blight and will be used for DNA testing in 2017. 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

Research in the current RosBREED2 project is identifying FBL contributing to the fire blight "tolerance" of 'Splendour'. A detailed genetic map of a 'Gala' X 'Splendour' seedling population has been constructed and the fire blight resistance of this seedling population was previously evaluated in the greenhouse. The results are currently being analyzed to identify FBL present in 'Splendour'. This 'Gala' X 'Splendour' population has been established in the field at USDA-ARS Kearneysville (2015) and will continue to be evaluated for its fire blight resistance under field conditions, along with approximately 200 test seedlings derived from a cross of 'Splendour' X 'Cripps Pink' that will be used to evaluate DNA tests designed from 'Splendour' FBL identified in RosBREED2.

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. This proposed research is partially supported by the RosBREED2 project aimed at combining disease resistance with horticultural quality in new fruit varieties. The support from the WTFRC for this project combined with the opportunity for Co-PIs to work together with other leaders in the field of fruit breeding in the RosBREED project is accelerating the progress of the project.

LITERATURE CITED

Durel C.E., Denancé C and Brisset M.-N. 2009. Two distinct major QTL for resistance to fire blight co-localize on linkage group 12 in apple genotypes 'Everest' and *Malus floribunda* clone 821. Genome 52:139-147.

Khan, M.A., Zhao Y. and Korban S.S. 2013. Identification of genetic loci associated with fire blight resistance in *Malus* through combined use of QTL and association mapping. Physiologia Plantarum 148:344-353.

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

WTFRC Project Number:

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

PI: Ken Johnson
Organization: Oregon State University
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2082 Cordley Hall
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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.

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

Item	2016	2017
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

- 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.
- Blossom Protect twice or Blossom Protect then OxiDate (2X) provided very good fire blight control in apple.
- In general, materials that suppress infection also reduce pathogen inoculum. Under weather conditions highly conducive for fire blight, numerous materials were only fair at both including Serenade Opti, three-quart rate of Cueva soluble copper, and experimental phage-based materials.
- Among various (mostly disappointing) phage-based materials, a preparation of phage pre-infected into *Pantoea agglomerans* show significant inoculum and infection suppression.
- Alum at 1% (8 lbs./100 gal) provided very good inoculum sanitation and excellent fire blight control.
- Late bloom treatments of lime sulfur (2%) provided good inoculum sanitation and fire blight control and improved fruit finish.
- Acidifying oxytetracycline with buffer protect (pH 4) appeared to improve the level of inoculum sanitation and fire blight control from this antibiotic.
- In both pear and apple, epiphytic populations of the fire blight pathogen on flowers were still increasing one week after 'petal fall'.

METHODS

Rationale. In recent years, there has been a rapid increase in the number of biopesticide materials available to control fire blight. Many have become EPA-registered with only a limited number of field trials that demonstrate efficacy. Consequently, we are making a comparative investigation of the various materials registered for fire blight control in conventional and organic systems. In addition, we are investigating several experimental materials near commercialization. We seek to better understand on a comparative scale: the effects of a material on epiphytic pathogen populations on inoculated trees, their ability to suppress infection, and the material dose/pathogen killing relationship.

Experimental design. Objectives were addressed in experimental orchards located at Oregon State University's Botany & Plant Pathology Field Laboratory in Corvallis. Experiments were arranged in a randomized complete block design with 4 replications. During early morning, treatment suspensions and pathogen inoculum were sprayed to near runoff with backpack sprayers.

Measurement of pathogen populations: Eight flower clusters were sampled from each replicate tree on each of three dates: full bloom, petal fall, and one-week post petal fall. Each flower cluster sample was washed in sterile phosphate buffer. After washing, dilutions of wash were spread on CCT medium to selectively enumerate *Erwinia amylovora*. We also spread the washes on Pseudomonas

agar F amended to enumerate total cultural bacteria populations and on potato dextrose agar to enumerate yeast (*A. pullulans*) populations.

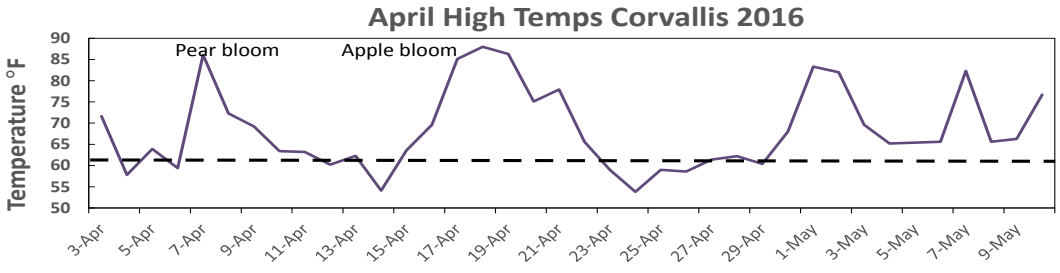
Disease and fruit assessment. Incidence of fire blight was determined by counting blighted flower clusters from each tree 2- to 4-weeks after bloom. Number of blighted flower clusters were divided by total clusters per tree, which was determined pre-bloom. In August, percent fruit russet was scored with a modified Horsfall-Barratt rating scale.

Lab-based dosed response experiments. Laboratory-based assays were designed to develop logistic-decline dose-response curves for effect of biopesticides on *E. amylovora* survival. The assay exposed pathogen cells to a dose of biopesticide for a period of time (60 min). Pathogen cells were recovered by filtration, rinsed in buffer, then dilution plated on nutrient agar to determine survivorship.

RESULTS & DISCUSSION

Weather in spring 2016. Temperatures were exceptionally favorable for epiphytic growth of *E. amylovora* on both pear and apple flowers. Fire blight risk as determined by the heat unit model, COUGARBLIGHT, was high to exceptional during bloom of both tree species. Average maximum daily high between 7 and 22 April was 72ºF with high temperatures of 86 and 87ºF on 7 and 19 April, respectively (Fig. 1). Consequently, epiphytic populations of the fire blight pathogen and incidence of fire blight were very high in all four orchard trials. For orchards used in objective 1, the number of strikes per tree on the water treated control averaged 673 and 315 in Bartlett pear and Golden Delicious apple, respectively; for orchards used for objective 2 and 3, strikes per tree on the water treated control averaged 319 and 197 in Bartlett pear and Gala apple, respectively.

Fig. 1



Obj. 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.

Fire blight control. For pathogen-inoculated trials, disease intensity was high with fire blight infections on water-treated trees averaging 76% and 55% of total clusters in Bartlett pear and Golden Delicious apple, respectively (**Table 1**). Antibiotic standards (streptomycin (FireWall) once, and oxytetracycline (FireLine) twice) were among the best performing materials in both trials (72 to 88% control). In apple, compared to the water-treated control, percent control from two applications of Blossom Protect plus Buffer Protect (88%), one application Blossom Protect plus Buffer Protect then OxiDate twice (70%), Previsto twice (78%) were statistically similar to the antibiotic standards. In pear, these materials showed an intermediate performance along with the soluble copper material, Cueva. In both trials, low to intermediate levels of control (<50%) were observed with Serenade Opti applied three times or Serenade Opti (once) in a program with Cueva (twice). In apple, disease control obtained with Previsto was statistically superior to control obtained with Cueva, which we speculate is attributable to the amount of metallic copper in each material (2.9 and 1.8%, respectively). In lab-based bioassays, copper-based materials, OxiDate (hydrogen dioxide, peroxyacetic acid), acidified solutions, lime sulfur, streptomycin and kasugamycin were effective materials for killing *E. amylovora* (data not shown).

Table 1. Evaluluation of EPA-register non-antibiotic materials for fire blight control in Bartlett pear and Golden Delicious apple, Corvallis, 2016.

Treatment	Rate per 100 gallons water	Bloom stage of treatment* 70%	Full	Petal Fall	BARTLETT PEAR Percent blighted floral clusters**		GOLDEN D. APPLE Percent blighted floral clusters**	
Water		**---§**	**X**	**X**	**76.3**	**a**	**55.0**	**a**
FireWall	8 oz.	---	X	---	10.4	f	7.3	ef
FireLine	16 oz.	---	X	X	20.9	ef	6.9	f
Serenade Opti (plus BioLink)	20 oz.	X	X	X	60.0	abc	30.5	cd
Serenade Opti (plus BioLink)	20 oz.	X	---	---	72.4	abc	41.6	bc
then Cueva (2 quarts)	64 fl. oz.	---	X	X				
Buffer Protect	150 oz.	X	---	---	65.2	abc	44.5	bc
Blossom Protect	21.4 oz.	X	---	---	69.7	abc	31.5	cd
Blossom Protect	21.4 oz.	X	---	---	54.3	bcde	23.6	de
Buffer Protect	150 oz.	X	---	---				
Blossom Protect	21.4 oz.	X	X	---	40.8	de	6.7	f
Buffer Protect (twice)	150 oz.	X	X	---				
Blossom Protect	21.4 oz.	X	---	---	42.4	cde	16.0	ef
Buffer Protect	150 oz.	X	---	---				
then OxiDate	128 fl. oz.	---	X	X				
Cueva (3 quarts)	96 fl. oz.	X	X	---	45.9	bcde	38.7	bcd
Previsto (3 quarts)	96 fl. oz.	X	X	---	35.6	ef	12.0	ef

*** Trees inoculated with *Erwinia amylovora* strain Ea153N (streptomycin-sensitive) at 5 x 10^5 CFU/ml on 30 March (pear) and 6 April (apple). ** Trees used in the experiments averaged 871 and 507 flower clusters per tree for pear and apple, respectively. For each treatment, percent blighted flower clusters was transformed arcsine($\sqrt{x}$) prior to analysis of variance; non-transformed means are shown. § X indicates material was sprayed on that specific date; --- indicates material was not applied on that specific date. # Means within a column followed by the same letter are not significantly different according to Fischer's protected least significance difference at $P = 0.05$.**

Epiphytic pathogen populations. Measured epiphytic populations of pear and apple flowers were generally correlated with incidence of disease. The highest epiphytic population size was measured on the water-treated control and the lowest was measured on flowers that received streptomycin. The soluble copper material, Previsto, applied at 70% and full bloom effectively suppressed *E. amylovora* populations through petal fall (**Fig. 2A&D**). In contrast, FireLine (oxytetracycline) and Cueva (soluble copper) provided intermediate levels of suppression of the fire blight pathogen. Serenade Opti showed only slight suppression of epiphytic pathogen populations (**Fig. 2B&E**), which also correlated with the low level of disease control obtained with this material. The exception to the correlation of pathogen population size and disease incidence occurred with Blossom Protect plus Buffer Protect treatments, either applied twice or applied once and followed in a program with OxiDate (applied twice). Populations of the pathogen were not markedly suppressed by these treatments (**Fig. 2C&F**), but the corresponding levels of disease control were intermediate (pear) to outstanding (apple) (**Table 1**).

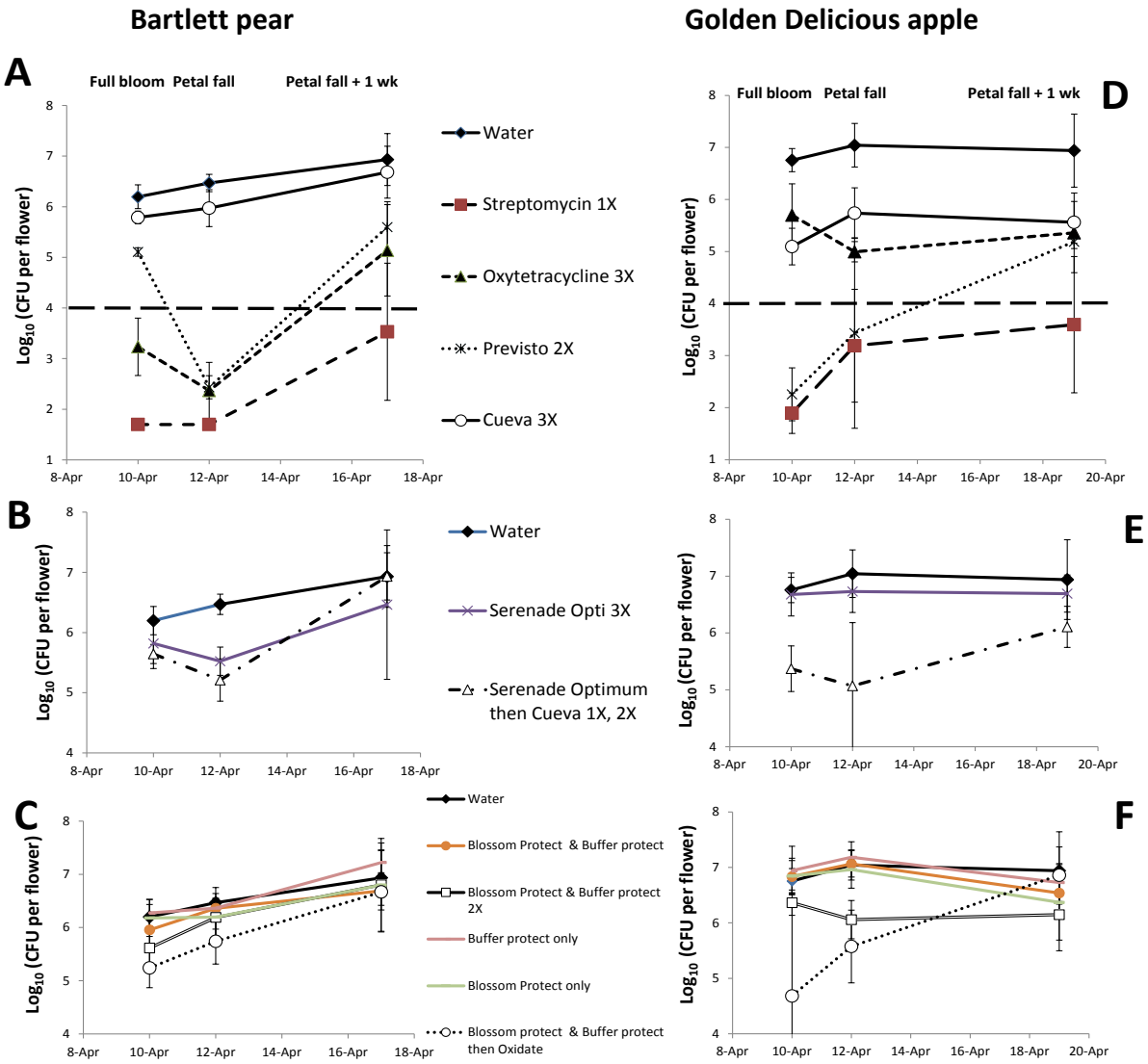


Fig. 2. Effect of treatments for fire blight suppression on *E. amylovora* populations on flowers during pear and apple bloom, April 2016. Orchards were located near Corvallis, OR with each treatment applied to four replicate trees. Pathogen populations were determined by bulk sampling five flower clusters (~25 flowers) from each tree; each sample was washed in 25-ml of sterile phosphate buffer followed by dilution plating onto tryptic soy agar. Panels A (pear) and D (apple): antibiotics and soluble copper materials; Panels B (pear) and E (apple): Serenade Opti with and without Cueva soluble copper; and Panels C (pear) and F (apple): Blossom Protect and Buffer Protect with and without OxiDate. Horizontal dashed line in Panels A and D indicate the bottom of y-axis scale in Panels B, C, E and F.

Obj. 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.

Obj. 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.

Fire blight control. In previous trials, alum ($KAl(SO_4)_2$), a low pH salt used at the rate of 2% (w:w), has effectively suppressed fire blight infection and has not contributed to fruit marking in several russet evaluations. In 2016, 2% alum (16 lbs. per 100) again effectively suppressed fire blight in both pear and apple. Moreover, 1% alum was nearly as effective as the higher concentration. In contrast, with 0.5% alum, fire blight suppression fell off compared to higher rates of the material.

Materials containing phage (bacterial viruses) to specifically attack *E. amylovora* generally provided poor fire blight suppression. Poor performance is possibly attributable to the need for phage to efficiently infect their prey, which is a difficult task when weather conditions allow the pathogen's epiphytic population size to expand rapidly. An exception was Ag Canada's #2 phage prep that was pre-infected into *Pantoea agglomerans* strain E325 (the EPA-registered strain in Bloomtime Biological first selected by Dr. Larry Pusey, ARS, Wenatchee). We term this strategy of phage deployment a 'Trojan horse' because the viruses that attack *E. amylovora* can increase inside a closely related, beneficial bacterium (with suppressive properties of its own) when populations of the fire blight pathogen are small. Research with Ag Canada's strategy will continue in 2017.

Table 2. Evaluluation of EPA-register altenative materials for fire blight control in Bartlett pear and Gala apple, Corvallis, 2016.

Treatment	Rate per 100 gallons water	Bloom stage of treatment*			BARTLETT PEAR Percent blighted floral clusters**		GALA APPLE Percent blighted floral clusters**	
		60%	Full	Petal Fall				
Water		---§	X	X	**57.3**	**a#**	**33.1**	**a#**
Streptomycin	8 oz.	---	X	---	1.0	h	6.4	ghi
Kasumin 2L	64 fl. oz.	---	X	X	2.4	h	6.4	hi
FireLine plus	**16 oz.**	---	**X**	**X**	-not tested-		**2.9**	**i**
Buffer Protect (half)	**75 oz.**	---	**X**	**X**				
Alum 2%	**267 oz.**	---	**X**	**X**	**4.6**	**gh**	**7.9**	**efghi**
Alum 1%	**134 oz.**	---	**X**	**X**	**13.1**	**fgh**	**6.8**	**fghi**
Alum 0.5%	67 oz.	---	X	X	37.1	bcde	19.9	abcd
Buffer Protect (full)	150 oz.	---	X	X	56.2	ab	18.0	bcde
Alum 0.5% plus	67 oz.	---	X	X	34.7	cde	17.9	cde
Buffer Protect (half)	75 oz.	---	X	X				
Serenade Opti (BioLink)	20 oz.	X	X	X	39.7	abcd	24.2	abcd
Pantoea agglomerans C9-1	10^8 cfu/ml	X	X	X	-not tested-		17.2	cde
Ag Canada *Pantoea* spp. #1	10^8 cfu/ml	X	X	X	-not tested-		23.1	abcd
Ag Canada *Pantoea* spp.	10^8 cfu/ml	X	X	X	32.7	cde	20.8	abcd
plus selected phage #1	~	X	X	X				
Ag Canada *Pantoea* spp.	**10^8 cfu/ml**	**X**	**X**	**X**	**19.3**	**ef**	**16.5**	**cdef**
plus selected phage #2	**~**	**X**	**X**	**X**				
Fire Quencher A	~	X	X	X	51.1	abc	29.4	abc
Fire Quencher A	~	X	X	X	35.5	cde	22.2	abcd
plus Serenade Opti (BioLink)	20 oz.	X	X	X				
Fire Quencher B (tryptophan)	~	X	X	X	48.7	abc	32.5	ab
Fire Quencher C	~	X	X	X	48.0	abc	23.5	abcd
plus Serenade Opti (BioLink)	20 oz.	X	X	X				
Lime sulfur 2%	**256 fl. oz.**	**---**	**X**	**X**	**21.9**	**def**	**12.2**	**defgh**
OxiDate 1%	128 fl. oz.	---	X	X	46.6	abc	12.7	defgh
Oxycom CA	64 oz.	---	X	X	35.7	cde	16.7	cdefg

*** Trees inoculated with *E. amylovora* strain Ea153N (streptomycin-sensitive) at 5 x 10^5 CFU/ml on 1 April (pear) and 6 April (apple). ** Trees used in the experiments averaged 584 and 574 flower clusters per tree for pear and apple, respectively. See footnote of Table 1 for other callouts and description of statistical analysis.**

Epiphytic pathogen populations. Again, measured epiphytic populations on pear and apple flowers generally correlated with incidence of disease. Alum showed a strong dose response relationship on pear but less so on apple (**Fig. 3**), and on both pear and apple, Ag Canada phage prep #2 stood out from other phage treatments and the water-treated control. Interestingly, on apple, suppression of the pathogen's population size with phage prep #2 was similar to observed with 1% and 2% alum, but alum had a larger effect on suppression of infection. We speculate that this is related to the lower pH of alum; corresponding levels of disease control with this alum were intermediate (pear) to outstanding (apple) (**Table 1**).

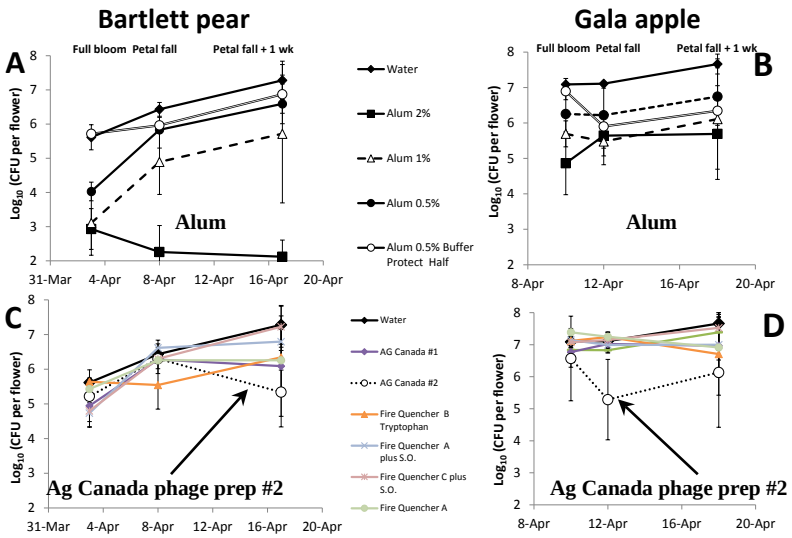


Fig. 3. Effect of alum and phage treatments for fire blight suppression on *E. amylovora* populations on flowers during pear and apple bloom, April 2016. Sampling protocol described under Fig. 2. Panels A (pear) and B (apple): various rates of alum ($KAl(SO_4)_2$) compared to water –treated control; panels C (pear) and D (apple): various formulations of phage materials including 'naked' phage prep with and without sunscreens (FireQuencher, BYU University) and 'trojan horse' preparations of phage in *Pantoea* spp. (Agriculture Canada).

Other notable observations. FireLine (oxytet) amended with a half label rate of Buffer Protect was the best treatment in the Gala apple trial (**Table 2**). This treatment is notable because the pathogen's population size at one week post-petal fall was lower than we have observed previously (compare oxytet result in **Fig. 4** to oxytet results in **in Fig. 2**). Acidification of select treatments with Buffer Protect (e.g., oxytet, Serenade Opti) will be a focus of 2017 experiments.

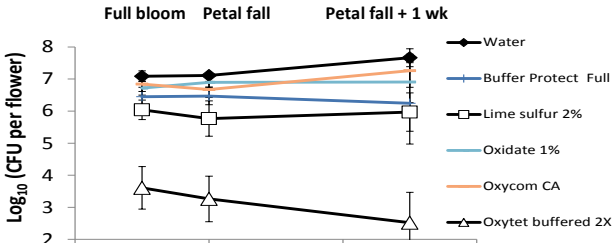


Fig. 4. Effect of alternative treatments for fire blight suppression on *E. amylovora* populations on flowers during pear and apple bloom, April 2016. Sampling protocol described under Fig. 2.

Lime sulfur (2%) was another notable observation as it significantly suppressed fire blight (**Table 2**), suppressed pathogen populations size (**Fig. 4**), and resulted in the least russeted pear fruit in the alternative materials trial (**Fig. 5**). In the other pear trial (**Table 1**), Blossom Protect treatments increased fruit russet (data not shown), thus we believe the effect of lime sulfur shown in **Fig. 5** is the result of suppressed yeast populations. In apple, some central WA advisors are now using lime sulfur (up to 4%) for fire blight control in late bloom. Higher rates of lime sulfur will be a research focus in 2017.

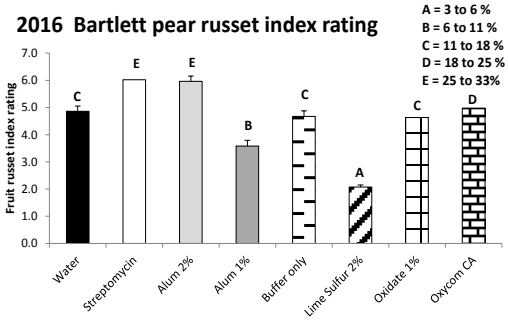


Fig. 5. Effect of alternative treatments for fire blight suppression on fruit russet of Bartlett pear, Aug 2016.

CONTINUING PROJECT REPORT **YEAR:** 1 of 3
WTFRC Project Number: CP-16-101

Project Title: Brown marmorated stink bug control in Washington

PI: Elizabeth H. Beers
Organization: WSU-TFREC
Telephone: 509-663-8181 ext. 234
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Address: 1100 N. Western Ave.
City/State/Zip: Wenatchee/WA/98801

Cooperators: None

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

Other funding sources
Agency Name: Washington State Commission on Pesticide Registration (WSCPR #16PN25)
Amt. Awarded: $16,356 (July 1, 2016 to June 30, 2017).
Agency Name: Washington State Commission on Pesticide Registration (WSCPR #17AN029; submitted, pending)
Amt. Requested: $18,733

WTFRC Collaborative Expenses: None

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

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 detected in 19 counties in Washington state
- Clark, Walla Walla and Yakima counties had the highest number of finds, although this reflects sampling efforts as well as population densities
- *T. japonicus* was recovered from 5 of 6 sites surveyed in Vancouver, WA, and appears to be well established there
- Stink bug and codling moth damage were lower in the net cages than in the conventional (airblast) treatment and the untreated control
- Woolly apple aphids, mites, and thrips were higher inside the cages; the greatest difference was for woolly apple aphid
- The most likely explanation for increased woolly apple aphid is the absence of syrphids and lacewings; predatory mites were unaffected by the cages, thus microclimate changes may be responsible for increases in spider mites

Methods, Results and Discussion:

Objective 1. Determine distribution of *Trissolcus japonicus* in Washington[1]*:* The discovery of a wild population of *T. japonicus* in a very limited survey in the Vancouver, WA area in 2015 indicated a much broader survey was warranted. The 2016 survey work was confined to the Vancouver area, but the number of sites was doubled, and the number of egg masses was increased. A sentinel egg mass survey was conducted for monitoring the presence of egg parasitoids, including *T. japonicus*, from 1 Jun to 19 Aug, 2016, at six sites in Vancouver, WA. Egg masses for the survey were taken from a BMSB colony maintained at the WSU Heritage Farm in Vancouver, WA. The colony was maintained at room temperature on a diet of fresh produce. The cages were provided with paper toweling as an oviposition substrate, and egg masses were collected every 24 h to ensure attractiveness to parasitoids. The egg masses were transferred to strips of cardstock, which was pinned to the underside of a leaf on favored BMSB host plants. A total of 134 fresh sentinel egg masses (3,430 total eggs) were deployed at the six sites from 1 Jun to 19 Aug, 2016. Egg masses were left in the field for 2-5 days, then returned to the lab, placed individually in Petri dishes to rear out parasitoids.

Obj. 1. Results and Discussion. *T. japonicus* was found at five out of six sites surveyed (Sites 1, 3, 4, 5, and 6), with a total of 26 egg parasitized egg masses found (Table 1). Site 6 was particularly productive, with over half of the 13 egg masses yielding *T. japonicus*. A total of 451 adult wasps were recovered from the 26 egg masses, of which 86% were females. This survey confirms the widespread presence of *T. japonicus* in the Vancouver area. The 2015 find, confined to a single site (Site 3) may have been indicative of only low levels of this parasitoid. To date, this represents one of the more evident *T. japonicus* populations in the nation; other areas have had repeat finds subsequent to the initial one, but some have had no further finds.

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

Table 1. Summary of sentinel egg mass survey, Vancouver WA, 2016

Site	Location	Host Plant	Common name	Distance from 2015 find (km)	Number of egg masses deployed	Number parasitized by *T. japonicus*
1	Burnt Bridge	*Rhamnus alnifolia*	Alder buckthorn	2.53	19	3
2	South Cliff	*Catalpa* sp.	Catalpa	2.17	22	0
3	Wintler Park	*Acer circinatum*	Vine maple	0	47	8
4	Marine Park	*Acer platanoides*	Norway maple	2.03	21	6
5	Columbia Way	*Acer circinatum*	Vine maple	5.27	12	2
6	St. James	*Catalpa* sp.	Catalpa	5.88	13	7
				Totals:	**134**	**26**

Objective 2. Maintain a laboratory culture of *T. japonicus* in preparation for release. Using adult *T. japonicus* from the sentinel egg survey in Vancouver, a laboratory culture of *T. japonicus* was initiated and kept in a controlled temperature growth room (22 °C [72 °F], 16:8LD, 60% humidity) (Fig. 1). A BMSB colony (see Obj. 1) was maintained to perpetuate the *T. japonicus* culture. The adult wasps were kept in 8 fl oz coated paper containers. Two fresh (<24 h old) BMSB egg masses per week were placed in the container, and left there to allow adults to emerge. The containers were provisioned with a 50:50 solution of honey:water applied to a Kimwipe taped to the lid. The Kimwipe was replaced frequently to avoid mold. We are currently exploring holding adults at cold temperatures (just above freezing) to determine survival during the winter. Keeping females in diapause during the winter months will save time and resources obviating the need to keep the BMSB colony in continuous production.

Fig. 1. Female *T. japonicus* scent-marking the egg mass after ovipositing.

Objective 3. Evaluate IPM-friendly management strategies for BMSB

3a. Physical exclusion, large field cages. 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, 2) conventional management, 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 Gala). 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 block maintenance pesticide applications (herbicides, 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 end of July, 2016 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 three 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 4-inch roll of cardboard tied to the trunk with flagging tape. Mite samples were a composite of 30 leaves per plot, with mites 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 assessment of parasitism by *A. mali* was made in late August by counting the number of live and parasitized WAA in a maximum of 15 colonies/plot. 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.

3a. Results and Discussion. Stink bug fruit damage was significantly reduced in the caged (1.4%) vs the other two treatments (airblast 5.8%; check 4.0%) (Fig. 2). Codling moth pheromone trap captures were reduced 4-5-fold inside the cages compared to the other two treatments; obliquebanded and pandemis leafroller captures were negligible throughout the season. Codling moth damage averaged 4.3% inside the cages, compared to 15.6% (airblast) and 77.1% (untreated check) (Fig .2). Surprisingly, thrips damage was higher in the cages (thrips data not shown). Spider mite densities were high in the June sample (maximum of 44 mites/leaf; 91% brown mites), but not significantly different among treatments, although they tended to be higher inside the cages. Woolly apple aphid densities (CID) were 35-42-fold higher inside the cages than in the airblast and check treatments, respectively (Fig. 2). *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. Percentage parasitism did not differ among the three treatments (11-16%). The captures of lacewings and syrphids inside the cages were greatly reduced relative 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 (0.2%); surprisingly, the airblast treatment (2.6%) also reduced sunburn relative to the check (8.9%).

3b. Physical exclusion, small field cages. The experimental design of the small cage experiment was similar to the large ones, 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 place 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.

3b. Results and Discussion. Codling moth pheromone trap captures were greatly reduced inside the cages (18 total) vs outside (334 total moths). Likewise, fruit damage by codling moth was 2% inside the cages, vs. 19% (airblast) and 60% (untreated) (Fig. 2). Scale damage was low overall (<1%), but significantly higher in the airblast treatment. Thrips damage was significantly higher inside the cages (5.5%). Woolly apple aphid densities were 133 to 157-fold higher inside the cages than in the airblast and check treatments, respectively (Fig. 2). Spider mites were low overall (<2.5 mites/leaf), but significantly higher inside the cages; 95% of the mites found were brown mite. Earwigs were significantly higher inside the cages, while spiders were significantly higher in the airblast treatment.

Lacewing and syrphid adult were effectively excluded by the cages. Sunburn was significantly reduced inside the cages (0.7%); however, as in the large cages, the airblast treatment (5.1%) also reduced sunburn relative to the check (12.7%).

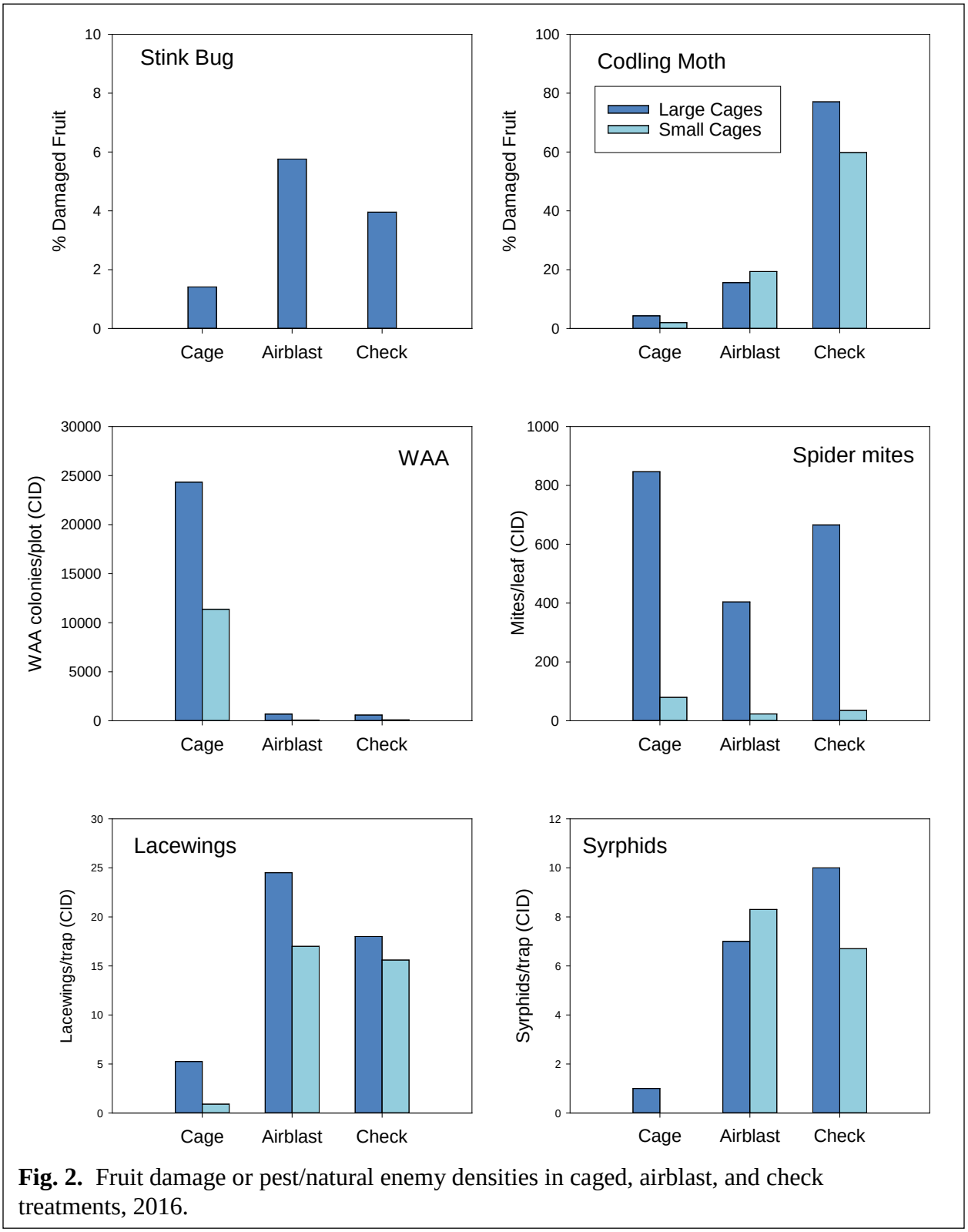

Fig. 2. Fruit damage or pest/natural enemy densities in caged, airblast, and check treatments, 2016.

3c. Physical exclusion, single-wall barriers. 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. 3) 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 bug populations were assessed with a beating tray, and fruit damage by visual inspection in late August/early September.

Fig. 3. Single wall net barrier between sagebrush habitat and orchard.

Obj. 3c. Results and Discussion. Stink bug densities and fruit damage were extremely low in 2016. We found an average of 0.52% fruit damage in the barrier section, and 0.72% damage in the check.

Obj. 3 Conclusions. The preliminary information on stink bug exclusion indicates there is potential for this method, but low 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. For the small cages, which have been in place for several years, sanitation efforts in 2015 were successful, and 0-6 moths/cage were captured during the entire season, with a correspondingly low level of fruit damage (2%). For the large cages, which were under construction in 2015 and not completely sealed until bloom during the 2016 season, codling moth damage ranged from 1-10% inside the cages. Sanitation efforts during 2016 (including spraying, banding, and fruit removal) should improve the level of control in 2017.

It is also clear that several secondary pests are increased by caging: woolly apple aphids, mites, and thrips (Fig. 2). In the case of woolly apple aphid, parasitism by *A. mali* was actually higher in the cages, indicating cages are 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.

Objective 4. Document the spread of BMSB within the state. We used direct surveys (beating trays, pheromone traps) in likely areas to determine the presence and relative abundance of BMSB in the state. We also solicited input from homeowners and Master Gardeners; the latter are most likely to receive questions and specimens about stink bugs invading homes. Verified finds were recorded in a database available to BMSB researchers, and the results available in map form (http://www.tfrec.wsu.edu/pages/bmsb/Home).

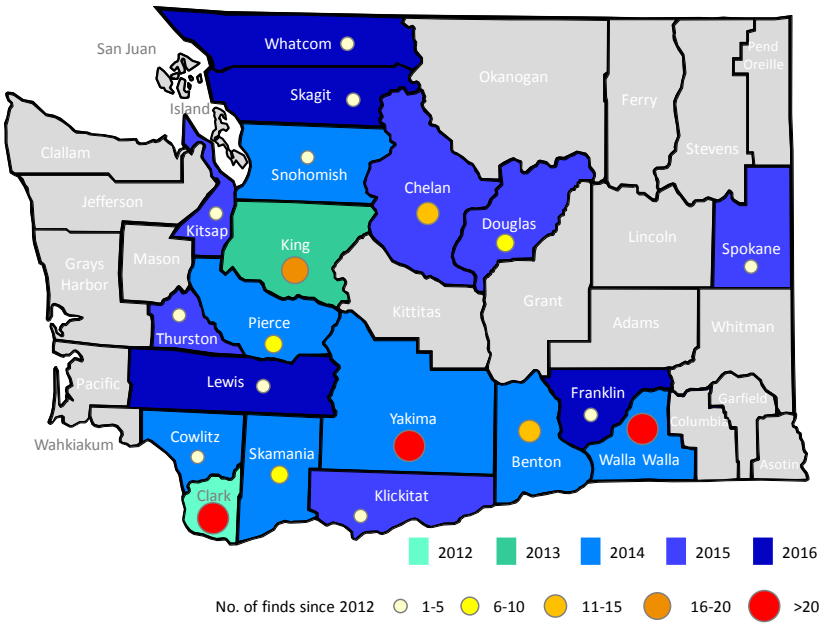

Fig. 4. Number of BMSB finds by county, Washington State, December 2016.

Obj. 4. Results and Discussion. A total of 19 counties have reported detections of BMSB (Fig. 4, Table 2). The highest numbers of BMSB reports came from Clark (Vancouver), Walla Walla, and Yakima counties. This reflects both the high degree of establishment in these counties, but also the higher level of sampling effort. Similarly, the numbers of reports in King and Pierce counties reflect the large urban/suburban population that is interested in this pest, and the amount of vehicular traffic that could deposit this invasive species. Snohomish, Skagit, and Whatcom counties are equally hospitable climates for BMSB, and will likely yield more finds if more sampling effort is expended. Conversely, Chelan and Douglas counties have a (relatively) lower human population and vehicular traffic, but a keen degree of awareness and interest in this species, possibly leading to a higher reporting incidence. Spokane and the Tri-Cities are also likely infested to a greater degree than has been reported. The current northern distribution in western North America is now defined by an apparently established population in Penticton, British Columbia, so it is reasonable to expect detections along the Hwy 97 corridor in Okanogan county in the near future. Other areas of the state may experience a temporal delay if human traffic is low; climate is less likely a limiting factor.

Objective 5. Determine suitability of native shrub-steppe plants as hosts for BMSB: This objective was deferred until the 2017 field season. In collaboration with Dr. Rodney Cooper, USDA-ARS Wapato, we will be working on methods for gut content analysis of BMSB. This, along with caged feeding studies, will help determine nutritional requirement and feeding habits, and provide an assessment of risk associated with certain plant habitats for buildup of BMSB populations.

Table 2. Number of BMSB finds (nymphs + adults) by county and year, Washington State, December 2016.

County	2012	2013	2014	2015	2016	Total
Benton			3	1	11	**15**
Chelan				1	12	**13**
Clark	1	1		3	11	**43**
Cowlitz			1	1		**2**
Douglas				5	1	**6**
Franklin					1	**1**
King		1	3	5	11	**20**
Kitsap				1	1	**2**
Klickitat				3		**3**
Lewis					1	**1**
Pierce			2	2	2	**6**
Skagit					1	**1**
Skamania				7		**7**
Snohomish			1			**1**
Spokane				1		**1**
Thurston				1		**1**
Walla Walla			1	25		**26**
Whatcom					1	**1**
Yakima			13	16	34	**63**

CONTINUING PROJECT REPORT **YEAR**: 1 of 2
WTFRC Project Number:

Project Title: Cold tolerance, diapause, and survival of Brown Marmorated Stink Bugs

PI:	Jason Irwin	**Co-PI (2):**	Elizabeth Rathburn
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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) Measure seasonal changes in lower lethal temperatures for overwintering nymphs and adults (e.g., determination of supercooling points).
2) 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., photoperiodic thresholds).
3) Identify the 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).

Timeline of activities for Spring 2017-Winter 2018.

Continue developing field and lab-based BMSB colonies:	Spring 2017 – Summer 2017
Identification/monitoring of field sites:	September 2017 – April 2018
Field collection for cold tolerance/diapause:	June 2017 – April 2018
Tests of photoperiod for diapause induction	Winter 2017 - Winter 2018
Data analysis/final report preparation	Spring 2018

SIGNIFICANT FINDINGS, YEAR ONE

- BMSB can supercool (that is, remain unfrozen) to an average temperature of 6.8 °F (-14 °C) in mid-December, with no significant decrease in supercooling ability throughout the seasonal sampling period (Sept-Dec). Individual adult BMSB supercooled as low as 1.4 °F (-17 °C). Yakima and Walla Walla populations were not statistically different in supercooling ability.
- Diapause induction was monitored through the seasonal change between fall and winter, and observed to show a significant decrease in metabolic rate throughout the sampling period (Oct-Dec). Metabolic rates fell significantly between Nov. 9th and 29th, suggesting that diapause is initiated between these dates.
- While both cold tolerance and diapause have been sampled throughout the fall and winter months, cold tolerance did not appear to be strongly linked to diapause inductions.
- The winter temperatures experienced by BMSB are being monitored on the outside of residential home near Pioneer Park in Walla Walla, WA, and within an enclosure in Yakima, WA (details in Facilities section below). Minimum temperatures recorded in exposed sites have already fallen below the minimum temperatures which BMSB could survive in laboratory testing.

METHODS

Objective 1 (Cold tolerance): A single cohort of BSMB will be used to measure seasonal changes in cold tolerance. To capture the natural timing of these events, a large colony of BMSB (n = ~750) will be established in spring and summer of 2016 in an outdoor enclosure located in a residential neighborhood in the West Valley of Yakima, WA (details of the site are in the *Facilities* and *Field monitoring* sections below). Through the fall, winter, and spring, samples (n = 10-12) will be taken at 3-week intervals and used for measurements of cold tolerance. If large natural populations are located in the fall, additional samples will be made to compare to those sampled from the enclosure.

Objective 2 (Diapause regulation): Seasonal changes in metabolic rate and developmental arrest will be measured on bugs sampled at the same time as the cold tolerance measurements. To assess whether BMSB are in diapause, we will measure the metabolic rates and reproductive condition of 8-10 individuals (equal number of males and females).

Photoperiodic threshold for diapause induction will be measured by exposing late-instar and adults from a laboratory colony to a series of progressively decreasing short-day photoperiods (light: dark -15.5:8.5h, 15:9h, 14.5:9.5h, 14:10h, 13.5:10.5h, 13:11h, 12.5,11.5h, 12:12h) to identify which

will induce diapause in adult and late-instar nymphs, as measured by reduced metabolic rate, and cessation of development and/or reproduction.

Objective 3 (Links between diapause and cold tolerance): The links between diapause and cold tolerance will be examined by comparing data from the seasonal collections outlined in Objectives 1 & 2. The relative timing of diapause induction and cold tolerance may have a large effect on the potential northward range expansion of this species.

Objective 4 (Overwintering site preferences/conditions): Although the overwintering sites of BMSB have been described in the Mid-Atlantic region, the places and conditions experienced in Washington State have not been carefully studied. We will locate overwintering aggregations of BMSB and monitor the temperatures experienced within these sites. A sub-population of BMSB (n=100) within the enclosure study will be monitored for mortality through the winter.

Detailed Methods

Collection of live specimens

Adult stink bugs will be sampled from naturally occurring aggregations from known sites in Washington State, generously shared with us by Pete Landolt (YARL) and Mike Bush (WSDA). Our own colony of BMSB may be augmented with individuals obtained from the existing colony at the Yakima Agricultural Research Laboratory in Wapato, Washington.

The laboratory colony will be reared under constant environmental conditions conducive for reproduction: 16L:8D photoperiod, temperature at 25°C (77°F), and 60% humidity (Lee Ream, YARL, pers. comm.). Upon hatching, nymphs will be fed raw carrots and water containing 0.05% sodium L-ascorbate and 0.025% L-cysteine (Noda, 1991) which will be refreshed every other day. "Sibling" nymphs within a single egg clutch will be housed together, and each cohort will be monitored for developmental rate and characteristics throughout each instar stage, as well as mortality the rate into full maturity.

Field-monitoring of BMSB

Field measurements of microclimate at natural and artificial (field enclosure) overwintering sites are necessary to understand how our local climate affects winter survival. These populations will also serve as a source for year-round collection of BMSB for tests of cold tolerance and diapause (as in Haye et al. 2014 and Šlachta et al. 2002).

The winter temperatures experienced by BMSB are being monitored on the outside of residential home near Pioneer Park in Walla Walla, WA. The site in Walla Walla is a house with cedar shingle siding where BMSB were found aggregating within the crevices underneath this exterior paneling of the structure. This house is within a few blocks radius of Pioneer Park (46.0662° N, 118.3178° W), a known and established site of BMSB during the growing season.

In the outdoor enclosure, BMSB will be kept in bug dorms (30 x 30 x 30 cm) filled with 10 cm of leaf litter, two pieces of wood, and five pieces of stacked bark for protection, but otherwise will be fed and maintained in the same way as the laboratory colony. Temperature dataloggers (HOBO Pro v2, Onset Computer Corporation) will be placed within the outdoor enclosure and in natural overwintering sites adjacent to overwintering BMSB.

Measurements of lower-lethal temperature (supercooling)

To measure the lowest temperatures survived by BMSB, we will measure the temperatures at which they freeze (in this case, we can refer to this as the supercooling point). Since freezing is lethal (Rathburn and Irwin, unpubl. data), the supercooling point represents the lower lethal temperature. To measure this, individual bugs are placed in a 2-ml microcentrifuge tube with the tip of a thermocouple probe (36 ga. copper-constantan) adjacent to its abdomen. The tube is plugged with foam, then

inserted into a larger test tube (also plugged with foam) that is partially submerged in a computer-controlled cold bath (Neslab RTE-740). The cold bath is programmed to cool at a rate of 1°C (1.8°F) per min. and the bug's temperature is monitored during cooling via a temperature datalogger (USB-TEMP, Measurement Computing). Freezing is easily detectable as a temperature increase which is caused by the release of the latent heat of fusion as water is converted to ice. Because supercooling is somewhat stochastic, we typically use 10-12 to determine an average supercooling point.

Measurements of metabolic rate

To characterize diapause within this species, the seasonal timing of diapause-induced metabolic suppression will be determined using respirometry following the methods of Irwin et al. (2001) and Lester & Irwin (2012). We will measure seasonal changes in metabolic rates by measuring CO_2 production and O_2 consumption of 8-10 BMSB collected every 3 weeks throughout the fall, winter, and spring from natural or enclosure populations. Measurements will be made using a flow-through, positive-pressure respirometry system (flow rate: 100 $mL \cdot min^{-1}$) (Sable Systems International), which includes an Oxzilla II oxygen analyzer and LiCor LI-6251 CO_2 analyzer. Metabolic rates of individual bugs will be made by placing pre-weighed bugs into a small glass respirometry chambers, which are placed in a double-walled beaker. Temperature of the chambers is controlled by fluid from a temperature-controlled bath (RTE-740, Neslab Instruments) flowing through the double-walled beaker. Baseline values measured from an empty control chamber will be subtracted to account for leakage. Respirometry will be performed at 0°C (32°F), 5°C (41°F), and 10°C (50°F) with a one-hour acclimation period prior to measurements at each temperature. Calculation of Q10 (factor by which metabolic rate increases of a 10°C [18°F] span), is a strong indicator of metabolic suppression associated with diapause (Irwin et al. 2001). Dr. Lisa Neven (YARL) has offered the use of her differential scanning calorimeter for measures of metabolic rate. We will explore this possibility as it would be more time efficient.

Photoperiodic threshold

Several photoperiods will be tested as a cue for diapause induction, following a quasi-natural progression of naturally occurring photoperiod regimes experienced in Yakima, WA (following https://www.timeanddate.com/sun/usa/yakima). All BMSB will be transferred from a LD regime experienced in the breeding colony used to induce reproduction (16L:8D), into the next lowest LD regime (15.5L:8.5D). Each LD regime will be run at constant temperature of 20°C (68°F). The LD regime will be incrementally reduced in light-hour duration by 30 min intervals beginning from 16:8 and ending on a LD cycle of 12:12. The duration of each LD regime will be determined by observed natural seasonal changes experienced in Yakima, WA (Table 1). At the transition of one LD phase to the next, a selection of individuals will be sampled (n = 8) consisting of individuals from each 'age group'. Cohorts of similar age (determined by a one week interval from when eggs were laid) will be labeled as one 'Age group' which will be identifiable by color coordinated labels. 4^{th} and 5^{th} instars will be kept within their specified egg clutch cohorts and monitored periodically for developmental rate. Once fully matured, these individuals will be labeled accordingly and placed within the general adult holding cages (e.g., "Bug Dorms" #1462C, BioQuip Products) After exposure to the experimental photoperiod for a total of 105 days, we will measure the proportion continuing development to adulthood, the reproductive condition of any adults produced (from nymphs), and the metabolic rates of all living individuals. Any adults will be dissected for reproductive status. Females without mature eggs or vitellogenic oocytes in the ovarioles, and males without secretory fluids in the ectodermal sacs of the accessory glands, will be classified as in diapause (Nakamura & Numata 1997).

Facilities/equipment available:

BMSB will be reared in captivity in Central Washington University's Vivarium. The facility was designed to contain animals and pathogenic material and is thus appropriate for quarantine of BMSB. The facility includes negative pressure air flow (filtered before venting to outside), multiple-door entry to prevent escapes, limited access security, and individually temperature- and photoperiod-controlled chambers.

BMSB will be reared/wintered indoors and outdoors within collapsible field cages (#1451D, BioQuip Products), with the outdoor cages housed within a large lumite outdoor cage (#1412A, BioQuip Products). The large outdoor cage will be in an outdoor shed fully sealed with 6mm plastic sheeting to further reduce risk of escape, along with a lock on the outside doors to enhance security of the outdoor enclosure. The shed is located on private residential property in the West Valley area of Yakima, WA.

Experiments will be performed in the laboratory of Dr. Jason Irwin which is fully equipped for measurements of metabolic rate on small insects via a Sable Systems respirometer (including the Oxzilla II oxygen analyzer and LiCor LI-6251 carbon-dioxide analyzer). Cold tolerance can be measured using a NESLAB RTE-740 recirculating cold bath and a number of dataloggers including a number of Omega DAQPRO-5300s and a Measurement Computing USB-TEMP. Thermocouple probes can be built in-house as required from materials on-hand. A walk-in refrigerated room and a number of photoperiod- and temperature-controlled incubators are available to house BMSB during winter and for measurements of metabolic rate. Dr. Irwin has a large number of field dataloggers (HOBO Pro v2, Onset Computer Corporation) available for temperature monitoring in the field and enclosures. The department has research vehicle available (Ford Expedition) and CWU faculty have access to other vehicles through the CWU motor pool.

RESULTS & DISCUSSION

While much research has been done on BMSB, particularly host-plant impacts and early developmental life history, there are no detailed studies regarding its cold tolerance and diapause regulation – both factors that will have a major impact on the success of BMSB as it spreads into Washington State. 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.

BMSB were observed to have suppressed metabolic rates, and therefore support for diapause induction, beginning in early November, as visualized particularly in the 50 °F (10 ℃) and 59 ˚F (15 ℃) temperature regimes (Fig. 1). These findings implicate apopulation of adult BMSB that are fully transitioned from a reproductive, to an overwintering population. 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 growing season. While the highest average observed metabolic rates were observed to occur at the beginning of this sample period, we have yet to determine an average metabolic rate that occurs during the growing season for comparative measures. Earlier seasonal sampling performed this upcoming season (Aug-Sept 2017) will greater distinguish when BMSB individuals begin to cease allocating resources to reproduction (feeding on agricultural crops) to reallocating those already acquired energy sources for winter survival.

Mathematical models predicting potential range expansion of BMSB demonstrated that minimum monthly temperature plays a significant role in determining range (Zhu et al. 2012), but even the most basic information regarding this species' cold-tolerance are lacking (for example, minimum temperature survived is unknown). The average supercooling point sampled from the outdoor enclosure ranges from 9.3 °F (-12.6 °C) in late-September to 6.8 °F (-14 °C) in late-December 2016 (Fig. 2). Males on average were observed to have a lower supercooling point, and

thus better cold tolerance than females. This difference in SCP could due to the smaller body size of males allowing them to supercool better - the ongoing data collection may reveal this pattern.

The outdoor enclosure located in Yakima, WA has been constructed to experience natural conditions including temperature and photoperiod. [While members of the WTFRC expressed concerns about large numbers of BMSB in an outdoor enclosure, permission to perform this work was given by Mike Willett given the outbreak of BMSB in Yakima this summer/fall.] Observed mortality within the outdoor enclosures indicates that not all individuals acquire the same level of cold tolerance. Field temperatures have been lower (0 °F/-17.8°C) than the minimum supercooling temperature for BMSB (1.4 °F/-17 or °C). Other studies expansion of a species can be limited in regions where cold weather arrives before the bugs are in a deep diapause (and thus do not supercool well). Our preliminary data suggest that this may be the case for BMSB in central Washington State. The survival of BMSB in our area may require artificially heated structures, sites which could be a target during winter as part of pest management strategies.

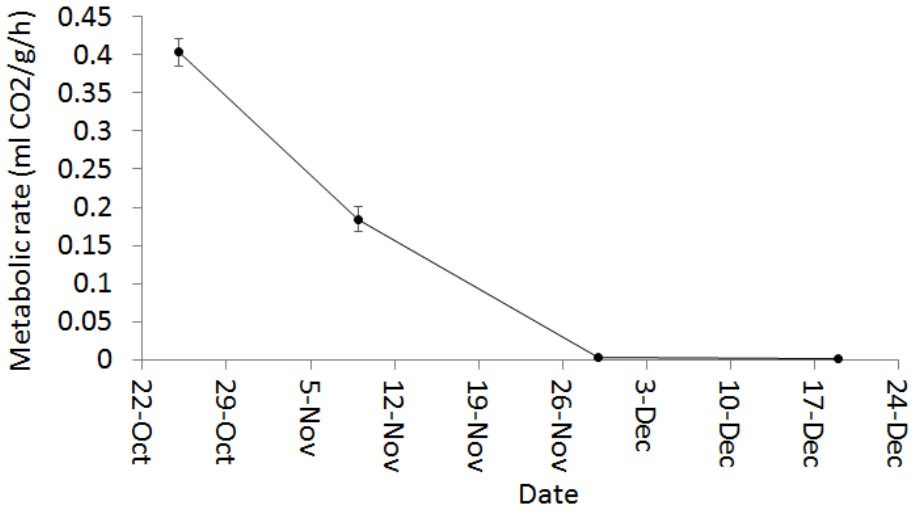

Figure 1. Seasonal reduction in the metabolic rates (measured at 15 °C) of adult BMS an outdoor enclosure in Yakima, WA through fall and winter. Data presented as mean +/- standard error.

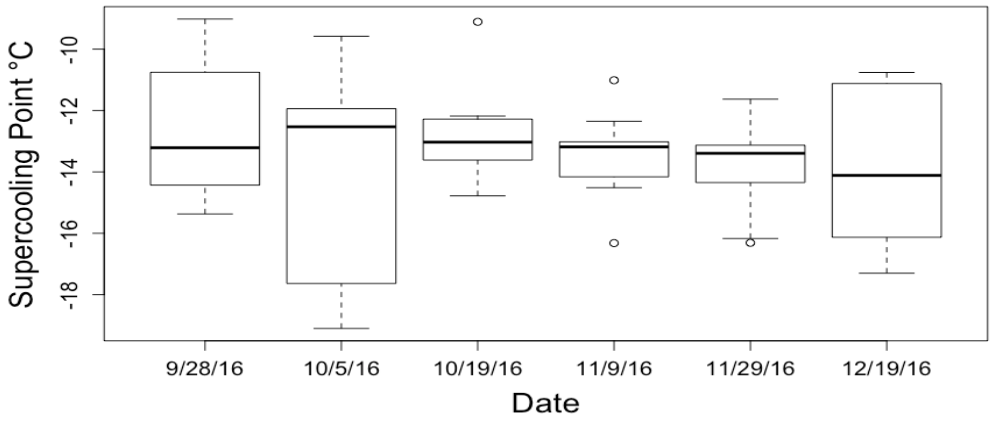

Figure 2. Supercooling points of adult BMSB sampled from an outdoor enclosure in Yakima, WA showing no significant seasonal shift in cold tolerance within the population.

CONTINUING PROJECT REPORT **YEAR:** 2 of 2 (No Cost Ext)
WTFRC Project Number: CP-14-104

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 expands 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. We will also include an economic analysis of how woolly apple aphids affect apple production systems.

WTFRC Collaborative expenses: None

Budget 1
Organization Name: WSU **Contract Administrator: Katy Roberts**
Telephone: 509 335-2885 **Email address: arcgrants@wsu.edu**

Item	2014	2015
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; 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

Objective 1

- Populations of woolly apple aphids, green lacewings, *Aphelinus mali* wasps, and earwigs were quantified on 8 conventional and 12 organically managed apple orchards in an area bounded by Chelan and Royal City on 3-5 visits throughout each of the 2014 and 2015 seasons.

Objective 2

- Physical, chemical, and biological factors of soil quality (bulk density, phosphorous, cation exchange capacity, pH, electrical conductivity, nitrate nitrogen, and microbial carbon) were measured at all study orchards in 2015.
- One measure of leaf nitrogen in 2014 and three measures of leaf nitrogen (on three separate visits) in 2015 were obtained for each study orchard.
- Perennial cankers were scouted in each orchard in 2015.

Objective 3

- Woolly apple aphid counts in organic and conventional orchards on average tended to be similar.
- Soil quality factors had no clear correlation to woolly apple aphid counts.
- Soil texture may be associated with fewer woolly apple aphids, 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.
- Relationships between woolly apple aphid counts and green lacewings, *A. mali* wasps, and earwigs were all difficult to interpret likely due to the limited resolution of our sampling strategy (only 3-5 data points per orchard per year).
- 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.

Other significant findings

Several follow-up studies were conducted which were not in our original objectives, but clarify aspects of the project goals.

- In a greenhouse study, sandier soil reduced movement of woolly apple aphids down to roots, as wood chip and paper slurry mulches, but migration was not completely blocked.
- 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, none of the soil quality measurements on average differed significantly between organically and conventionally managed orchards. Neither did overall soil quality when all measurements were summarized into a normalized score from zero to one (one being the best) representing overall soil quality. Though organic orchards are expected to have higher soil quality, our results could be explained by the low number of years some locations were managed organically (i.e., < 5), and by overlap in management practices between styles (such as use of manure fertilizer in one of the conventional orchards).

There was also no clear relationship between soil quality and woolly apple aphid abundance in either year of study (Fig. 1). However, three of the orchards had especially high sand content (>60%) and these orchards also had low woolly apple aphid counts (Fig. 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.

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 significantly reduced woolly apple aphid infestation of roots (Fig. 3). Thus, use of mulches on an orchard could be expected to reduce woolly apple aphid infestations. Similarly, sandy soil may reduce woolly apple aphid risk.

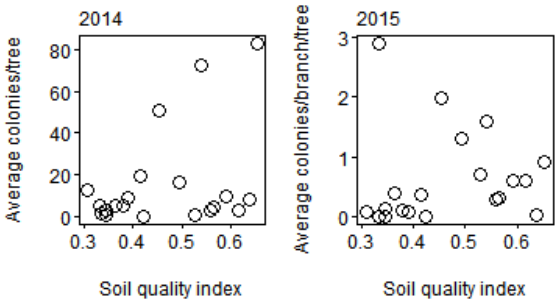

Figure 1. Relationship between woolly apple aphids and soil quality. Woolly apple aphid colonies were counted on entire trees (2014) or on each of 10 approx. 1' long branches on 16 trees per orchard (2015) on each of 3 to 5 visits throughout the growing season. Counts averaged to one number per orchard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

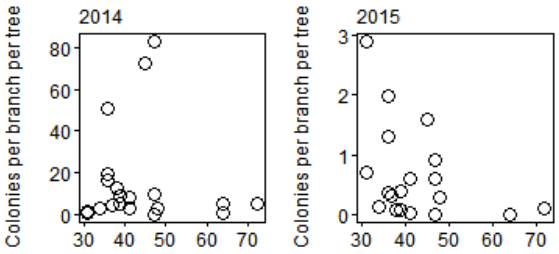

Figure 2. Relationship between woolly apple aphids and soil texture

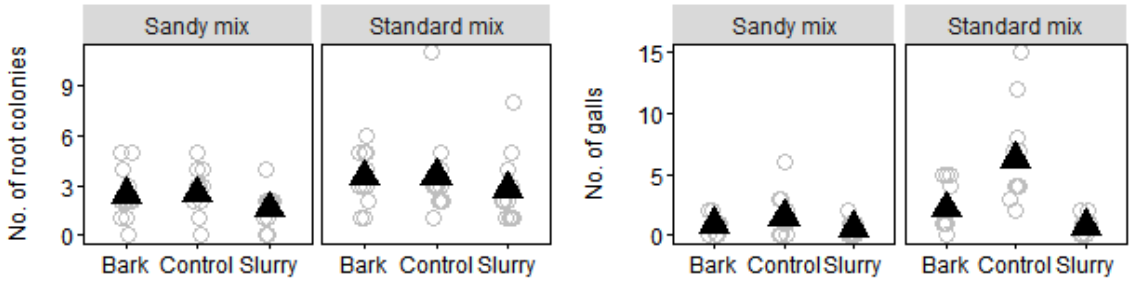

Figure 3. Soil and mulch greenhouse experiment. Woolly apple aphids were introduced to canopies of potted trees with different soil and mulch combinations. After two months trees were pulled for root inspection and colonies and galls were counted. Circles represent counts on individual trees and triangles 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.

Plant nitrogen content

There was no clear relationship between leaf nitrogen and woolly apple aphid abundance in either year of study (Fig. 4). 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, 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 what aphids actually feed upon.

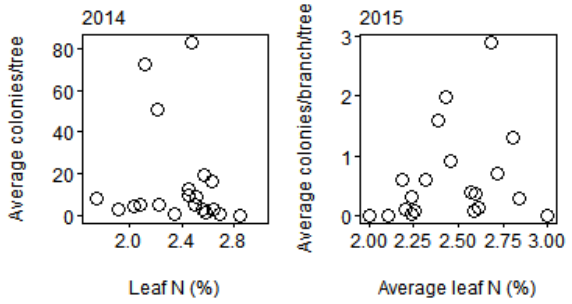

Figure 4. Woolly apple aphids and leaf nitrogen. Woolly apple aphids were counted as in Figure 1. Leaf nitrogen measurements were obtained from samples of mid-terminal fully expanded leaves. Leaf samples were collected on one visit (2014) or three separate visits to each orchard throughout the growing season and (2015).

Pesticide use intensity

We found that aphids had slighly lower densities in orchards that had reduced pesticide-use intensity. We believe this is because softer spray programs conserve natural enemies which are essential for woolly apple aphid management. More work is needed to relate timing of applications to wooly apple aphid populations, however, and we still need data from the 2015 field season to finalize these results.

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 overall a weak connection between woolly apple aphid and perennial canker because of the lack of perennial cankers at other orchards. The high infestation rate of perennial cankers suggests that they are great feeding sites that are preferred by aphids. This may cause some growers to falsely conclude that aphids are vectors of perennial canker. However, woolly apple aphids do not spread or cause first-year cankers. It was previously demonstrated that woolly apple aphids do not transmit the perennial canker fungus. Yet, trees with canker may provide suitable feeding sites for aphids, and it is possible that incidence of canker in an orchard could amplify aphid outbreaks.

Natural enemies

Orchards with higher abundance of natural enemies (green lacewings, *Aphelinus mali* wasps, earwigs) tended to have lower woolly apple aphid counts. Interestingly, we noted that earwigs appeared to be particularly important natural enemies for woolly apple aphids. To follow-up on these surprising results, a controlled experiment was conducted with earwigs. The experiment clearly shows that the addition of this generalist predator resulted in fewer woolly apple aphids (Fig. 5). Therefore, earwig conservation through timing and selection of pesticide sprays, and timing of cultivation (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 overripe splitting apples in the cup, but there was no evidence of any damage initially *caused* by earwigs and overall damage was not higher in the earwig sections.

We are following-up on this research to investigate the feeding habits of earwigs in orchards to determine situations where biological control by this natural enemy can be optimized.

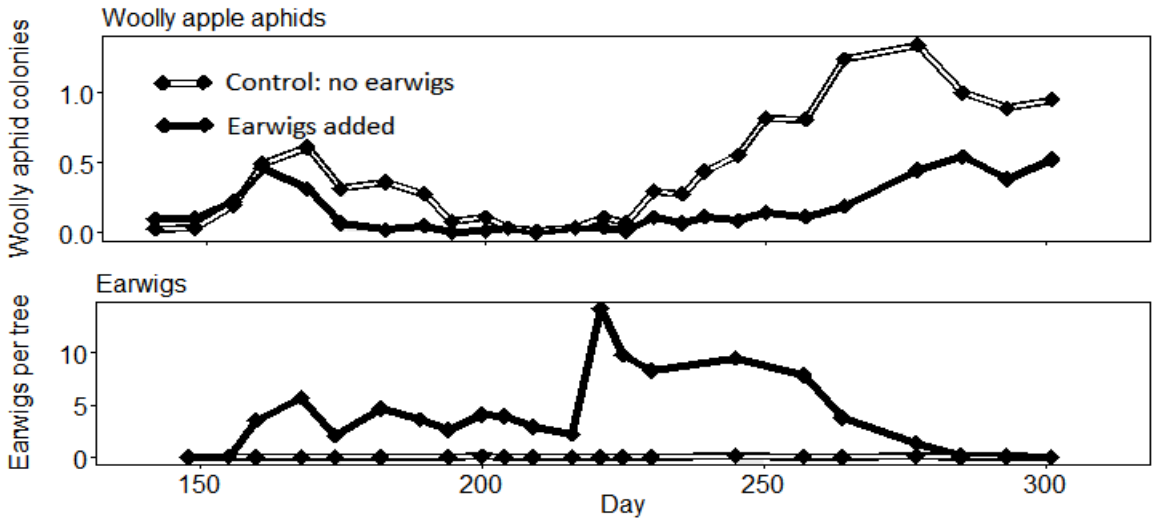

Figure 5. Results of earwig biocontrol experiment. Earwigs were first introduced to five 10 × 10 meter sections of the block on Ordinal Day 155 (see bottom chart) and were monitored each visit with counts in rolls of corrugated cardboard. A total of 675 earwigs per section were released between Ordinal Day 155 and 176. On Ordinal Day 217, 1,000 earwigs per section were released. The number of woolly apple aphid colonies (top panel) 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, and clearly show that earwigs had incredibly strong impacts on woolly apple aphids. The dropoff 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 (Fig. 6). We expect that in future years woolly apple aphids will also decline during extreme summer heat and any management actions during such periods would be superfluous.

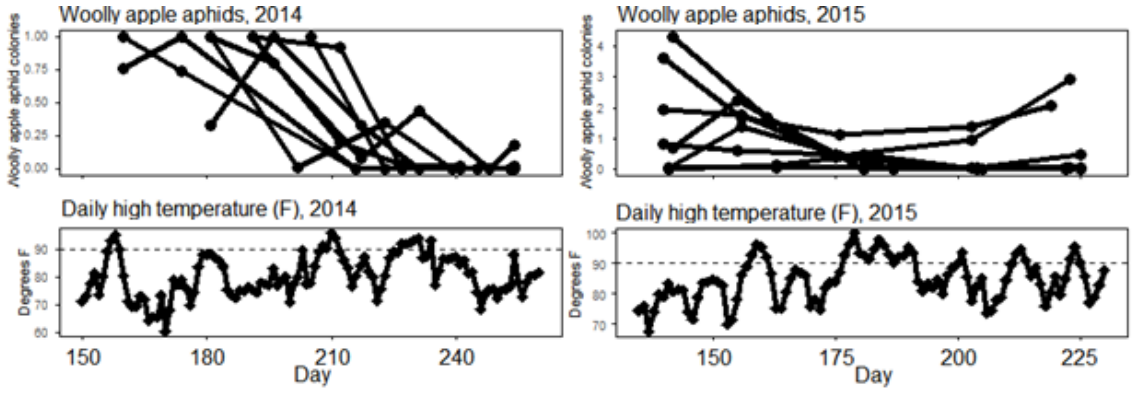

Figure 6. Temperature and season-wide woolly apple aphid counts. Woolly apple aphid counts (as in Fig. 1) at orchards near Quincy, WA over time are presented along with daily high temperatures in degrees F. The dotted lines in temperature graphs are at 90F, above which woolly apple aphids die. Because 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, but the difference was not statistically significant. 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 aphids (Fig. 7).

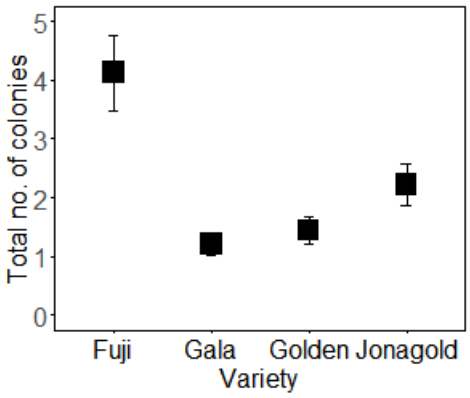

Figure 7. Woolly apple aphid counts on different apple varieties. In 3 mixed-planted blocks at the WSU Sunrise Research Orchard (containing alternating rows of Fujis, Galas, Goldens, and Jonagolds, the number of woolly apple aphids on October 6, 2015 were counted on ten ~1' long twigs on each of 312 total trees. Average counts per tree for each variety are shown with standard errors.

CONTINUING PROJECT REPORT **YEAR**: 1 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, WSU/UCR, Dr. Kate Evans; WSU-TFREC, Dr. David Gang, WSU

Total Project Request: 149.599 **Year 1**: 58,710 **Year 2**: **49,079** **Year 3**: 40,477

Other funding sources

None

Budget 1
Organization Name: University of California-Riverside (new organization because of move)
Contract Administrator: Teeny Ellis
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Item	2016	2017	2018
Salaries	$32,836	**$20,646**	$21,369
Benefits	$3,424	**$16,463**	$17,118
Wages			
Benefits			
Equipment			
Supplies**	$22,450	**$11,970**	$1,990
Travel			
Plot Fees			
Miscellaneous			
Total	$58,710	**$49,079 (↓$601)**	$40,477 (↓$732)

Footnotes: *Salaries and Benefits are to support one MS student; Changes to the original amount proposed in 2016 reflect adjustments for salary through UCR.
**RNA sequencing services, lab supplies for tissue assays or extractions, and high performance computer server access.

NOTE regarding move: At the end of 2016 the Nabity lab moved to UCR to begin a new faculty position. The lab will continue its focus on plant-insect interactions, especially those of agricultural significance. We plan to continue working on the WAA project through its completion in 2018, if funding is continued. As a result of this move, costs for the student salaries decreased slightly reducing the overall cost of the project. Because this project uses greenhouse space for insect colonies and plants, and lab space for assays, no work will be affected by the move. Cooperator Zhao will join UCR in late spring and begin collaborating again to help complete this project. Cooperators established at WSU will remain as colleagues and advise when appropriate.

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 will be to combine transcriptomic information on the apple genes induced by aphid colonization with the genes and proteins active in aphids. Comparing across susceptible and resistant cultivars will identify the processes in common and the different networks that define resistance. Linking insects to plants will help identify how aphids overcome resistance 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. Our project also aims to train one MS 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. We are collaborating with Dr. David Gang (WSU) to screen these salivary secretions for proteins. The whole body transcriptome assembly and assessment is complete. The dissected salivary gland sequencing is underway and will be complete by the time of the presentation. The protein samples have been submitted and will be run during January.

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. This work will begin in spring 2017.

3. **Identify functional plant traits that confer immunity to WAA.**

Once gene networks are identified, we can infer the functional plant defense chemistry and signaling that result from aphid attack. We will measure reactive oxygen formation (e.g., peroxides) and callose growth, which are known to provide chemical and structural defense against aphids in other plants. This work will also begin spring 2017.

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 RosBreed scientists including K. Evans (WSU-TFREC) will enable the linkage of genes to chromosomes or nearby markers.

Timeline. Our expected timeline is outlined below. We collected and assessed WAA during year 1,

and will complete Objective 1 this winter. We plan to assess plant immune functions during year 2, and link this information together in a final summary during year 3.

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

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).**

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 will compare these to those in salivary glands currently being sequenced. Genes found only in salivary glands will be matched to the proteins secreted. This year we will characterize the transcriptomes 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 be assessed concurrently to link gene expression to immune response 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 sent to the Tissue Imaging and Proteomics Lab at WSU where Dr. David Gang and Dr. Jing Wang prepared samples for an LC-ESI-MS/MS run in January. These protein data will be analyzed using a freely available online search engine for protein identification, MASCOT, and compared to our transcriptome and the NCBI database.

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 will profile the transcriptomes in resistant (e.g., MM106) and susceptible (e.g., Fuji) cultivars. If no feeding occurs as can occur with complete resistance, we will add an additional cultivar that is partly susceptible as defined by low WAA colonization (e.g., M.9 Pajam). After 24-48h we will harvest plant tissue. RNA extraction and sequencing will commence as described above except that alignment of reads will be done by using free software (TopHat2; Kim et al. 2013) and the apple reference genome (Velasco et al. 2010). Also, sequencing will be performed at the UCR Core Genome sequencing facilities.

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). 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 PI P. Nabity has extensive experience in analyzing plant transcriptomic networks and resolving physiological responses of plants to herbivory (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 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.

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, 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.

Table 1. Summary of WAA effector proteins predicted from the whole body transcriptome

RNAseq reads	Total proteins	Effector proteins	Effector families	Effectors specific to WAA
344 million	**85,925**	**184**	**61**	**140 (76%)**

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CONTINUING PROJECT REPORT **YEAR**: 2 of 3
WTFRC Project Number: CP-14-102

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

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Cooperators: None

Total Project Request: **Year 1:** 10,000 **Year 2:** 0 **Year 3: $3,000**

Other funding sources: None

Budget 1
Organization Name: WSU **Contract Administrator: Katy Roberts**
Telephone: 509 335-2885 **Email address: katy.roberts@wsu.edu**

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**

Footnotes: No expenses were expended in 2014 due to inability to develop collaborative arrangements for germplasm collection prior to the end of the season of drone-availability. Funds requested for 2017 are for bee keeping supplies and equipment for queens.

OBJECTIVES

1. Collect, cryopreserve and import semen from a diverse selection of *A. m. pomonella* honey bee colonies made in the apple forests of Kazakhstan and Kyrgyzstan.

2. Following USDA-APHIS quarantine procedures, recover *A. m. pomonella* stocks through backcrossing, make selections under Washington conditions and distribute to California queen producers for distribution

SIGNIFICANT FINDINGS

1. Competed all aspects of Objective 1 in summer of 2015. Honey bee semen was collected in Kazakhstan from the Aksul-Jabagly reserve in the western Tien Shan Mountains and from a known original collecting site in eastern Kazakhstan approximately 800 km (500 miles).

2. Submitted imported semen to USDA-APHIS quarantine procedures and 50% pomonella queens were overwintered in winter 2015. In 2016, initial backcrosses were made and 75% pure pomonella queens are being overwintered in winter 2016.

METHODS

Semen was collected from drones originating from hives identified as *A.m. pomonella*. Fresh semen was held in 100 µl glass capillary tubes for transport back to the US. Half of the semen collected was cryopreserved using a portable programmable freezing unit. The cryopreserved semen was held in 0.25 ml semen cryo-straws and stored in a dry-shipper for transport of the frozen semen back into the US.

Upon arrival in summer 2015 the fresh semen was used to inseminate virgin queens. The inseminated queens were overwintered in Pullman in winter of 2015-2016 and were used to produce daughters in spring 2016. The daughters (50% A.m. *pomonella*) were inseminated with the previously frozen semen. The resulting daughter queens produced from the insemination with frozen semen (now 75% *A.m. pomonella*) will be inseminated with previously frozen semen in 2017. The daugher queens from the second round of inseminations with frozen semen will be 87.5% *A.m. pomonella*.

RESULTS & DISCUSSION

The continued backcrossing using frozen semen will produce 97% pure *A.m. pomonella* subspecies at the end of the three year project. We will continue to distribute daughters to the queen producing partners. Based on experience we have with our other importations and stock recovery, we do not see any specific problems that would interfere with development of the >97% *A. m. pomonella* stock within the three year period of the grant. We will continue work beyond the term of this grant to further evaluate and select honey bee stocks that work well in tree fruit pollination under Washington conditions.

ADDITIONAL ITEMS

The original approved grant request was for $16,000 ($10,000 year 1, $3,000 year 2 and $3,000 year 3). We requested and received a one-year delay to start the project to due to logistical issues with travel to Kazakhstan. We conducted the initial year one field work in 2015 and did not receive any funding in 2016. We will continue the work as planned, although we have not received year 2 or year 3 funding.

LITERATURE REVIEW

Ancestor to the modern domestic apple, pear and cherry can all be traced back to the Tian Shan Mountains in Western Asia (Hokanson et. al. 1997). Government and University researchers have made great efforts in traveling to this region to collect germplasm to improve domesticated stocks. Sheppard and Meixner (2003) identified a new subspecies of honey bee that has evolved in the ancient fruit forests and has unique morphological and physiological characteristics that separate it from the other honey bee subspecies. This subspecies could serve as an excellent pollinator of Washington's fruit trees but more importantly, the importation of the germplasm and introduction into the current US breeding stock will provide a much needed influx of genetic diversity. Research has demonstrated that greater genetic diversity in honey bee colonies improves disease resistance and increases fitness and productivity (Mattila and Seeley 2007, Seeley and Tarpy 2007). The increase in genetic diversity will also provide a greater array of genetic tools from which queen breeders will make their selections, ultimately helping to stabilize honey bee populations and pollination services.

Our lab is the only lab in the world to initiate and develop a honey bee genetic repository. This has been made possible through recent improvements in the cryopreservation of honey bee semen (Hopkins and Herr 2010). We have demonstrated the ability to perform multiple backcrosses using cryopreserved semen (Hopkins et. al. 2012). Using these methods we have been utilizing cryopreserved semen collected in the Republic of Georgia's Caucasus Mountains in 2011, 2012, 2014, Slovenia in 2011 and Italy in 2012 and 2013.

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CONTINUING PROJECT REPORT **YEAR**: 1/3
WTFRC Project Number: 1087 (internal account, general food safety)

Project Title: WTFRC internal program – food safety efforts

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Cooperators: Laura Grunenfelder and Kate Woods (NHC), Jacqui Gordon (WSTFA), Manoella Mendoza and Mackenzie Perrault (WTFRC), Lauren Walter and Kyu Jeong (WSU), Missy Partyka, Ronny Bond, Jennifer Chace, Jayanti Das (UC Davis), Bonnie Fernandez-Fenaroli (CPS)

Acknowledgement: WTFRC seasonal crew efforts are acknowledged and appreciated. We would like to thank Harold Schell, Jake Gutzwiler, and Brent Milne (all WTFRC board members) for their assistance in reviewing CPS grant proposals.

Other funding sources

Agency Name: WA SCBGP
Amt. requested/awarded: $ 216,682 Title: Enhanced food safety education and training for tree fruit producers (awarded to WSTFA with Jacqui Gordon as PI)
Notes: In 2016 a total of six workshops (three topic areas) were organized for tree fruit producers, with WTFRC participation

Agency Name: FDA
Amt. requested/awarded: $243,651 for FY17 awarded to WCFSS (Atwill et al.) Title: Facilitating implementation of FSMA regulations for agricultural water quality
Notes: This budget covers sampling in both California and Washington and includes staff salaries. The budget for Washington alone is estimated at ~$140K. WTFRC participated in site selection, experimental design, and planning for 2017

Agency Name: CPS
Amt. requested/awarded: $334,252 Title: Evaluation of an alternative irrigation water quality indicator; PI: Trevor Suslow, UC Davis
Notes: In 2016 WTFRC sampled 14 locations and filtered samples (Mohr swabs) in monthly intervals from May-Sept. as part of a multi-state study (CA, AR, WA)

Agency Name: WSU/UI School of Food Science
Amt. requested/awarded: $30,000 (awarded)
Notes: Funds were utilized to continue food safety research in a microbiology lab in Pullman, former Killinger lab, and to help develop industry training

WTFRC internal program expenses:

Item	2016	2017[3]
Salaries	27,146	27,689
Benefits	5,322	5,428
Wages	2,257	2,584
Benefits	855	979
RCA Room Rental		
Shipping		
Supplies[1]	177	200
Travel[2]	1,622	5,000
Plot Fees		
Miscellaneous		
Total	**37,379**	**41,880**

Footnotes:

[1]Supplies include three posters (2 for IAFP, 1 for ASHS)

[2]Travel includes: CPS in Seattle, trip to WSU in Pullman, in state day travel to attend trainings, IAFP in St. Louis, NW Food Safety and Sanitation Conference in Portland; projection of 2017 travel costs is significantly higher, since Dr. Hanrahan cannot guarantee that she will be an invited speaker and/or can purchase flight tickets on WTFRC credit card miles; an effort will be made to reduce the projected costs similar to 2016

[3]Wages and salaries have been calculated as follows: salaries = 2% increase, wages = 14.5% increase based on projected state minimum wage increase and assuming that workload does not further increase

OBJECTIVES

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

SIGNIFICANT ACCOMPLISHMENTS IN 2016

Research: Dr. Hanrahan started her year with a six-week ***sabbatical at UC Davis***. From February 4 until March 13, 2016, she spent time as a visiting scholar at the Western Center for Food Safety (WCFS) with the team of Robert Atwill (Center Director; www.wcfs.ucdavis.edu). Primarily, Dr. Hanrahan received basic and advanced microbiology training and participated in ongoing projects (including a trip to Mexico to investigate the cause of *Salmonella* infection in Papaya). A second focus of her leave was the chance to network and foster or expand professional connections in California, explore ways to provide training and answer questions related to FSMA implementation, and to deepen existing and forge new collaborations with several research teams (Suslow, Linke, Walse, Holcroft). A full report and detailed ppt descriptions of activities are available upon request.

Secondly, Dr. Hanrahan continued to lead the former Killinger lab staff at WSU in Pullman, and successfully finished all on-going projects, while transitioning staff to new positions. Kyu Ho Jeong remains on the team until July 2017 to help finish data analysis and preparation of manuscripts.

Third, we participated in a number of on-going collaborative projects, funded by WTFRC, CPS, and FDA (see Table 1).

Lastly, the WTFRC, under leadership of Ines Hanrahan, has served as a partner in research for the Center for Produce Safety (CPS). Tree fruit specific research priorities are developed and integrated into the annual CPS call for proposals, with the help of the NHC Food Safety Committee. As a result, six proposals addressing industry needs were originally submitted, and ultimately one was funded: 'Control of Listeria monocytogenes on apple through spray manifold-applied antimicrobial intervention' (Zhu/Suslow; $290,000). WTFRC staff (Hanrahan) was also involved in planning of the annual CPS conference held in Seattle this year. As a result, CPS devoted a half day of the two-day conference to the experience and research needs of tree fruit (specifically *Listeria*). Dr. Hanrahan was invited as a speaker to explain our industry response to the *Listeria* outbreak (Washington Tree Fruit Industry Response to *Listeria monocytogenes* Caramel Apple Outbreak).
Further, we supplied pictures of our crops to be used in advertising material, supplied names of local students (including Manoella Mendoza) to help at the meeting, and WTFRC designed and assembled centerpieces themed around tree fruit for the VIP dinner. Following the meeting, Hanrahan organized a visit of the entire staff of the Produce Safety Alliance (PSA) to the Yakima Valley for a combination of field trips (orchards and warehouses) and discussions (incl. NHC, WSTFA). The PSA is responsible for providing training for the Produce Rule.

Table 1: Summary of WTFRC collaborations* in food safety research in 2016 and pending research for 2017

Keyword	PI's	Affiliation(s)	Funding Source	Amount
Continuing in 2016				
Evapor. cooling	Hanrahan/Zhu	WSU, UW, WTFRC	WTFRC	190,000
List. packing	Hanrahan/Suslow	WSU, WTFRC, UC Davis	WTFRC	66,000
Imp. Dryer	Ganjyal/Zhu	WSU	WTFRC	57,000
Bacteroides	Suslow	UC Davis; UoA, WTFRC	CPS-SCBG	336,000
New in 2016				
Listeria storage	Zhu/Amiri/Hanrahan	WSU, WTFRC	WTFRC	195,414
Water sampling	Partyka/Bond	UC Davis, WTFRC	FDA	243,651
Pending for 2017				
List. cleaning	Zhu/Hanrahan	WSU, WTFRC	WTFRC	306,285
Water sampling	Atwill/Partyka/Bond/Hanrahan	UC Davis, WTFRC	CPS	367,000
Equipment	Linke/Das/Chase/Hanrahan	UC Davis, WTFRC	WTFRC	66,000
Education	Ganjyal	WSU	WTFRC	95,000
Brush beds	Blakey	WSU	WTFRC	55,000

*collaborations may involve a WTFRC internal budget or utilize Dr. Hanrahan as a consultant/co-PI or collaborator

FSMA implementation: In order to best serve the needs for timely information distribution related to new laws pertaining to food safety, WTFRC staff (Hanrahan) lead an effort to coordinate all outreach activities by the various industry organizations. Specifically, NHC (policy), WSTFA (education) and WTFRC (research) efforts were combined and talking points coordinated to prevent further confusion, when learning how to implement the already complicated laws. The entire team (Grunenfelder, Woods, Gordon, Hanrahan) has developed a uniform slide set to be used by each group member when addressing groups. This is a living document and has been updated numerous times.

Development of industry training modules: In collaboration with WSTFA, NHC, and WSU we developed and executed two workshops with a total of 112 participants in 2016:

a. Putting Cleaning and Sanitation Programs into Practice (two locations)
b. Verification of Cleaning and Sanitation for Tree Fruit Packinghouses

These workshops provided a combination of classroom and hands-on activities and took place in collaborating packing facilities (Table 2). Dr. Hanrahan's contributions to these workshops included: leading of general curriculum development, being a trainer, delivering talks, and helping with logistical support (including staff). Secondly, the same group, in collaboration with UC Davis held three workshops named: FSMA water quality testing. This module was also the first of it's kind in the nation to address practical considerations for water testing under FSMA. Workshops were designed to give participants theoretical background in combination with outdoor activities geared towards learning based on examples coupled with hands on training (Table 2). After conclusion of the workshop, a permanent document was prepared collaboratively between Melissa Partyka, Ronny Bond, and Ines Hanrahan to detail considerations for water testing and placed onto the WSU Tree Fruit Extension team webpage as a living document (http://treefruit.wsu.edu/news/water-sampling-for-fsma-compliance-done-simply/). For 2017, we plan on repeating all workshops as needed, or as soon as guidance for water sampling is released by FDA. In addition, WTFRC is collaborating with the WSFTA to develop hand washing training materials including a video and practical training material.

Table 2: WTFRC staff involvement in WSTFA sponsored food safety trainings in 2016

Name of Workshop/Training	**Date**
2016 FSMA Water Quality Testing Workshop Wenatchee	5/17/2016
2016 FSMA Water Quality Testing Workshop Selah	5/18/2016
2016 FSMA Water Quality Testing Workshop Yakima	5/19/2016
Putting cleaning and sanitation programs into practice - Zillah	7/20/2016
Putting cleaning and sanitation programs into practice - Wenatchee	7/21/2016
Verification of cleaning and sanitation programs for tree fruit packinghouses: a hands-on environmental monitoring workshop for food safety managers	11/1/2016

Food Safety outreach: Ines served as the session manager for the food safety session during the WSTFA 112th Annual Meeting (HortShow) in December 2016. As an added service to conference attendees, the team (incl. Jacqui Gordon, Mackenzie Perrault and Ines Hanrahan) collected all questions asked during the session that did not get answered, prepared written responses and sent them out via email.

Dr. Hanrahan also served on the search committee for the WSU Food Safety Extension position. To date, the position remains open, because no suitable candidate has been found yet. In addition, Ines was asked to join the WSU School of Food Science as an adjunct faculty member. She is currently serving on one MsSc. Committee.

Further, Dr. Hanrahan is serving on the stirring committee of the PNW Food Safety and Sanitation Conference, a regional conference with 450 attendees annually. Other outreach activities included: three posters at national/international meetings, and ten invited talks. The press covered WTFRC food safety activities in five Good Fruit Grower articles and three blogs/videos.

CONTINUING PROJECT REPORT **YEAR**: 2 of 3
WTFRC Project Number:

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

PI: Alan Knight
Organization: USDA, ARS
Telephone: 509-454-6566
Email: alan.knight@ars.usda.gov
Address: 5230 Konnowac Pass Rd
City/State/Zip: Wapato, WA 98951

Co-PI (2): Gary Judd
Organization: Agriculture and Agri-Food Canada
Telephone: 250-494-6372
Email: gary.judd@agr.gc.ca
Address: Box 5000, 4200 Hwy 97
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
Salaries			18,000[a]
Benefits			
Wages	17,500	20,000	22,000
Benefits	1,500	1,500	2,000
Equipment			
Supplies	6,000	7,500	8,000
Travel (local to research plots)	1,000	3,000	3,000
Miscellaneous			3,000[b]
Plot Fees	3,000	3,000	3,000[c]
Total	29,000	35,000	**59,000**

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.

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
Salaries	0	0	0
Benefits	0	0	0
Wages[1]	18,000	0	0
Benefits	2,500	0	0
Equipment	0	0	0
Supplies	4,000	4,000	6,000
Travel[2]	1,000	0	0
Miscellaneous	500	0	0
Total	26,000	4,000[a]	**6,000[a]**

Footnotes: [a] The drop in requests to WTFRC for 2016-17 was due to the awarding of Canadian funds to support this project.

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

- Effective lures (10 mg red septa and long-lasting proprietary closed membrane cups) loaded with either 2-phenylethanol (PET) or phenylacetonitrile (PAN) were developed and tested with a new proprietary closed membrane cup lure loaded with acetic acid (AA) in collaboration with Trécé Inc (Adair, OK). All lures were found to be highly effective.
- PET+AA is more attractive for leafrollers and PAN+AA is more attractive to eye-spotted bud moth (ESBM). PET/PAN+AA was effective for both moth groups.
- Volatile captures by Valentino Giacomuzzi (Free University of Bolzen, Italy) of apple volatiles from foliage untreated or subjected to OBLR larval feeding were analyzed by Jim Mattheis (ARS, Wenatchee, WA) and provided a complete description of the array of important volatiles to be considered as attractants.
- No major discoveries of new attractive plant volatiles were made in extensive field trials in 2016. However, several compounds were found to reduce moth catch, and one compound caused a marginally significant increase in catch.
- Orchards treated with sex pheromone dispensers for OBLR were effectively monitored all season with PET+AA-baited traps.
- We learned that the new LR lures can be used in the same trap with lures for codling moth to monitor both pests with a single trap.
- We learned that the new LR attractants significantly reduce male but not female catches with OBLR and ESBM sex pheromone lures.
- Bucket traps using a solution of propylene glycol to retain moths were found to be the most effective low-maintenance trap for use in mass trapping.

Goals and activities for next year: Our 2017 work in Washington is focused on developing improved monitoring and mass trapping for codling moth and leafrollers, while the work in Canada is focused on the same goals for ESBM and leafrollers (because they have the sterile CM program established).

We intend to have Dr. Valentino Giacomuzzi from the Free University of Bozen in Bolzano, Italy visit the ARS laboratories in Yakima and Wenatchee for six months in 2017. He will again be tasked with collecting and analyzing volatile data from apple, pear, and cherry hosts, from the lures, and from yeast isolates collected from tree fruits. Drs. Esteban Basoalto from University of Chile in Valdivia, Chile and Valentina Mujica from INIA in Uruguay will also be working with us for a period of time this coming year to help process the various field collections, e.g. moth sexing and mating status dissections. Dr. Judd will expand his collaborations with Dr. Gerhard Gries at Simon Frazier University (EAG studies to characterize antennal responses of both sexes to apple volatiles and volatile collections from apple and cherry) and Dr. Maya Evenden at University of Alberta (flight mill studies to characterize the relationship of adult's nutritional status with dispersal) by helping to

supervise one or more graduate students associated with his expanded Canadian project. Replicate mass trapping studies will be conducted in both countries.

Schedule of Activities:

1. Flight tunnel tests are ongoing at YARL and Ag Canada to study the adult behavior of virgin and mated male and female OBLR and PLR with the key volatiles. These tests will likely continue until the start of the field season and then will start back up in October.
2. Volatile analyses will begin in June with Drs. Giacomuzzi and Basoalto working with Dr. Mattheis and collaborating with the Canadian group including students of Drs. Gries.
3. Field testing of lures, new kairomones, and new kairomone blends will begin in May in both Washington State and British Columbia and will continue through the summer.
4. Seasonal monitoring of ESBM, OBLR, and PLR in conventional and sex pheromone-disrupted orchards with our most advanced kairomone lures will begin in May.
5. Replicated mass trapping evaluations will be established in the spring and run all season.

METHODS

Volatile collection. Headspace of host plants (apple, pear, and cherry) will continue to be sampled from shoots with feeding larvae of various species in the field and laboratory. We are now working in Dr. Mattheis laboratory using his advanced analytical equipment to analyze these data.

Flight tunnel studies. Various 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 identified from volatile captures. Mated and virgin males and females of Pandemis and OBLR are being tested for their response to the key volatiles. Flight tunnel tests are also being used to evaluate traps' relative catch efficiency of both moth sexes and to develop lure-and-kill technologies.

Field trials. A few specific studies remain to be completed. First, we want to further examine whether the leafroller sex pheromone can be used with PET plus AA to increase the total moth removal capacity of traps. Second, we want to examine the potential attraction or repellency of various plant volatiles using oil-coated clear pane traps. These traps allow us to compare moth behavior in the canopy in relation to an unbaited passive interception trap. Thus we can measure both attractancy and repellency with this technique. Third, we are looking at the effect of the trap liner's adhesive type on whether female moths lured to the new attractants can then attract male moths into the trap. Fourth, we will look more closely at the use of a binary lure loaded with both PET and PAN for each of the key species because this binary lure might be a more successful commercial product. Fifth, we will again review the use of the new leafroller lures in combination with codling moth lures in the same trap. All studies will be conducted with standard protocols using complete randomization of lure treatments with 5-20 replicates per experiment.

Formulation development. Effective lures have been developed within this study in close collaboration with Trécé Inc; however, one or more modifications of lures are still needed. For example, we need to establish how long the 10 mg lures are effective and compare this with the proprietary membrane lures; and we need to tweak the loading of the membrane lures, i.e. reduce the loading of PET and PAN lures to reduce costs; and to extend the activity of the new AA lure by either increased loading or the use of a different membrane.

Monitoring populations. Effective lures are now available to distribute to a number of field men for *Beta* testing. This will be conducted with cooperation and help from one or more large growers and related Ag companies providing monitoring services for both conventional and organic orchards.

Data will be collected on the phenology of both male and female catches in traps, and these data will be compared with the current phenology models for OBLR and PLR in DAS. Traps with 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.

Development of mass trapping. Our results in 2016 suggest that the reusable (+10-year warranty) bucket traps with the inclusion of a food-grade propylene glycol (PPG) to retain moths was likely the best approach to maximize moth catch over a season with a minimal labor and material cost. A similar trap and lure set can be used in organic orchards but will need to use a certified mineral oil instead of PPG and a 30% organic vinegar instead of AA. Studies focused on organic orchards will be expanded with a request for additional funding for organic leafroller management.

Studies will initially be conducted in replicated 1 acre blocks at the USDA Research Farm and in 2.5 acre blocks in a Wapato orchard. Our goal is to identify other sites prior to the season and similar studies will be established. Treated blocks will receive an array of 16 (U.S.) or 25 (Canada) traps per acre. Traps will be baited with a Combo CM+PET+AA lure set (U.S.) or the PET/PAN+AA lure set (Canada). Leafroller (budmoth) larval populations will be sampled in the spring and mid-summer, and fruit injury will be assessed midseason and just prior to fruit harvest. Codling moth fruit injury will be assessed mid-season and prior to harvest. All moths will be sexed and females will be dissected to establish their mating status.

Development of 'Lure and Kill'. Flight tunnel bioassays are being conducted to evaluate male and female contact with an insecticide-treated substrate (lure station) surrounding the new lures. Both direct mortality and sublethal (subsequent mating success and resulting fecundity) effects are being measured. Next summer the toxicity of variously aged lure stations will be assessed with the standard 10-sec touch test.

RESULTS & DISCUSSION

Weight loss over a 14-d period at varied >7-fold among the AA and PET lures. The rate of weight loss for lures used in the seasonal field trials also varied widely over a 12-wk period. For example, the membrane cup lure TRE1256 had a consistent weight loss over this longer time period, slope = 0.0023, $P = 0.54$. The AA vials with 1.0-mm and 3.2-mm apertures clearly had different mean weight losses (Table 1), and the vial with the larger aperture which was the one used in seasonal field trials had a consistent weight loss over a 12-week period at 25ºC, slope = -0.8794, $P = 0.21$. In contrast, the AA membrane cup lure TRE1468 had a significant linear decline in weight loss as a function of time, slope = -4.069, $R^2 = 0.90$, $P < 0.001$. After six weeks the mean daily weight loss of the TRE1468 membrane cup lure was reduced to a level (5.09 mg d^{-1}) similar to that of a new (< 2-weeks-old) TRE3321 lure. Red rubber septa loaded with 10.0 mg of PET had a relatively short active release phase with only a minimal weight loss (< 0.1 mg d^{-1}) measured during the third week of aging at 25ºC. While, the proprietary membrane cup lure loaded with PAN TRE1381 was purported to have the same plastic membrane and to be loaded with the same volume of active compound (800 mg) as the PET lure (TRE1256); its mean weight loss was nearly 4-fold higher over the initial two-week period of aging (Table 1). TRE1381 also had a consistent weight loss over the extended 12-week assessment period, slope = -0.0218, $P = 0.27$.

The type of PET or AA release device used in delta traps had a significant effect on trap performance across a series of experiments. Traps with vials losing higher amounts of AA caught significantly more total moths than traps with the membrane cup lure TRE3321 (Expt1). Catch of female moths was significantly higher with the use of the 3.2-mm aperture vial than TRE3321 and the vial with the

smaller aperture caught an intermediate number of female moths. Total or female moth catch in Expt. 2 did not vary with the use of either the 1.5-mm aperture vial or the membrane cup TRE1256 lure loaded with PET when used with the 3.2-mm aperture vial with AA. However, moth catch was significantly reduced in Expt3 when traps were baited with the larger membrane cup lure TRE1278 loaded with PET compared with TRE1256 when used in combination with the 3.2-mm aperture AA vial. Septa lures loaded with either 5.0 or 10.0 mg PET caught similar number of total moths as traps with TRE1256 when the 3.2-mm aperture vial with AA was used in Expt4. All three lures outperformed the 1.0-mg septa lure in this experiment. Differences in female moth catches among lure treatments were more variable with the TRE1256 and 10.0-mg septa lure catching similar numbers and the 5.0-mg septa caught fewer females than the TRE1256-baited traps. No significant difference in total or female moth catch occurred in Expt5 between traps baited with TRE1256 and either the membrane cup lure TRE1468 or the vial with a 3.2-mm aperture loaded with AA. Similarly, no significant differences in total or female moth catch occurred in traps baited with TRE1468 loaded with AA and either the 10-mg septa or TRE1256 lures loaded with PET in Expt6. However, the lure combination of TRE1256 plus a vial with a 3.2-mm aperture caught significantly more total moths than traps with a 10-mg septa and TRE1468 in Expt7.The difference in female moth catch between these two lure combinations was not significant, $P = 0.08$.

Experiments 8 and 9 compared moth catches with membrane cup lures loaded with either PET or PAN. First, significant differences in both total and female moth catches were found between traps baited with AA plus either PET or PAN. Second, the addition of either PET or PAN significantly reduced the catch of male moths in Multipher traps baited with sex pheromone.

Results in Experiments 10 and 11 were similar for the dual captures of *C. pomonella* and *C. rosaceana* when different lures for each species were combined in traps. For example, the addition of vials with either PAN or PET to traps baited with a codlemone and pear ester loaded septum plus the AA 3.2-mm aperture vial did not affect moth catches of *C. pomonella*. Similarly, catch of *C. rosaceana* was not impacted with the two vial lures when the binary septa were added with either aromatic.

Monitoring under mating disruption

Similar and significant differences in catches of *C. rosaceana* were found among lures in orchard plots treated with MD dispensers in both study years (Table 1). A significant interaction of MD dispenser treatment × lure type was found in the ANOVA for total moth catches in both years. Moth catch was highest in sex pheromone-baited traps placed in untreated plots. Total moth catch in traps baited with PET and AA in both untreated and treated plots had the second highest total moth catch, and moth catch in either the sex pheromone-baited traps placed in MD-treated plots or catch in traps baited with codlemone, pear ester and the membrane cup lure TRE3321 in either the untreated or MD-treated plots were the lowest. Cumulative mean female moth catch were similar and significantly higher in both the untreated and MD-treated plots then in traps baited with the codlemone plus pear ester septa and the membrane cup TRE3321 AA lure. Few unmated *C. rosaceana* females were caught in traps baited with PET and AA in untreated and MD-treated plots in either year: 18 and 22% in 2015 and 5 and 10% in 2016, respectively. No fruit from any orchard in either year were found to have leafroller larval injury.

Table 1 Catches of oblique banded leafroller (OBLR), *C. rosaceana* in traps baited with either sex pheromone (OBLR PH), PET (PET) plus AA (AA) or the sex pheromone of *C. pomonella* and pear ester (CM) plus AA in season-long trials conducted in six and seven paired apple blocks treated with or without sex pheromone mating disruption (MD) for *C. rosaceana* in Washington State during 2015 and 2016, respectively

Year	Dispenser treatment	Mean (SE) total moth catch per trap			Mean (SE) female catch	
		OBLR PH	PET + AA	CM + AA	PET + AA[b]	CM + AA[c]
2015	None	51.9 (7.0)a	13.3 (2.2)b	2.0 (0.7)c	4.3 (0.9)A	0.6 (0.2)B
	OBLR MD	0.1 (0.1)c	11.2 (1.7)b	2.1 (1.0)c	3.0 (0.8)A	0.7 (0.3)B
2016	None	59.1 (19.7)a	18.3 (7.2)b	1.0 (0.7)c	5.3 (1.7)A	0.6 (0.6)B
	OBLR MD	1.0 (0.4)c	14.4 (4.1)b	0.4 (0.3)c	5.7 (1.5)A	0.1 (0.1)B

Mean total catch followed by a different lowercase letter and mean female catch followed by a different uppercase letter within each year were significantly different, $P < 0.05$, Tukey's test.

Volatile collections The emission rates of 24 volatiles were recorded from intact and herbivore-infested apple foliage (Table 2). Emissions of 17 of these volatiles were significantly increased due to herbivore damage. Three of the four GLVs measured were significantly increased with herbivore damage. (*Z*)-3-Hexenyl acetate was the most abundant volatile in this class of compounds. Six of the nine aromatics were significantly increased with herbivore feeding compared with intact leaves. PAN was the most abundant aromatic compound and its emission rate increased nearly 50-fold from intact foliage. The emission of four of the six terpenes measured were significantly higher with herbivore damage than the intact treatment. DMNT, (*E*,*E*)-α-farnesene and β-caryophyllene were the three most abundant terpenes. (*Z*)-jasmone was not detected in intact foliage but was measured at a relatively low level from foliage with herbivore damage.

Table 2. Estimated mean emission rate (pmol dm^{-2} h^{-1}) of volatile compounds detected in the headspace of apple foliage that were either or subjected to *P. pyrusana* larval feeding.

Compounds	Emission released from foliage sampled		Moth catch with AA
	Intact	LR-feeding	
Acetaldehyde	10.2 (3.2) b	96.1 (2.0) a	
Acetone	19.1 (5.0)	31.3 (5.0)	
Ethanol	29.5 (9.4)	81.1 (26.9)	
Acetic acid	632.5 (92.6)	660.5 (143.3)	
GLVs			
(*E*)-2-Hexenal	4.9 (2.0) b	139.2 (27.1) a	0
(*Z*)-3-Hexen-1-ol	29.8 (4.6) b	263.5 (35.1) a	0
(*Z*)-3-Hexenyl acetate	564.7 (94.6) b	1709.0 (447.9) a	0[a]
(*E*)-2-Hexenyl acetate	25.0 (3.6) b	144.0 (51.4) a	
Aromatics			
Benzaldehyde	6.6 (3.4) b	22.8 (2.4) a	0
Phenylacetaldehyde	3.7 (1.1)	10.6 (3.0)	0
Methyl salicylate	2.7 (0.5)	23.1 (5.3)	-
2-Phenylethyl acetate	0.3 (0.1) b	1.2 (0.4) a	0
Benzyl alcohol	6.9 (2.1)	10.1 (1.3)	0
2-Phenylethanol	5.8 (2.9) b	17.8 (1.8) a	+
Phenylacetonitrile	1.2 (0.3) b	60.1 (7.9) a	+
Indole	0.1 (0.1) b	4.2 (0.7) a	0
(*Z*)-3-Hexenyl benzoate	1.4 (0.5) b	6.3 (1.8) a	0
Terpenes			
Myrcene	1.8 (0.5)	3.3 (0.4)	0
(*E*)-β-Ocimene	1.4 (0.4) b	21.6 (9.2) a	0
DMNT	1.9 (1.0) b	41.7 (8.6) a	-[b]
Linalool	3.3 (1.3) b	21.5 (4.3) a	0
β-Caryophyllene	2.9 (1.1) b	36.3 (7.1) a	-

(*E*,*E*)-α-Farnesene	6.7 (2.5) b	39.5 (4.1) a	0
Ketones			
(*Z*)-Jasmone	0.0 (0.0) b	6.6 (2.2) a	0

'0' denotes no attraction, '-' denotes repellency, and '+' denotes attraction

[a] Increase in moth catches when used with PET+AA. [b] Decrease iwhen used with PET or PAN+AA

Mass trapping. Several studies assessed the use of different traps (Multipher, Unitrap, Conestoga, and Green Pitfall bucket traps and delta traps with removable sticky liners). Delta traps with sticky liners have a relative short operational longevity that is defined by the magnitude of their targeted catch plus the numbers of non-targets that foul the liner's surface plus the accumulation of dust that reduces the tackiness of the surface. Hard shell plastic bucket style traps are considered to be non-saturating. No-pest strips placed in these traps can last all season and initially knockdown and then kill moths that enter into traps. Thus this type of trap should not require any seasonal maintenance However, it is not been clear what proportion of moths can escape these traps prior to the initial knockdown effect. Interestingly, our studies found that bucket traps with propylene glycol were superior (> 4x increase in catch) to the use of either kaolin powders or the No-pest strip when used in several bucket trap designs. This effect was most clearly seen in a seasonal study we conducted in replicated (N = 4) 1-acre unsprayed blocks using the CM Combo plus AA lure set. In this study the Multipher bucket traps caught on average 3.6X more codling moths than delta traps with four liner changes (Multipher trap caught 5,188 males/4,801 females; versus the Delta trap catching 1,797 males/1,000 females) per acre. These data show that the catch of female moths was even more pronounced (nearly a 1:1 sex ratio and 4.8X more moths) with the use of the Multipher versus Delta traps, and this response may be due to an advantageous chemical interaction of the AA with the propylene glycol. Similarly, Multipher traps caught 6.1X more leafrollers than Delta traps (62 males/17 females versus 6 males/7 females). Multipher traps with propylene glycol baited with the PET+AA leafroller lures consistently outperformed similarly-baited Delta traps with sticky liners in several shorter lure-comparison trials. Multipher traps baited with the PET+AA leafroller lures caught similar numbers of moths using either an organic mineral oil or propylene glycol.

Attract and Kill. Studies were conducted with both an insecticide-impregnated cloth and PVC material provided by AlphaScents for both leafrollers and codling moth. A 10-sec exposure to either the cloth or PVC caused nearly 90 and 85% kill of codling moth and leafrollers, respectively. Field aging tests found that the cloth lost a significant level of toxicity to moths after two months in the field. The PVC material has not yet been tested in the field. Experiments (6 minutes of flight) in a flight tunnel with male moths found that the use of the cloth (proportion dead within 24 h) was as effective as the use of a delta trap (caught on liner) when baited with a sex pheromone lure. Studies were also conducted in a flight tunnel with codling moth using a piezo sprayer releasing pear ester and acetic acid and similar results were recorded with females. Studies have not yet been conducted with female leafrollers in the flight tunnel. Sublethal tests with codling moth and OBLR male and female moths exposed to various times to the treated cloth found that > 95% of moths which were still alive after 24 h did not subsequently mate (either sex) or lay viable eggs (females).

CONTINUING REPORT Year 1 of 2

Project Title: Prevalence, biology and management of bull's eye rot in apple

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Total Project Request: $50,000 **Year 1:** $25, 000 **Year 2: $25,000**

Other funding sources: Technical Assistance for Specialty Crop **(**TASC) $1000 per annum (2016-17) [funding dedicated only for information transfer from previous and future research on management of bull's eye rot].

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

Item	2016	2017
Salaries[1]	$18,000	$18,000
Benefits	$2,000	$2,000
Wages		
Benefits		
Equipment		
Supplies	$3000	$3000
Travel[2]	$1000	$1000
Miscellaneous		
Plot Fees	$1,000	$1,000
Total	$25,000	**$25,000**

Footnotes: [1]Funding is requested to support a Research Assistant; [2]Travel to field sites, packing sheds, etc.

Objectives:

1. Conduct a survey of packing houses and orchards to assess the prevalence of bull's eye rot and perennial canker in Washington State.
2. Prepare a collection of *Neofabraea* spp. isolates from different geographic regions in Washington State and assess fungicide sensitivity/resistance of the pathogen population.
3. Test and compare the efficacy of fungicide treatments and orchard management for the control of bull's eye rot in commercial orchards.
4. Develop methodology for control of bull's eye rot in storage and in orchards both post-harvest and pre-harvest.

Significant Findings:

1. Fungicide containing fluopyram and trifloxystrobin (Luna Sensation) as an active ingredient inhibited *Neofabraea* spore germination and mycelial growth *in situ* at a concentration as low as 1 ppm.
2. The EC_{50} value (effective concentration to reduce mycelial growth or spore germination by 50% in comparison to control) for Luna Sensation was less than 0.1 ppm.
3. *Neofabraea perennans* was detected in orchards located in north, central and southern districts of the Washington State apple production region.
4. Isolates of *N. perennans* were recovered that expressed *in vitro* resistance to postharvest fungicides containing pyrimethanil (Penbotec), solatenol (Aprovia) and thiabendazole (Mertect).
5. The findings were presented at the 2016 annual meeting of the American Phytopathological Society.

Methods:

Objective 1: Conduct a survey of packing houses and orchards to assess the prevalence of bull's eye rot and perennial canker in Washington State. It is important to define the geographic distribution and prevalence of bull's eye rot, as well as determine the dominant causal pathogen species present in the various tree fruit production areas of Washington State. In 2016 we identified and surveyed orchards that exhibited high incidence of perennial canker. The survey of packing houses will be initiated in January 2017. Isolates collected during a separate survey conducted from 2012- 2015 (Aguilar 2015) were included in this study and provided preliminary information on distribution of bull's eye rot and will be incorporated with data obtained in the current study. Certain isolates collected during the 2012-2015 survey were included in the fungicide sensitivity assay (objective 2). Fruit exhibiting symptoms of disease were collected from various packing houses in 2015 and information regarding the source orchard from which fruit were harvested, packing house location, and pre-harvest and/or postharvest fungicide treatments applied, were recorded. Fruit were inspected visually and symptoms were recorded photographically. Fruit were rinsed using 70% Ethanol, and tissue was aseptically excised from the margin of diseased and healthy tissue on symptomatic fruit. This tissue was transferred onto Potato Dextrose Agar (PDA) media and incubated at 70°F (20°C) in dark for 2-3 weeks. Morphology of fungi recovered from symptomatic fruit tissue when cultivated on PDA was recorded and identifications were determined based upon previously published taxonomic descriptions. DNA was isolated from putative *Neofabraea* spp. isolates and DNA extracts were used for species identification in PCR amplification reactions conduct using species-specific primer pairs.

Objective 2: Prepare a collection of Neofabraea spp. isolates from different geographic regions in Washington State and assess fungicide sensitivity/resistance of the pathogen population. Fifty four isolates collected and identified to species level during surveys conducted in 2012-2015 were used in

studies to examine relative fungicide resistance/sensitivity across *Neofabraea* species and populations. We will continue to conduct resistance/ sensitivity assays in 2017 using additional isolates collected in the current study. Initial fungicide chemistries used for study in this trial were selected based upon efficacy trials that were completed from 2012-2015 (Aguilar et al., 2015). Six fungicide chemistries were employed in the initial trial and additional chemistries will be added as needed. The initial fungicides screened include:

- Penbotec (Pyrimethanil)
- Mertect (Thiabendazole)
- Topsin (Thiophenate methyl)
- Luna Sensation (Fluopyram + Trifloxystrobin)
- Inspire Super (Difenoconazole+ Cyprodinil)
- Aprovia (Solatenol®/Benzovindiflupyr)

Sensitivity of the fungal isolates to the different chemistries was examined *in vitro* using agar-based assays that include media amended with a specific individual fungicide chemistry. For each test fungicide, the active chemistry was incorporated into media at five different (10-fold dilution series) concentrations, with each trial including a no fungicide control growth medium. At each fungicide concentration, each isolate was represented by three replicate test agar plates. Fungal growth and development traits that were measured in these studies include spore germination and mycelial growth across the agar surface, with comparison to fungal growth and sporulation recorded on media without fungicide amendment. Fungicide sensitivity was expressed in terms of minimum inhibition concentration (MIC; the minimum concentration that inhibits visible growth of the fungus) and EC_{50}, which is the concentration that reduces fungal growth by 50% compared with growth attained on media in the absence of the fungicide. These data will be useful in determining whether geographic or species differences exist among elements of the causal fungal community, and will also serve in selection of materials to be evaluated in subsequent field studies.

Objective 3: Test and compare the efficacy of fungicide treatments and orchard management for the control of bull's eye rot in commercial orchards. Preliminary *in vitro* assays indicated that the pre-harvest fungicide treatments fluopyram + trifloxystrobin and thiophenate methyl and postharvest fungicide treatments pyrimethanil and thiabendazole are effective in suppressing spore germination and mycelial growth of *Neofabraea* spp. Hence, fluopyram + trifloxystrobin (Luna Sensation) and thiophenate methyl (Topsin) as preharvest fungicide treatment and pyrimethanil (Penbotec) and thiabendazole (Mertect) for post-harvest fungicide treatment were examined for control of postharvest bull's eye rot in storage. One commercial orchard located at Orondo, WA possessing a high incidence of bull's eye rot and perennial canker in trees as identified during our 2016 orchard inspection, was selected for use in this study. The cultivars Golden Delicious, Red Delicious and Fuji were included in this evaluation.

In **pre-harvest trials**, each fungicide selected was represented by 4 replicates, and each replicate consisted of 5 trees in a completely randomized design, and the study included a no fungicide treatment control. All fruit were naturally infected in the orchard. Artificial inoculations of fruit were not included in this study as previous trials using such an approach have previously been reported (Aguilar et al. 2015). Our goal was to study efficacy under disease pressure likely to be encountered in a commercial orchard setting. Treatments were applied to fruit 2 weeks prior to harvest and fifty fruit per replicate were harvested on commercial harvest dates from this orchard. Golden Delicious fruit were harvested on September 6th, 2016, Red Delicious fruit harvested on September 14th 2016 and Fuji fruit were harvested on September 30th 2016. Fruit was stored in boxes lined with plastic liners and on fiber board trays in a cold room at regular atmosphere and 32° F (0° C) for up to 8

months post-harvest (mph). Fruit will be monitored monthly for bull's eye rot symptom development starting at 3 mph and continuing for the duration of the storage period. Incidence of bull's eye rot in control fruit versus fungicide treated fruit will be compared to assess fungicide efficacy.

In the study of **post-harvest fungicide** treatments, fruits were harvested from the same orchard and same block as the pre-harvest trial. A buffer zone of two rows was employed between trees that were treated with pre-harvest fungicide and those trees possessing fruit to be harvested for use in postharvest drench or no fungicide control treatments. There were 4 replicates per treatment and per cultivar of fruit. We harvested 10 fruit per tree, from a total of 5 trees per replicate. Fruit were harvested on the same dates as noted above and received a drench of the representative fungicide treatment. Fruit were stored in boxes in the same manner and under the same conditions as described above. Fruit will be inspected monthly for the incidence of bull's eye rot from 3 mph to 8 mph.

Orchard sanitation plays a key role in reducing the inoculum load of *Neofabraea* spp. during the fruit growing season. As bull's eye rot infection occurs in the field and the pathogen remains latent until the post-harvest period, it is important to ensure the use of effective orchard sanitation practices to limit potential disease development. From our initial experiments conducted in the Washington State University-Sunrise research orchard, we observed that canker development can be initiated through the year, however the rate of canker expansion on infected limbs (and thus subsequent inoculum production sites) is greatest during the spring and fall (Aguilar et al., 2014). Orchards identified in this current study exhibited high incidence of perennial canker. However, most of the cankers existed on the tree trunk and canker removal would likely result in a need for tree removal and subsequent replanting. Hence in this study canker removal was not included as a practice for the control of Bull's eye rot in storage.

Objective 4: Develop methodology for control of bull's eye rot in storage and in orchards both post-harvest and pre-harvest. This research is directed towards controlling bull's eye rot in commercial orchards. Information from fungicide trials and orchard management trials will be incorporated towards development of grower bulletin / handouts that will be circulated online or in print.

Results and Discussion:

Objective: 1 ***survey of packing houses and orchards to assess the prevalence of bull's eye rot and perennial canker in Washington State.*** Isolates that were collected from 2012-2015 and also some from 2005 were included in this study as a means to provide an initial assessment regarding the distribution of the pathogen across Washington State (Table1). Starting in January 2017, additional samples will be collected from packing houses in Washington State and isolates recovered from symptomatic fruit will be added to this survey.

Table 1: Initial distribution of *Neofabraea* spp. complex, with information of pre-harvest or post-harvest treatment, variety of apple, location of orchard and identity of species.

Year Collected	Location of Orchard	Variety	Treatment	Species identified
2015	Brewster	Granny Smith	_[a]	*N. perennans*
2015	Chelan	Gala (twig)	_	*N. perennans*
2015	Orondo	Golden Delicious	Scholar Max (MCP)[b]	*N. perennans*
2015	Wenatchee	Gala	Pyrimethanil	*N. perennans*
2015	East Wenatchee	Golden Delicious	Scholar Max (MCP)	*N. perennans*
2015	Wenatchee	Cripps Pink	Organic	*N. perennans*
2013	Manson	Golden Delicious	no PH treatment	*N. perennans*
2013	Manson	Red Delicious	no PH treatment	*N. perennans*
2012	Yakima	Granny Smith	_	*N. perennans*
2005	Wapato	Golden Delicious	_	*N. perennans*
2005	Mattawa	Fuji	DPA [c] drenched	*C. kienholzii*
2005	Tonasket	Red Delicious	Drenched	*C. kienholzii*

[a] '_' represents no information about treatment applied available; [b] PH meaning postharvest; [c] DPA diphenylamine

[b]MCP= 1-methylcyclopropene

Objective 2: Prepare a collection of Neofabraea spp. isolates from different geographic regions in Washington State and assess fungicide sensitivity/resistance of the pathogen population. Fifty four *Neofabraea* spp. isolates were screened *in vitro* for sensitivity to the selected fungicide chemistries noted above. Inhibition of fungal spore germination was recorded after 48 hours incubation on fungicide augmented culture media and inhibition of mycelial growth after three weeks.

Below are the significant observations noted with respect to spore germination and mycelial growth of fungal isolates cultivated on fungicide amended media. Sensitivity is expressed as EC_{50} and MIC and assessing spore germination after 48h in comparison to no fungicide augmented control agar plate.

- All 54 isolates tested were sensitive to the pre-harvest fungicide containing fluopyram+ trifloxystrobin (Luna Sensation) with EC_{50} values lower than 0.1 ppm among 47 isolates, EC_{50} ranged from less than 0.1 ppm to 1 ppm for all 54 isolates and MIC ranged from 0.1 ppm-100 ppm.
- EC_{50} values recorded in assays using pre-harvest fungicide containing Thiophenate methyl (Topsin) ranged from 1 ppm to greater than 1000 ppm (among 54 isolates tested), and MIC values greater than 1000 ppm were recorded for three isolates, indicating the presence of resistant strains in the *Neofabraea* collection.
- The majority of test isolates were sensitive to the post-harvest fungicide Pyrimethanil (Penbotec) with 38 isolates exhibiting an EC_{50} lower than 0.1 ppm. However, an EC_{50} greater

than 480 ppm was noted for one isolate. EC_{50} ranged from less than 0.1 ppm to 482 ppm. MIC value was under 1000 ppm (effective concentration) for all 54 isolates.

- EC_{50} values for the post-harvest fungicide containing thiabendazole (Mertect) ranged from 4775 ppm to less than 0.1 ppm. EC_{50} was less than 0.1 ppm for 22 isolates. MIC was higher than 1000 ppm for 1 isolate thus indicating existence of resistant strain.

Results from the mycelial growth assay exhibited similar trends as the spore germination assay. These assays are ongoing with the three week observation yet to be recorded for many of the strains; thus final EC_{50} and MIC values will be forthcoming.

Additional strains collected from surveys in 2015 and those collected during the 2017 survey will be included in upcoming studies. A total of 100 strains will be subjected to analysis in the course of these studies. A final EC_{50} and MIC calculation will be completed by the end of 2017. As EC_{50} values less than 0.1 ppm were observed in 2016, spore germination assays will employ a minimum fungicide concentration of less than 0.1ppm.

Objective 3: Test and compare the efficacy of fungicide treatments and orchard management for the control of bull's eye rot in commercial orchards. Fruit harvested during the 2016 growing season will be examined starting from 1st week of November 2016 to May 2017. Fungicide efficacy for the control of bull's eye rot will be analyzed at the end of May 2017. Year 2 of the fungicide efficacy trial will be conducted in the same orchard at Orondo in 2017 using the methods as described for the 2016 trial, and fruit inspection will be completed in 2018.

Objective 4: Develop methodology for control of bull's eye rot in storage and in orchards both post-harvest and pre-harvest. Initial results from the fungicide sensitivity assays (objective 2) were presented at the 2016 annual meeting of American Phytopathological Society.

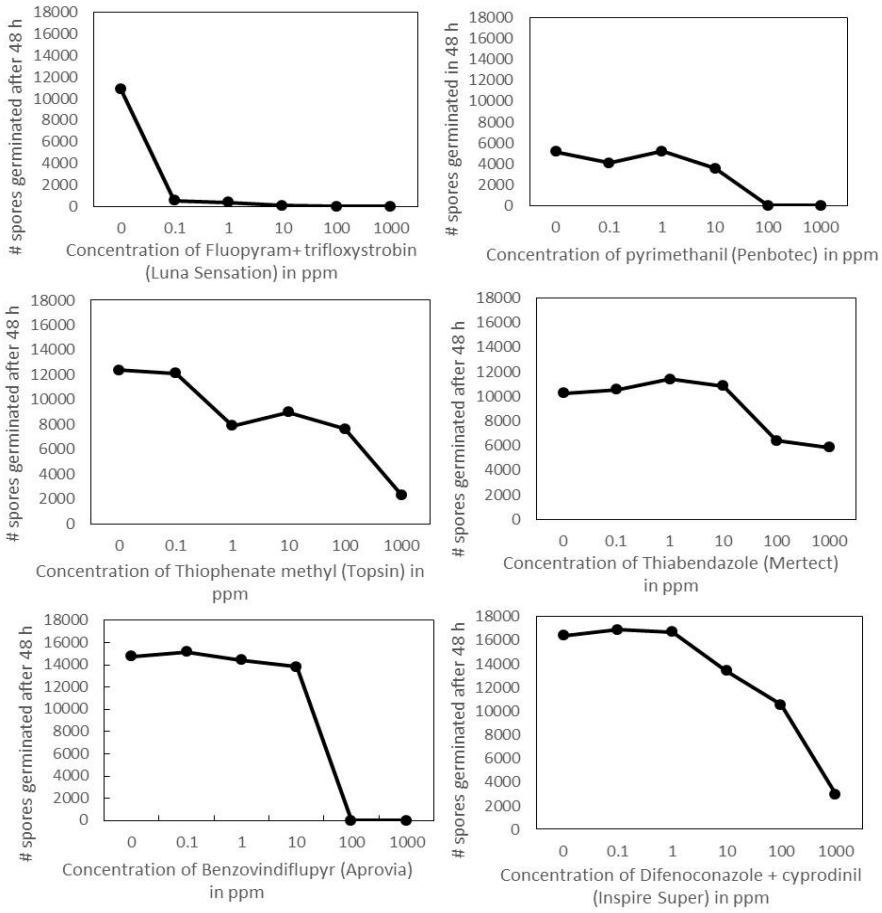

Fig 1: Sensitivity of *Neofabraea perennans* isolate #4471 to different fungicide chemistries as determined by *in vitro* assessment of spore germination on agar media amended with a specific individual fungicide chemistry. Fungicide concentration is expressed as parts per million (ppm).

Continuing work:

- Complete survey of incidence/severity of bull's eye rot and perennial canker across Washington State.
- Continue screening of isolates to determine EC50 and MIC of test fungicides.
- Continue evaluation of harvested fruit to determine efficacy of fungicide treatments for control of bull's eye rot in storage.
- Develop a working methodology for management of bull's eye rot in storage and publish the work in peer reviewed journals.

Citations:

Aguilar, C.G., Mazzola, M., and Xiao, C.L. 2014. Seasonality of canker induction and expansion by *Neofabraea perennans* and *Cryptosporiopsis kienholzii* in apple trees. Phytopathology 104:S3.4.

Aguilar, C.G., Xiao, C.L., and M. Mazzola. 2015. Management of bull's-eye rot of apple using pre- and postharvest fungicides. Phytopathology 105:S4.4.

CONTINUING PROJECT REPORT **YEAR**: 1 of 2
WTFRC Project Number: CP-16-104

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

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Cooperators: Gennaro Fazio, apple rootstock breeder, USDA ARS

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

Other funding sources *None*

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

Item	2016	2017
Salaries	38,790	38,790
Benefits	13,577	13,577
Wages		
Benefits		
Equipment		
Supplies	1,733	1,733
Travel		
Plot Fees		
Miscellaneous		
Total	55,000	**55,000**

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

OBJECTIVES

The potential commonality of the response between resistance to *P. ultimum* and other members of the ARD pathogen complex will be investigated. The consistency of the resistance phenotypes under greenhouse environment and field conditions will be examined.

1. Evaluate resistance response to multiple components within ARD pathogen complex. Based on the protocol established in phenotyping the resistance response to *P. ultimum* infection, characterization of resistance responses will expand to other key components within the ARD pathogen complex including *Rhizoctonia solani* and *Pratylenchus penetrans*.

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

- Wide spectrum of resistance levels was observed among individual apple rootstock germplasm lines from 'Ottawa 3 x Robusta 5' (O3R5) cross population. Most of the lines have been assayed at least once by *P. ultimum* infection. More than half of these lines have been evaluated in response to infection by *Rhizoctonia solani* and one third of them have assayed for their ability to repel/attract nematodes *Pratylenchus penetrans*.

- To the more advanced dataset for the resistance response to *P. ultimum* infection, the survival rate ranging from 95% to 8% were observed between most resistant and most susceptible lines, and root biomass reduction due to *P. ultimum* infection varied from 12% to 80%, respectively.

- Contrasting patterns of necrosis progression were observed between resistant and susceptible lines based on examination under dissecting microscope. Not surprisingly, rapid spreading of necrosis was often observed along the root of susceptible lines, and delayed and hindered spread of root necrosis is commonly associated with resistant lines.

- The preliminary data support the notion that there is substantial overlap between resistance mechanisms to *P. ultimum* and that to *R. solani*; but no significant correlation was found between resistance to *P. ultimum* and *P. penetrans* (nematode) so far.

- Field evaluation for selected lines at replant site in Columbia View suggests that in many cases susceptible lines benefit more from fumigation than resistant lines do.

- Current phenotyping dataset are being summarized for resistance QTL mapping analysis in collaborator's lab.

METHODS:

The systematic and quantified evaluation of resistance phenotypes were performed on O3 x R5 population, which consists of over 90 genotypes or lines using plants with uniform genetic background and comparable age through our in-house micro-propagation procedures. Single pathogen challenge experiments were evaluated using *P. ultimum*, *R. solani* or *P. penetrans*.

1. Tissue culture-based micro-propagation to prepare genetically uniform and age defined plants

The in-house micro-propagation procedure were used to supply the plants for selected genotypes of O3 x R5 population. Briefly, from proliferated shoots, individual shoot tip was transferred to Magenta™ boxes containing root elongation media for root generation for 4 weeks, with one plant per box.

2. In-soil acclamation for tissue culture generated plants

Plants with good root system were transferred into soil (Sunshine® potting mix) for root system further development or in-soil acclimation for 7 days at 25°C (77 °F) under 12 hr dark and 12 hr light conditions before infection assay.

3. Pathogen inoculum preparation

Preparation of *P. ultimum* inoculum

The *P. ultimum* isolate used in this study was originally isolated from the roots of 'Gala'/M26 apple trees at Moxee, WA, USA. Inoculum of *P. ultimum* were prepared by cultivating in potato carrot broth (20 g of carrots and 20 g of potatoes in 1 L of medium) with two drops of wheat germ oil added per liter of medium. The *P. ultimum* cultures were grown in the broth in Petri dishes at 22°C (71.6 °F) for 4-5 weeks. The resultant mat consisting of oospores and mycelium were ground in a blender for 30 s., and then the *P. ultimum* oospore/mycelia suspension in 0.5% methyl cellulose were adjusted to a final concentration of approximately 2000 colony forming unit/mL and used to inoculate plant roots systems into this inoculum solution for 5 seconds. Then plants were then transferred into pots containing pasteurized Sunshine® potting mix.

Soil infestation with *R. solani*

Inoculum of *R. solani* AG-5 strain 1007 were prepared by growing *R. solani* in 500-ml Erlenmeyer flasks containing 250 ml of oat bran and 80 ml of distilled water. The flasks were sterilized by autoclaving for 90 min on two consecutive days and inoculated with a culture of *R. solani* growing on 1/5-strength PDA. Flasks were incubated at 20°C (68 °F) for 14 days, and then inoculum were air-dried in a laminar flow hood. Ground oat grain inoculum of *R. solani* AG-5 were homogeneously mixed into soil at a rate of 0.25% (wt/wt), and plant were transferred to this *R. solani*-infested soils after 24 h of incubation at 20 to 23°C (73.4 °F) (Mazzola, 1997).

Amplification, inoculation and recovery of *P. penetrans*

A natural population of *P. penetrans* that is resident to an orchard soil located near Manson, WA (Mazzola et al., 2009) was used in this study. To amplify the density of *P. penetrans* in this soil, subsamples of 900 g of well mixed GC orchard soil were decanted into 1.2 L plastic pots, and soils will be planted with five 8-week-old 'Gala' apple seedlings. Seedlings were grown in environmental growth chambers using a day/night temperature regime of 24/18°C (75.2/64.4 °F) with a 12-hour

photoperiod over a five-week period. *P. penetrans* inoculum potential will be estimated by extraction from 0.5 g root and 50 g soil samples for each of 5 randomly selected pots per previously described methods (Mazzola, 1998). All seedlings were removed after the nematode amplification treatment and two pots were randomly assigned to each rootstock genotype. At completion of a twelve-week growth period, plants were harvested and roots were washed to remove adhering soil particles. Plant growth response including shoot length and root biomass will be measure and normalized to control plants. The number of lesion per root system will be determined. *Pratylenchus penetrans* were extracted and counted from 0.5 g root samples and 5 grams of soils.

4. Assays for plant responses and microscopic characterization of necrosis progression among rootstock germplasm

Systematic phenotypic analyses of resistance response were focused on the individuals of O3 x R5 population using *P. ultimum* initially. Then, based on the results of responses to *P. ultimum*, selected genotypes were placed in two extreme groups of "resistant" and "susceptible", and each group will consist of 10-15 genotypes. The data of overall survival rated, root length, shoot and root biomass, necrosis progression examined and documented. Plants were carefully excavated from soils to minimize mechanical damage at designated time points. Soils associated with root tissues were gently removed by rinsing under tap water. Roots for both mock inoculated control and *P. ultimum* inoculated plants were kept in water until microscopic examination was conducted within two hours. Images were obtained using a DP73 digital camera installed on the Olympus SXZ12 dissecting microscope, and the accompanied software suite of celSense (Olympus, Center Valley, PA).

5. Field evaluation of resistance phenotypes in greenhouse at replant site for selected apple germplasm.

The control rootstock germplasm included the susceptible cultivars M.26 and B.9 and the tolerant cultivars G.935 and G.41. Among the accessions from O3R5 population, ten genotypes designated as either resistant or susceptible based on greenhouse evaluation were tested under field conditions. Field tests were carried out at the CV orchard replant site. The genotype-specific survival rate under ARD pressure, tree height, tree stem diameter, and total biomass increase were assayed after three months.

RESULTS AND DISCUSSION

1. Wide range of resistance responses among O3R5 lines to *P. ultimum* infection

Using in-house plant micropropagation procedure and standardized infection protocol, a total of 60 out of 90 available O3R5 lines have been assayed for their resistance levels towards three ARD pathogens. The resistance phenotypes to *P. ultimum* infection are at a more advanced stage, compared to other two ARD pathogens, i.e. *Rhizoctonia solani* and *Pratylenchus penetrans*. The overall resistance levels were primarily evaluated by the survival rate of infected plants at 14 dpi (day post inoculation) for *P. ultimum* and 28 dpi for *R. solani*. Reduction of plant biomass and the features of root necrosis patterns by microscopic observation are the other parameters used to evaluate their resistance traits. Currently, about 30 lines among O3R5 apple rootstock cross population have been assayed at least twice for their responses to *P. ultimum* (**Table 1**). A wide range of resistance level was demonstrated by our standardized phenotyping protocol, as shown in **Figure 1** as examples. Specific to infection by *P. ultimum*, "resistance" phenotypes were generally assigned to O3R5 lines with 80% or higher plant survival rate, while "susceptibility" was assigned for those lines with 30% or lower plant survival rate. Those lines with repeated mediocre survival rates were not included in

Table 1. As shown in **Table 2**, there is a general trend that a larger values of biomass reduction were commonly observed for susceptible lines as compared to those from resistant lines.

In general, the consistent survival rates were observed for most of the O3R5 lines by *P. ultimum* infections, however, variations of survival rate and biomass reduction were observed for some O3R5 lines between repeated assays, or among the same response group (resistant or susceptible). Although the assays were conducted under "controlled" environments and effort was made to maintain the consistency of experimental condition, some yet to identified factors seem to influence the measured biomass values between infection events. Among these potential factors are minor variations at the age of tissue culture generated plants, slight fluctuation of temperature or the presence of other forms of abiotic stress.

Table 1. Survival rates from *P. ultimum* inoculation in resistant or susceptible categories

Susceptible lines			Resistant/tolerant lines		
Line #	Assayed times	survival rate	Line #	Assayed times	survival rate
75	3	15.7 ±13.6	**58**	4	89.75 ±14.6
115	5	24.3 ±13.5	**G935**	4	89.5 ±15.8
31	2	24.9 ±11.5	**173**	3	89.3 ±10.1
106	2	29.0 ±5.7	**134**	2	88.5 ±4.9
5257	2	31.9 ±21.4	**161**	4	87.5 ±14.4
132	3	32.0 ±1.0	**164**	3	85.3 ±4.0
122	2	36 ±15.6	**142**	3	82.3 ±17.0
55	1	26	**63**	2	75.5 ±13.4
80	1	10	**27**	2	75 ±34.4
M9	1	20	**135**	3	76 ±21.9

Survival rates were scored at 14 dpi. Control plants by mock inoculation from the same line remain healthy under the same growing condition.

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

Line #	% reduction of root biomass	% reduction of shoot biomass
115 (S)	**45** ±14.3	**28** ±11.2
132 (S)	**63** ±16.8	**40** ±8.0
103 (S)	**78** ± 8.6	**46** ± 9.1
134 (R)	**4.8** ±4.4	**29** ±6.5
173 (R)	**28** ±7.6	**32** ±4.7
63 (R)	**25** ±4.9	**35** ±3.9

The percentage biomass reduction is calculated by comparing the values from survived plants with those of control plants at 28 dpi. Numbers of survived plants from *P. ultimum* infection varied widely across germplasm lines. R: denotes resistant line; S: denotes susceptible line.

Figure 1. Distinctive resistance phenotypes among O3R5 lines in response to *P. ultimum* infection. Image was taken at 14 dpi. Top row is the image of control plants from mock inoculation. Bottom rows are plants which were inoculated with *P. ultimum*; left: a line showing susceptible phenotype; middle: a line showing medium level of resistance; right: a line showing resistance phenotypes.

2. Contrasting necrosis patterns between resistant and susceptible apple rootstock lines

Between resistant/tolerant and susceptible lines, contrasting patterns of root necrosis progression were routinely identified under dissecting microscope. For example, at 7 dpi a large portion of the root systems remained healthy for the resistant G.935, as indicated by the mostly white-color root tissues (**Fig 2A**). Also, a relatively well-defined zone (arrow) separating healthy (white color) from necrotic sections (brown color) can be easily observed on the roots of resistant G.935. On the other hand, the susceptible B.9 plants exhibited root system-wide necrosis with very few or no healthy root sections at 7 dpi (**Fig 2**B).

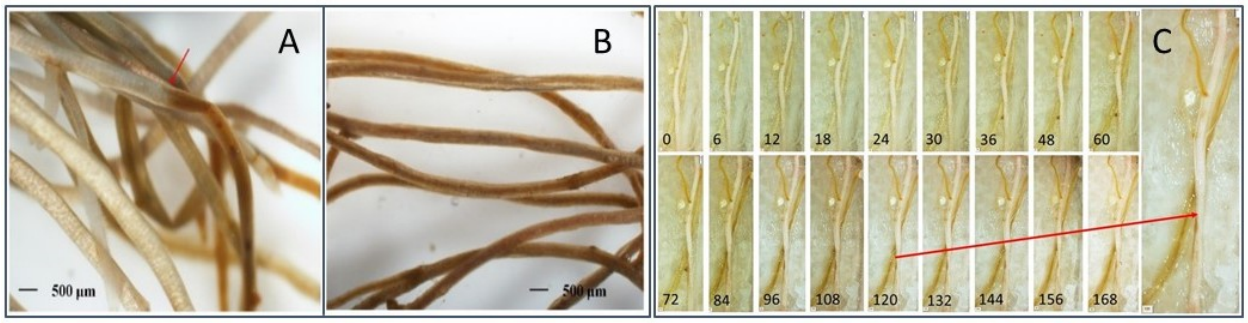

Figure 2. Patterns of root necrosis progression from *P. ultimum* infection. A, necrosis development at 7 dpi for G.935; The arrow denotes the clear zone separating healthy and necrotic tissues; **B,** necrotic tissues at 7 dpi for susceptible B.9, virtually all roots are necrotic as shown by the brown-colored roots. **C,** time-lapse images of root necrosis progression for #161 line in response to infection by *P. ultimum*; the number at the bottom of each image denotes at hour post inoculation (hpi) when the image was taken. An enlarged image of roots at 120 hpi was on the right side demonstrating the details of the "edge" or "line" separating healthy and necrotic tissues.

As shown in **Figure 2C**, the root of resistant #161 line demonstrated hindered or impeded necrosis progression, which resemble the observation on root of G.935. Although necrosis was detected on younger root or lateral root as early as 12 hpi, tissue necrosis initiated from this lateral root seems to be localized at the lateral root junction without spreading into primary root. While one of the primary root became necrotic as early as at 30 dpi, the lower section of another primary root (on the top in the image) only started to show sign of necrosis at 60 hpi; Furthermore, the necrotic lesion on this root

failed to expand to whole root from 60 to 168 hpi, in an extended period of over 100 hours. From the enlarged image at 120 hpi (far right side of the figure), a clearly defined "line" was easily identifiable at the lower section of root between healthy and necrotic tissues. This is distinct from what was observed on the susceptible root from B.9 and 115 (data not shown). In our opinion, the existence of such a "clearly defined line" demonstrated ability to prevent or delay the fast-growing *P. ultimum* from spreading the necrosis throughout the entire root system of the resistant #161 line. This individual root (within an infected root system) with the ability to impede the necrosis progression strongly suggest that a functional resistance mechanism, or active "chemical warfare", operates in the root of resistant #161 line. Such delayed necrosis progression could be a major factor contributing to the survival of this plant.

3. Field evaluation in both fumigated and non-fumigated rows

Field growth evaluation was carried out to examine the possible link between resistance response to *P. ultimum* observed in greenhouse assay and the overall field performance, as judged by biomass increase between growing in fumigated and non-fumigated rows. As shown in **Table 3**, almost all the tested *P. ultimum*-susceptible lines showed increased values of biomass in fumigated soil, but only half of *P. ultimum*-resistant lines show increased biomass values. The preliminary data seem to support the notion that susceptible lines benefit more from chemical fumigation as indicated by larger increased values of biomass. On the other hand, most of the resistant lines did not show the benefit from fumigation treatment, i.e. no increase of biomass by growing in fumigated row. In fact, many resistant lines showed suppressed growth in fumigated soil as indicated by the negative values of biomass changes. The reason behind this observation is unknown. The possible contributing factors include genotype-specific growth habit under field condition, nutrient utilization efficiency, or the slight age variations between the tested germplasm lines at the time of transplanting into soil. The conclusive answer regarding the variation of field performance of these tested lines will require a more carefully designed experiment, which is out of the scope of this study.

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

#line	Phenotype	total biomass (g); non-F	total biomass (g); F	% Net increase non-F vs F
55#	S	19.3 ±4.7	8.2 ±3.3	-135
77#	R	64.3 ±9.7	29.9 ±6.4	-115
114#	R	75.0 ±11.2	47.7 ±9.3	-57
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

Numbers are the average values for the measured total biomass from 5 plants if all survived; F: plants grown in fumigated row, non-F, plant grown in non-fumigated row. R (resistant) or S (susceptible) ratings were based on the result of *P. ultimum* infection in greenhouse assay. Those germplasm lines with undecided or mediocre resistance phenotypes were not included in this table. Fumigant was applied 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.

Summary

Apple rootstock germplasm with experimentally-defined resistance levels to ARD pathogens are pivotal for investigating the genetic controls over the resistance traits. Prior to this study, no protocol exists for evaluating the resistance phenotypes in apple root *per se*. Using micro-propagated plants, root resistance responses of over 60 apple rootstock germplasm lines from O3R5 cross population have been carefully evaluated for their resistance response to *P. ultimum* inoculation. Furthermore, based on their resistance phenotypes to *P. ultimum* infection, the potential consistency in response to inoculation by other ARD pathogens, such as *R. solani* or *P. penetrans*, are being investigated. The preliminary data support the notion that there is substantial overlap of resistance mechanisms to both *P. ultimum* and *R. solani*; but it appears that no correlation exists between resistance to *P. ultimum* and number of recovered nematodes from roots or soils among tested lines. The results from this study are the basis for subsequent genetic analysis for uncover the genetic controls over apple rootstock resistance to ARD.

CONTINUNING PROJECT REPORT **YEAR**: Ongoing

Project Title: Pesticide residues on apple

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Budget 1:
Organization Name: WTFRC **Contract Administrator:** Kathy Coffey
Telephone: (509) 665-8271 **Email address:** kathy@treefruitresearch.com

Year	**2016**	**2017**
Salaries	3500	3500
Benefits	1000	1000
Wages	1000	1000
Benefits	300	300
Equipment	200	200
Supplies	200	200
Travel	1000	1000
Analytical lab fees	5670	5670
TOTAL	$12,870	**$12,870**

Footnotes: Supply costs primarily covered by private industry cooperators
Travel includes costs for hauling spray equipment to trial site and delivery of samples to Portland

NOTE: Budget for informational purposes only; research is funded through WTFRC internal program