APPLE CROP PROTECTION RESEARCH REVIEW

29-Jan-15

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FINAL PROJECT REPORT

Project Title: *Amblydromella caudiglans*: A new predatory mite for Washington apples

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Other funding sources
Agency Name: Washington State Commission on Pesticide Registration
Amt. awarded: Awarded $13,690 (2013), Awarded $11,750 (2014)

Total Project Funding: **Year 1:** 23,419 **Year 2:** 23,275

Budget History:

Item	2013	2014
Salaries	14,198	14,766
Benefits	2,029	2,110
Wages	4,733	4,922
Benefits	459	477
Equipment	0	0
Supplies	0	0
Travel	0	0
Miscellaneous	2,000	1,000
Plot Fees	0	0
Total	23,419	23,275

OBJECTIVES

1. Conduct a survey of predatory mite (Phytoseiidae) species in Washington apple orchards to determine species prevalence and biodiversity.

2. Assess the effects of various factors, including climate, landscape, available prey species, and type of pesticide regime (conventional or organic) on the species composition of predatory mites found at each site using Geographic Information Systems (GIS) analysis. This will allow us to form a model which can be used to predict the species composition of individual orchards. The ability to inform growers which predator species they are likely to find in their orchard will allow them to adapt their management strategy to best suit the needs of that particular predator to maximize biological control of spider mites.

3. Compare the biology, pesticide tolerance, and predatory ability of *A. caudiglans* and *G. occidentalis*. This will provide growers with the information needed to adapt current integrated mite management (IMM) practices to their dominant predator.

SIGNIFICANT FINDINGS

1. Predatory mite survey:

- 22% of identified mites were *Amblydromella caudiglans,*
- *Amblydromella caudiglans* was the dominant species in 20% of samples,
- Predatory mite species found in the survey include: *Amblydromella caudiglans*, *Amblyseius andersoni, Euseius finlandicus, Galendromus flumenis, Galendromus occidentalis*, *Neoseiulus fallacis,* and *Typhlodromus pyri,*

2. Factors effecting predatory mite populations:

- *Galendromus occidentalis* populations were higher in apple blocks where bifenazate was used, in conventional blocks (vs. organic), and where carbaryl was used as a fruit thinner
- *Amblydromella caudiglans* populations were higher in apple blocks where bifenazate was not used and in blocks with weedy herbicide strips
- The main variety planted in the block also affected *A. caudiglans* populations; fewer *A. caudiglans* were found in 'Golden Delicious' blocks

3. Biology, pesticide tolerance, and predatory ability of *A. caudiglans* and *G. occidentalis:*

- *Galendromus occidentalis* spent significantly more time in the egg and larval stage than *A. caudiglans*, but *A. caudiglans* spent significantly more time in both nymphal stages, thus *G. occidentalis* has an overall shorter development time.
- Survivorship for both species was similarly high at all life stages, except for the egg stage; fewer *G. occidentalis* eggs hatched.
- The sex ratio for both species was similar (74% female for *G. occidentalis*, 78% female for *A. caudiglans*).
- *Amblydromella caudiglans* had higher than 25% corrected mortality when treated with bifenazate, azinphosmethyl, imidacloprid, carbaryl, and spinetoram, whereas *G. occidentalis* had >25% mortality only when treated with imidacloprid and spinetoram.

- All pesticides tested reduced *A. caudiglans* fecundity by more than 25% relative to the untreated control.
- *Amblydromella caudiglans* consumed more *Tetranychus urticae* eggs than *G. occidentalis*, but consumed a similar number of protonymphs.
- However, on a diet consisting solely of *T. urticae* eggs, *A. caudiglans* laid fewer eggs than *G. occidentalis*. The predators laid similar numbers of eggs on the protonymph diet. This indicates that *T. urticae* eggs may be a poor source of nutrition for *A. caudiglans*.

RESULTS & DISCUSSION

Survey. *Amblydromella caudiglans*, *Amblyseius andersoni*, *Euseius finlandicus*, *Galendromus flumenis*, *Galendromus occidentalis*, *Neoseiulus fallacis*, and *Typhlodromus pyri* have been identified from the locations surveyed (Fig. 1). The majority of identified individuals were *G. occidentalis* (Fig. 1), but *A. caudiglans* was also present in significant numbers. Although *G. occidentalis* was the dominant predator in the majority of orchards, *A. caudiglans* was dominant at 20% of the sites surveyed (Fig. 2). It was suggested by Downing and Moillet (1972) that a movement away from chemical control, especially organophosphates, could result in the replacement of *G. occidentalis* by *A. caudiglans*. *Galendromus occidentalis* has historically been considered to be highly resistant to pesticides compared to other phytoseiid species (Downing & Moillet 1972). Additionally, European red mite has replaced the McDaniel mite as the common outbreak pest mite species in Washington apple orchards (Beers and Hoyt 1993). While spider mites that spin copious webbing, like the McDaniel mite and twospotted spider mite, are the preferred prey of *G. occidentalis* (McMurtry and Croft 1997), *A. caudiglans* has difficulty moving through webbing and prefers spider mites like *P. ulmi* that produce little webbing (McMurtry and Croft 1997, Putman 1962).

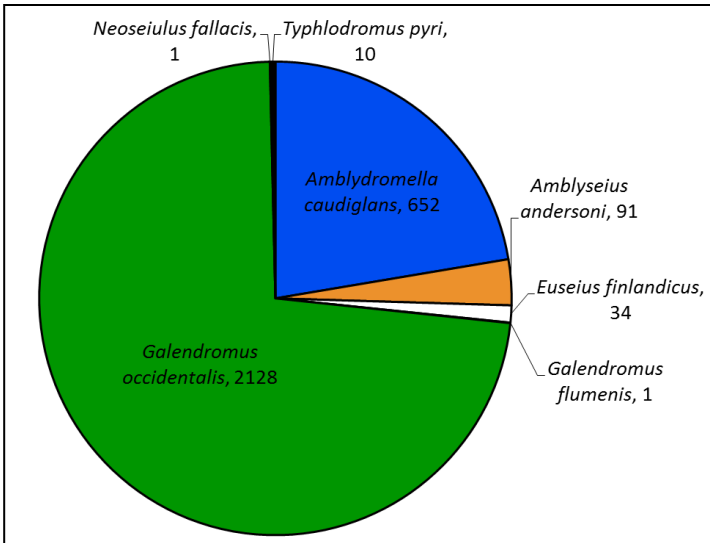

Figure 1. Number of female phytoseiids found in survey (total *n*=2917)

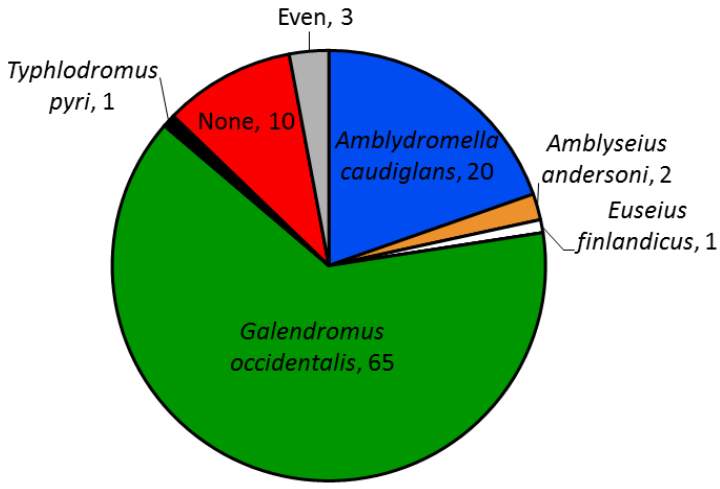

Figure 2. Number of sites where each species was dominant (n=102)

Therefore, the transition to a new predominant pest mite species may have promoted the increase in *A. caudiglans* populations.

Survey Analysis. Seventy-nine surveys regarding grower practices have been completed. The answers to these surveys, as well as the latitude, elevation, surrounding landscape, and prey species present at each site were modelled separately against the abundance of *G. occidentalis* and *A. caudiglans*. For each model, degree days were used as a covariate to account for effect of sampling date. The use of bifenazate positively affected *G. occidentalis* abundance, but negatively affected *A. caudiglans* abundance. *Galendromus occidentalis* populations were higher in conventional blocks than organic blocks and in blocks where carbaryl was used for fruit thinning. *Amblydromella caudiglans* populations were higher in blocks with weedier herbicide strips. The main variety of apple planted in the block was also found to effect *A. caudiglans* populations; fewer *A. caudiglans* were found in 'Golden Delicious' blocks than other varieties (Fig. 3). These findings indicate that *G. occidentalis* populations thrive in environments with higher levels of agricultural disturbance, whereas, *A. caudiglans* can increase in abundance where agricultural inputs are less intense. Organic or "soft" conventional programs should take into consideration that the dominant mite predator that they find in their orchards may be *A. caudiglans* instead of *G. occidentalis* and take measures to conserve this predator. This may include preserving habitat (weeds) in herbicide strips and avoiding the use of certain pesticides. Additionally, these findings support previous research that indicated that generalist predators (like *A. caudiglans*) are more effected by the surrounding environment (dominant apple variety and weediness) than specialist predators (*G. occidentalis*) (Camporese and Duso 1996; McMurtry and Croft 1997). The identification of pesticides that may affect phytoseiid populations (bifenazate, carbaryl) is also a useful tool for determining future bioassay targets.

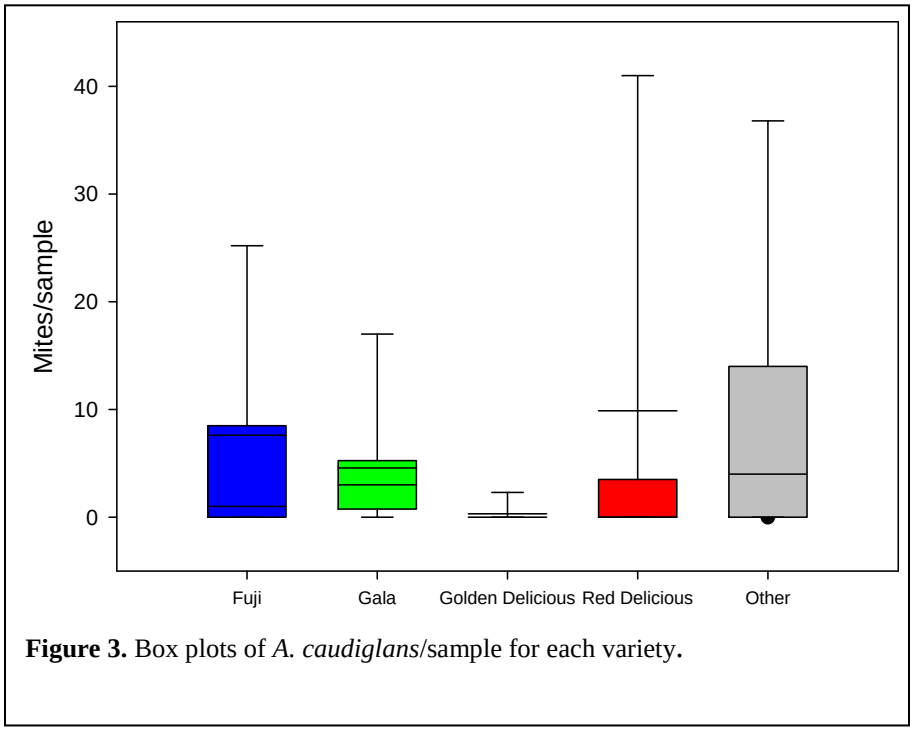

Figure 3. Box plots of *A. caudiglans*/sample for each variety.

Pesticide Bioassays. Eight pesticides were tested for nontarget effects on *A. caudiglans* and *G. occidentalis*. Due to previous difficulties establishing a colony of *A. caudiglans*, individuals of both species were collected from two experimental orchards where each species was previously found to be dominant. These field collected individuals were used in the assays. After the completion of an assay, individual mites were identified to species to ensure that the correct species was used. Corrected mortality (percent difference from check) for both species was calculated (Table 1). More pesticides were found to be toxic to *A. caudiglans* than *G. occidentalis*, including the two pesticides flagged by the survey data (bifenazate and carbaryl). Spinetoram and imidacloprid were toxic to both species, but azinphosmethyl, bifenazate, and carbaryl caused >25% correct mortality to only *A. caudiglans*. As previously mentioned, resistance to organophosphates like azinphosmethyl is well-known in *G. occidentalis* and its use may be a driving factor in the abundance of these two phytoseiid species. Some carbaryl resistance in *G. occidentalis* has also been reported (Babcock and Croft 1988), so its use may tip any competition in favor of *G. occidentalis* over *A. caudiglans*. The negative effects of bifenazate on *A. caudiglans* are surprising, as this pesticide is specifically marketed as safe for beneficial mites. However, *A. caudiglans* is not one of the phytoseiids specifically mentioned on the label (*Amblyseius fallacis, Phytoseiulus persimilis, Galendromus occidentalis, Typhlodromus pyri*). This indicates that this species may differ

	Abbott's Corrected Mortality (%)	
Pesticide	*A. caudiglans*	*G. occidentalis*
Bifenazate	43.29	0.00
Novaluron	18.48	-9.09
Azinphosmethyl	81.88	21.21
Chlorantraniliprole	-8.70	-0.36
Imidacloprid	83.70	76.28
Carbaryl	100.00	9.88
Spinetoram	95.06	90.08
Spirotetramat	13.04	1.30

Table 1. Abbott's corrected morality for each pesticide tested. Negative numbers indicate a lower mortality than the check. Box shading represents the relative strength of the effect, where dark gray is the most harmful (>75%), white is the least harmful (<25%), and light gray is intermediate (≥25 and ≤75).

	Eggs/female		Live larvae	
	A. caudiglans	*G. occidentalis*	*A. caudiglans*	*G. occidentalis*
Bifenazate	47		60	
Novaluron	64	39	81	94
Azinphosmethyl	77	49	91	46
Chlorantraniliprole	56	13	46	24
Imidacloprid	70	68	87	83
Carbaryl		52	100	97
Spinetoram	100	100	89	100
Spirotetramat	77	87	65	100

Table 2. Percent reduction from check in fecundity and live larvae for each pesticide tested. Values for *G. occidentalis* were taken from Beers and Schmidt 2014. Box shading represents the relative strength of the effect, where dark gray is the most harmful (>75%), white is the least harmful (<25%), and light gray is intermediate (≥25 and ≤75). Black boxes indicate data was not collected (no females survived treatment by carbaryl to lay eggs).

significantly in pesticide resistance and tolerance when compared to current model organisms.

The field collected *G. occidentalis* used in these pesticide trials had an unusually low rate of oviposition (even in the check), so these data are not reported. A recently published study (Beers and Schmidt 2014) can be consulted for the effects of the pesticides tested here (except for bifenazate) on a Washington population of *G. occidentalis*. All pesticides reduced *A. caudiglans* fecundity by >25% and resulted in reduced live larvae in the second generation (Table 2). Compared to data for *G. occidentalis* (Beers and Schmidt 2014, shown in Table 2), azinphosmethyl and chlorantraniliprole seem to negatively affect *A. caudiglans* more than *G. occidentalis*, whereas spirotetramat reduced live larvae less in *A. caudiglans* than *G. occidentalis* (although live larvae were substantially reduced in both). Because these sublethal effects assays were conducted in separate experiments, comparisons between species may not be valid. However, the negative effects of bifenazate and chlorantraniliprole on *A. caudiglans* (vs. *G. occidentalis*) were also seen in the mortality data.

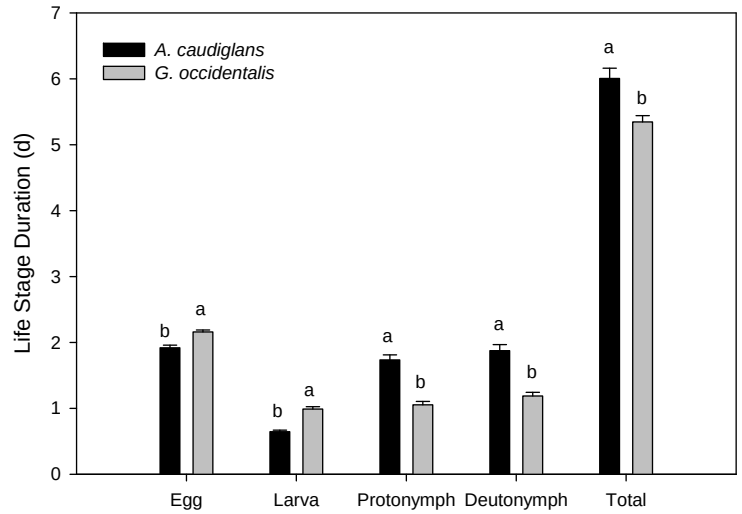

Figure 4. Life stage duration of predatory mite species.

Life table studies. *Galendromus occidentalis* has a shorter egg to adult development time than *A. caudiglans* at 75-89° F and 16 h day length (Fig. 4). Although *A. caudiglans* has shorter egg and larval stages, *G. occidentalis* has shorter nymphal stages. Unlike *G. occidentalis, A. caudiglans* larvae do not feed, thus rapid progression more quickly through its early life stages is advantageous. One implication of this life history trait is that *G. occidentalis* is capable of providing biological control earlier in its life cycle than *A. caudiglans.* This makes it less detrimental that it spends a greater amount of time as a larva. Both species develop quickly, allowing for their success as predators of rapidly growing pest populations. Both predators also had similar proportions of individuals survive through each life stage, except as eggs (Fig. 5). At the specified conditions, *G. occidentalis* has lower percentage egg hatch. This could indicate that *A. caudiglans* eggs are more likely to remain viable in the warmer climates found in central Washington. Therefore, like *G.*

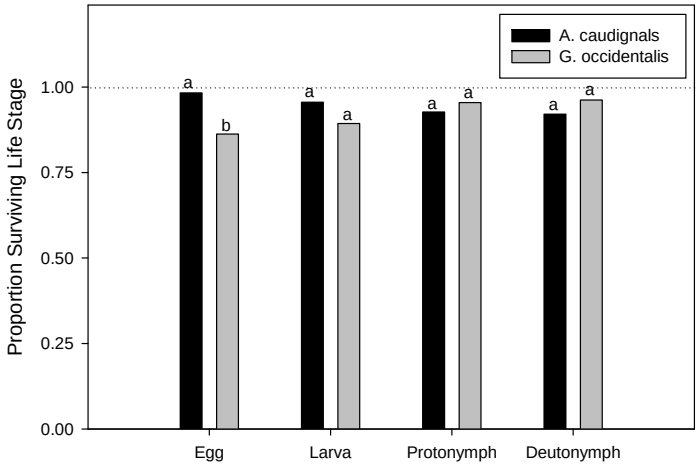

Figure 5. Survivorship at each life stage for predatory mites.

occidentalis, this predator matures quickly, increasing its ability to control pest mite populations. Both species have similar sex ratios (74% female for *A. caudiglans*, 78% female for *G. occidentalis*).

Prey Consumption. Predation of *T. urticae* eggs was much higher for *A. caudiglans* than *G. occidentalis* (Fig. 6a). However, this diet resulted in decreased oviposition in *A. caudiglans* compared to *G. occidentalis* and to *A. caudiglans* fed on a diet of *T. urticae* protonymphs (Fig. 6b). This supports previous findings that indicated that *A. caudiglans* may not derive adequate nutrition from *T. urticae* eggs, resulting in compensatory feeding (Putman 1962). Both species consumed a similar number of protonymphs and oviposited at similar rates on this diet (Fig. 6). Unfortunately, prey consumption studies comparing both species feeding on European red mite could not be performed, as we were unable to establish this species in a laboratory colony. However, these data indicate that *A. caudiglans* does not perform as well as *G. occidentalis* on one stage of the former breakout spider mite pest (*T. urticae*). Reduced fecundity on this prey may be another contributing factor to previous findings that *G. occidentalis* is the sole predator in Washington apple orchards.

This study highlights potential factors that may have limited *A. caudiglans* populations in the past. This species is more sensitive to agricultural disturbances, including pesticides, than *G. occidentalis*. Additionally, it is also less capable of reproducing on one stage (eggs) of *T. urticae*, which may have limited its ability to reach high abundances when *Tetranychus* spp. were more common pests of orchards than they are at present. However, the discovery that it is presently the dominant phytoseiid species in many apple orchards warrants its conservation in these systems. It is more likely to be found in organic or "soft" conventional programs where key pesticides (like carbaryl and bifenazate) are avoided. Additionally, we are likely to see more of this predator in the coming years as organophosphate use is phased out.

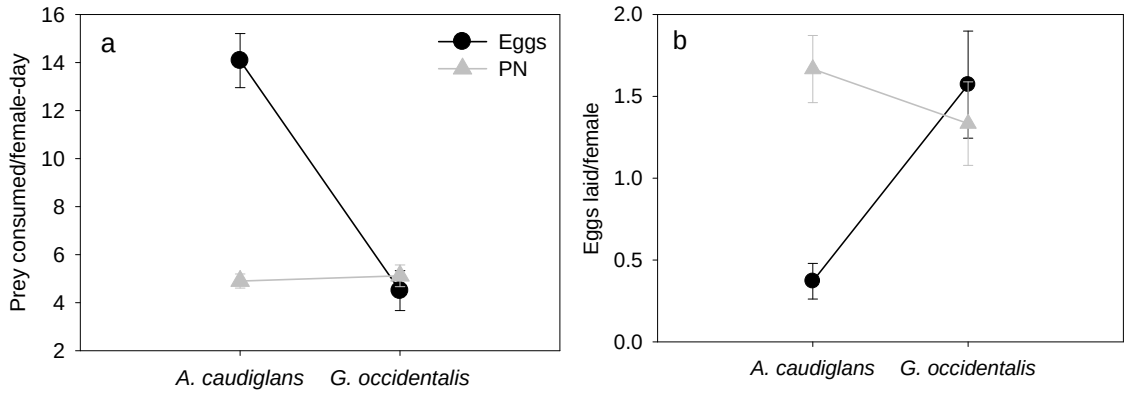

Figure 6. a) Average number of prey consumed by females per day in a two day evaluation period, b) average number of eggs laid per female on each diet four days after starting diet.

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Babcock J.M. and Tanigoshi L.K. 1988. Resistance levels of *Typhlodromus occidentalis* (Acari: Phytoseiidae) from Washington apple orchards to ten pesticides. Experimental and Applied Acarology 4: 151-157.

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EXECUTIVE SUMMARY

This two year project focused on increasing current knowledge of predatory mite populations in Washington apple orchards. The integrated mite management program in Washington is based on the assumption that *Galendromus occidentalis* is the only predatory mite in orchards, but this idea has not been tested. To improve our understanding of predatory mite diversity, three objectives were addressed: 1) a survey of predatory mites to determine the available diversity within orchards, 2) analysis of the effects of various factors, including location, landscape, pesticide use, and other agricultural inputs on predatory mite abundance, and 3) a comparison of the biology, prey consumption, and pesticide tolerance of the two most commonly found species of predatory mite in the survey.

Although it was found in high abundance (73% of collected individuals), *G. occidentalis* was not the only predatory mite found in apple orchards. *Amblydromella caudiglans, Amblyseius andersoni, Euseius finlandicus, Galendromus flumenis, Neoseiulus fallacis,* and *Typhlodromus pyri* were also present. *Amblydromella caudiglans* was the second most abundant species and was dominant at 20% of sites.

Galendromus occidentalis populations were higher at sites that used bifenazate and carbaryl, and in conventional (vs. organic) orchards. *Amblydromella caudiglans* populations were higher at locations where bifenazate was not used and in blocks with weedy herbicide strips. The counts of this species were also lower in 'Golden Delicious' blocks as compared to other varieties. These results indicate that increased agricultural inputs may favor *G. occidentalis* and that changing management practices may promote *A. caudiglans* populations.

Galendromus occidentalis was also more tolerant of most of the pesticides tested in bioassays than *A. caudiglans*. Bifenazate, azinphosmethyl, imidacloprid, carbaryl, and spinetoram caused >25% corrected mortality in *A. caudiglans*, whereas, only imidacloprid and spinetoram reached these levels of toxicity in *G. occidentalis*. Chlorantraniliprole, which did not have significant sublethal effects on *G. occidentalis* in previous studies, reduced fecundity in *A. caudiglans*. These results support previous findings that *G. occidentalis* is much more resistant to broad-spectrum insecticides (especially organophosphates) than *A. caudiglans*.

The duration of life stages for both species is fairly similar. The egg and larval stage were slightly longer for *G. occidentalis*, while the nymphal stages were longer for *A. caudiglans*. This resulted in *G. occidentalis* having a slightly shorter development time. The prey consumption of these two predators significantly differed. *Amblydromella caudiglans* consumed more *Tetranychus urticae* eggs than *G. occidentalis*. However, when fed exclusively on this life stage, *A. caudiglans* laid relatively few eggs. Both predators consumed similar numbers of *T. urticae* protonymphs and feeding on this prey did not depress the fecundity of either species. This indicates that *T. urticae* eggs are nutritionally poor host for *A. caudiglans*; although it eats more prey items, it is unable to adequately reproduce.

These findings indicate that there are two key factors that may have influenced historically unnoticed populations of *A. caudiglans* in apple orchards. Previous pesticide regimes, involving frequent sprays of broad-spectrum insecticides were not conducive to conserving this predator. Additionally, the fairly recent switch from a less suitable prey (*T. urticae*) to European red mite may also have allowed for *A. caudiglans* populations to flourish. As this predator is more likely to be dominant in organic or "soft" conventional programs, measures should be taken to conserve its populations for the purposes of biological control

FINAL PROJECT REPORT **Year** 3 of 3

Project Title: Models to assess pesticide impacts on CM, OBLR and *C. nigricornis*

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Other funding sources

We have submitted and received a new grant ($21,438, one year) to the Washington State Commission on Pesticide Registration to leverage some of the work being done on this grant. That grant is "Evaluating low dose insecticide residues on codling moth flight and behavior".

Total Project Funding: **Year 1:** $74,266 **Year 2:** $79,287 **Year 3:** $82,378

WTFRC Collaborative expenses:

Item	2012	2013	2014
Miscellaneous[1]	2300	2392	2488
Total	2300	2392	2488

Budget 1
Organization: WSU-TFREC **Contract Administrator**: Carrie Johnston/Joni Cartwright
Telephone: 509-335-4564/509-663-8181 x221 **Email:** carriej@wsu.edu / joni_cartwright@wsu.edu

Item	2012	2013	2014
Salaries	46,783	49,233	51,889
Benefits	18,429	19,317	19,494
Wages	3,200	4,500	4,680
Benefits	554	437	454
Equipment	0	0	0
Supplies	2,500	2,600	2,704
Travel	800	1,200	1,248
Miscellaneous (plot charges at WSU Sunrise: 2 acres@$1,000/acre)	2,000	2,000	0
Total	74,266	79,287	80,468

Objectives:
1. Develop life history information needed for the *Chrysopa nigricornis* model.
2. Develop mortality versus residue age curves for the three species to six commonly used pesticides.
3. Develop demographic models that will estimate the pesticide effects on *C. nigricornis*, OBLR, and CM.

Significant Findings:
- Life history information was reported on last year and was used to develop the demographic models in Objective 3.
- OBLR bioassays from two seasons found larval mortality >80% with residues up to 60-days old for Altacor, Delegate and Warrior. Residue activity was shorter for Proclaim and Entrust with OBLR larval mortality <60% by day 20 and for Assail where mortality was below 30% by residue day 7. The data was used in developing the demographic models for this species.
- Lacewing residue mortality bioassays are completed for five of the six pesticides for leaves from 2012. Bioassays using leaves from 2013 are in progress for all six pesticides.
- Lacewing larval mortality was ≤20% for Altacor, Assail, Delegate, Entrust and Proclaim with residues that were four-days old. Twelve-day old residues for these five materials were virtually no different from the control. Warrior four-day old residues had >70% lacewing larval mortality but was <30% by residue day 25.
- Extra leaves from field applications were collected, sealed and frozen for use in residue mortality bioassays in 2012, 2013 and 2014. This has allowed us to continue testing residue effects beyond the field season.
- The models for *Chrysopa nigricornis*, OBLR, CM, and *Chrysoperla carnea* were completed.
- Simulations looking at the greatest period of susceptibility to pesticides were run for all four models.
- Optimal treatment programs for codling moth and OBLR were evaluated and compared to currently recommended programs.
- Lacewing populations were relatively unaffected by organic treatment options for either codling moth or OBLR, but tend to be susceptible to conventionally based programs even with the short residual activity found in our residual assays.

Results & Discussion:
Objective 1. *Note: This objective was completed last year, so this narrative is the same as last year's*. Newly emerged adult *C. nigricornis* were selected from the colony and used in experiments to gather life history information such as adult female longevity and oviposition rates. A single male and female lacewing were paired together in a large plastic deli container covered with mesh netting for aeration and containing a 2-ounce deli cup with 10% honey water. Each pair was fed weekly with Mediterranean flour moth eggs and three times per week with frozen wooly apple aphids collected from low input or non-sprayed orchards. Each pair was held in a growth chamber with 16L:8D photoperiod, at 77°F and 50-60% relative humidity. Containers were checked daily for egg clusters, which were counted and removed once recorded. A subset from the removed eggs were placed in a 4-ounce deli cup, given *E. kuehniella* eggs and placed in a growth chamber to evaluate progeny survival rates.

Results: Life history data was analyzed and used as the basis for the demographic models (Obj. 3). The data showed that this species (which is predaceous as an adult) is extremely long lived. They also have an extremely long oviposition period and high reproductive rate that results in the mortality corrected female egg production being 739 female eggs/female and 95% egg production does not occur until 79 days (2120 DD). Comparing these results to *Chrysoperla carnea* (that is not

predaceous as an adult), the adult longevity is much longer (nearly 2x as long) and 95% of the total egg production occurs nearly 1.5 times later. Even without the initial demographic model, it is clear that overlapping generations are the rule (even if we assume the mortality rate in the field is 2-3 x what happens in the lab), and that the phenology models are primarily telling us when the next generation's reproduction starts to occur. If adult lacewings do have the sort of mortality rates found in the lab (very unlikely), some of the overwintering generation of adults could last until late July or early August.

Objective 2. OBLR mortality was obtained for the aged residues of six pesticides over two seasons. In 2012, the late summer trials (August 4 application) were successful, while in 2013 residue mortality bioassays were completed for both an early season (May 31 application) and summer season (July 19 application). In general, Altacor, Delegate and Warrior had activity that resulted in >80% larval mortality at older residue ages compared to Entrust, Proclaim and Assail. The timing of the application (early vs. later season) also appeared to affect the longevity of the residue activity for most pesticides tested with summer applications lasting significantly longer. Additional bioassays were also conducted in 2014 to look at the effects of horticultural oils on OBLR 1st instar larval mortality. Both organic and conventional orchards use horticultural oils in their pest management programs and it was important to get a good estimate of mortality rates for the demographic models. For these assays, clean leaves were dipped into a 1% oil solution and set under a fume hood to dry. Five larvae were then confined to 2 cm leaf discs from treated leaves, similar to method used for the pesticide residue assays. Mortality was 64% higher on oil treated leaves compared to the control. This information and the residue mortality data have been incorporated into the demographic models (Obj. 3) for OBLR.

The first round of lacewing residue bioassays were finished for Altacor, Assail, Delegate, Entrust and Proclaim and almost complete for Warrior using frozen leaves from 2012. Results indicate that all of the pesticides, with the exception of Warrior, have low mortality (≤20%) on 1st instar lacewing larvae for residues between 4 and 12 days old (Table 1). The corrected mortality for Warrior with 4 day old residues was >70% and does not decline below 20% until residue day 47. Bioassays for Warrior residues between 7 and 25 days old are in progress and should be finished by the end of the year. We are also in the process of running the second round of bioassays for all six pesticides using previously frozen leaves from 2013 to confirm our results.

We focused much of our efforts during the spring and summer of 2014 on CM bioassays using apples with field-aged residues for the 6 pesticide treatments. We obtained unsprayed, non-infested apples for these experiments at the Columbia View research orchard where mating disruption was applied over two 1-acre blocks in late April. Pesticide treatments were applied in one block on June 6 (early season) and on July 18 (summer season) for the second block. The summer season block was also treated with several applications of codling moth virus in May and June to reduce infestation during the first generation flight. Apples were collected twice a week for the first two weeks after spray applications and then every week thereafter (40 day old residue early season; 55 day old residue summer season). For the assays, CM neonate larvae <24h old (five in early season; two in summer season) were confined to a single apple in a plastic deli cup. A total of 125 (early season) or 80 (summer) CM larvae were evaluated for each pesticide and residue sample day. Larvae were checked for mortality at 7 days after exposure to each residue sample.

CM mortality for a majority of the control treatments was highly variable and ranged between 40-80% over the season. We attempted to address this issue by running simultaneous second control tests during the early season where only two larvae were confined to a single apple and replicates included more apples. In comparison, this alternative appeared moderately better with 10% lower mortality rates on average, thus this methodology was used during the summer bioassays. Unfortunately, these

assays continued to yield control mortality that was inconsistent and above the acceptable 20% threshold to conduct a meaningful analysis. The data shows activity for all pesticide materials for residues 30 days and older, however, since we are unable to correct the data for natural mortality it is difficult to determine the actual treatment mortality and its relevance to pest management decisions. We are continuing to look into other natural mortality correction factors that could be applied to this data set.

Table 1. Corrected lacewing mortality (%) for six pesticides testing activity of field aged residues.

Residue	% Corrected mortality					
age (days)*	Altacor	Assail	Delegate	Entrust	Proclaim	Warrior
4	18.2	9.2	14.0	7.2	20.5	72.8
7	6.5	7.7	7.1	11.7	5.3	*In progress*
12	1.3	4.2	8.5	11.8	2.0	*In progress*
25	-	-	-	-	-	20.0
32	-	-	-	-	-	26.8
47	-	-	-	-	-	18.0

* Not all treatments were tested for all residue ages. When mortality rates were within control levels (< 15% mortality; Altacor, Assail, Delegate, Entrust, Proclaim) pesticide residue effects are low to none.

Objective 3. We completed four models this year (codling moth, oblique-banded leafroller, and the lacewings *Chrysopa nigricornis* and *Chrysoperla carnea*). These models are unique because they not only provide the phenology, they also allow estimation of pesticide effects when treatments are applied at different times, have different activities (e.g., ovicide, larvicide) on the target population, and last different amounts of time. In addition, the obliquebanded leafroller accounts for the fact that the number of instars is variable, allowing individuals in any generation to go through either five or six instars. The codling moth model was radically modified to allow better estimates of the effect of mating disruption, and uses seasonal environmental conditions to estimate how mating disruption effects vary over the season. All the models now use real weather data to drive longevity of residues and population effects; for this report we used data from WSU-TFREC.

Each of the models has a control population (no treatments), and allows the user to specify eight different treatment programs, each of which can have up to 18 different sprays applied. The treatment programs can be specified based on degree-days, and the residue length is specified on a calendar date basis. Each of the type of treatments (Table 2) is based on either literature data or data collected in our lab. Combination treatments are also possible (e.g., CM granulosis virus + oil, pesticide + oil, *Bt* + oil); if oil or an oil combination is specified, the oil effect only acts on those individuals present at the time of application; there is no residual effect. The user needs to specify which AWN station and which year is used to drive the model. The models also allow the user to specify a particular codling moth treatment program and evaluate the effect of that program not only on codling moth, but also leafrollers and both lacewing species (and for OBLR treatments assess effects on CM and the two lacewings).

We evaluated the models in several ways: (1) we applied a single simulated pesticide application every 90 DD whose residue lasted only 25 DD throughout the season to evaluate the timing of susceptibility of the target species; (2) we evaluated several different codling moth treatments (using mating disruption or not) and OBLR treatments using the normal and optimal recommended timings

Table 2. Sensitivity of codling moth, obliquebanded leafroller, and two species of lacewings to pesticides used in simulations. "Pesticide" is a combination of several different efficacious compounds and uses the average efficacy of those materials.

	Treatment						
Stage Affected	**CM virus**	***Bt***	**Pesticide**	**oil****	**virus +oil****	***Bt* + oil****	**Pesticide + oil****
CM egg	–	–	–	80%	80%	80%	80%
CM larva (neonate only)	75%/6d*	–	90%/14d	30%	82.5%	30%	93%
OBLR egg	–	–	–	56%	56%	56%	56%
OBLR larva	–	85%/7d	90%/20d	71%	71%	84%	93%
lacewing eggs	–	–	0%	18%			18%
lacewing larvae	–	–	67%/7d	0%			67%
lacewing adults	–	–	99%***	0%			99%

* % mortality/longevity of residue

**all oil treatments only affect the susceptible stages at the time of application, they have no residue combination treatments immediately revert back to the activity/residue of the other component or either *Bt* + oil on CM or virus + oil on OBLR, effects default to oil alone; for both lacewings, *Bt* and virus have no activity, so combinations default to oil alone

***caused 99% mortality in 108 DD; adults normally live ≈1440 DD

(Tables 3 & 4) and evaluated the non-target effects the other three species; (3) we used temperature data from 2014 (very warm year) and 2011 (cool year).

General Results:

1. Pesticide effects operate on a calendar date basis (primarily from UV light degradation or by being partially washed off by rainfall). There is also a plant growth issue over longer periods of time (e.g., new leaf production or growth in the fruit diameter); these effects are predictable on a degree-day basis but are not included in the model (the project to evaluate this was rejected last year by the technology committee).

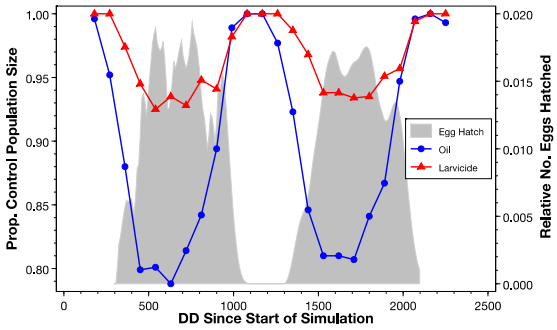
2. Pesticide effects on either pests or natural enemies are greater during warmer years, warmer times of the year, or at warmer locations. This is because for a given length of the residue (e.g. 7 days), in warmer situations more of the population will go through the sensitive stage (because more DD are accumulated) and thus more of the population is exposed to the pesticide in the susceptible stage (assuming the pesticide is put on at the correct time). The corollary of this is that during colder years normal spray programs (e.g., two codling moth sprays per generation) may leave the later part of the generation untreated. The greater suppression during the warm weather needs to be balanced with the longer season and more generations that might come through.

3. Mating disruption also is more effective when it is warm. This is because for a given number of days before mating occurs (e.g., 3 days), in warmer years the delay on degree-day basis is greater which reduces the reproductive rate compared to individuals not exposed to a delay in mating.

4. The best conventional treatment (with no MD) for CM for the first two generations is not as good as a relatively weak treatment only in the first generation when mating disruption is used.

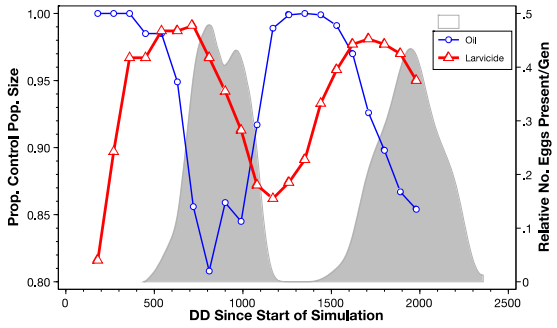
Times of greatest susceptibility to sprays:
CM – The effect of a single spray of oil and a larvicide effects are similar in respect to time of maximum effect with the larvicide effect offset slightly to account for egg hatch (Fig. 1). Oil acts as an ovicide and kills 80% of the eggs present at the time of application and 30% of the neonates present, and thus has a greater effect than the larvicides, which only affect the neonate larvae present at the time of application and 25 DD later. The larvicide effect would be larger if we had a longer residue, but for this simulation, we were investigating times of peak susceptibility and not which treatment was better.

Fig. 1. Seasonal effect of a single pesticide application with a 25 DD residual activity for codling moth.



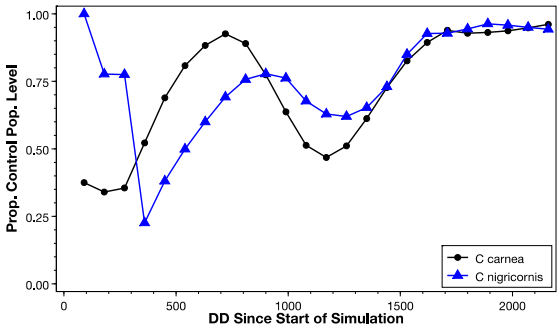
OBLR – The use of oil has only a minor effect until between 400–550 DD when the adults of the overwintering generation begin to lay eggs (Fig. 2). Similarly, the larvicide treatment has a strong effect on the overwintering larvae (after diapause is broken), which quickly is lost after ≈ 270 DDF (Fig. 2); then increases as the next generation of larvae starts to increase between 1080–1350 DD. The new overwintering larvae are never well controlled by sprays, since they feed only briefly and then enter diapause in the 1st through early 3rd instars. As with the codling moth, remember that these simulations have only a very short residual so that we can evaluate the timing of the sprays. Another thing to consider is that the larval effects are at the maximum level (i.e., there is no correction for larvae feeding within the rolled leaf). In addition, the long residual effect of the sprays after leaf growth decreases in the summer would increase the efficacy of the larvicide compared to the oil (ovicide) treatment.

Fig. 2. Seasonal effect of a single pesticide application with a 25 DD residual activity for obliquebanded leafroller.



Lacewings – Although both species of lacewings share the same responses to pesticides in each of the different stages (Table 2), that does not mean that they share the same sensitivity to application timing. *C. nigricornis* overwinters in a silken pupal case, and emerges later in the season than *C. carnea*, which overwinters as a diapausing adult. Thus, even before any models are made, it is apparent that *C. nigricornis* would not be greatly affected by the earliest spring sprays, whereas *C. carnea* would be more likely to be suppressed. Simulations showed the expected trend, with *C. carnea* heavily impacted by the initial spray (at 90 DD), whereas *C. nigricornis* was not affected at all until emergence started around 180 DD (Fig. 3). The sensitivity of the two species was offset and is related to the early season emergence times. The latter part of the season (after 1200 DD) should not be taken as showing that there is no effect with sprays at that time – the low effect there is caused by the fact that two generations of both species had

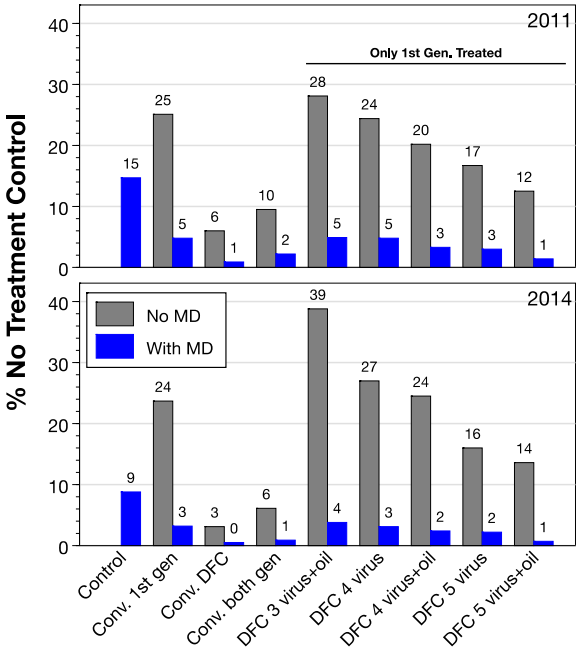
Fig. 3. Seasonal effect of a single pesticide application with a 25 DD residual activity for the lacewings *Chrysopa nigricornis* and *Chrysoperla carnea*.



already occurred, so the full effect sprays at that time would not be reflected until the next (overwintering) generation occurs.

Optimal Codling Moth Timing: Conventional treatments for codling moth generally call for two sprays in the first two generations. The first spray is put on about 250 DD after first emergence (175 DD) or at 425 DD. The second spray in the first generation is then put on 14-16 days later, depending on the residual effectiveness for the spray used. The second-generation sprays start at 1425 DD and the second is also put on 14-16 days later. Another treatment program is called the delayed first cover (DFC) program, where and ovicide (generally oil) can be applied at 375 DD – this kills most of the unhatched eggs, and allows the first cover spray to be delayed until 525 DD, the second cover is then again delayed 14-16 days depending on residual of the material used. The second generations are treated the same, with the first oil spray applied at 1375 DD and the first cover spray delayed until 1525 DD and the second applied at 14-16 days later.

Fig. 4. Comparison of different treatment programs under mating disruption or not in 2011 (cold year) and 2014 (warm year). Numbers represent the population size as a percentage of the no MD control.



Organic management must be done in conjunction with mating disruption because of the short residual of all the materials (other than Entrust) registered. We can use the same tactics of delayed first cover (an oil applied at 375 DD), then either virus + oil or virus alone for subsequent treatments. Table 3 has the timings investigated.

Results:

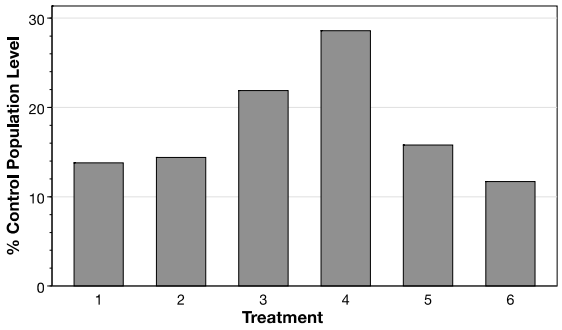
All the efficacy information is based on the percentage of the control (no treatments applied) population level. In the no-mating disruption treatments, the comparison between the conventional treatment (#3) and the delayed first cover (#2) treatment showed that the delayed first cover decreased the CM population level to 3.1% of the control compared to 6.1% in 2014 and in 2011 to 6.0% compared to 9.5% of the control (Fig. 4). The difference between the two programs is a result of the residues dissipating at the end of the generations earlier in the conventional treatment program.

Table 3. Pesticide timings for codling moth treatments.

1. 2 sprays in 1st gen, conventional timing, conventional pesticide	5. Same as #4, using 4 virus sprays
2. Delayed first cover (375 and 1375 DD) both generations treated with two conventional pesticides as described above.	6. Same as #5, but using virus + oil sprays
3. 2 sprays in first 2 generations, conventional timing and pesticides	7. Same as #4, but using 5 virus sprays
4. Delayed first cover oil (360 DD) then 3 virus sprays 6 days apart (1st gen only) starting at 525 DD	8. Same as #7, but 5 virus + oil sprays

The organic treatments (without mating disruption) generally performed equivalent to the conventional treatment restricted to the first generation (#1), with several performing better, particularly when five treatments were applied (Fig. 4). However, when mating disruption was used, all the treatments (except #4) performed better than the best no mating disruption treatment (#2); treatment 4 was similar to the delayed first cover conventional treatment (#2) and better than the traditional timing with just two conventional sprays per generation. Thus, even a very soft organic program (#4) is roughly equivalent to the best non-MD program.

Fig. 5. Comparison of different treatment program timings on OBLR population levels. Treatment numbers correspond to treatment numbers in Table 4. Treatment 4 is the current recommendation.



The simulations clearly show that MD makes any treatment program better than in its absence and even in situations with an out of control population, MD makes a conventional program more effective and cheaper than excessive treatments. The addition of oil to the virus treatments reduced the population about 1-2% compared to the no oil treatments when mating disruption was used. Mating disruption by itself resulted in population levels that were 8.8 (2014) vs 14.7% (2011) of the no MD control.

OBLR optimal timing

The timing of leafroller sprays has always been more vague than codling moth sprays, partially because fruit damage is a result of feeding on leaves that touch the fruit, rather than the insect requiring fruit resources to complete development. In addition, the phenology of OBLR is more complicated with some individuals completing five instars and some requiring six instars. The exact targets for control are not so narrow as with CM, essentially the eggs and larvae are targets, with some larvae protected by feeding within the feeding shelters and not being exposed to pesticides. The simulations we ran did not take this into account, thus the treatment efficacy is optimistic compared to what would be found in the field.

The current guidelines state control of the overwintering larvae should be done by 370 DD when less than 10% of the population has entered the pupal stage (which is unaffected by pesticides). If the population is high in the spring, the summer generation control is recommended to occur between 700-750 DD, and if populations remain high, treatments should occur between 1800-1880 DD so that overwintering larvae don't damage the fruit. It is important to realize that the programs listed are only needed when the population sampling indicates things are out of control, and can cease if populations are adequately reduced – the intensive programs listed are not always needed.
Simulations showed that the timings in the previous paragraph are suboptimal for control of OLBR (see Fig. 5, treatment 4), with the 370 DD figure treatment catching only a portion of the sixth instar and the latter half of the fifth instar. Treatment in the overwintering generation needs to be on by

Table 4. OBLR treatments corresponding to Figure 5.

1.	90 DD Pesticide, 720 DD oil, 900 DD 2 *Bt* + oil sprays 7 days apart, *Bt* spray 7 days later
2.	90 DD 2 *Bt* sprays, 900 DD 2 *Bt* + oil sprays 7 days apart, *Bt* spray 7 days later
3.	90 DD Pesticide, 900 DD pesticide + oil
4.	**370 DD Pesticide, 1350 DD pesticide + oil, 1800 DD Pesticide + oil**
5.	90 DD Pesticide, 900 DD 2 *Bt* + oil sprays 7 days apart, *Bt* spray 7 days later
6.	90 DD 2 *Bt* sprays, 900 DD 2 *Bt* + oil sprays 7 days apart, *Bt* spray 7 days later, 950 DD pesticide + oil

about 100 DD to affect the earlier instars and to prevent leafrollers that skip the sixth instar from getting to the pupal stage. The summer generation larval treatments between 700-750 DD occur too early for maximum efficacy, essentially wasting any residual effect of larvicides on only the first part of the first instar, although ovicides at this point in time are efficacious and all sprays applied between 900 –1145 DD should include oil to increase efficacy by killing eggs. We found the optimal timings were 90 DD (pesticide only or two *Bt* sprays 7 days apart), and either a pesticide + oil combination at 900 DD or two *Bt* + oil sprays 7 days apart followed by a *Bt* spray 7 days later. Simulations treating the new overwintering generation showed additional population suppression, which might be useful if populations are not under control later in the season. Simulations not in Figure 5 showed that treating the (spring) overwintering generation resulted in an additional reduction in the population size, compared to treatments that only target the second generation.

Fig. 6. Effect of CM treatments (Table 3) on OBLR, *C. carnea*, and *C. nigricornis* population levels.

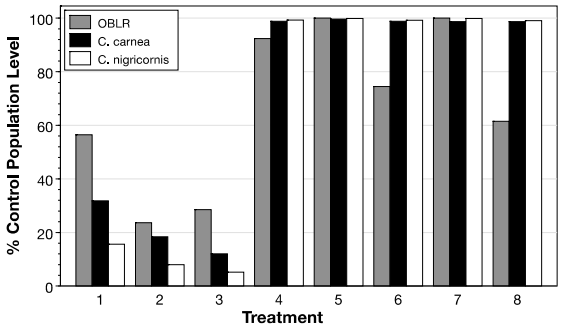
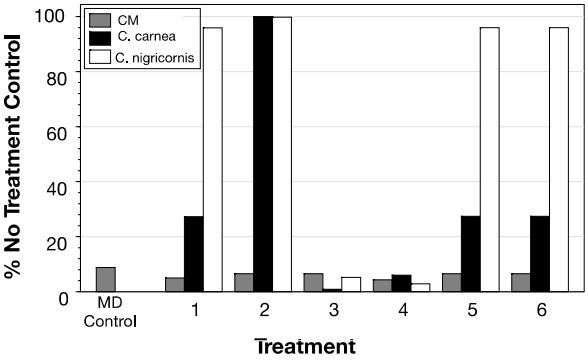


Fig. 7. Effect of OBLR treatments (Table 4) on CM, *C. carnea* and *C. nigricornis* population levels.



Non-target effects

The timings for codling moth can be tested against the two lacewings as well as OBLR. Conversely, the effects of the OBLR treatments can be run against the two lacewings and CM. The three conventional codling moth treatments (#'s 1-3) suppress both lacewing species to between 10 and 27% of the control population size for *C. carnea* and 9-17% for *C. nigricornis* (Fig. 6). In contrast, the organic treatments (#'s 4-8) had virtually no effect on either lacewing species. OBLR population suppression from codling moth sprays was minimal for most treatments although the delayed first cover caused a 72.2% reduction and the conventional two treatments per generation caused a 68.5% reduction. The organic treatments with oil included did reduce the populations, but generally by only 25-35%.

OBLR sprays caused little additional suppression of CM if mating disruption was not used, except for the current recommendations (e.g., treatment 4 above), which reduced the population ≈80%. When mating disruption was used, the OBLR treatments generally caused less than 5% change from the mating disruption treatment with no pesticides applied (Fig. 7). The lacewings showed differences between the treatments with *C. nigricornis* surviving better than *C. carnea* in treatments 1, 5, and 6 and no differences in treatment 2. Survival was very low in treatments 3 and 5 which were combinations of pesticide + oil treatments. Not all the treatments using conventional pesticides were toxic; treatment 1, 5 and 6 had pesticide either very early or very late, so that *C. nigricornis* was not yet emerged (treatments 1 and 5) or most had already emerged by the time of the last application (treatment 6).

Executive Summary:

This project developed the basic life history information needed to model pesticide effects on the most abundant lacewing predator (*Chrysopa nigricornis*) and also synthesized the information for codling moth (CM), obliquebanded leafroller (OBLR), and the second most abundant lacewing predator in tree fruits in Washington (*Chrysoperla carnea*) and developed models for all four species. The residual assays provided us with good estimation of the longevity of residues for lacewings and OBLR. The work on CM residual assays was not useful because control mortalities were too high and inconsistent.

The models for the four insects are incredibly valuable as a way to evaluate different pesticide treatment programs on both pests and two key natural enemies. The CM simulations clearly showed that the new delayed first cover treatment program recommended the past 3-5 years is significantly better (≈1.5 fold better) than the old conventional treatment program using only two sprays per generation. The simulations also showed that even very soft organic programs applied only during the first generation when used in conjunction with mating disruption reduced the population levels as much or more than the best conventional treatment programs spanning multiple generations when mating disruption was not being used. Several of the organic treatment programs with mating disruption show that even high populations can be reduced quickly as long as external, uncontrolled populations are not migrating into the orchard. Any situation where CM populations are considered to be out of control would be controlled better using mating disruption along with a suitable spray program.

The OBLR simulations suggest that the current recommendations for control of OBLR populations are not optimal and need to be adjusted. Early season applications need to be moved earlier (to ≈100 DD), and larvicide treatments for the summer generation should start at ≈900 DD and protect the crop during the 900-1260 DD period. OBLR field-aged residue studies showed many of the materials lasted much longer than most people realize, although the growth of new foliage reduces the potential for control as the larvae often move to younger (and untreated) leaves.

The non-target effects of both OBLR and CM treatments could be broadly characterized as organic treatments (excluding Entrust, which would behave similarly to a conventional pesticide) having minor effects on the two lacewings. In general, OBLR treatments (both conventional and organic) made little difference for the CM populations, especially if mating disruption was used. However, in the two conventional codling moth treatments that were applied in both CM generations did reduce OBLR population levels approximately 80%. Two of the organic CM treatments that used heavy doses of oil reduced population levels about 25-30%.

FINAL PROJECT REPORT

Project Title: Olfactory proteins as targets for enhanced codling moth control
WTFRC Project Number: CP-12-101

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Other funding sources: *None*

Total Project Funding: $125,134

Budget History:

Item	2012	2013	2014
Salaries	$24,958	$25,714	$26,470
Benefits	$ 4,292	$ 4,333	$ 4,367
Wages			
Benefits			
Equipment			
Supplies	$10,000	$10,000	$10,000
Travel			
Plot Fees			
Miscellaneous	$ 5,000		
Total	$44,250	$40,047	$40,837

ORIGINAL PROJECT OBJECTIVES

1) Express and characterize proteins involved in codlemone detection. Proteins thought to be involved in the detection and regulation of pheromone (codlemone) signaling include pheromone binding proteins (PBP), sensory neuron membrane proteins (SNMP), pheromone receptors (PR) and odorant degrading enzymes (ODE). The purpose of this objective was to clone and produce material we could functionally analyze.

2) Determine which odorant binding proteins, nerve membrane receptors, and odorant degrading enzymes are involved in the codlemone signaling pathway using *in vitro* protein expression and binding assays. Using materials generated in objective 1, the goal of this second objective was to determine which proteins interact with codlemone.

3) Determine expression of olfactory protein gene transcripts and detect GOBP that are expressed in antennae using immunofluorescent detection methods. General odorant binding proteins (GOBP) and PBPs have been shown to bind pheromones in other moths. The goal of this objective was to generate antibodies that bind to GOBPs so that we would have a tool to detect these proteins in codling moth antennae. Once antibody detection was confirmed another goal of this objective was to determine if gene expression and protein production can be correlated.

4) Determine if codlemone signaling can be disrupted using various odorant degrading enzyme inhibitors and parapheromones in flight tunnel studies. Degradation of pheromones (codlemone) is thought to be important for maintaining the sensitivity of pheromone signaling and behavioral responses in male moths. The first goal of this objective was to use enzyme inhibitors to determine the class of ODE involved in degradation of codlemone. Parapheromones, molecules that are strong agonists or antagonists of pheromones (codlemone), have been developed for other moth species. The second goal of this objective was to determine if a parapheromone could be developed to disrupt codlemone activity in male moths.

SIGNIFICANT FINDINGS

Objective 1:
1) Identified five additional odorant binding proteins (OBP) including 2 PBPs (5 total) and 3 GOBPs (6 total), that have potential to serve as codlemone binding proteins.
2) Identified 2 additional SNMPs (4 total) that have the potential to function in codlemone signaling.
3) Identified 3 additional PRs (8 total).
4) Identified two additional ODEs (24 total).
5) Identified 25 chemosensory binding proteins (CSP). CSPs have been shown to bind pheromones in other moths.
6) Discovered a potentially new mechanism for regulating olfactory protein (odorant receptors including PRs, PBPs, GOBPs) production in antennae.

Objective 2:
1) Generated a new cell line to use in high-throughput assays for odorant receptor/ligand identification assays.

Objective 3:
1) Generated antibodies that can be used to detect GOBPs in antennae.
2) Quantitated PR gene expression using qPCR.

Objective 4:
1) Synthesized a parapheromone derivative of codlemone.
2) In a preliminary field trial determined that the parapheromone may be a codlemone antagonist.

RESULTS AND DISCUSSION

The overall goal of this project was to characterize olfactory proteins that interact with codlemone in an attempt to identify proteins that could be targeted for codling moth control. When we first started this project we had limited information with only a handful of proteins to evaluate. Through the WTFRC-funded codling moth transcriptome, we have substantially increased the number of proteins that need to be evaluated. We have now in total 54 transcripts encoding odorant receptors (8 belonging to the pheromone receptor sub-family), 48 transcripts encoding odorant binding proteins (5 pheromone binding proteins and 6 general odorant binding proteins), 25 chemosensory binding proteins, 24 odorant degrading enzymes and 4 sensory neuron membrane proteins. This result demonstrates the complexity of the codling moth olfactory system and is now providing more target proteins that need to be evaluated. Because the methods currently used for evaluation of each of these proteins are not set up to handle these numbers, new methods will be needed for thorough characterizations. To address this issue, I have initiated a collaboration with Dr. Sindhuja Sankaran, a professor in the Ag Engineering department at WSU, to develop a biosensor system that can be used as a high-throughput method to determine odorant ligands of the codling moth olfactory proteins that have been identified in this project. In addition, we are developing a gene knock out system (CRISPR/Cas9) that if successful, will provide us with a rapid method to evaluate olfactory protein function directly in the codling moth.

Figure 1. Diagram of messenger RNA structure. Messenger RNA (mRNA) has 5 structural features. The coding sequence is the portion of mRNA that is translated to produce the protein. The 5' and 3' ends of the mRNA are untranslated regions, but have features that protect mRNA from being degraded (Cap and Poly(A)). The untranslated regions are also involved in regulating localization and translation of the mRNA into protein.

An unforeseen result of this project, and perhaps the most exciting, was the discovery of a potential mechanism that insects use for the regulation of olfactory protein production. For years, researchers have been trying to use quantitative PCR (qPCR) as a method for determining which proteins are important in pheromone (and other odorant) detection. Using qPCR to examine olfactory protein production has had little or no success. We discovered that gene transcripts (messenger RNA; see Figure 1 for a diagram of mRNA structure) encoding olfactory proteins produced anomalous results. Because we are comprehensive in our analyses of PCR amplified gene transcripts, we found that multiple transcripts with identical coding regions but differ in the length of the 3' untranslated region of mRNA are produced for both odorant receptors and pheromone binding proteins. Why might this be important? Normally, when proteins are needed by cells, they generate an mRNA encoding that particular protein and the mRNA is translated into a protein right away for immediate use. It is now becoming clear, in mammals at least, that nerve cells (neurons) use a different mechanism for protein production. In this mechanism, mRNA is produced, transported to a region of the nerve cell where the protein is needed and then the mRNA is locally translated when the protein is needed. Because of this mechanism, quantification of mRNA does not correlate with protein production, explaining why qPCR is not a viable method for evaluating olfactory proteins. Therefore,

alternative methods will be needed in the future to evaluate protein production in response to pheromones. We are currently developing a method using 2-dimensional gel electrophoresis to evaluate proteins that are produced in response to codlemone exposure. Because this method will be using techniques and tools developed in Objective 3 of this project, I hope to provide you with further information in the future.

EXECUTIVE SUMMARY

The use of codlemone for mating disruption has had major impact in codling moth control programs. The major sensors that regulate codlemone detection and behavior are proteins that reside in the olfactory neurons located in codling moth antennae. With a greater understanding of how this detection system functions, new compounds or methods might become apparent for enhanced control of codling moth through disruption of olfactory proteins. Therefore, the main goal of this project was to identify and characterize proteins that participate in the detection and regulation of codlemone.

In previous WTFRC-funded projects, we used a PCR-based method to identify five putative pheromone receptors, and most recently, we mined a codling moth transcriptome which led to identification of three additional receptors. Through quantitative PCR analyses of transcripts expressed in male and female antennae, one transcript was determined to have male biased expression at extremely high levels. The transcript encoding this receptor was cloned and expressed in a mammalian cell line to determine if it is a codlemone receptor. In cell-based assays, addition of codlemone elicited a cellular response indicating it is a codlemone receptor. However, we do not know if it is the codlemone receptor used by codling moth males to locate female mates. We are working to develop a genome editing system where we can knock out our putative codlemone receptor and then use flight tunnel bioassays to determine receptor function in behavior response. In addition to the pheromone receptors, the codling moth transcriptome has been mined to identify 54 transcripts encoding odorant receptors, 48 transcripts encoding odorant binding proteins, 22 transcripts encoding odorant degrading enzymes, 25 transcripts encoding chemosensory binding proteins and 4 transcripts encoding sensory neuron membrane proteins. In the future, projects to determine the roles of these proteins in codling moth will be needed to gain a fuller understanding of olfaction mechanisms.

An interesting finding in this project was that a high proportion of transcripts encoding olfactory proteins contain modified 3' untranslated regions. Two mechanisms we found that cause these modified 3' untranslated regions are alternate polyadenylation and differential splicing. In both of these mechanisms, nucleotide sequence is deleted, perhaps modifying response elements present in the 3' untranslated regions that regulate transcript localization or have effects on translational control. In mammals, similar mechanisms are used in nerve cells to regulate mRNA localization and translation of nerve proteins. We will be exploring the significance of modified 3' untranslated regions in codling moth olfactory protein transcripts to determine the relevance of this observation in regulation of protein production in olfactory neurons.

The results produced from this project have generated several other ideas for future research. We produced a codlemone analog that in preliminary field trials appears to act as a codling moth deterrent. We will continue pursuing this line of research to determine if there are field applications for this compound in codling moth control efforts.

FINAL PROJECT REPORT

Project Title: Incorporating fire blight resistance into Washington apple cultivars

PI:	Jay Norelli	**Co-PI (2):**	Kate Evans
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City:	Kearneysville	**City:**	Wenatchee
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Cooperators: Cameron Nursery, LLC, Eltopia, WA donated 4,000 MM.111 EMLA rootstocks to project for tree propagation.

Other funding sources

Agency Name: USDA-ARS-National Plant Germplasm System
Amt. awarded: $10,000
Notes: *Title:* 'Genotyping By Sequencing (GBS) *Malus sieversii* accessions to identify and characterize new sources of resistance to *Erwinia amylovora'.* Funds were provided to identify genetic markers in the accessions of *M. sieversii* used in this WTFRC project.

Total Project Funding: **84,479**

Budget History:

Item	2012	2013	2014
Salaries		$11,990[1]	$13,760[1]
Benefits		$1,079	$1,216
Wages			
Benefits			
Equipment			
Supplies		$1,760	$1,800
Travel	$500	$2,650	$3,070
Plot Fees	$2,200	$2,200	$14,600[2]
Miscellaneous	$500		$27,154[3]
Total	$2,200	$19,679	$62,600

Footnotes: 1: 2 summer students to assist with fire blight inoculation, recording data and plant maintenance; **2:** plot fees higher in year 3 due to planting of orchard associated with obj. 2, **3:** propagation of trees for obj. 2.

OBJECTIVES

1. Identify the best *M. sieversii* (wild apple) accessions to be used as sources of fire blight resistance in the WSU apple breeding program.

2. Establish planting of RosBREED apple Crop Reference Set and Washington State Breeding Pedigree Set in Wenatchee, WA for future fire blight evaluation.

The goal of this project was to develop the genetic resources necessary to incorporate the selection of fire blight resistance into the Washington State University (WSU) apple breeding program.

The goal of Objective 1 was to identify the best sources of fire blight resistance within *Malus sieversii*, the wild large-fruited progenitor of domesticated apple, for use in scion breeding.

The goal of Objective 2 was to establish a planting of the RosBREED reference germplasm for future fire blight evaluation. This will allow us leverage advances made by the RosBREED project to enable marker-assisted breeding of fire blight resistance in existing seedlings and selections of the WSU apple breeding program.

SIGNIFICANT FINDINGS

- Field plantings of 194 wild apple accessions and 8 control cultivars were established at WSU's Columbia View Orchard and USDA-ARS, Kearneysville, WV in 2012 for the purpose of identifying the best wild apple accessions to be used as sources of fire blight resistance in the WSU apple breeding program.

- Based upon controlled challenge with the fire blight pathogen in 2013 and 2014 at both the Wenatchee, WA and Kearneysville, WV plantings, 12 wild *Malus sieversii* accessions were identified as highly resistant to fire blight and will serve as good sources for introducing strong fire blight resistance into the WSU apple breeding program.

- The RosBREED reference germplasm (approximately 600 cultivars) was budded onto MM.111 rootstock at Willow Drive Nursery and 3 replicate trees of each cultivar will be planted at WSU's Columbia View Orchard in spring 2015.

RESULTS & DISCUSSION

Objective 1: Identify the best *M. sieversii* accessions to be used as sources of fire blight resistance in the WSU apple breeding program.

The approach we used in evaluating resistance to fire blight in these wild apple (*Malus sieversii*) accessions was to use a severe inoculation procedure with a high dose of the fire blight pathogen (*Erwinia amylovora*) to ensure that a fire blight infection will be initiated, if possible, and then evaluate resistance based upon how far fire blight disease progresses in the infected shoot. An alternative approach would be to evaluate the trees based on their resistance to the incidence of infection, such as determining the number of blossom infection that occurred after flowers are sprayed with a relatively low dose of the fire blight pathogen. These types of evaluation methods are appropriate when evaluating chemical or biological control treatments, but they are not appropriate when selecting sources of resistance for breeding. Although resistance to disease progression, or severity of infection, is usually correlated with resistance to the incidence of infection, there is not a one to one association between these two types of resistance. We believe that growers could tolerate a relatively high level of fire blight blossom infection if those infections do not progress systemically through the tree. Because economic losses from fire blight are the result of the death of young trees and woody tissue, we believe rating cultivar resistance based upon progression of disease in shoot tissue is the most useful and appropriate method of accessing fire blight resistance.

As expected, after challenging vigorously growing shoot tips with the fire blight pathogen we observed diverse responses among the wild apple accessions ranging from highly susceptible to highly resistant. After challenge with the fire blight pathogen, typical fire blight infections developed on many of the wild apple accessions. In some cases the infections progressed through the current season's shoot growth into the previous season's wood or into 2 or 3year-old wood (highly susceptible response) and in a few cases into 4 year-old wood causing the death of trees. In other cases the infections progressed through much of the current season's shoot growth but did not penetrate into the previous season's growth (intermediate response). In several cases only minor evidence of disease was observed in the challenged leaves and infections did not progress from the leaf into the shoot (highly resistant response).

The amount of disease observed after challenge with a pathogen is the result of an interaction between the pathogen, the host plant and the environment. Fire blight severity is strongly influenced by environment and when evaluating plant material for its resistance it is important to separate the effect of the host's genetic resistance from the effect of environment. By evaluating the *M. sieversii* accessions in 2 very different environments, Wenatchee, WA and Kearneysville, WV, over 2 different years, we in-effect evaluated the material under 4 different environmental conditions. In many cases, an accession may have appears highly resistant in some of the tests, but appeared susceptible in others. This is the result of resistance that is strongly influenced by environment and not useful resistance to use in the breeding program. Although approximately 10-30% of the accessions may have appeared highly resistant in any individual test, only 6%, or 12 accessions, were consistently, highly resistant in multiple tests.

Table 1. The percent of the current season's shoot growth that developed fire blight symptoms following controlled challenge with the fire blight pathogen. Twelve *M. sieversii* accessions were consistently rated as highly resistant when evaluate in Wenatchee, WA (WA) or Kearneysville, WV (WV) in 2013 and 2014. "PI#" indicated *M. sieversii* accessions that are Plant Introductions into the permanent US National Plant Germplasm System. "GMAL#" indicated *M. sieversii* accessions that have not yet been assigned to the permanent collection. "N" is the number of shoots challenged in the test.

Genotype	WA 2013	N	WA2014	N	WV2013	N	WV2014	N
Robusta 5					0.00%	20	0.00%	27
GMAL4002.k			0.0%	4	0.00%	20	0.00%	40
PI657115			0.0%	4	0.00%	20		
PI657116			0.1%	4	0.00%	15	0.00%	15
GMAL3616.o			0.0%	4	0.00%	15	0.20%	31
GMAL4002.m					0.00%	20	0.30%	38
GMAL4211.d			0.0%	5	0.70%	20	0.00%	39
PI657054					0.00%	20	0.40%	40
PI657085					0.00%	19	0.50%	37
GMAL4211.a					0.00%	20	0.90%	28
GMAL3975.c			0.0%	4	0.30%	20	2.00%	38
GMAL3688.c	0.0%	5			0.00%	14	2.80%	27
GMAL3989.c			0.0%	4	0.60%	19	3.30%	38
Delicious					1.10%	18	3.80%	38
Golden Delicious			5.6%	17				
Gala							24.50%	31
Jonathan					74%	19		

Robusta 5 is a wild apple with small, bitter and astringent fruit that has been used as a source of fire blight resistance in the Geneva rootstock breeding program. The fire blight resistance of GMAL4002.k, PI657115, and PI657116 appeared equivalent to Robusata 5 in more than one test. GMAL3616.o, GMAL4002.m, GMAL4211.d, PI657054, PI657085 and GMAL4211.a had some slight fire blight development in one of the tests, but also appear comparable with Robusta 5 in resistance. GMAL3975.c, GMAL3688.c and GMAL3989.c did develop fire blight comparable with 'Delicious', which is a resistant cultivar, in the WV 2014 test. However, these accessions were also judged as useful sources of resistance since they performed well in other tests and cultivars with fire blight resistance comparable with Delicious would be desirable.

Not all accessions were evaluated in every test. The *M. sieversii* (wild apple) planting at WSU Columbia View Orchard sustained heavy deer damage in the spring and early summer of 2013. This resulted in a limited number of usable shoots for fire blight challenge in 2013 and only 32 of the 194 wild apple accession had a sufficient number of shoots for reliable fire blight evaluation. The orchard has since been protected by deer fencing and 2014 tests at Columbia View Orchard were more extensive. However, the number of replicate shoots evaluated at the Columbia View Orchard was lower.

Initially, the wild apple accessions were quantitatively ranked based upon the average distance the fire blight infection progressed in the shoot and then assigned into categories of highly resistant,

resistant, intermediate, susceptible or highly susceptible based upon comparison to known control cultivars in the trial. Those that responded similarly to Robusta 5 were rated as highly resistant; those similar to 'Empire' or 'Golden Delicious' were rating intermediate and those similar to 'Gala' or 'Jonathan', which was severely damaged by the fire blight challenge, were rated highly susceptible.

In addition to the average distance the fire blight infection progressed in the shoot, several other measures of resistance were evaluated and the ratings of the wild apple accessions were adjusted in a conservative manner. Other measures of resistance included the average age of the oldest tissue infected and the percent of the current season's shoot length infected. The analysis of these other measures sometimes led to the "conservative" adjustment of the resistance based on an accession's worst performance, not its best performance or average performance. For example, if an accession was rated as resistant based upon distance of disease progression but intermediate based upon age of wood infected, its rating would be adjusted down from resistant to intermediate; however if the cultivar ranked higher based upon the analysis of another measure of resistance, its rating would not increase from resistant to highly resistant. Similarly, if an accession was rated differently in the WA and WV trials, or in 2013 and 2014, the accession would be given the lower of the two ratings. Although this conservative adjustment of resistance rating may be considered somewhat "unscientific" or unfair, it should help to ensure that the accessions selected as sources of resistance for the breeding program are in fact resistant.

Other observations of fire blight development were also considered in adjusting the resistance rating of the wild apple accessions. Because we were looking at a genetically diverse collection of wild apples we looked for the unexpected. Atypical from observations in most domesticated apple cultivars, we observed several wild accessions that appeared quite resistant to the initiation of fire blight infection, but when an infection did occur it progressed rapidly into older wood. In the case of GMAL4028.h, only one of 35 shoots challenged with the fire blight pathogen in 2013 and 2014 developed into a shoot infection but that infection progressed through 2 year-old wood into the central leader, destroying the young tree (Fig. 1). Because only 1 of 34 challenged shoots were infected, the accession's average distance of progression and average age of wood infected was still quite low, however this is obviously not a useful type of resistance to incorporate into the breeding program. To eliminate this type of resistance from consideration, any accession with a single infection that progressed into 2 year-old wood was rated as highly susceptible and an accession with an infection that progressed into the previous season's growth was ranked as susceptible.

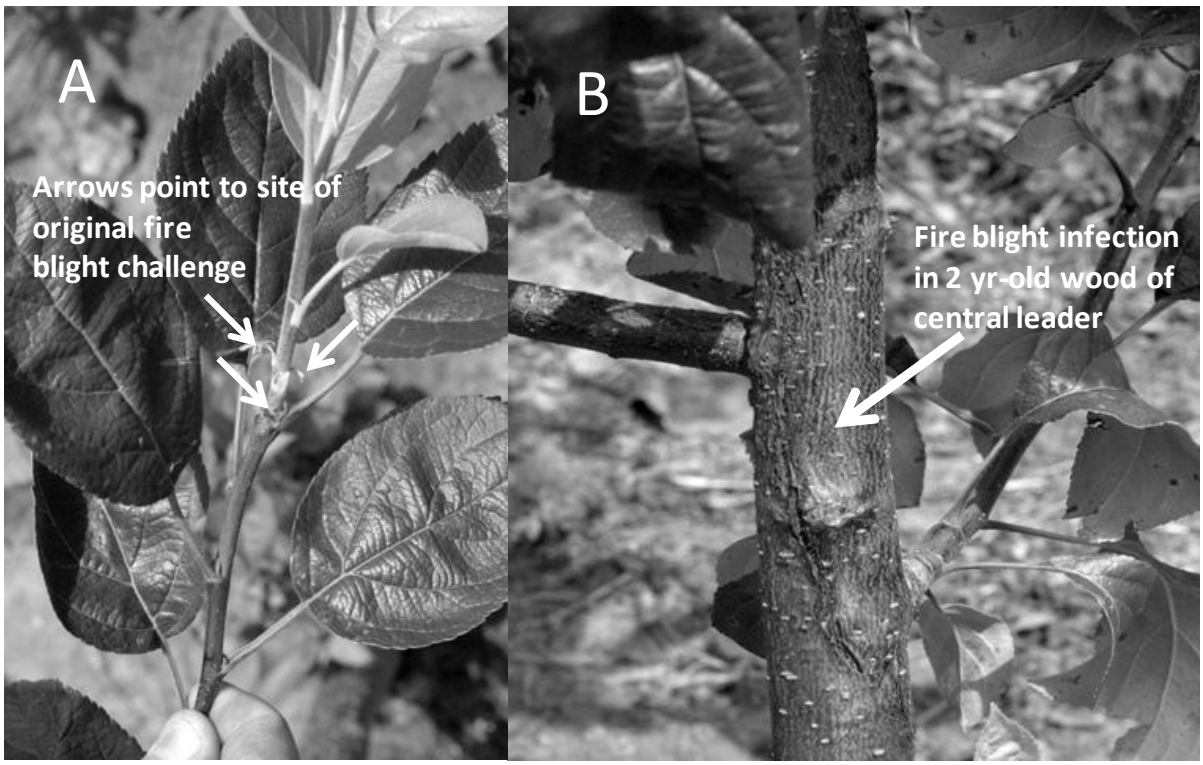

Figure 2: Evaluation of fire blight resistance of *M. sieversii* GMAL4028.h. **A**: In 34 of 35 fire blight shoots challenged in 2013 and 2014 no evidence of infection could be observed 6 weeks after inoculation. **B**: In 1 of 35 fire blight challenged shoots, fire blight progressed into 2 year-old wood of central leader.

In addition, naturally occurring blossom and shoot infections (infection that were not the result of our controlled fire blight challenge) were recorded and monitored, and resulted in downward adjustment to an accession's resistance rating if infection resulted in significant fire blight damage to the tree. In 2013, after adjustment 26 wild apple accessions were rated as highly resistant, 38 were rated resistant and 128 were rated intermediate, susceptible or highly susceptible (Fig. 2). Similar results were obtained in 2014.

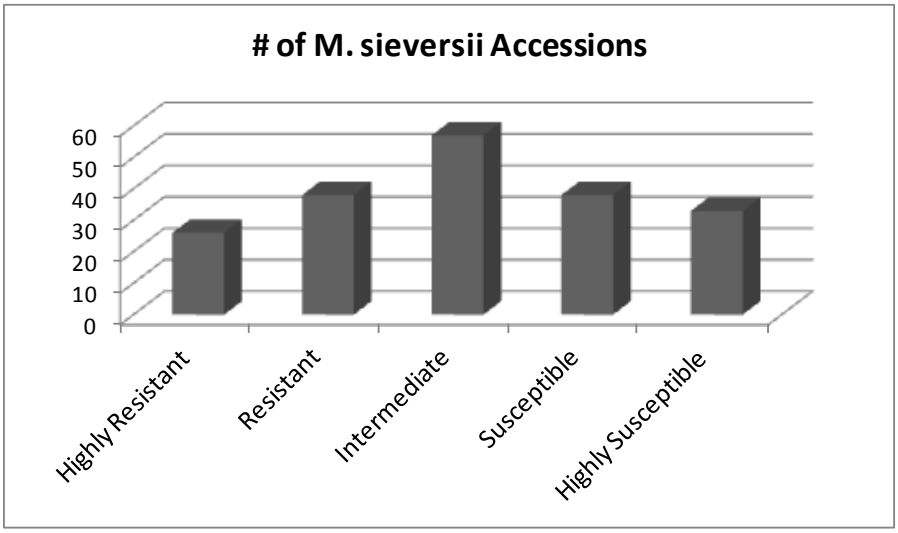

Figure 2. Number of wild apple accessions rated in different classes of resistance to fire blight.

This project has allowed us to identify excellent sources of fire blight resistance for use in the WSU apple breeding program. Several years ago the USDA-ARS Plant Genetic Resources Unit established a large collection of *M. sieversii*, the main progenitor of the domestic apple, collected from Central Asia (Kazakhstan). The 194 *M. sieversii* accessions used in the project were selected from over 1,000 *M. sieversii* seedling based upon their fire blight resistance and performance in an orchard grown at the USDA-ARS facility in Geneva, NY. The 12 accessions we have now identified as highly resistant to fire blight by replicated controlled pathogen challenge at multiple locations probably represent the best available sources of strong fire blight resistance available for apple scion breeding. Although many wild apples have been identified that are highly resistant to fire blight, most have extremely poor fruit quality. *M. sieversii* is the only wild species with large, edible fruit. Kate Evans thinks the most effective way to select the 1-3 accessions to start using in the WSU apple breeding program will be to evaluate the fruit of the 12 accesions in the standardized fruit quality evaluation protocols of the WSU apple breeding program. These trials are planned for 2015.

Objective 2: Establish planting of RosBREED apple Crop Reference Set and Washington State Breeding Pedigree Set for future fire blight evaluation**.**

The goal of this objective is to determine the fire blight resistance of the RosBREED reference germplasm so that we can utilize RosBREED resources to identify markers for fire blight resistance. Although we identify excellent sources of fire blight resistance in Objective 1 to be used in future crosses, it will not facilitate selection of fire blight resistance among the existing seedlings and selections of the WSU apple breeding program. Evaluating the RosBREED apple reference germplasm for its resistance/susceptibility to fire blight will allow us to leverage the significant financial investment of RosBREED in marker and software development to enable marker-assisted breeding of fire blight resistance in existing seedlings and selections in the WSU apple breeding program. Because fire blight disease can result in major structural damage of trees, and in some cases tree death of susceptible cultivars, existing plantings of the RosBREED apple Crop Reference Set and WSU Breeding Pedigree Set established to evaluate fruit quality traits cannot be used to evaluate fire blight resistance. In order to keep the cost of this project as low as possible, a single planting located at WSU Columbia View Orchard was established.

Trees for this planting have been propagated at Willow Drive Nursery in Ephrata, Washington. Budwood of the RosBREED apple Crop Reference Set and WSU Breeding Pedigree Set was collected at WSU-TFREC Wenatchee, WA or obtained from the other RosBREED core breeding programs at the University of Minnesota and Cornell University, or the USDA-ARS-Plant Genetic Resources Unit in Geneva and budded onto M.111 rootstock during the 2013 growing season. MM.111 rootstock was selected because of its tolerance to fire blight to prevent tree loss due to rootstock infection. Trees will be planted this spring within the current grant cycle at the WSU Columbia View orchard with protective deer fencing.

Because fire blight resistance will be determined on shoots, the tests for resistance can be conducted on young trees and the planting is expected to be of short term duration (3 to 4 years), allowing for planting at high density. Because fire blight challenge of the planting will be required for future evaluation of fire blight resistance, the planting will be situated on the Columbia View Orchard just north of Wenatchee.

Executive Summary

Project Title: **Incorporating fire blight resistance into Washington apple cultivars**

This project had two goals: 1) identify the best sources of fire blight resistance within *Malus sieversii*, the wild large-fruited progenitor of domesticated apple, for use in scion breeding; and 2) establish a planting of the RosBREED reference germplasm for future fire blight evaluation. Both of the goals were successfully completed.

Twelve *M. sieversii* accessions were determined to be highly resistant to fire blight shoot infection in multiple tests conducted in 2013 and 2014 at WSU's Columbia View Orchard in Wenatchee, WA and the USDA-ARS Appalachian Fruit Research Station Farm in Kearneysville, WV. A total of 194 *M. sieversii* accessions were evaluated in the trial that had been selected from over 1,000 seedling accessions collected in Kazakhstan. Many of the accessions appeared as resistant as *Malus x robusta* Robusta 5 which has been successfully used as a source of fire blight resistance in the Geneva rootstock breeding program. However, unlike Robusta 5 which has small, inedible fruit, the *M. sieversii* accessions have more typical apple fruit that are larger and edible. This will make the *M. sieversii* accessions a much more suitable source of resistance for scion breeding. Although the fruit are edible, they are not of commercial quality and their resistance will need to be bred with elite selections to improve fruit quality while maintaining fire blight resistance. The resistance of the 12 *M. sieversii* accessions is far stronger than the type of resistance normally observed in fire blight resistant cultivars, such as 'Delicious' or 'Enterprise'. To choose the best of these 12 accessions to start incorporating into the WSU apple breeding program we plan to evaluate the accessions for their fruit quality. Although some fruit quality data exists in the USDA database of these accessions (which was used as a factor in selecting them for the trial), Kate Evans thinks that seeing the performance of the fruit in the WSU breeding program's standardized fruit quality evaluation protocols will give her a much better knowledge base for selecting the accession(s) to work with. We plan on using the current *M. sieversii* plantings in Wenatchee and Kearneysville to evaluate the fruit of the 12 highly resistant accessions in 2015 and 2016.

The project also established a planting of the RosBREED apple reference germplasm set (elite cultivars and their seedlings, 3 replicate trees, total n=3,500) at WSU's Columbia View Orchard for the purpose of evaluating their fire blight resistance. A vast dataset was developed for this germplasm in the previous RosBREED project, including comprehensive fruit quality evaluations, high-resolution genome scans, and predictive genotypes at fruit quality-influencing loci. Determining the fire blight resistance of this reference germplasm will allow us to leverage the significant financial investment of RosBREED to enable marker assisted breeding of fire blight resistance of existing seedlings and selections in the WSU apple breeding program. The Columbia View Orchard will be planted spring 2015 and evaluated for resistance to fire blight shoot infection in 2016-2017 (Obj. 2). This should allow us to identify predictive genotypes for fire blight resistance loci 2017-2018. Markers for these loci would then be developed and evaluated. Once validated they would be used in breeding.

FINAL PROJECT REPORT

Project Title: Improving the management of two critical pome fruit diseases

PI: Timothy J. Smith
Organization: Washington State University
Telephone/email: 509-667-6540 / smithtj@wsu.edu
Address: 400 Washington Street
City: Wenatchee,
State/Zip Washington, 98801

Cooperators: Travis Allan, Allan Bros. Fume Trial Site; Mike Conway, Trident Ag Products

Total Project Request: Year 1: $15,155 **Year 2:** $15,737 **Year 3:** $16,343
Three year total: $47,235

Other funding sources

Trident: provided in kind support (fumigation) $9000. Other necessary financial support was received from companies supplying products tested for effect on fire blight or orchard replant during this project. I was Co-PI on the project "Development of Non-Antibiotic Programs for Fire Blight Control in Apple and Pear," from the USDA Organic Agriculture Research and Extension Initiative (OREI). My three year sub-award was a total of $89,661 2012-13-14. The TFRC project funding helped to justify and aquire the OREI grant.

Budget

Organization Name: WSU **Contract Administrator:** Jennifer Jansen
Telephone: 509-335-2867 **Email address:** jjansen@wsu.edu

Item	2012	2013	2014
Salaries	$10,125	$10,660	$11,086
Benefits	4,759	4,477	4,656
Wages			
Benefits			
Equipment			450
Supplies			
Travel	600	600	600
Plot Fees			
Miscellaneous			
Total	$15,484	15,737	$16,792
Three Year Total			**$48,013**

Footnotes: Salaries and benefits are in support of 0.28 FTE of a full time technician. Travel is to plot sites. Equipment is for a backpack mist sprayer.

Original OBJECTIVES- *Fire blight of apple and pear:*

1. We will continue to test fire blight control materials in the orchard, on both apple and pear, to assess efficacy and aid registration of effective fire blight control alternatives.
2. We will further study the relationship of temperatures to fire blight infection risk.

Significant Findings- Fire Blight:

Objective 1. We tested a wide range of fire blight control materials, rates and timings in the orchard.

- In 2014, twenty-five products were tested in 42 different timings and/or sequences.
- In 2013, thirteen products were tested in 36 different combinations, series and timings.
- In 2012, ten products were tested with 24 different timings and/or sequences.
- The treatments included the antibiotics streptomycin, oxytetracycline, and the newly registered kasugamycin, also "Blossom Protect" (*Aureobasidium pullulans*, a yeast-like biocontrol), many formulations and compounds of copper bactericides, *Bacillus subtilis* compounds (such as Serenade, Double Nickle and Companion), dihydrogen peroxide (Oxidate) and Actigard or other SAR treatments. See Table for 2014 results summary.
- Of the total102 treatments tested over the past three seasons, 39 usually protected the flowers at 80% or higher level, compared to the inoculated check. This level of control in an inoculated plot indicates potential for excellent control under orchard use conditions. These products did not induce russet in a large scale russet trial that was carried out in 2013 at the WSU Sunrise Research Orchard, but significant concerns remain about potential for russet induction with some of these products. Most forms of copper fungicides and "Blossom Protect" have a potential to mark or russet fruit if applied during or for several weeks after bloom, especially during wet, rainy or high humidity weather. Application during good drying conditions is critical.
- The sequence of application copper fungicides and other non-fire blight related sprays and the pH of the water in the spray tank must be considered carefully. Oils and acid buffers are commonly applied in early season fruit thinning and pest control sprays. Application of oils or products with low (acid) pH within a few days after application of copper products may increase the rate of active copper ion release. This increase could mimic the application of a much higher rate of the copper fungicide, leading to russeting and other fruit damage. Most "Buffers" commonly used in Washington orchard spray mixtures are intended to buffer alkaline water to acidic levels of 4.0 to 5.0. The potential for any product causing fruit finish problems is reported to be relatively higher east of the Rocky Mountains and is experienced much less frequently in low spring rainfall regions of the Pacific Northwestern states, USA.

Product and Timing (* = Organic use)	Average Control %	Highest %	Lowest %	Number of trials used (x) in average and Comments
Blossom Protect* 1,25 to 1.34 lbs. / 100/ A + Buffer Protect* @ 9.35 lbs. / 100 2 or more applications pre-bloom	83.1	95.2	73	(23) Most effective if applied starting at least 3-4 days before infection period begins. Potential for fruit russet when applied during cool, wet conditions.
Cueva* or Provisto 3 or 4 quarts / 100 / A Applied day before infection + ASAP after	Provisto 82.1 Cueva 80.7	98 83.6	62 77.6	(5) Cueva is a copper soap. (14) Provisto is a liquid copper material not yet registered for apples or pears.
Oxytetracycline (FireLine, or Mycoshield) 1 lb. / 100 gal. / Acre Applied day of infection.	79.4	96	62	(17) The standard effective product used in Washington since 1975.
Kasugamycin (Kasumin) Applied day of infection.	79	89	62	(8) An effective product. Use in rotation with others. Newly registered.
Serenade* (recent versions) Double Nickle* Higher label rates.	60.9	81	47	(17) Products have varied in strength, formulation and rate.
Copper Bactericides / Fungicides of Various Chemistries. Check for organic status.	58	70	30	(23) All copper products tested are effective, but less so than other available choices. Useful as part of a control program when applied to dormant trees.

Table 1. Most Consistent Effective Products in Multiple Trials. Summary of average percent control of blight infection (compared to an inoculated untreated check) in similar trials conducted on pears and apples, highest and lowest percent control and comments. Use of trade names does not imply endorsement by author.

Objective 2. Completed the first year, and the fire blight risk model "CougarBlight" is available through WSU DAS, and has been provided to an increasing number of states and other countries.

Significant Findings- Fire Blight Management Products:

1. ***Objective 1***.

Results and Discussion – Fire Blight: 2014 Products, Rate, Timing and Sequence Efficacy Trial.

Note: Some of the products reported below are not yet registered for use in orchards. They are listed only to report research results. Check the label for the crop details prior to any use.

Products	Rate/100 gal./Acre, Timing	% Infection	% Control
Blossom Protect + BP buffer, then Serenade Optimum	BP+BP (1.25 lb & 9.35 lb) 50 & 100% bloom, then 20 oz / 100 Serenade Opt. @ Petal Fall	0.98	97.8
GWN 10373 (Provisto version)	4 qt. / 100 + 64 oz wetter, day before & day after 100% bloom inoculation.	1.75	96.0
Blossom Protect + BP Buff. +Actigard	BP+BP (1.25 lb & 9.35 lb) + Act. 2 oz./ A, twice, 50 & 100% bloom	2.47	94.4
Blossom Protect + BP Buffer standard	BP+BP (1.25 lb & 9.35 lb), twice, 50 & 100% bloom	2.78	93.7
GWN 10074 (Provisto version)	4 qt. / 100 + 64 oz wetter, day before & day after 100% bloom inoculation.	3.0	93.2
GWN 10073 (Provisto)	3 qt. / 100 + 64 oz wetter, day before & day after 100% bloom inoculation.	4.23	90.4
Cueva (Copper soap)	4 qt. / 100, day before & day after 100% bloom inoculation.	4.25	90.4
Cueva	3 qt. / 100, day before & day after 100% bloom inoculation.	5.8	86.8
Blossom Protect + BP Buffer, Cueva	BP+BP (1.25 lb & 9.35 lb), twice, 50 & 100% bloom, then Cueva at petal fall	6.8	84.6
Streptomycin – half rate	0.5 lb./100 gal (100 ppm) applied @ 100% Bloom before inoculation	7.5	83.0
Oxidate	2 gal. /100 gal. / A on day of inoculation, 1 gal. next day, and 1 gallon @ PF	11.7	73.5
Champ Ion	0.5 lb. / 100 / Acre applied at 50 & 100% bloom	11.9	73.0
Phyton27	40 fl. oz. / 100, day before & day after 100% bloom inoculation.	12.1	72.6
Actigard, then oxytet.	Actigard at 50% bloom, oxytet. at 100% bloom	14.5	67.1
Kocide 3000	0.5 lb. / 100 / Acre applied at 50 & 100% bloom	14.7	66.7
Actigard then Oxytet PF, then Act. 6-8 " shoots	Actigard 2 oz. / 100 / A @ 50 & 100% bloom, oxytet. @ PF, then Actigard 2 oz. when shoots 6 – 8 inch	15.2	65.5
Tech Flow NutriCop 20 then CopoCal	Tech Flow NutriCop 20, 2 qt. at Del. Dormant, then CopoCal 3 qt., day before & day after 100% bloom inoculation, again at petal fall.	19.1	56.7
OxiPhos, Oxidate, then oxytet.	OxyPhos 1 gal. on Day of Inoc, 1 gal. OxiDate the day after, then 1 lb./100 oxytet.(Mycoshield) at Petal Fall	19.6	55.6
Italipollina Copper EXL-880	21 fl. oz. / 100 / A, day before & day after 100% bloom inoculation.	20.1	54.4
CopoCal	3 qt/ 100 / A, day before & day after 100% bloom inoculation.	21.6	51.0

Serenade Optimum	32 fl.oz. / 100 / A, at 50%, 100% bloom and at petal fall	22.8	48.3
Badge SC	20 fl.oz. / 100 / A, the day before & 1 day after 100% bloom inoculation.	23.8	46.0
Taegro, then Oxytet., then Taegro	Taegro 5.2 oz. / 100 / A @ Pink, Oxytet. 1 lb/ 100 / A @ 50% bloom, then Taegro 5.2 oz. at 100% bloom.	24.8	43.8
Bacteriophage mixture B	The day before and the day of inoculation.	26.2	40.2
Serenade Optimum then Oxytet, then Serenade Optimum	Serenade Optimum 24 oz. @ pink, then Oxytet. 1 lb/100 / A @ 50% bloom, then Seren. Opt. 24 oz. 100%	26.90	39.0
CopoCal (with 2nd bloom timing)	3 qt./100/A the day before and day after 100% bloom inoculation, again at Petal Fall and PF+10 days	26.92	39.0
BioAtlantis Resistance Blend	35 fl.oz./100/A at 50% bloom open and the day before full bloom and again at Petal Fall	27.4	38.9
Bacteriophage mixture A	The day before and the day of inoculation.	39.5	10.4
Untreated check, inoculated	No treatment, inoculated at 100% bloom open.	44.1	0

Table 2. 2014 Fire Blight Control Product Efficacy trial on Apples.

Treatment	**Number of Treatments**	**Highest Percent Control**	**Lowest Percent Control**	**Average Percent Control**
Strep + ASM*	6	100	90.6	95.1
Copper (new forms)	24	98	76.7	86.9
Streptomycin	12	90	75	85.9
BP + Buffer Protect	19	97.8	72	92.6
Oxytetracycline	18	93	53	79.0
Kasugamycin	8	89	62	77.5
Gentamycin	6	88	51	74.5
Serenade	18	84	38	60.1
Copper (old forms)	17	80	26	54.0
Fungicides	6	57	33	48.6
Acid Buffers	4	39	19	30.5
SAR (Claims)	10	46	0	30.2
Nutrient minerals	3	36	5	18.8

Table 3. Summary of author's current and past fire blight control efficacy trial results. Plots all inoculated. *ASM = Actigard, BP = *Aureobasidium pullulans*, "Blossom Protect." Average of 46.8 percent blight infection in inoculated untreated checks.

Orchard Replant Treatment Trial

Original OBJECTIVES – *Orchard Replant Disease:*
We will demonstrate the effect on soil fumigation on the productivity and quality of apples grown under a very modern production system.

1. We will document apple productivity over a range of chloropicrin and 1, 3-DCP rates.
2. We will provide this information to the fruit growers of Washington in the effort to increase the practice of pre-plant soil fumigation from its current 60% of replanted acres.
3. We will calculate the extrapolated economic impact of the various treatments.
4. We will provide this information to the Northwest Hort. Council, the US EPA, the fumigant registrants, or anyone else involved in the 2013-15 re-registration of soil fumigants.

Significant Findings- Orchard Replant Treatment

Objective 1. Tree growth and size were measured after the first and second year. Growth of all fumigated trees was similar, and much greater than in the unfumigated checks (Table 4). Production and fruit size were documented in 3rd through 5th leaf (2011-2014), (Tables 5 and 6.) The yields in all fumigated treatments greatly exceeded those in the untreated checks. Fruit size was not significantly different after the first year of production (Table 7). It became apparent that the 1, 3-DCP (Telone) part of the standard fumigant mixture (DCP + chloropicrin) plays an important role in the efficacy of the fumigants most commonly applied on old orchard sites. While chloropicrin (the "C" in C-17 and C-35, also applied in "PicPlus" and "Pic 60" in this trial) is necessary to the treatment of replant disease, the treatment of high relative levels of chloropicrin with no 1, 3-D (Treatment A), while much better than the untreated areas, it was the least productive of the fumigation treatments. The moderate 1, 3 DCP + moderate chloropicrin rate treatment was superior. This lower rate of chloropicrin will require much reduced "buffer zone" distances.

Objective 2. The gross economic differences continue to increase (Table 6). Since the orchard was planted as a "sleeping eye in" in spring 2009, the most productive treatment has grossed about $32,000 more per acre than the untreated check, after taking into account the cost of fumigating, picking and packing. This has returned over $50 for each $1spent on the cost of fumigation.

Objective 3. These results have been presented to growers and advisors at numerous times in many venues. The data and results will be published in both popular and scientific texts. Unlike the situation in apples, there are no pear or cherry fruit rootstocks that have been proven resistant to orchard replant disease. In the past, pears and cherries have responded to soil fumigation in a manner similar to the response in apples.

Objective 4. The data from this project was submitted to the US EPA on November 14, 2013 in support of the continued registration and availability of 1, 3-dichloropropine, one of the two active ingredients in pre-planting soil treatments for orchard sites (products such as Telone C-17 and C-35.) Re. Docket ID No. EPA-HQ_OPP_2013-0154.

Treatment:	*A: PicPlus (150 lbs./A Chloropicrin) 0 DCP*	*B: PC60 (144 lbs./A Chloropicrin) 94 lb/A DCP*	*C: Telone C-35 (25 GPA, 98 lb/A chloropicrin) 178 lb/A DCP*	*D: Telone C-17 (30 GPA, 51 lb/A chloropicrin) 260 lb/A DCP*	*Untreated*
Tree Height (inches)	86a	85a	86a	88a	74b
Trunk X-sec. mm^2	249a	249a	236a	253a	139b
Total Shoots (inches)	155a	120a	139a	185a	29b

Table 4. 2010 (second season) tree growth data: Average inches height, cross section area of trunk 4 inches above the graft union and total current season shoot growth of second season Cripp's Pink apples planted as a "sleeping eye" on M9, planted after fumigation on a replant site.

Year	Treatment A PicPlus	B PicClor 60	C Telone C-35	D Telone C-17	Untreated
2010	0	0	0	0	0
2011	12,808	12,826	15,935	15,529	6,286
2012	28,333	32,500	32,437	38,920	17,585
2013	25,556	32,862	30,734	36,591	16,792
2014	26,480	24,422	22,458	29,182	19,003
Total	**93,177**	**102,610**	**101,564**	**120,222**	**59,666**

Table 5. Gross yield per acre in pounds during first six years of growth. See Table 4 for treatment details.

Treatment A	***PicPlus (175 lbs. per ac: 150 lbs./A chloropicrin, NO 1,3-DCP)***						
	average box size 2014	Tree yield (lb.)	Gross wt. lbs./ Acre	90% pack wt.	Packed boxes	$ Value*	$ / Acre by Treatment
	91.81	15.50	26480	23832	567	20	11,389
		**Minus 2014 costs, adjustments of: $4,479				**Adjust 2014: $6,910**	
							Total Adjusted Gross / A in 2011 +12 + 13 + 14 crops $41,764
Treatment B	***PicClor 60 (20 GPA: 144 lbs./A chloropicrin, 94 lb/A 1,3-DCP)***						
	average box size 2014	Tree yield (lb.)	Gross wt. lbs./ Acre	90% pack wt.	Packed boxes	$ Value*	$ / Acre by Treatment
	93.0	14.3	24,442	21,998	524	20	10,475
		**Minus 2014 costs, adjustments of: $4,188				**Adjust 2014: $6,287**	
							Total Adjusted Gross / A in 2011 + 12 + 13 + 14 crops: $46,883
Treatment C	***Telone C-35 (25 GPA: 98 lb/A chloropicrin, 178 lb/A DC)***						
	average box size 2014	Tree yield (lb.)	Gross wt. lbs./ Acre	90% pack wt.	Packed boxes	$ Value*	$ / Acre by Treatment
	91.7	13.15	22,458	20,212	481	20	9,625
		**Minus 2014 costs, adjustments of: $3,841				**Adjust 2014: $5,784**	
							Total Adjusted Gross per acre in 2011 through 2014 crops: $46,966

Treatment D	***Telone C-17 (30 GPA, 51 lb/A chloropicrin 260 lb/A DCP)***						
	average box size 2014	Tree yield (lb.)	Gross wt. lbs./ Acre	90% pack wt.	Packed boxes	$ Value*	$ / Acre by Treatment
	91.3	17.1	29,182	26,264	625	20	12,507
		**Minus 2014 costs, adjustments of: $4,996				**Adjust 2014: $7,512**	
Total Adjusted Gross per acre, 2011 through 2014 crops: $58,823							
Treatment E	***Untreated***						
	average box size 2014	Tree yield (lb.)	Gross wt. lbs./ Acre	90% pack wt.	Packed boxes	$ Price*	$ / Acre by Treatment
	93.1	11.13	19,003	17,103	407	20	8,144
		**Minus 2014 costs, adjustments of: $3,470				**Adjust 2014: $4,674**	
Total Adjusted Gross per acre 2011 through 2014 crops: $25,969							

Table 6. **Yield per acre, box size grouping and rough estimate of fruit gross economic value per acre.** *Approximate FOB average on 11/17/2012. ****Costs, adjustments**: picking @ $20/bin, and packing @ $7 / box. Fumigation @ $650-750/acre accounted for in 2011 cost adjustments. Credit applied for 12 cents/lb. for cull fruit, except 2 cents in 2014.

Year	Treatment A PicPlus	B PicClor 60	C Telone C-35	D Telone C-17	Untreated
2010	0	0	0	0	0
2011	204 (94.1)	220 (86.3)	216 (89.0)	222 (86.3)	195 (98.3)
2012	207.1 (92.1)	198.6 (96)	200.7 (95)	200.5 (95.1)	196 (97.3)
2013	186.5 (102)	195.5 (97.5)	190.3 (100)	191.7 (99.5)	183.9 (103.7)
2014	208.9 (91.8)	205.4 (93)	207.9 (91.7)	208.8 (91.3)	204.8 (93.1)
Average	201.6	204.9	203.7	205.8	194.9

Table 7. Average size of fruit in grams (average number in 42 lb. box in parenthesis).

Executive Summary - Improving the Management of Two Critical Pome Fruit Diseases.

This project was actually two separate efforts, with entirely different sets of goals and expected outcomes.

The replant treatment portion of the project was designed:

- To provide scientifically valid research into the efficacy and necessary rates of chloropicrin as a component of soil fumigants used as a treatment of orchard replant disease. Data was not available on this subject, and the EPA wanted data for re-registration.
- To determine the effect of 1, 3 dichloropropene (1, 3 DCP – "Telone") at various rates when added to chloropicrin.
- To provide information about the lowest effective rate per acre of both products. This information was critical, as the "buffer zones" distances in the new label regulations were determined by rate per acre and acres treated. If these rates were set too low, growers would lose production efficiency and experience seriously reduced returns.
- To provide this data from a trial carried out in high-value cultivar growing under intensive modern system and management.

Results: After six seasons of intensive data collection and analysis, we could support the following conclusions:

- The most effective treatment was a blend of the lowest rate of chloropicrin in the trial (51 lbs. /A) blended with a moderately high rate of 1, 3 DCP (260 lbs./A), a mixture that is identical to the current industry standard of 30 gallons per acre of Telone C-17.
- Chloropicrin, when used at highest rates as the sole soil fumigant, was not as effective as when used at low standard rates blended with 1, 3 DCP.
- Under conditions of this trial (high-value cultivar and intensive management) the standard fumigation treatment increased economic returns by about $32,850 per acre, a return of about $50 for every $1 spent on the cost of fumigation.

Information from this trial is used in reregistration process for both chloropicrin and 1, 3 DCP.

The fire blight treatment portion of the project was designed:

- To research the efficacy, application timing and necessary rates of products used for fire blight blossom infection management.
- To find alternative products acceptable for organic production.

  Results: at the inception of this trial, one effective product, oxytetracycline (Mycoshield), was used for fire blight control in the state of Washington. The results of these trials, supported by others, were instrumental or part of the registration of and use of at least three new products (Blossom Protect, Kasumin and Provisto) that are at least as effective as oxytetracycline. The use and efficacy of other products that may play an important role is now better understood, and more registrations of useful control products are impending.

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

Project Title: Fungicide evaluation for the control of bull's eye rot of apple

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Total Project Request: $40,700 **Year 1:** $19,500 **Year 2: $21,200**

Other funding sources: none

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

Item	2014	2015
Salaries*	$14,000	$15,000
Benefits	$2,000	$2,200
Wages		
Benefits		
Equipment		
Supplies	$2,000	$2,500
Travel	$500	$500
Miscellaneous		
Plot Fees	$1,000	$1,000
Total	$19,500	**$21,200**

Footnotes: *Funding is requested to support a Research Assistant

OBJECTIVES

The overall objectives of this program are to 1) evaluate the efficacy of select pre-harvest fungicides and post-harvest fungicide drenches for the control of bull's-eye rot of apple incited by *Neofabraea perennans* and *Cryptosporiopsis. kienholzii* and 2) determine the effectiveness of fungicide applications in the control of early, mid and late season apple fruit infection by *N. perennans* and *C. kienholzii* occurring in the field.

The specific objectives addressed during the current funding period were:

- Identify pre-harvest applied fungicides that provide adequate postharvest control of bull's-eye rot infection in stored apples
- Identify post-harvest applied fungicides that provide adequate postharvest control of bull's-eye rot infection in stored apples
- Determine whether fungicide efficacy remains consistent regardless of the timing of fruit infection in the field (i.e. early season versus late season infection)
- Determine whether fungicides applied pre-harvest or post-harvest are more effective at controlling bull's-eye rot in stored apples
- Determine whether the efficacy of a single fungicide is consistent across the spectrum of bull's-eye rot causing pathogens used in this study

Significant Findings:

- Among the pre-harvest fungicides examined in this study, Topsin-M provided the most effective control of bull's-eye rot caused by either *N. perennans* or *C. kienholzii*.

- Among the post-harvest fungicides, both Penbotec and Mertect provided control of bull's eye rot, but Mertect exhibited a greater degree of efficacy than Penbotec. Inconsistent results were observed on Penbotec-treated fruit between the fruit inoculated 2 and 5 weeks before harvest. This needs to be confirmed during 2014-15 storage season.

- Disease incidence incited by either pathogen was consistently higher when fruit were inoculated two weeks compared to five weeks prior to harvest. Correspondingly, fungicide efficacy appeared to be greater in trials when fruit were inoculated five weeks prior to harvest, which may correspond with increased fruit susceptibility.

- When comparing disease incidence for the no treatment control, *N. perennans* and *C. kienholzii* incited similar levels of Bull's eye rot, though *N. perennans* demonstrated a greater level of aggressiveness based on its capacity to incite rot on immature fruit (five week prior to harvest inoculation).

- Disease control was attained using either pre-harvest or post-harvest fungicide applications, though post-harvest application with Mertect was marginally better than pre-harvest application with Topsin-M.

- Multiple post-harvest fungicides demonstrated efficacy for the control of Bull's eye rot.

METHODS

Objective 1:

Evaluation of pre-harvest fungicides:
All studies were conducted on 'Fuji' variety apples growing at block 10 of the WSU-Sunrise Research Orchard near Rock Island, Washington.

Apple fruit were inoculated in the orchard five weeks prior to commercial harvest to represent "early season" infection and two weeks prior to infection to represent 'late season' infection periods. A spore suspension of either *N. perennans* or *C. kienholzii* inoculum at a concentration of 5 x 10^5 spores mL^{-1} was used to inoculate the fruit. Fruit were inoculated at the orchard in the evening to minimize spore exposure to extreme heat and/or sunlight. The inoculum suspension was applied to the fruit surface to a drip point using a plastic spray bottle. Immediately following the inoculations, apples were covered with pre-moistened white plastic bags of varying size in order to maintain high relative humidity to encourage pathogen spore germination. Plastic bags were removed from the fruit the morning after spore application (approximately 15 hours post inoculation) and fruit remained on the tree to the appropriate harvest date..

Four pre-harvest treatments (Table 1) were employed in this study and were randomly assigned to trees following a completely randomized design with four tree replicates per treatment. Inoculated apples were treated with select pre-harvest fungicides following manufacturer application rates and recommendations. Fungicides were applied to fruit using a backpack sprayer. A plastic screen was placed between trees to prevent chemical drift from one tree to the other. Four replicate trees were included per treatment combination and twenty apple fruit from each tree were harvested following commercial harvest dates. Fruit were sorted and tray packed into cardboard apple boxes and stored under regular atmosphere conditions at 0°C for up to nine months after harvest. Disease incidence was monitored on a monthly basis beginning in month three of cold storage. Tissue from symptomatic fruit were cultured onto nutrient rich artificial medium (potato dextrose agar – PDA) in order to isolate causal fungi of fruit rot and confirm infection by *N. perennans* or *C. kienholziii*.

Table 1. Selected pre-harvest fungicides with corresponding application rate information

Fungicide Trade Name	Chemical Ingredient	Application Rate	Application Timing
No fungicide control	N/A	N/A	N/A
Ziram	Zinc	6.0 lb/acre	14 days before harvest
Pristine	Pyraclostrobin + Boscalid	14.5 oz/acre	2 days before harvest
Topsin M	Thiophanate-Methyl	1.0 lb/acre	2 days before harvest

Evaluation of post-harvest fungicide drenches:

Fruit were inoculated in the orchard as described above. Fruit were harvested according to commercial harvest dates and treated with post-harvest fungicides (Table 2) immediately after harvest. Fruit were submerged in a specified fungicide solution for 30 seconds to simulate drenching. Fruit were subsequently allowed to air dry for two hours prior to being sorted and tray packed into cardboard apple boxes. Fruit were stored at 0°C under regular atmosphere conditions for nine months post-harvest. Disease incidence was monitored monthly beginning at month three of cold storage and

continuing through month nine of storage. Tissue from bull's-eye symptomatic fruit was isolated from lesions and cultured onto PDA to isolate the suspected bull's-eye rot pathogen and confirm infection.

Table 2. Selected post-harvest fungicide drenches with corresponding application rate information.

Fungicide Trade Name	Chemical Ingredient	Application Rate
No fungicide control	N/A	N/A
Mertect	Thiabendazole	16 fl oz/100 gal water
Scholar	Fludioxonil	12 fl oz/100 gal water
Penbotec	Pyrimethanil	16 fl oz/100 gal water

Objective 2:

Early versus late season fruit infection:

During the second year of funding, apple fruit will be inoculated at three specific intervals during the growing season representing early (late-May/early June), mid and late season infection periods (eighteen, five and two weeks before anticipated commercial harvest, respectively). Inoculations will be conducted as described under objective 1. Application of chemical treatments will depend on disease incidence data collected from the study conducted during the previous growing season. Pre-harvest and post-harvest chemicals giving adequate bull's-eye rot control in the previous study will be retested to determine whether timing of fruit infection can influence fungicide control efficacy. Fungicide application will proceed as described in the previous objective.

After fruit have been treated with the respective fungicide treatments, fruit will be sorted and tray packed into cardboard apple boxes and stored under regular atmosphere conditions at 0°C for up to nine months. Fruit will be checked on a monthly basis beginning in month three to record disease incidence. Any fruit suspected to be infected with bull's-eye rot will be removed. Tissue along the margin of fruit lesions will be excised from symptomatic fruit and placed onto PDA in order to culture the suspected bull's-eye rot pathogen and confirm infection.

RESULTS & DISCUSSION

To date, collection of data from trials representing the 2013 growing season has been completed. Field conducted fruit inoculations and fungicide applications for the 2014 field season have also been completed. The fruit from the 2014 growing season is currently in cold storage. Evaluation of fruit from these trials will commence in mid-January and will be completed in September 2015.

2013 Growing Season

Effect of pre-harvest fungicide treatments on disease incidence:

Pre-harvest application of Pristine or Ziram had no significant effect on incidence of disease caused by either *N. perennans* or *C. kienholzii* relative to the no treatment control (Table 3). Fruit inoculations conducted two weeks prior to harvest (late season infection) resulted in disease incidence

that was numerically higher than incidence resulting from inoculations conducted five weeks prior to harvest (early season infection). In general, disease incidence resulting from inoculations with *N. perennans* was similar to that cause by *C. kienholzii*.

In contrast to the aforementioned fungicide treatments, pre-harvest application of Topsin-M significantly reduced the incidence of post-harvest fruit rot incited by either pathogen relative to the no fungicide treatment controls (Table 3). As observed for the no treatment control, resulting disease incidence was significantly higher for fruit inoculated two weeks prior to harvest compared to fruit inoculated at five weeks before harvest. Disease incidence resulting from fruit inoculations did not differ between the two pathogens.

Effect of post-harvest fungicide treatments on disease incidence

Post-harvest application of the fungicide Scholar had no significant effect the incidence of fruit rot that developed in response to inoculation with either *N. perennans* or *C. kienholzii* (Table 4). Post-harvest application of either Penbotec or Mertect significantly reduced the incidence of bull's eye rot relative to the control, but application of Mertect resulted in significantly lower disease incidence relative to the Penbotec treatment. Although *N. perennans* and *C. kienholzii* appeared to incite similar levels of disease as evidenced by results from the no treatment control, both Penbotec and Mertect demonstrated greater efficacy in reducing rot caused by *C. kienholzii* than that resulting from inoculation with *N. perennans*. As was observed in the pre-harvest trials, resulting disease incidence was significantly higher when inoculations were conducted two weeks prior to harvest than observed on fruit inoculation five weeks prior to harvest. Penbotec did not effectively control bull's eye rot when fruit inoculations were conducted two weeks prior to commercial harvest.

Table 3. Effect of pre-harvest fungicide applications on incidence of bull's eye rot resulting from fruit inoculation with *Neofabraea perennans* or *Cryptosporiopsis kienholzii* during 2013 season.

Preharvest Chemical	**Inoculation Time-Point**	**Pathogen**	**Average Disease Incidence**
No Fungicide Control	Five weeks before harvest	*N. perennans*	64%
		C. kienholzii	49%
	Two weeks before harvest	*N. perennans*	79%
		C. kienholzii	89%
Ziram	Five weeks before harvest	*N. perennans*	63%
		C. kienholzii	38%
	Two weeks before harvest	*N. perennans*	81%
		C. kienholzii	65%
Pristine	Five weeks before harvest	*N. perennans*	59%
		C. kienholzii	60%
	Two weeks before harvest	*N. perennans*	81%
		C. kienholzii	90%
Topsin-M	Five weeks before harvest	*N. perennans*	15%
		C. kienholzii	3%
	Two weeks before harvest	*N. perennans*	28%
		C. kienholzii	18%

Table 4. Effect of post-harvest fungicide applications on the incidence of bull's eye rot resulting from fruit inoculation with *Neofabraea perennans* or *Cryptosporiopsis kienholzii* during 2013 season.

Postharvet Chemical	Inoculation Time-Point	Pathogen	Average Disease Incidence
No Fungicide Control	Five weeks before harvest	*N. perennans*	61%
		C. kienholzii	51%
	Two weeks before harvest	*N. perennans*	60%
		C. kienholzii	88%
Scholar	Five weeks before harvest	*N. perennans*	53%
		C. kienholzii	63%
	Two weeks before harvest	*N. perennans*	74%
		C. kienholzii	86%
Penbotec	Five weeks before harvest	*N. perennans*	18%
		C. kienholzii	3%
	Two weeks before harvest	*N. perennans*	61%
		C. kienholzii	1%
Mertect	Five weeks before harvest	*N. perennans*	9%
		C. kienholzii	1%
	Two weeks before harvest	*N. perennans*	20%
		C. kienholzii	3%

Studies will continue to assess fungicide efficacy during 2015 and will be modified to include an additional inoculation time point (18 weeks prior to harvest) to assess the capability of these pathogens to cause very early season fruit infections that are expressed during the storage season. An additional fungicide treatment (mixture of difenconazole and fludioxonil) will be evaluated as a post-harvest treatment for control of bull's eye rot.

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

Project Title: Non-antibiotic fire blight control that minimizes fruit russet risk

PI: Ken Johnson
Organization: Dept. Botany and Plant Pathology, Oregon State University, Corvallis
Telephone/email: 541-737-5249 johnsonk@science.oregonstate.edu

Cooperators: Tim Smith, WSU, Wenatchee, WA; Rachel Elkins, UC-ANR, Lakeport, CA
David Sugar, OSU, Medford, OR

Budget: **Year 1:** $25,000 **Year 2: $25,750**
Annually**:** FRA 3.5 mo plus fringe, 2K M&S, 1K local travel & plot fee, 3% inflation

Other funding sources

Agency Name: USDA NIFA OREI
Amt. awarded: $476K to Johnson, Elkins, and Smith 10/11 - 9/14
Notes: Objectives 1 and 2 of this proposal are matching objectives for the above NIFA OREI project
Agency Name: USDA NIFA ORG
Amt. awarded: $495K to Johnson, Elkins, Granatstein and Smith 10/14 - 9/17
Notes: Objectives 1 and 2 of this proposal are related to objectives for the above NIFA ORG project

WTFRC Collaborative expenses: None

Budget
Organization Name: OSU Agric. Res. Foundation **Contract Administrator:** Kelvin Koong
Telephone: (541) 737-4066 **Email address:** .j.koong@oregonstate.edu

Item	**2014-15**	**2015-16**	
Salaries Faculty Res. Assist.	14,000	14420	
Benefits OPE 58%	8,120	8364	
Wages undergrads	900	927	
Benefits OPE 12%	108	111	
Equipment			
Supplies	1,000	1030	
Local Travel	372	383	
Miscellaneous			
Plot Fees	500	515	
Total	$25,000	**$25,750**	

Footnotes: Annually**:** FRA 3.5 mo plus fringe, 90 hr undergrad labor, 2K M&S, 1K local travel & plot fee, 3% inflation.

OBJECTIVES

1) Develop non-antibiotic fire blight control programs that minimize fruit russet risk.

1a. Understand the specific risks to fruit from biological material, Blossom Protect (*Aureobasidium pullulans*).

2) **Continued evaluation of alternative, organic-approved materials for fire blight suppression.**

SIGNIFICANT FINDINGS

- Blossom Protect applied once at 70% bloom provided outstanding fire blight control in a pear trial with light disease pressure.
- Under severe disease pressure, fire blight control in Gala apple with Blossom Protect was enhanced by two oversprays Cueva soluble copper between full bloom and petal fall.
- After full bloom, *Aureobasidium pullulans* was detected on nearly 100% of flowers sampled from trees treated with Blossom Protect, and on most flowers (> 90%) sampled from non-treated trees.
- Blossom Protect induced russetting on Bartlett pear fruit in a wet climate (Corvallis) but not in semi-arid Lakeport, CA.
- Cueva induced russetting on Bartlett pear fruit in a wet climate (Corvallis), on Comice pear fruit in a semi-arid climate (Medford), but not on Bartlett pear fruit in a semi-arid climate (Lakeport).
- Molecular methods to identify Blossom Protect strains of *Aureobasidium pullulans* were verified and used to confirm that *A. pullulans*-induced damage to cherry and apple fruit from orchards not treated with Blossom Protect was not caused by Blossom Protect strains of *A. pullulans*.
- Several additional materials - Oxidate, Taegro, R42014 and Previsto – show potential to contribute to non-antibiotic fire blight control programs in certified organic orchards.

METHODS

Objective 1 hypothesis: We know from prior research that integrated programs of Blossom Protect followed by Serenade Optimum and/or a soluble copper (e.g., Cueva) will provide good to excellent fire blight control. We also know that there is a fruit russetting risk with both Blossom Protect and with soluble coppers. These russetting risks are poorly understood, and therefore, we want to more clearly define the risk when these materials are used in integrated blight control programs (hypothesized russeting risk is shown in **Fig. 1**).

Experimental design. Objectives were addressed in experimental orchards located at Oregon State University field stations in Corvallis and Medford, and an organic pear orchard in Lake County, CA. Experiments were arranged in a randomized complete block design with 4 replications. Treatments were applied to trees during early morning (dates and bloom stages provided in results). Treatment suspensions and pathogen inoculum were sprayed to near runoff with backpack sprayers or with a motorized 25-gallon tank sprayer equipped with a hand wand.

Microbial colonization and disease assessment. Microbial populations were measured by washing flowers sampled from the experimental trees followed by dilution plating the wash onto a semi-selective culture medium to enumerate microbial populations. Fire blight was measured by counting the number of blighted flower clusters (strikes) on each tree during weekly inspections in May and early June. Microbial populations on flowers (log-transformed), total number of blighted flower clusters per tree, and disease incidence (diseased clusters divided by total clusters (based on prebloom counts)) were subjected to analysis of variance.

Fruit russet evaluation. Prior to harvest, 30 to 50 fruit were sampled from each replicate tree. For each fruit, percent surface russetting was graded using a modified Horsfall-Barratt rating system.

Molecular identification of Blossom Protect strains of Aureobasidium pullulans. The fire blight biocontrol product, Blossom Protect, consists of strains CF10 and CF40 of *A. pullulans* mixed

in a 50:50 ratio. In recent situations of fruit rot of cherry (R. Kim, postharvest cherry lots, Yakima 2012) and fruit russet of apple (J. Pscheidt, Braeburn apple orchard, Corvallis 2014), *A. pullulans* was implicated as the cause of the fruit damage. This led us to investigate published molecular PCR protocols for specific identification of the Blossom Protect strains of *A. pullulans*.

RESULTS & DISCUSSION

Obj. 1. Non-antibiotic fire blight control programs that minimize fruit russet risk.

Fire blight control. *Bartlett pear trial.* Integrated programs of the non-antibiotic materials

Table 1. Bartlett pear, non-antibiotic fire blight trial, Corvallis, 2014.

Treatment	Rate per 100 gallons water	Date treatment applied* 7 Apr 80% bloom	10 Apr Full bloom	14 Apr Petal Fall	Number of blighted clusters per tree**		Percent blighted floral clusters***	
Water		---§	**X**	**X**	**11.8**	**a#**	**1.7**	**a#**
FireWall 100 ppm	8 oz.	---	X	---	1.3	cd	0.2	cd
FireLine 200 ppm	16 oz.	---	X	X	1.0	cd	0.2	cd
Serenade Optimum	20 oz.	---	X	X	6.0	ab	1.0	ab
Blossom Protect	21.4 oz.	X	---	---	0.3	d	0.1	d
plus Buffer Protect	150 oz.	X	---	---				
Blossom Protect	21.4 oz.	X	---	---	1.8	cd	0.3	cd
plus citric acid	150 oz.	X	---	---				
Blossom Protect	21.4 oz.	X	---	---	1.0	cd	0.1	cd
plus Buffer Protect	150 oz.	X	---	---				
then Serenade Optimum	20 oz.	---	X	X				
Blossom Protect	21.4 oz.	X	---	---	2.5	bc	0.4	bc
plus Buffer Protect	150 oz.	X	---	---				
then Serenade Optimum	20 oz.	---	X	X				
plus Cueva (one pint)	16 fl. oz.	---	X	X				
Blossom Protect	21.4 oz.	X	---	---	1.8	cd	0.3	cd
plus Buffer Protect	150 oz.	X	---	---				
then Serenade Optimum	20 oz.	---	X	X				
plus Cueva (one quart)	32 fl. oz.	---	X	X				
Blossom Protect	21.4 oz.	X	---	---	1.8	cd	0.3	cd
plus Buffer Protect	150 oz.	X	---	---				
then Serenade Optimum	20 oz.	---	X	X				
plus Cueva (1.5 quarts)	48 fl. oz.	---	X	X				
Blossom Protect	21.4 oz.	X	---	---	1.5	cd	0.2	cd
plus Buffer Protect	150 oz.	X	---	---				
then Serenade Optimum	20 oz.	---	X	X				
plus Cueva (two quarts)	64 fl. oz.	---	X	X				
Blossom Protect	21.4 oz.	X	---	---	2.8	bc	0.4	bc
plus Buffer Protect	150 oz.	X	---	---				
then Cueva (3 quarts)	96 fl. oz.	---	X	X				
Blossom Protect	21.4 oz.	X	---	---	1.0	cd	0.2	cd
plus Buffer Protect	150 oz.	X	---	---				
then Serenade Optimum	20 oz.	---	X	X				
plus Actigard	2 oz.	---	X	X				
Luna Sensation	4 oz.	---	X	X	10.8	a	1.7	a

*** Trees inoculated with *Erwinia amylovora* strain Ea153N (streptomycin-sensitive) on an evening 1 to 2 days before the full bloom treatment applications; total inoculum concentration was 1 x 10^6 CFU/ml. ** Transformed log(x + 1) prior to analysis of variance; non-transformed means are shown. *** 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.**

depicted in Figure 1 were evaluated for fire blight control in a 54-yr-old 'Bartlett' pear orchard near Corvallis, OR. Trees used in the study averaged 597 flower clusters per tree. Fire blight risk as determined by the heat unit risk model, COUGARBLIGHT, was moderate to high during the primary bloom period. Disease intensity was low with fire blight infections on water-treated trees averaging 12 strikes per tree (**Table 1**). Compared to the water-treated control, each of the treatments reduced significantly ($P < 0.05$) total strikes per tree and incidence of disease; the exception treatment was Luna Sensation, which performed similar to the water treated control (and was included as a control to suppress non-target floral colonization by *A. pullulans*). Antibiotic standards and programs that began with one treatment of Blossom Protect provided a very high level of control including Blossom Protect by itself. Serenade Optimum by itself provided an intermediate level of control.

Gala apple trial. Integrated programs of the non-antibiotic materials depicted in Figure 1 were evaluated for fire blight control in a 15-yr-old 'Gala' orchard Corvallis, OR. Trees used in the study averaged 572 flower clusters per tree. Fire blight risk as determined by the heat unit risk model, COUGARBLIGHT, was low to moderate during the bloom period. Perhaps owing to a high dose of pathogen inoculum, disease intensity was very high with fire blight infections on water-treated trees averaging 389 strikes per tree (**Table 2**). Compared to the water-treated control, each of the treatments reduced significantly ($P < 0.05$) incidence of disease; the exception treatment was

Table 2. Gala apple, non-antibiotic fire blight trial, Corvallis, 2014.

Treatment	Rate per 100 gallons water	Date treatment applied* 13 Apr 80% bloom	15 Apr Full bloom	19 Apr Petal Fall	Number of blighted clusters per tree**		Percent blighted floral clusters***	
Water		---§	**X**	**X**	**389**	**a#**	**69.8**	**a#**
FireWall 100 ppm	8 oz.	---	X	---	129	c	25.2	b
FireLine 200 ppm	16 oz.	---	X	X	245	b	43.8	b
Serenade Optimum	20 oz.	---	X	X	215	bc	32.6	b
Blossom Protect	21.4 oz.	X	---	---	258	b	41.5	b
plus Buffer Protect	150 oz.	X	---	---				
Blossom Protect	21.4 oz.	X	---	---	269	ab	44.5	b
plus citric acid	150 oz.	X	---	---				
Blossom Protect	21.4 oz.	X	---	---	213	bc	37.5	b
plus Buffer Protect	150 oz.	X	---	---				
then Serenade Optimum	20 oz.	---	X	X				
Blossom Protect	21.4 oz.	X	---	---	252	b	45.0	b
plus Buffer Protect	150 oz.	X	---	---				
then Serenade Optimum	20 oz.	---	X	X				
plus Cueva (one pint)	16 fl. oz.	---	X	X				
Blossom Protect	21.4 oz.	X	---	---	205	bc	34.8	b
plus Buffer Protect	150 oz.	X	---	---				
then Serenade Optimum	20 oz.	---	X	X				
plus Cueva (one quart)	32 fl. oz.	---	X	X				
Blossom Protect	21.4 oz.	X	---	---	192	bc	33.1	b
plus Buffer Protect	150 oz.	X	---	---				
then Serenade Optimum	20 oz.	---	X	X				
plus Cueva (1.5 quarts)	48 fl. oz.	---	X	X				
Blossom Protect	21.4 oz.	X	---	---	203	bc	37.8	b
plus Buffer Protect	150 oz.	X	---	---				
then Serenade Optimum	20 oz.	---	X	X				
plus Cueva (two quarts)	64 fl. oz.	---	X	X				
Blossom Protect	21.4 oz.	X	---	---	142	c	28.1	b
plus Buffer Protect	150 oz.	X	---	---				
then Cueva (3 quarts)	96 fl. oz.	---	X	X				
Luna Sensation	4 oz.	---	X	X	406	a	71.4	a

See table 1 for footnotes.

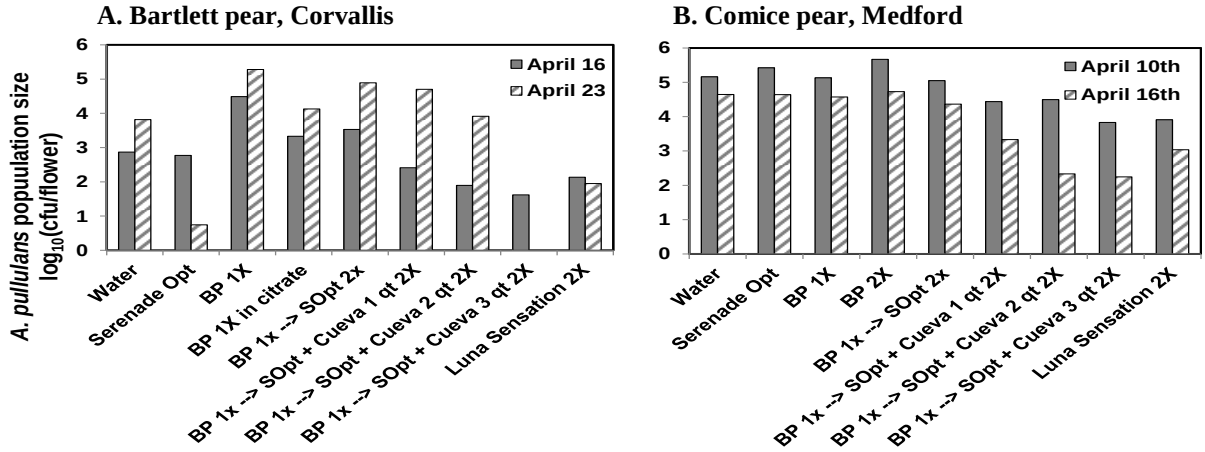
Luna Sensation, which performed similar to the water treated control. Based on ANOVA of total strikes per tree, Blossom Protect followed by Cueva twice at 3 quarts provided improved control compared to Blossom alone (64% control versus 34% control, respectively).

Discussion of fire blight control. With respect to fire blight severity, our 2014 trials yielded contrasting data with light disease pressure in Bartlett pear (water control averaged 12 strikes/tree) and severe disease pressure in gala apple (water control averaged 389 strikes/tree). In Bartlett pear, the 70% bloom treatment of Blossom Protect accounted for nearly all of the observed fire blight suppression. In apple, the high disease pressure resulted in Blossom protect alone providing an intermediate level of control in spite of nearly all flowers being colonized by *A. pullulans*. In apple, following Blossom Protect with Cueva (3qts/100 gallon) resulted in a level of control comparable to streptomycin.

Yeast populations on flowers oversprayed with Serenade Optimum and Cueva copper.

In spray trials with Serenade Optimum and Cueva soluble copper, Blossom Protect was applied once at 70% bloom; populations of the Blossom Protect organism, *A. pullulans*, were measured on two sampling dates between full bloom and petal fall. Trials included those inoculated with the pathogen (**Tables 1 & 2**) and russet evaluation trials in southern Oregon (Comice pear, Medford) and northern California (Bartlett pear). Over all trials, *A. pullulans* was detected on nearly every flower (> 99%) from trees treated with Blossom Protect, and was detected on most flowers (> 90%) sampled from trees not treated with this material. In some trials (**Fig. 2A**), the measured population sizes of *A. pullulans* on non-treated trees was smaller than the population size of this organism on trees treated with Blossom Protect only. In contrast, in other trials (**Fig. 2B** and Gala apple, Corvallis (not shown)), the measured population size of *A. pullulans* on flowers from non-treated trees was statistically similar to the population size of this organism on trees treated with Blossom Protect only. Oversprays of Serenade Optimum after Blossom Protect did not significantly ($P > 0.05$) suppress *A. pullulans* populations compared to the population size of this organism on trees treated with Blossom Protect only. In contrast, mixing Serenade Optimum with 2 or 3 quarts of Cueva significantly suppressed *A. pullulans* populations ($P \leq 0.05$) compared to Blossom Protect only (i.e., Cueva copper caused a 10- to 1000-fold reduction in yeast population size).

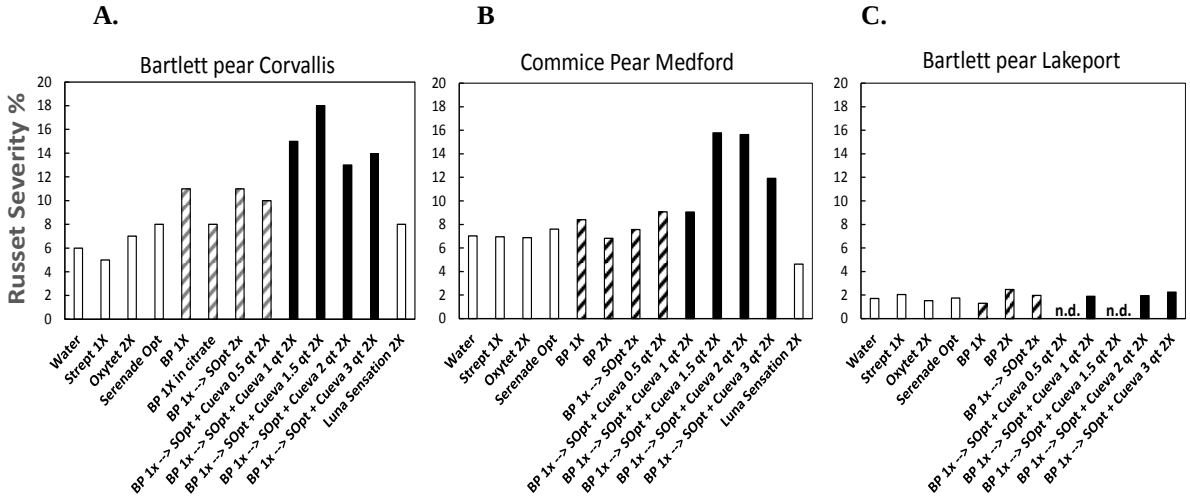
Fig. 2. Population size on *Aureobasidium pullulans* (Blossom Protect organism) on pome fruit flower treated with integrated non-antibiotic fire blight control programs.



Fruit russeting associated with Blossom Protect, Serenade Opt and Cueva copper programs.

Russetting data was collected from all pear trials: Bartlett-Corvallis, Bartlett-Lakeport, Comice-Medford. The Gala apple trial had too much fire blight and apple scab to provide useful fruit russetting data. Within location, the Corvallis location showed significantly ($P \leq 0.05$) elevated

Fig. 3. Fruit russet severity on pear fruit flower treated with integrated non-antibiotic fire blight control programs. Open bar: low russet potential, hatched bar: Blossom Protect, solid bar: BP then Cueva.



russeting in Blossom Protect treatments (either alone or with Serenade Optimum) compared to treatments that received water only (Fig. 3A). Also in Corvallis, following Blossom Protect with Cueva treatments resulted in a significant ($P \leq 0.05$) enhancement in russetting severity compared to Blossom Protect by itself. Similary, at Medford, Cueva treatments after Blossom Protect significantly enhanced russetting of Comice pear compared to Blossom Protect alone, but severity of russetting on the Blossom Protect only treatments was not different than the water-treated control. At Lakeport, mean russetting severity was low (< 2% severity) and not effected by any of the treatments.

Discussion of A. pullulans populations and fruit russetting potential. Pears were chosen for the trials because they are more susceptible to russetting than apple, with Comice pear being exceptionally susceptible compared to the moderately susceptible, Bartlett pear. In addition, the trial locations represented two types of spring climate: semi-arid (Medford & Lakeport) and wet (Corvallis). Russetting was apparently influenced by climate with Bartlett pear in Corvallis showing a higher mean severity than the drier locations. Within drier climates, russetting was apparently influenced by cultivar with Comice pear in Medford showing a higher mean severity than Bartlett pear in Lakeport. In the semi-arid climates, Blossom Protect showed a little potential to enhance russet. In contrast, Cueva showed more potential to induce russetting; although based on the Lakeport data, this material appears relatively safe on tolerant cultivars as long as conditions remain dry during the period of high susceptibility (petal fall to plus 3 wk). In 2015, we intend to focus russet evaluation trials on apple.

In the upcoming 2015 season, implementation of non-antibiotic fire blight control is required for certified organic pome fruit. Based on the data above (and previous results), we have been communicating the following recommendations:

- Early bloom apple and pear Blossom Protect:
  - One full, or two half apps, or two full apps if blight in orchard last year
  - In apple, Blossom Protect immediately after 2nd lime sulfur thinning treatment
  - In smooth-skinned pears in wetter areas, russet risk might be unacceptably high
  - Bloomtime Biological is an alternative, fruit-safe biological material
- Full bloom to petal fall, depending on cultivar russet risk/CougarBlight model risk:
  - Serenade Optimum every 2 to 5 days (most fruit safe)
  - Improved control: Mix Serenade Opt with Cueva (2 to 3 qts/A)
  - Cueva every 3 to 6 days (3 to 4 qts/A) (good blight control but least fruit safe)

Obj. 1a. Understand the specific risks to fruit from the biological material, Blossom Protect: Molecular identification of *Aureobasidium pullulans* strains.

DNA extracted from CF10 and CF40 (positive controls) yielded their respective PCR products (Table 3). PACE- and BRAE-isolates (Table 3), which were recovered from cherry and apple fruit, respectively, all had pink coloration and filamentous edges consistent with *A. pullulans*. Amplification of DNA extractions using the general *A. pullulans* primers (CF40 ITS) resulted in positive amplification of all isolates and positive controls with the exceptions of isolate PACE-A758 and the no-DNA control. Amplification of isolate DNA with the specific CF10 and CF40 primer sets resulted in no PCR products for any of the PACE- or BRAE-isolates.

Table 3.

		PCR Primer set		
Isolate designator:	**Source:**	**CF40 ITS**	**SCAR6**	**SCH3RAPD**
CF10	Blossom protect	100 bp	307 bp	-
CF40	Blossom protect	100 bp	-	962 bp
PACE-A625, -A626, -A705, -A721, -A723, -A724, -A725, -A728, -A731, -A732, -A733, -A735, , -A759, -A760, -A762	cherry fruit, Yakima	100 bp	-	-
PACE-A758	cherry fruit	-	-	-
BRAE-1, -2, -3, -4, -5, -6, -7, -8, -9, -10	apple fruit, Corvallis	100 bp	-	-

Discussion. Specific PCR primers for the amplification of *A. pullulans* strains CF40 and CF10 (Blossom Protect) were used to successfully detect these strains from the product package and from treated pear and apple flowers (data not shown). We used these tools to investigate strain identity of *A. pullulans* isolates from cherries in Washington and Braeburn apple in Oregon; i.e., fruit damaged by *A. pullulans* from orchards that were not treated directly with Blossom Protect. Based on the general and specific primer sets, isolates from damaged fruit were identified as *A. pullulans* but not the Blossom Protect strains of this organism. We are continuing investigation into other methods for specific identification of Blossom Protect strains of *A. pullulans*.

Obj. 2. Evaluation of alternative, organic-approved materials for fire blight suppression.

Gala apple trial. Non-antibiotic materials for fire blight control were evaluated in a 15-yr-old 'Gala' orchard near Corvallis, OR. Trees used in the study averaged 572 flower clusters per tree. Fire blight risk as determined by the heat unit risk model, COUGARBLIGHT, was low to moderate during the bloom period. Perhaps owing to a high dose of pathogen inoculum, disease intensity was very high with fire blight infections on water-treated trees averaging 389 strikes per tree. Compared to the water-treated control, most treatments reduced significantly ($P < 0.05$) incidence of disease; exceptions were Luna Sensation, OxiPhos, BmJ alone, BMJ and Double Nickel combination, Double Nickel alone, and Double Nickel and Cueva Combination. Intermediate levels of control (36 to 53%) were provided by FireLine, Serenade Optimum, Taegro, Oxidate, R42014, and Blossom Protect. The highest levels of control (60 to 64%) were provided by FireWall (streptomycin), the Blossom Protect and Cueva combination, and the mineral material, LMA.

Discussion. From the perspective of certified organic production, the materials Oxidate, Taegro, R42014, and Previsto either have or are expected to be placed on the National Organic Program's approved material list. We will re-evaluate most of these materials in 2015 within integrated programs with other materials (e.g., Blossom Protect). The mineral material, LMA, is being used for fire blight control in Europe, but it is not yet clear if this material will receive organic approval. In addition to the above materials, we used our Golden Delicious block in 2014 to evaluate a mix of experimental phage (bacterial viruses) from Brigham Young University. The mix contained phage that specifically infect the fire blight pathogen. The phage material did not provide significant fire blight control, but will be looked at again in 2015.

Continuing Project Report **YEAR**: 1 of 2
WTFRC Project Number: CP-14-103

Project Title: Identify apple genes associated with apple replant disease resistance

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Cooperator: Gennaro Fazio, apple rootstock breeder, USDA ARS

Total Project Request: Year 1: $54,000 **Year 2: $55,000**

Other funding sources: none

Organization Name: USDA, ARS

Contract Administrator: Charles Myers, Extramural Agreements Specialist		
Email:cwmyers@pw.ars.usda.gov		
Item	**Year 1: 2014-2015**	**Year 2: 2015-2016**
Salaries *	38,790	38,790
Benefits	13,577	13,577
Wages		
Benefits		
Equipment		
Supplies	1,633	1,733
Travel		
Miscellaneous		
Total	54,000	**55,000**

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

OBJECTIVES

1. Select "resistance associated" candidate apple genes based on the results of two previous genomic studies.
2. Screen these genes to assess differential expression behaviors between tolerant and susceptible apple rootstocks.
3. Validate gene-trait associations within an expanded germplasm collection of a rootstock breeding population.

The goal of this proposed study is to identify specific apple genes that are robustly associated with the resistant phenotype of apple rootstocks to ARD pathogens. These genes should provide the foundation for development of DNA markers that will allow screening of germplasm for ARD resistance. The proposed project relates to the highest WTFRC priority subject of "replant disease".

SIGNIFICANT FINDINGS

- Forty apple candidate genes were selected based on previous transcriptome analysis; most of these genes are associated with the production of anti-microbial metabolites in response to pathogen infection.

- Gene expression analysis of candidate genes among M26, B9, G41 and G935 rootstocks suggest an elevated level of expression in the tolerant G935 and G41 in response to *Pythium ultimum* infection and no change in expression level in susceptible B9 and M26.

- Genetically identical plants from a diversity of genotypes, including those from an Ottawa 3 x Robusta 5 breeding population, were generated by tissue culture-based micro-propagation for use in the second year of this study.

METHODS:

The expression patterns of selected candidate genes during pathogen-root interaction were assessed at 1, 2, 3, 7 and 14 days after inoculation with replant pathogens. In the first year, 35-40 candidate genes were screened against field-evaluated tolerant and susceptible apple rootstocks, i.e. susceptible B9 and M26, and tolerant G935 and G41.

1. Selection and expression analysis of candidate apple genes

Expression patterns of candidate genes (35-40) known to function in the biosynthesis of anti-microbial compounds and other defense responses were analyzed after pathogen inoculation using quantitative reverse transcription polymerase chain reaction (qRT-PCR) method.

2. Apple rootstock micro-propagation

Tolerant G41 and G935 and susceptible B9 and M26 apple rootstocks were the core selections for testing the gene-trait relationship in the first year. Tissue-culture generated plants were utilized to examine gene-trait relationships.

3. Pathogen inoculum preparation, inoculation and root tissue collection

Pathogen inoculum preparation and quantification followed established procedures developed in the Mazzola lab. Plants about 2-months old were inoculated by dipping 1/4 to 1/3 of the root system for 5 seconds in a *Pythium ultimum* suspension, or directly planting in pre-prepared inoculum-infested soils. Non-inoculated control was included at each time point. Root tissues collected at designated time points were immediately frozen in liquid nitrogen and stored until used for total RNA isolation, cDNA conversion and gene expression analysis.

RESULTS AND DISCUSSION

1. Selection of candidate genes functioned in root defense response to the ARD pathogen

Based on the results of a recent transcriptome analysis, which focused on the genome-wide gene activation in apple rootstocks during infection by the ARD pathogen *P. ultimum*, and assisted by the knowledge of plant defense mechanisms identified in other patho-systems, forty (40) candidate genes (Table 1) were selected for investigation in this study. The annotated function of the selected genes is shown in Table 1. In most cases, more than one gene was included under each name listed in the first column.

Table 1. List of selected apple genes for testing their association with rootstock resistant phenotypes

Apple gene name	Assigned functions
Aminocyclopropane-1-carboxylate synthase (ACS)	Ethylene biosynthesis
Allene oxide synthase (AOS)	JA biosynthesis
Linoleate 9S-lipoxygenase	JA biosynthesis
Cytokinin hydroxylase	Cytockinin biosynthesis
Ethylene response factor (ERF)	ET/JA signaling
Endochitinase (PR-4) CHIB	Pathogenesis-related protein
MYC2	Transcription factors
WRKY33	Transcription factors
NahG	Pathogenesis-related protein
Chalcone synthase	Phenylpropanoid biosynthesis pathway
Flavonol synthase/flavanone 3-hydroxylase	Phenylpropanoid biosynthesis pathway
Beta-glucosidase	Phenylpropanoid biosynthesis pathway
Squalene monooxygenase	Terpenoid biosynthesis
Biphenyl synthase BIS3	Phenolics biosynthesis
Biphenyl synthase BIS2	Phenolics biosynthesis
Spermidine synthase SPDS	Defense response to pathogen
Cytochrome P450	Cellular oxidation and reduction
2-Oxoglutarate/Fe (II)-dependent dioxygenase	Cellular oxidation and reduction
NAD(P)-linked oxidoreductase	Cellular oxidation and reduction
Mandelonitrile lyase	Hydrogen cyanide generation
Cyanogenic beta-glucosidase	Hydrogen cyanide generation

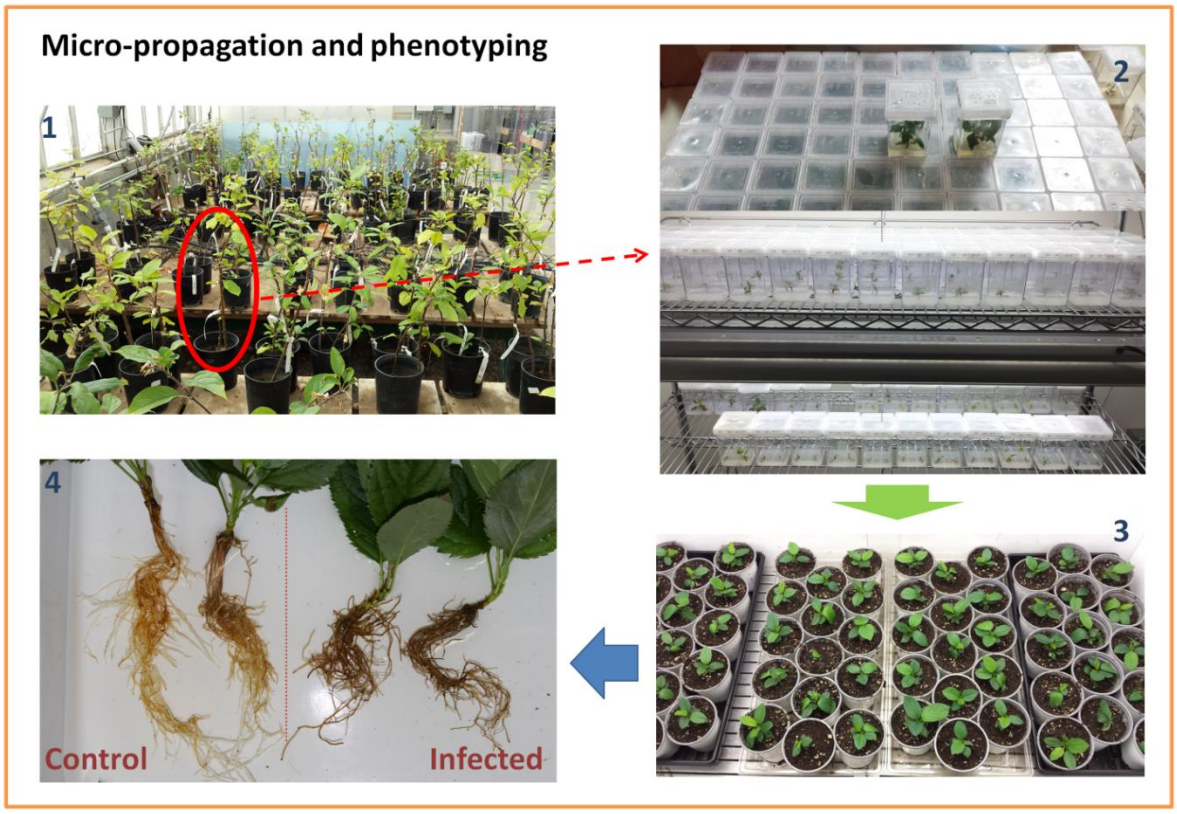
Note: multiple homologous genes or gene family members were included for the genes listed in first column.

2. Micro-propagation of genotype-specific and age-defined plants for apple rootstock varieties

Tissue culture procedures were implemented for micro-propagation of plants for selected apple rootstock varieties. These genotype-specific and age-defined plants are crucial for designing experiments to test gene expression features and resistant phenotype in response to the infection by ARD pathogens (Figure 1). Currently, young plants for the B9 and M26 (susceptible varieties) and

tolerant varieties (G935 and G41) are routinely generated in our lab. Micro-propagation for all 90 plus genotypes in the Ottawa 3 x Robusta 5 population has been initiated; and three-quarters of these genotypes are currently in the "secondary proliferation" stage. Careful and reliable evaluation of the resistant/susceptible phenotypes for the individuals of Ottawa 3 x Robusta 5 population is crucial for examining the gene-trait associations using an expanded rootstock germplasm collection in the second year.

Figure 1. The process of micro-propagation and infection assay for phenotyping the individuals in Ottawa 3 x Robusta 5 population.



3. Cultivar-specific gene expression patterns of selected candidate genes in response to infection by *P. ultimum*.

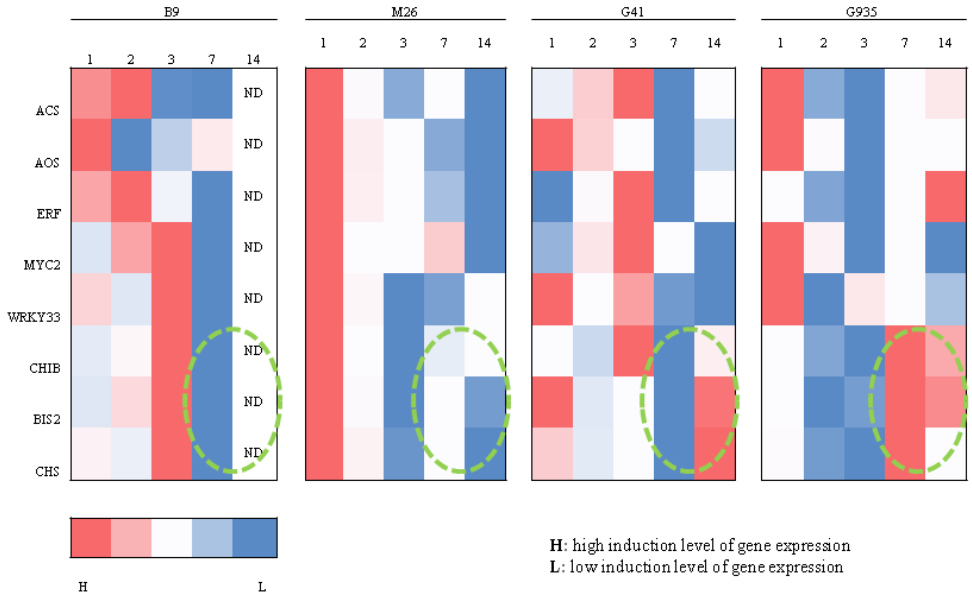
The contrasting gene expression patterns in root tissues among four rootstock varieties were observed using a group of eight candidate genes (Table 2). These candidate genes represent those functioning in the mid- and late-stages of defense responses during root and pathogen interaction, and are primarily responsible for generating antimicrobial proteins and phenolic compounds. The analysis of gene expression values was based on the contrast between control and inoculated tissues at each time point. The results demonstrated different trends in gene expression between resistant and susceptible varieties: i.e. a continuously elevated level of gene expression, particularly at the later stages of 7 and 14 days after infection in the root tissue of tolerant varieties of G935 and G41, compared with those observed in susceptible varieties of B9 and M26.

Table 2. Relative gene expression values of selected genes among various rootstock varieties

	B9					M26				
	1	2	3	7	14	1	2	3	7	14
ACS	11.6*	12.4	6.8	6.7	ND	15.8	2.0	0.7	1.7	0.3
AOS	10.5	1.6	3.0	4.7	ND	58.9	8.4	2.4	0.8	0.2
ERF	13.1	13.7	11.4	0.4	ND	15.1	4.1	2.9	1.7	0.6
MYC2	1.5	1.8	1.9	0.9	ND	2.6	1.1	1.1	1.6	1.0
WRKY33	4.0	3.1	5.2	1.0	ND	12.6	1.9	0.4	0.6	1.4
CHIB	8.8	11.4	39.1	1.0	ND	9.0	1.3	0.4	1.1	1.2
BIS2	14.6	20.6	30.0	0.7	ND	5.4	0.9	0.2	0.8	0.3
CHS	45.7	37.0	98.3	1.5	ND	12.3	2.1	0.3	1.2	0.2
	B9					M26				
	1	2	3	7	14	1	2	3	7	14
ACS	3.0	5.7	11.0	1.3	3.2	9.6	1.4	0.6	3.4	4.3
AOS	14.1	5.2	1.5	0.8	1.3	22.5	1.2	0.4	0.9	0.9
ERF	0.7	2.6	65.1	0.7	1.2	5.2	1.3	0.1	5.3	13.2
MYC2	0.7	1.8	5.0	1.2	0.4	2.6	1.4	0.6	1.3	0.6
WRKY33	4.3	1.6	3.3	0.4	0.2	4.3	0.6	1.3	0.8	0.7
CHIB	4.4	3.4	38.6	1.1	7.8	1.0	0.4	0.2	8.1	5.0
BIS2	5.4	2.3	2.6	0.8	5.2	1.8	0.6	0.8	4.4	3.8
CHS	7.8	3.5	3.8	1.9	16.0	2.7	0.9	0.6	9.4	2.6

*These values represent the comparison between measured gene activity between non-infection control and *Pythium* infected root tissues along the infection process at 1, 2, 3, 7, 14 days post infection (dpi). Data for many other selected genes are being tested.

Figure 2. Heatmap visualizing gene activity during *P. ultimum* infection in each genotype of tested rootstocks



ND indicated no data available due to root tissue necrosis from pathogen infection in the root of B9 plants at day 14.

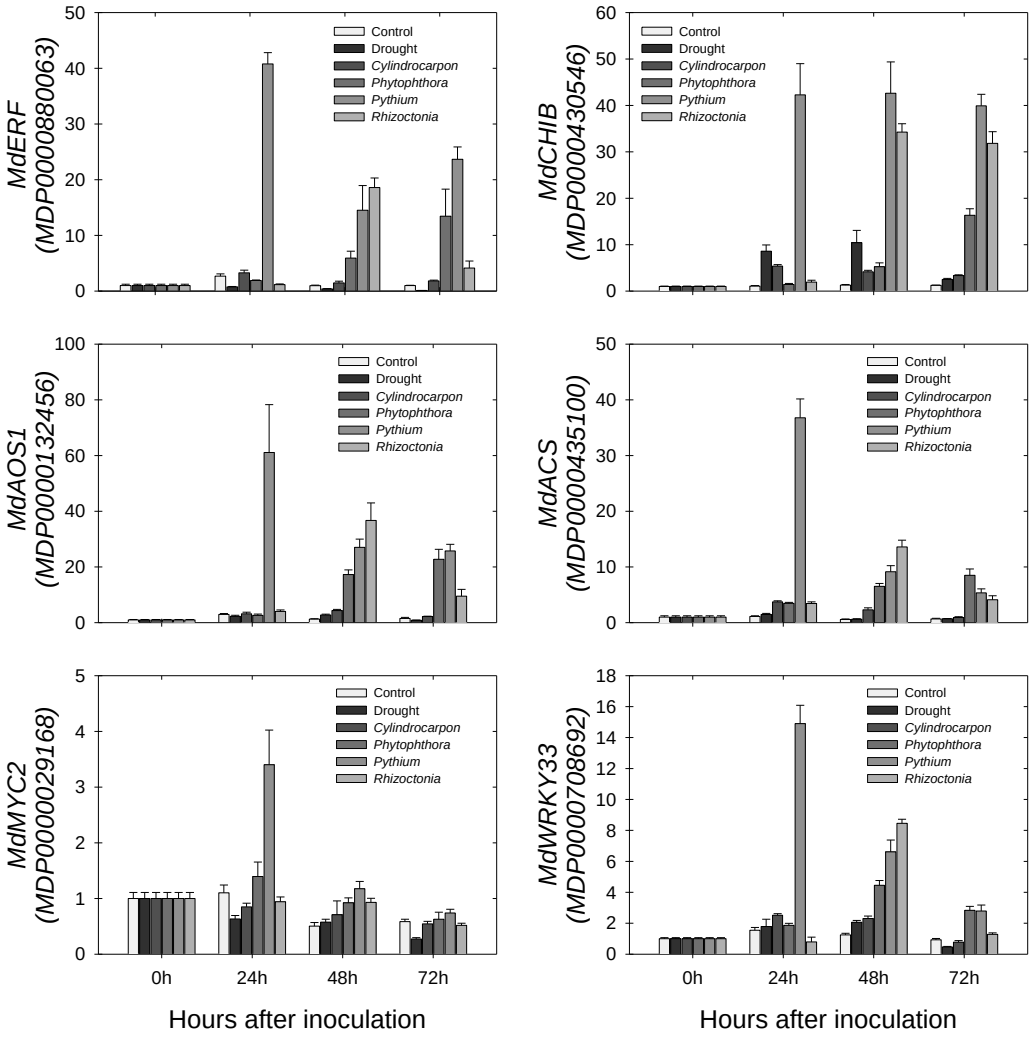
As Figure 2 demonstrates, highlighted with oval shape at bottom right corner of each panel, those genes encoding beta-endochitinase (CHIB), biphenyl synthase (BIS2) and chalcone synthase (CHS), which are involved in the biosynthesis of antimicrobial proteins and phenolic compounds, are continuously up-regulated in the tolerant varieties (G935 and G41) at 7 and 14 days post infection (dpi), but not in the roots of susceptible rootstocks (B9 and M26).

There is a great deal of analogy between the real-world warfare and plant-pathogen interaction. Once plant roots detect the presence of the pathogen (enemy), plants activate defense system and launch a counter-attack with the aim to deter and eliminate the pathogen and/or to repair damaged tissues. In molecular and biochemical terms, there are basically three stages in plant-pathogen interactions, i.e. pathogen detection, activation of defense mechanisms and production or release of anti-pathogen proteins and chemicals. Moreover, the production of these metabolites at the right time, right place and with sufficient strength are critical components of the induced resistant response. It is generally accepted that the quickness, the intensity and the duration of defense responses, in particular in the aspect of producing anti-pathogen reagents, could determine the outcome of plant pathogen interactions (resistance or susceptibility). Finding the key genes control these reactions are crucial to better utilize the genetic potential of natural resistance from apple rootstocks. Although it is still preliminary, these data represent the first observation of differential expression of these genes in the root tissue of different apple rootstock varieties in response to ARD pathogen. Our data are consistent with our working hypothesis regarding the potential role of secondary metabolites in defending invading pathogens in apple root tissues.

4. Differential responses for selected candidate genes in response to various pathogens

In addition to infection by *P. ultimum*, selected candidate genes were also characterized during infection by three other ARD pathogens. In most cases, *P. ultimum* incited the strongest expression of selected candidate genes, particularly at 24 hour post infection.

Figure 3. The comparison of expression patterns in response to the infection to *P. ultimum* and other ARD pathogens for a set of selected candidate genes.



Summary

Forty candidate genes were selected based on their annotated roles in root defense response to ARD pathogen. For a few candidate genes functioning in the production of antimicrobial compounds, the preliminary results indicate the contrast expression patterns between susceptible and tolerant apple rootstocks. The main focus for the second year of the project is to test selected genes among expanded rootstock genotypes, i.e. the individuals from Ottawa 3 x Robusta 5 population. The expected results will reduce the number of candidate genes to those with more robust association between gene expression patterns and resistant traits. The results from this study should contribute to the exploration of the beneficial gene pools for more efficient and precise utilization of natural resistance to tackle the apple replant disease.

CONTINUING PROJECT REPORT **YEAR**: 2 of 3
WTFRC Project Number: CP-13-102A

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

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Cooperators: Max Suckling, Plant and Food Research, P. O. Box 51, Lincoln 7608, New Zealand and Jim Walker, Plant and Food Research, Cnr Crosses and St. Georges Roads, Havelock North, Hawkes Bay, New Zealand 4130

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
Wages		$16,000	$16,000
Supplies		$3,000	$3,000
Total	0	$19,000	**$19,000**

OBJECTIVES

The overall objective or goal of the project is to develop and demonstrate control of codling moths in orchard plots using the attract-and-kill approach (A & K). Prior research has led to the use a sticky trap as the A & K 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 and 2014 field seasons).

1. We determined specifications for a sachet system to replace the vials used in prior work.
2. 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.
3. 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.
4. One-acre Washington field tests showed strong reductions in catches of female codling moth in kairomone traps but not males in pheromone traps.
5. Four-acre Washington field tests showed much less infestation of apples in a heavily infested orchard, following 30 days of kairomonal trapping with 50 traps per acre.

METHODS (for 2015 field season).

1. 4-acre plot tests of effect of A & K on fruit infestation rates.
   Three four-acre field plot tests will be conducted to determine consistency and range of reductions in apple fruit infestation. Treated plots will receive 50 A & K traps per acre along with 2 pheromone, 2 kairomone, and 1 blacklight monitoring traps. Control plots will receive pheromone, kairomone, and blacklight monitoring traps, but no A & K traps. All traps will be checked and maintained weekly for four weeks. Treatment and control plots will be separated by a buffer equivalent to another 4-acre plot, and treatment and control plots will be paired within orchards. Infestation rates will be determined at the start and the finish of the 4 week test, by visually inspecting 1000 apples per plot, as 40 apples per tree for 5 trees in each of 5 rows.
   a. The first of these tests will be conducted during the summer flight in New Zealand, which will be from late January to late February.
   b. The second test will be conducted during the spring flight in Washington.
   c. The third test will be conducted during the summer flight in Washington
2. One-acre plot tests of effect on numbers of female codling moth in mating disruption orchards. This experiment will involve 5 pairs of treated and control plots in commercial apple orchards. Each one-acre treated plot will receive 50 A & K traps, along with 2 pheromone, 2 kairomone, and 1 blacklight monitoring trap. Control plots will receive only the three monitoring traps, but no A & K traps. Monitoring traps will be deployed for three days to assess relative population levels, followed by deployment of A & K traps and monitoring traps for an additional 7 days. Traps will be checked at days 1, 3, 5, 7, and 10.

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.

Earlier 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 refer to as "knockdown".

Much of the effort in 2014 replicated and confirmed knockdown of female codling moths in orchard plots, and then tested the hypothesis that the knockdown of moths results in reduced infestation of apples.

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.

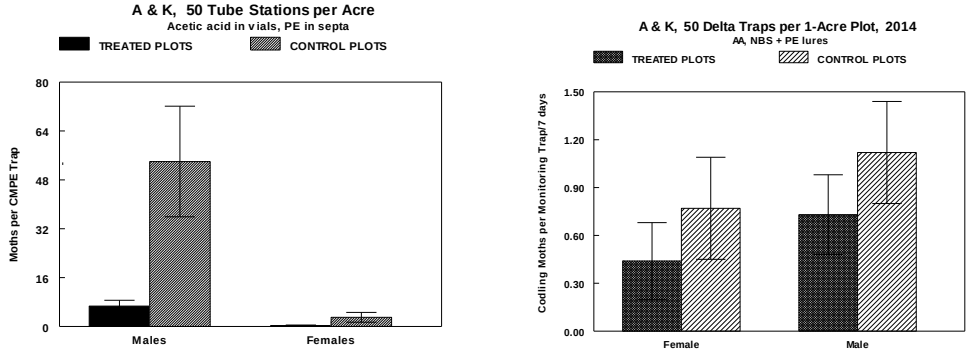

Figure 1. Mean numbers of codling moths captured in monitoring traps in A & K plots. On the left, in 2013, numbers of moths were greatly reduced in kairomone-baited monitoring traps in plots with tube shaped A & K traps. On the right, in 2014, numbers of moths in kairomone-baited monitoring traps were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

Four acre plot infestation reduction. A single pair of 4-acre plots were set up and maintained for four weeks in August of 2014; to compare codling moth infestation rates in apples with and without deployment of attract-and-kill. A & K traps baited with acetic acid + pear ester + N-butyl sulfide, were evenly spaced at 47 per acre. Treated and control plots were each monitored with four blacklight traps, 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. These samples were 20 apples inspected per tree, for 10 trees per row, for 10 rows of trees per plot.

Numbers of codling moths 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 and numbers of both sexes in blacklight 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 (Figure 3). Over the course of the four weeks of the test, 383 female and 383 male codling moths were captured in the A & K traps.

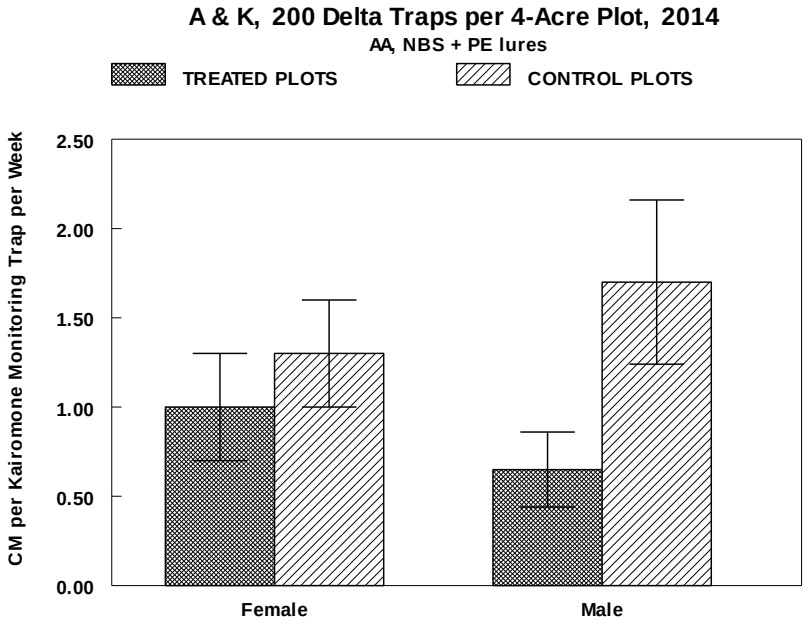

Figure 2. Mean numbers of male and female codling moths captured on monitoring traps baited with kairomone lures, in 4 acre plots treated with 200 A & K traps in untreated control plots.

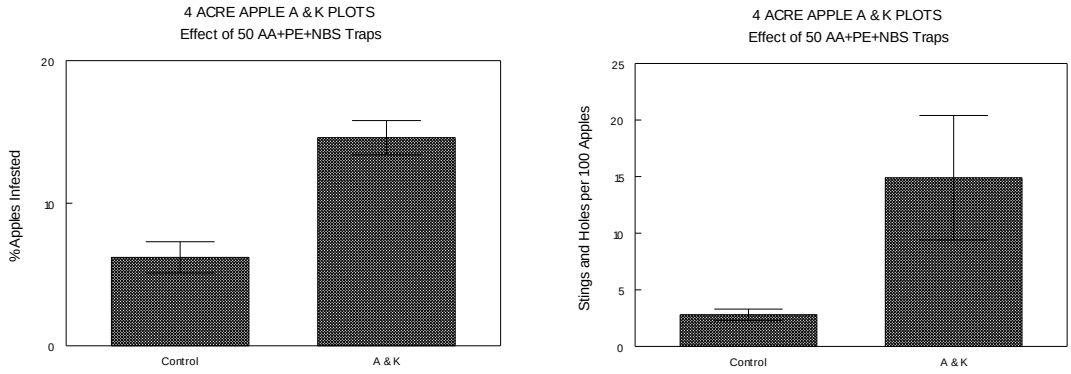

Figure 3. Mean percentages of apple fruit damaged by codling moth, and mean percentages of stings and holes per 100 apples, in apple blocks that were untreated controls, or were treated with attract and kill traps.

Results of the field plot tests consistently indicate an impact of the A & K traps on codling moth; both reducing the numbers of moths in plots, and reducing the codling moth damage to fruit. However, the larger scale plot tests need to be replicated before firm conclusions can be drawn. This is underway in New Zealand and planned in Washington for the coming field season.

One might ask why the results are not more consistent and dramatic. That is, with ca 50 A & K traps per acre, using a bisexual lure, why do we not see a complete elimination of codling moths in the plot, including in pheromone and light traps. 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. *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 what is important is protecting 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 2015 is the replicating of the 4 acre plot tests of the 50 kairomone A & K traps per acre, to provide rigor to any conclusions regarding efficacy in protecting fruit.

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

CONTINUING PROJECT REPORT **YEAR**: 2 of 3
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, OR

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

Other funding sources: **SCRI grant, $10,000**

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	$12,500	$12,500	$12,500
Benefits	1,500	1,500	1,500
Supplies	5,000	5,000	5,000
Travel	1,000	1,000	1,000
Total	$20,000	$20,000	**$20,000**

Footnotes: Personnel costs are for a ¼ time GS-5 technician to rear insects, conduct assays and field tests, and a summer student to assist with plant sampling and assays, and field tests. Supplies needed are GC gases, solvents, chemicals for EAD, assays and field tests, olfactometer glassware, and materials for traps for field testing of chemicals. Travel costs are for trips to multiple field sites in Oregon and Washington.

Budget 2
Organization Name:Univ. California, Riverside **Contract Administrator: Robert Chan**
Telephone: (951) 827-7986 **Email address: rchan@ucr.edu**

Item	2013	2014	2015
Wages	$12,035	$12,276	$12,522
Benefits	4,828	4,915	5,013
Supplies	3,137	2,809	2,465
Total	$20,000	$20,000	**$20,000**

Footnotes: Personnel costs are for a 30% time organic chemist postdoctoral scientist. It will be essential to have a highly trained organic chemist for rapid identification and synthesis of possible attractants, to provide test materials as rapidly as possible and avoid holding up the biological/ecological parts of the project. Salary and benefit rates are mandated by the state of California, and include a 2% projected increase each year. We also request funds in each year for chemistry supplies, to include solvents, columns, reagents, disposables, and equipment maintenance costs.

OBJECTIVES

The overall objective or goal of the project is 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 are 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 both signal and response 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. This provides the opportunity to isolate a male-produced sex attractant, and a bioassay method to use for that purpose.
8. 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.
9. 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.
10. An EAD system was modified for the BMSB antennae and its effectiveness was demonstrated using BMSB antennae and published pheromone chemicals. This system will be used in further efforts to identify both pheromones and kairomones.
11. The volatile chemistry of BMSB males and females was characterized and compared, to provide a baseline from which to detect and determine chemical signaling.
12. The volatile chemistry of BMSB defensive/alarm secretions was characterized.

METHODS (for 2015)

Sex attraction. We will use the sex attraction assay developed and demonstrated in 2014 to pursue isolation and characterization of the attractive compounds involved in female attraction to males. This will involve a series of experiments to 1) demonstrate BMSB attraction to a solvent extract of a volatile collection trap, followed by 2) GC-EAD to determine compounds detected by the BMSB, followed by 3) bioassays to determine attractiveness of individual and combined EAD-active compounds. The results of this effort will be compared to published pheromones, to determine the nature of subsequent experiments to test chemical blends.

Alarm pheromone and defensive chemistry. We will use the results of our alarm pheromone experiments to design a bioassay for alarm pheromone responses by BMSB. Samples of volatiles emitted by disturbed BMSB will be analyzed by GC-EAD to determine which compounds are detected by BMSB antennae and determine putative alarm pheromone compounds. Each EAD active compound and combinations of those compounds will be tested for alarm pheromone activity.

Similarly, we will use the ongoing tests of repellency of paper wasps to design assays to determine which odorants of disturbed BMSB have a defensive role. Volatile compounds produced by disturbed BMSB will be tested using GC-EAD using *Polistes* paper wasp antennae, followed by olfactometer bioassays to determine repellency to paper wasps.

Host Finding. When the characterization of the male-produced pheromone is completed, these experiments will be modified to evaluate BMSB response to that pheromone blend in combination with the odors of preferred plants, to test the hypothesis of positive interaction. In collaboration with WSU Wenatchee, we will be determining field preferences of BMSB for host plants. Results of that field work may suggest additional plant species to evaluate in the laboratory for attractiveness to the stink bug.

Chemical blend testing. The results of the laboratory work will suggest chemical blends and modification to blends to evaluate in the field. Those chemical blends will be tested at sites in western Oregon and in West Virginia. Blends of interest will be compared to unbaited traps and to a standard commercial lure produced by Sterling International, Spokane. These blends should involve a male-produced pheromone resulting from work described above, and there may be a need to test blends of compounds claimed to be sex and aggregation pheromones but with alarm pheromone or defensive chemical activities.

RESULTS AND DISCUSSION.

We have been able to demonstrate several behaviors in the BMSB that relate to aggregation and orientation responses. 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 response, and thigmotaxis which is 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; Guedot et al. 2009; MacKenzie et al. 2009), was used to determine BMSB responses to plant odor. 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. We also determined that the bug responses are much faster in a vertical orientation compared to a horizontal orientation.

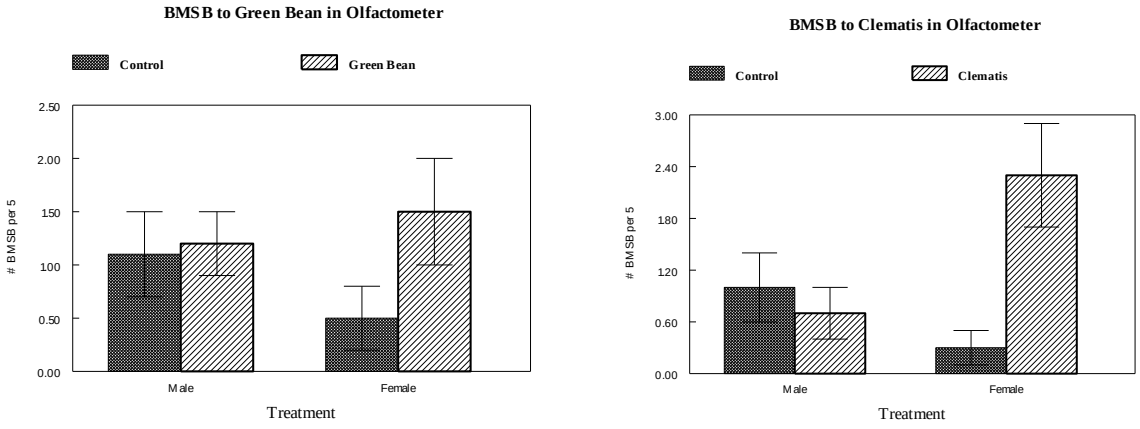


Figure 1. Numbers of female BMSB responding to airflow from over plant material in a Y-tube or choice olfactometer. N = 60, as 12 groups of 5. Control is airflow through an empty jar.

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 the olfactometer assay. Each assay involved the testing of 60 stink bugs one at a time, as 6 batches of 10, with the treatments replaced for each set. Responses of males to either male or female shelters was weak, while responses of females to male shelters was strong (Figure 2). A much stronger yet response was seen when the assays were conducted in the scotophase under red light (Figre 2). This provides the opportunity to isolate and identify a male produced sex attractant, and a bioassay method to use for that purpose.

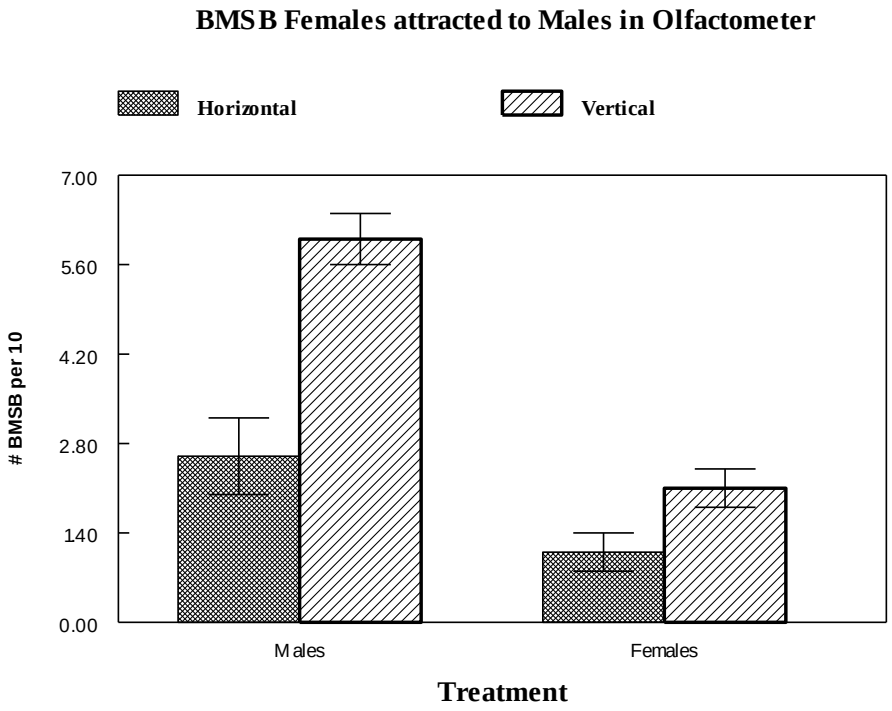


Figure 2. Numbers of female BMSB responding to airflow from over a male occupied shelter versus a female occupied shelter in a Y-tube or choice olfactometer. N = 60, as 12 groups of 5. The experiment was conducted twice; once with the olfactometer tubing horizontal, and again with the tubing vertical.

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. This work is being pursued to determine the functions alarm pheromone, and to understand the roles of complex BMSB body odor chemistry.

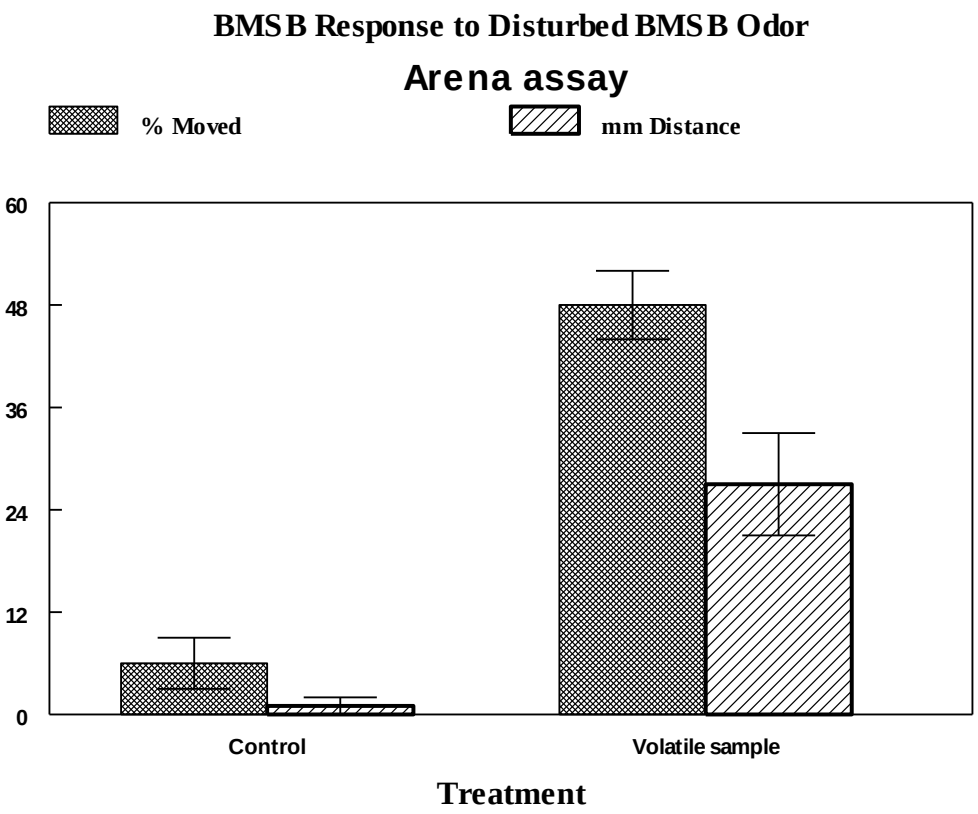
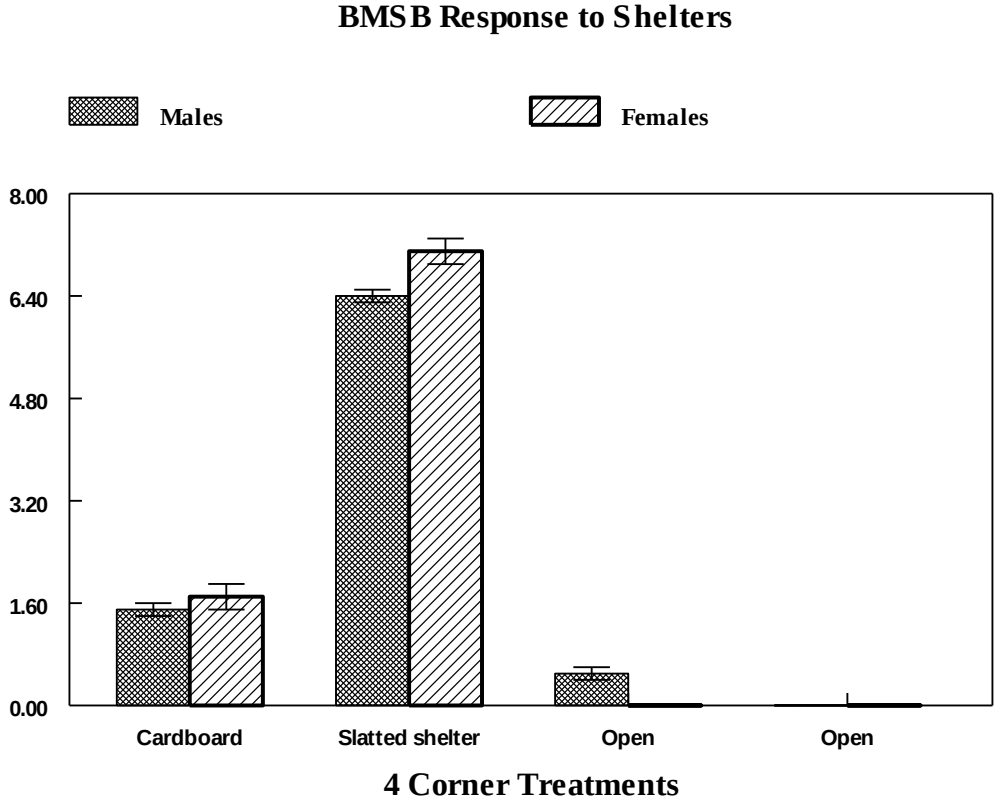


Figure 3. Numbers of male and female BMSB (combined) responding to airflow from a single disturbed BMSB. Movement was noted, as well as the distance moved in the 20 second long test. N = 100 as 20 groups of 5. Control is air from an empty jar.

BMSB Thigmotaxis. Like cockroaches, BMSB appear to prefer seeking out and hiding 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 4). This behavior may be an important aspect of one type of aggregation behavior and was the basis for our assay for sex attraction.



Coupled Gas Chromatographic-Electroantennographic Detection (GC-EAD) Analysis. Coupled GC-EAD analysis was performed using an Agilent 6890N gas chromatograph equipped with a DB-5 capillary column (30 m×0.25 mm ID, 0.25 μm film thickness; Agilent Technologies, Wilmington, DE, USA) in the splitless mode with 1 min sampling. The oven temperature was programmed for 5 min at 40 °C, 15 °C/min increase to 250 °C, and then held for 5 min. Injector temperature was set at 250 °C. Helium gas was the carrier at a constant flow rate of 2 ml/min. The column effluent was split 1:1 in the oven via an outlet splitter system (OSS-2, SGE Analytical Science, Austin, TX,USA) with nitrogen as a make-up gas (15ml/min). One arm of the splitter led to the flame ionization detector (FID) (260 °C) and the other to the heated EAD port (260 °C) (Syntech, www.syntech.nl) introduced into a humidified air stream (300 ml/min) directed toward the mounted antennae of the 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 will be used in further efforts to identify both pheromones and kairomones, using the 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.

Field sampling of stink bugs. For an additional year, about 100 separate 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 in Washington, principally in Yakima County, yielded nearly 700 stink bugs, all which were identified to species. Two BMSB were collected in pheromone traps in the city of Yakima, and several BMSB were found in two beating sheet samples made in the city of Sunnyside.

REFERENCES CITED

Cha, D. H., T. Adams, P. J. Landolt, and H. Rogg. 2012. Identification and field evaluation of wine and vinegar volatiles that mediate attraction of spotted wing drosophila, *Drosophila suzukii*. J. Chem. Ecol. 38: 1419-1431.

Guedot, C., D. R. Horton, and P. J. Landolt. 2009. Attraction of male winterform pear psylla to female-produced volatiles and to female extracts and evidence of male-male repellency. Entomologia Experimentalis et Applicata 139: 191-197.

Landolt, P. J., J. Brumley, C. Smithhisler, L. Biddick, and R. W. Hofstetter. 2000. Apple fruit infested with codling moth are more attractive to neonate codling moth larvae and possess increased amounts of E,E-alpha farnesene. Journal of Chemical Ecology 26: 1685-1699.

MacKenzie, J. K., P. J.Landolt, and R. S. Zack. 2009. Attraction to ornamental peony, *Paonia*, Paoniceae) by *Polistes dominulus* Christ (Hymenoptera: Vespidae) demonstrated using olfactometers. J. Kansas Entomol. Soc. 79:231-238.

Medal, J., T. Smith, A. Fox, A. S. Cruz, A. Poplin and A. Hodges. 2012. Rearing the brown marmorated stink bug *Halyomorpha halys* (Heteroptera: Pentatomidae). 95: 800-802.

Nielsen, A. L., G. C. Hamilton, and D. Matadha. 2008. Develomental rate estimation and life table analysis for *Halyomorpha halys* (Hemiptera: Pentatomidae). 37: 348-355.

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

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

PI: Vincent P. Jones
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Address: 1100 N. Western Ave
City/State/Zip: Wenatchee, WA 98801

Co-PI (2): Ute Chambers
Organization: WSU-TFREC
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City/State/Zip: Wenatchee, WA 98801

Collaborator: Betsy Beers, WSU-TFREC

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

Percentage time per crop: Apple: 50% Pear: 20% Cherry: 20% Stone Fruit: 10%

Other funding sources

Agency Name: WSU-Extension
Amt. awarded: $266,344
Notes: The amount funded is the contribution that WSU-Extension provides for DAS support and maintenance + an additional 1 FTE for a second programmer for one year.

Budget 1
Organization: WSU-TFREC **Contract Administrator:** Carrie Johnston/Joni Cartwright
Telephone: 509-335-4564/509-663-8181 x221 Email: carriej@wsu.edu / joni_cartwright@wsu.edu

Item	2014	2015	2016
Salaries[1]	42,129	43,814	45,567
Benefits[2]	14,983	15,582	16,205
Wages	12,480	12,979	13,498
Benefits[3]	262	273	283
Equipment	0	0	0
Supplies[4]	2,500	2,600	2,704
Travel[5]	2,800	2,912	3,028
Miscellaneous	0	0	0
Plot Fees	0	0	0
Total	75,154	**78,160**	81,285

Footnotes:
[1] U. Chambers Y1-3 (0.5 FTE); T. Melton Y1-3 (0.25 FTE)
[2] 33.5%
[3] 2.1%
[4] includes lab and field supplies
[5] w/in state travel

Objectives:
1. Develop models for mites and aphids using literature data and validate the information as needed.
2. Validate natural enemy models already developed in the SCRI biological control grant.
3. Re-evaluate the San Jose scale model and its biofix and accuracy.

Significant Findings:

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

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

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

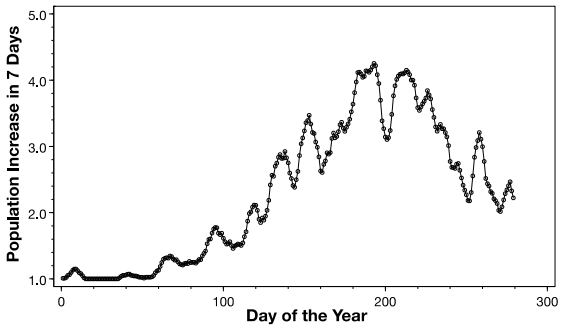
We obtained data for the egg hatch of ERM this past year by collecting overwintering eggs on twigs and bringing them to the lab, placing them into growth chambers and recording daily when egg hatch occurred. The ERM eggs were held at either 61ºF or 72ºF. In addition, we evaluated sticky tape as well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

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

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

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

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

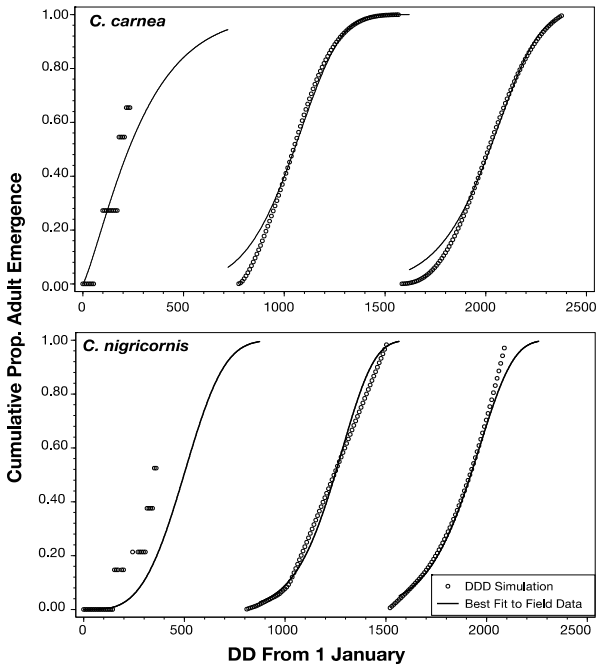


Woolly apple aphid: The literature data on WAA has been synthesized and a model that shows the population growth rate over the season has been developed. As with the ERM and TSSM, the literature review actually discovered more information than we thought and it is possible that we might be able to develop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

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

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

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



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

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

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

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

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

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

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

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

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

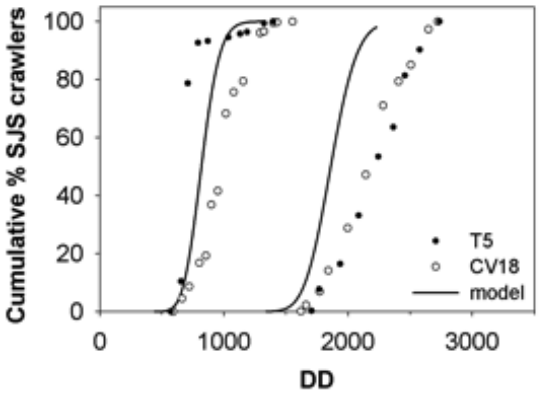


Fig. 3. Comparison of the current SJS model and field data for SJS crawlers in two experimental orchards.

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

pheromone traps as well as on sticky tapes and whether it is necessary to continue using the 275 DD as biofix.

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

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

Reference:

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

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

Project Title: Study of molecular mechanisms to preserve codling moth control agents

PI: Stephen F. Garczynski
Organization: USDA-ARS YARL
Telephone: 509-454-6572
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Address: 5230 Konnowac Pass Rd
City/State/Zip: Wapato, WA 98951

Cooperators: Tom Unruh, YARL; Rodney Cooper, YARL; Ron Nachman, USDA-ARS, Texas

Total Project Request: **Year 1**: $39,000 **Year 2**: $42,000 **Year 3**: **$45,000**

Other funding sources : None

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

Item	(2013)	(2014)	(2015)
Salaries[1]	26,100	27,000	28,000
Benefits	1,900	9,000	9,000
Wages			
Benefits			
Equipment			
Supplies	11,000	6,000	8,000
Travel			
Miscellaneous			
Plot Fees			
Total	39,000	42,000	**45,000**

Footnotes: [1]Salaries are to support one part time GS-6 level technician (30 hrs / wk)

OBJECTIVES

1) Determine the effects of Altacor, Delegate, Calypso and granulosis virus on gene expression levels of codling moth heat shock (stress response) proteins and detoxification enzymes. The purpose of this objective is to identify gene transcripts that are elevated in response to sublethal doses of Altacor, Delegate, Calypso, and granulosis virus. We will focus on the most likely candidates based on studies performed in other moths, which include transcripts encoding detoxification enzymes (cytochrome P450s, esterases, and glutathione *S*-transferases), or increased expression of stress response proteins (heat shock proteins). Induced expression of gene transcripts encoding these proteins has been correlated with their potential as "insecticide resistance" factors. To complete this objective we will first clone gene transcripts encoding codling moth heat shock proteins and detoxification enzymes. From previous codling moth transcriptome data, we have identified gene transcripts encoding 24 different heat shock (stress response) proteins, 20 cytochrome P450s, seven esterases and 10 glutathione *S*-transferases. Once these transcripts are cloned and their nucleotide sequences verified, we will design oligonucleotide primers for use in quantitative PCR (qPCR). Once qPCR conditions are established, we will then quantify the expression levels of gene transcripts encoding heat shock proteins and detoxification enzymes from untreated eggs, neonates, and adults or those exposed to heat, cold, or sublethal doses of Altacor, Delegate, Calypso and granulosis virus. This will allow us to determine if any of these proteins have a potential role in resistance.

2) Determine the effectiveness of PBAN antagonists to inhibit codlemone production by codling moth females. Another way to prevent insecticide resistance is to use control agents that utilize a different mode of action to help control insect pests. Dr. Ron Nachman (USDA-ARS, Texas) has developed and synthesized several PBAN antagonists that reduce or eliminate pheromone biosynthesis in the tobacco budworm, *Heliothis virescens*. Because of the amino acid diversity of codling moth PBAN vs tobacco budworm PBAN (only 45 % similarity), it will be prudent to determine the effectiveness of the PBAN antagonists **before** they are fully developed into commercial products. This collaboration provides us the unique opportunity to test the PBAN antagonists on codling moth; first to determine if they work, then to determine dosage and timing of applications. To complete this objective, we will clone gene transcripts encoding codling moth PBAN receptors (PBANR) and then to express the cloned receptors in mammalian cell lines. We will then use cell based assays to verify PBANR activity and determine if PBAN antagonists block receptor activity. We will also test PBAN antagonists on female codling moth to determine biological activity in pheromone biosynthesis inhibition and to see if these compounds disrupt mating.

SPECIFIC OBJECTIVES FOR YEAR 3

1) Complete cloning of transcripts encoding detoxification enzymes and heat shock proteins.
2) Determine expression levels of gene transcripts encoding detoxification enzymes (Glutathione S-transferases and cytochrome P450s) in response to sub-lethal doses of Altacor (Rynaxypyr), Delegate (Spinosad) and Calypso (Thiocloprid).
3) Determine if PBAN antagonists prevent codling moth male attraction to females in flight tunnel and mating bioassays.

SIGNIFICANT FINDINGS (ACCOMPLISHMENTS)

- Cloned and confirmed detoxification enzymes/heat shock proteins
    - 8 transcripts encoding putative esterases
    - 10 transcripts encoding putative cytochrome P450 monooxygenases
    - 26 transcripts encoding putative glutathione *S*-transferases
    - 12 transcripts encoding putative heat shock proteins
- Cloned and confirmed protein targets of insecticides

- Nicotinic acetylcholine receptor subunits (targets of neonicotinoids and spinosads)
- Ryanodine receptor (target of rynaxypyr – Altacor)

METHODS (PROJECT APPROACH)

Methods for Objective 1

1) Cloning gene transcripts that encode codling moth heat shock (stress response) proteins and detoxification enzymes. Through prior WTFRC funding, two codling moth transcriptomes have been generated. Using bioinformatics approaches, we will identify gene transcripts encoding heat shock (stress response) proteins, cytochrome P450 monooxygenases, carboxylesterases, glutathione *S*-transferases and protein targets of insecticides. We will use the nucleotide sequences determined from the codling moth transcriptomes to design gene specific primers that can be used in PCR reactions to clone full length mRNA molecules that encode each protein of interest.

2) Determination of gene transcript expression by quantitative PCR. Quantitative PCR (qPCR) will be used to determine the relative amount of gene transcripts expressed in codling moth. Sequence specific primers will be designed for each gene transcript of interest, and the amount of transcript will be determined for each transcript using real time qPCR detection. This technique has been developed in my lab for analysis of heat shock protein expression differences in untreated and heat treated insects. First, we will optimize qPCR conditions for each gene transcript of interest. After optimization, we will determine basal levels of gene transcripts expressed in codling moth eggs, neonates, and adults. Once the baseline expression levels are determined, we will perform the qPCR analysis on untreated or treated (sublethal doses of Altacor, Delegate, Calypso, and granulosis virus) codling moth to determine the effects on gene transcript levels. Of particular interest will be gene transcripts in which expression levels are significantly increased.

Methods for Objective 2

1) Cloning and expression of the codling moth PBAN receptor (PBANR). Oligonucleotide primers will be designed against the nucleotide sequence we have previously obtained for the codling moth PBANR and PCR will be used to amplify the full-length transcript. Once the full-length transcript is obtained, we will clone the protein encoding portion into a mammalian expression vector and incorporate this clone into a mammalian cell line.

2) Determine if the codling moth PBANR is biologically active. Cell based assays will be used to confirm the identity of the codling moth PBANR. Mammalian cell lines expressing PBANR will be exposed to synthetic PBAN (based on the amino acid sequence of codling moth PBAN) and will be monitored for activation of the cells' second messenger pathways. Once we confirm that the synthetic PBAN activates PBANR signaling, we will use the cell based assay to determine the effectiveness of the various PBAN antagonists (provided by Dr. Ron Nachman, USDA-ARS, Texas).

3) Biological assays to determine the effectiveness of PBAN antagonists. We will use pheromone production and mating assays to determine if the PBAN antagonists inhibit codlemone production and reduce female attractiveness to codling moth males. Pheromone production will be determined by injecting females at the 2^{nd}-4^{th} hour of photophase with either synthetic PBAN or with the antagonists. After treatment, pheromone glands will be dissected and placed in hexanes and pheromone quantified by gas chromatography. Mating bioassays will be performed in two ways; first we will use flight tunnels to determine the attractiveness of untreated controls or PBAN antagonist-treated with females to codling moth males. Secondly, we will set up mating bags pairing codling moth males with either untreated or PBAN antagonist-treated females. We will then quantitate egg

production and viable offspring to determine the effectiveness of the antagonists in close quarter matings.

RESULTS AND DISCUSSION

Cloning codling moth gene transcripts encoding detoxification enzyme and heat shock proteins

The main goal of this project is to provide information that will help prolong the use of successful codling moth control agents (Altacor, Delegate, Calypso) in the orchard. A major mechanism of insecticide resistance in the field is through detoxification of control agents by the esterases (EST), cytochrome P450 monooxygenases (CYP) or glutathione *S*-transferases (GST). Detoxification usually results from increased expression of one or more of these proteins. Originally, we proposed to clone and characterize gene transcripts encoding 20 CYPs, seven ESTs and 10 GSTs, however last year we reported the presence of transcripts encoding at least 80 CYPs, 50 ESTs and 22 GSTs. Because of the increase of potential transcripts we need to clone and characterize, we are now focusing on CYPs and GSTs. The rationale for this choice is that CYPs and GSTs have been the enzymes most often implicated in resistance to neonicotinoids, spinosads and rynaxypyr.

In this past year, we have cloned codling moth gene transcripts encoding CYPs and GSTs. We first focused on GSTs to verify the 22 transcripts identified from the transcriptome. Specific oligonucleotide primers were designed and used in PCR amplification reactions to clone each of the GSTs. From the sequences obtained from cloning efforts, we now have the complete sequences of 18 GSTs and partial sequence for eight others (we now have a total of 26 GST encoding transcripts). This information has been used to design oligonucleotide primers for assays to quantitate expression levels of the GSTs, and we will be performing those experiments in this upcoming year. In addition to the GSTs, we have also cloned and sequenced gene transcripts encoding 10 of the 80 CYPs. We are continuing these efforts to verify the remaining 70, and expect to have all CYPs cloned by Spring. The information we generate from the cloning efforts will then be used to proceed with assays to quantitate expression levels of these transcripts. We expect all cloning and expression experiments will be completed in this upcoming year. When we complete the cloning and expression aspects of this project, we will have a method that can be used to determine if resistance to Altacor, Delegate or Calypso (and other insecticides past or future) is caused by CYPs or GSTs.

Cloning codling moth transcripts encoding protein targets of Altacor, Delegate and Calypso

The protein targets of Delegate and Calypso are nicotinic acetylcholine receptors (nAChR), proteins that function in nerve transmission. Last year we provided data characterizing eight nAChR subunits, and this year completed the characterization of four others. Of particular relevance to this project, nucleotide sequences for two of the nAChR subunits (α6 and β1) implicated in neonicotinoid and spinosad resistance have been cloned. This sequence information comes with some good news; we were not able to detect any mutations in these nAChR subunits that are known to cause resistance. From the results of the nAChR portion of this project, we now have a method that can be used to monitor target site resistance to Delegate or Calypso. A manuscript presenting the results of our nAChR work has been accepted for publication (Martin and Garczynski, Putative nicotinic acetylcholine receptor subunits express differentially through life cycle of codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae)).

The ryanodine receptor, a protein that is important in nerve and muscle function, is the protein target of Altacor. The gene transcript that encodes this extremely large protein (4000 – 5000 amino acids) is over 15,000 nucleotides in length. Because it is almost impossible to clone the full length sequence of the ryanodine receptor transcript in one piece, we are using the strategy of cloning this transcript in 500 – 1000 nucleotide overlapping portions that can be assembled to provide us with the full length sequence. We started our cloning efforts with a region of the transcript encoding the

portion of the ryanodine receptor implicated in target site resistance to Altacor. With this sequence information we can now develop an assay that can be used to monitor codling moth for target site resistance to Altacor.

SUMMARY

We have now identified (by data mining the WTFRC-funded codling moth transcriptome) and confirmed the presence of (by PCR amplification and cloning) gene transcripts encoding protein targets for Altacor (ryanodine receptor), Delegate and Calypso (nAChRs for both). This information has allowed us to develop methods that can be used to monitor field populations for target site resistance. Through the codling moth transcriptome, we have also identified 80 CYPs and 22 GSTs and have completed cloning 10 CYPs and 26 GSTs. This information is currently being used to develop assays to monitor expression levels of CYP and GST transcripts that will be used in the future to monitor detoxification mechanisms of insecticide resistance. We expect that this assay will be completed by the end of the year. In addition to characterization of insecticide targets and detoxification, we will be determining the effects that PBAN antagonists on codling moth female attractiveness to males in flight tunnel bioassays

CONTINUING PROJECT REPORT: *No Cost Extension* **YEAR**: 1 of 3
WTFRC Project Number: CP-14-102

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

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Total Project Request: **Year 1:** $10,000 **Year 2: $0** **Year 3:** $3,000 **Year 4:** $3,000

Other funding sources: None

Budget 1
Organization Name: WSU **Contract Administrator:** Carrie Johnston
Telephone: 509 335-4564 **Email address:** carriej@wsu.edu

Item	2014	2015	2016	2017
Salaries				
Benefits				
Wages				
Benefits				
RCA Room Rental				
Shipping				
Supplies	2000		3000	3000
Travel	8000			
Plot Fees				
Miscellaneous				
Total	$10,000	**0**	$3,000	$3,000

Footnotes: *No expenses were expended in 2014 due to delay of germplasm collection prior to the end of the season of drone-availability. Arrangements for honey bee germplasm with our in-country collaborator Dr. Jashenko are now in place for 2015. A no-cost extension is requested to expend expenses and conduct Year 1 objectives in 2015.*

JUSTIFICATION

Central Asia is the center of origin for the domestic apple and pear. As such it represents a region where germplasm for apple breeding and parasites of apple pests have often been collected. Thus, both USDA and University researchers have made numerous expeditions for these purposes to Kazakhstan, Kyrgyzstan and Uzbekistan. These efforts have resulted in increased disease resistance and greater cross breeding opportunities for domestic apple production. Like apples, honey bees are not native to the US and the US honey bee population has limited genetic diversity. Only a few of the 28 unique subspecies of honey bees were ever imported into the US. WSTFRC funded research in Central Asia led to discovery of a new honey bee subspecies (*Apis mellifera pomonella*) in the wild apple forests of Kazakhstan. Despite early indications that this bee would be highly suitable for apple pollination, we were unable to import this subspecies into the US due to USDA-APHIS restrictions.

Honey bee subspecies have unique characteristics and life histories that have evolved to maximize their ability to take advantage of local floral sources. A majority of US breeding stock is currently derived from a Mediterranean subspecies (the Italian honey bee) that does very well in California and Florida and in managed migratory operations. Washington State beekeepers do not have enough hives to meet the annual pollination needs of the Washington tree fruit crop and the industry depends on migratory pollination services. With continuing hive losses each year, the stability of migratory pollination services is under constant threat. One area identified for improved stock development in bees is increasing genetic diversity from which to breed. The importation of germplasm from the subspecies that evolved in apple forests would improve the genetic diversity of commercial honey bees and introduce novel alleles that could improve pollination efficiency in Washington tree fruit orchards.

Honey bee colonies are under constant threat around the world, not just here in the US. In some cases, unique subspecies that evolved specialized characteristics over hundreds of thousands of years are in danger of being lost. The utilization of only a few of the subspecies by beekeepers and the human tendency to think "the grass is always greener on the other side" has increasingly driven the movement of bees in many European countries that, over time, replaces various native subspecies. The native apple forests in the Tien Shan Mountains are currently under threat of deforestation. Losses in the ancient tree fruit forests combined with increasing displacement of native honey bee subspecies means that there is only a limited time to collect and preserve germplasm from this unique subspecies.

We now have a unique opportunity to bring in germplasm for bees, similar to what has been done for apples. We are currently the only University in the country to have a permit from USDA-APHIS to import honey bee semen for breeding. We have also developed a reliable cryopreservation process for honey bee semen that allows us to perform multiple backcrosses from a single collecting trip. We have been successful in importing, cryopreserving and utilizing honey bee semen from three subspecies: *A.m. ligustica* (Italy), *A.m. carnica* (Slovenia), and *A.m. caucasica* (Republic of Georgia) since 2008. In partnership with California queen breeders, we are introducing novel germplasm from these three subspecies into the US commercial honey bee population. If funded, this proposal would provide bee breeders with novel genetic material and potential benefits analogous to those derived from apple germplasm importations. Gains in bee breeding for disease resistance, foraging capabilities and winter survival, to name a few, will help stabilize US honey bee populations and the pollination industry.

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, *A. m. pomonella* stocks will be recovered through backcrossing, undergo selection under Washington conditions and distributed to California queen producers for propagation

The goal of this research is to import germplasm from the honey bee subspecies that is endemic to the ancient tree fruit forests in the Tien Shan Mountains and to distribute their genetics to commercial queen producers. The queen producers supply US beekeepers with the queens that, in turn, head the colonies that pollinate Washington fruit trees. The expectation is that honey bees that have been pollinating apples and pears for tens of thousands of years in their original homeland, have a high likelihood to express apicultural traits that would be useful to current tree fruit production. Some of these traits may include foraging abilities that are better synchronized to tree fruit seasonality in cooler climates (low temperature, high moisture, diverse nectar conditions).

METHODS

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

To prepare for our arrival we will arrange with our California queen producing partners to prepare virgin queens to arrive in Pullman at the same time we return from our collection trip. Once we arrive the fresh semen will be used to inseminate the virgin queens. A portion of the instrumentally inseminated queens will go back to the partnering queen producers so that they can make their selection and begin production of daughters for sale and distribution to commercial beekeepers.

A portion of the inseminated queens will remain at WSU; were we will produce daughters from these queens the following spring. The daughters (now 50% A.m. *pomonella*) will be inseminated with the frozen semen. The resulting daughter queens produced from the insemination with frozen semen (now 75% *A.m. pomonella*) will be inseminated with frozen semen and they too will produce daughters. The daugher queens from the second round of inseminations with frozen semen will be 87.5% *A.m. pomonella*. 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.

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. Shepaprd 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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