APPLE CROP PROTECTION RESEARCH REVIEW

Thu 28 Jan 2016

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NEW PROJECT PROPOSAL **PROPOSED DURATION**: 3 years

Project Title: Spotted wing drosophila management in stone fruit

PI: Elizabeth H. Beers
Organization: WSU-TFREC
Telephone: 509-663-8181 ext. 234
Email: ebeers@wsu.edu
Address: WSU-TFREC
Address 2: 1100 N. Western Ave.
City/State/Zip: Wenatchee, WA 98801

Cooperators: stone fruit growers

Total Project Request: **Year 1: $17,658** **Year 2:** $33,667 **Year 3:** $34,900

Other funding sources: None

Budget 1
Organization: WSU **Contract Administrator:** Joni Cartwright, Carrie Johnston
Telephone: 509-663-8181x221; 509-335-4564 **Email:** joni.cartwright@wsu.edu, carriej@wsu.edu

Item	2016	2017	2018
Salaries[1]	10,695	22,245	23,135
Benefits[2]	4,128	8,587	8,930
Wages	0	0	0
Benefits	0	0	0
Equipment	0	0	0
Supplies[3]	1,000	1,000	1,000
Travel[4]	1,835	1,835	1,835
Miscellaneous	0	0	0
Plot Fees	0	0	0
Total	**17,658**	33,667	34,900

Footnotes: [1]Salaries 0.40 FTE Research Intern, [2]Benefits, Research Intern 38.6%; [3]SWD rearing supplies, traps and lures, office supplies/electronics; [4]Travel to plots, $0.575/mile x 3,192 miles/year.

Justification:

Spotted wing drosophila (SWD) was first detected in eastern Washington in 2010, and has been considered one the key direct pests of sweet cherry. The host range description was originally quite broad, with no differentiation as to the degree of risk to various crops as they are produced commercially. The designation of non-cherry stone fruits (peaches, nectarines, apricots, plums and pluots, or PNAPP) as hosts was based on both reports in the literature, and reports from a U-pick operation in the Willamette valley that sustained damage to peaches. Similarly, damage to nectarines was detected in nectarines in a U-pick operation in Chelan County. More recently, reports of more significant damage occurring to PNAPPs have come from commercial orchards in Franklin County, possibly associated with later harvested varieties or organic production.

Previous field and laboratory work done in Washington and California indicated that to PNAPPs are at low risk from SWD, when harvested 'firm-ripe'. In three years of sampling peaches, apricots, and nectarines, no field infestation occurred during the preharvest or harvest period. Nectarines were the most susceptible crop in laboratory bioassays, when female SWD were caged with fruit. Oviposition occurred at low levels on uninjured fruit, but was generally higher on fruit with some type of injury the broke the skin. Similarly, fruit became more susceptible as it became more mature. Maturity is coincident with a number of changes, but softening the flesh and reduction in skin penetration force are two of the changes that may affect ovipositional cues and/or success by SWD. These characteristics vary by cultivar, and thus conclusions drawn from one cultivar may not apply to all cultivars. In addition, SWD densities increase in the late summer and fall, and it is probable that later-maturing cultivars are at greater risk of SWD damage, regardless of skin/flesh characteristics.

Objectives:

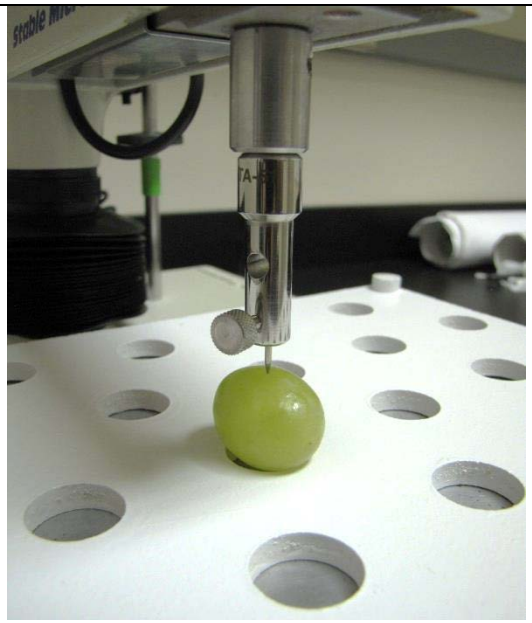
1. Determine skin penetration force and flesh firmness levels necessary to allow SWD oviposition
2. Test the use of synthetic lures to predict damage by SWD
3. Determine the number of traps per unit area needed to provide accurate prediction of damage risk.

Methods:

1. Previous research has shown that peaches and nectarines are low risk host crops for SWD. However, this insect can oviposit in them readily if they are over-mature; thus there must be a point in fruit development when they become susceptible. We will determine where along this continuum this point lies in terms of fruit maturity characteristics.

Fruit in multiple stages of maturity will be assayed in the laboratory using a lab culture of SWD. Fruit will be picked in the morning, transferred directly into individual plastic containers, and exposed to females the same day. Mated females SWD (10 days old) will be deprived of an oviposition substrate for 24 h, then exposed to a single peach or nectarine fruit for 2 h. At the end of this period, the female will be removed, and the fruit will be examined for oviposition (internal and external), recording the location of such oviposition if found. After scoring for oviposition, fruit maturity measurements will be made, including skin penetration force, flesh firmness, skin color, and brix. Within a single cultivar, assays will be performed on fruit collected at various points in time in the fruit maturation cycle, choosing the samples that represent all possible stages on the tree at that time. The number of ovipositions will be correlated with each variable to determine which one is most closely related to successful attack, and

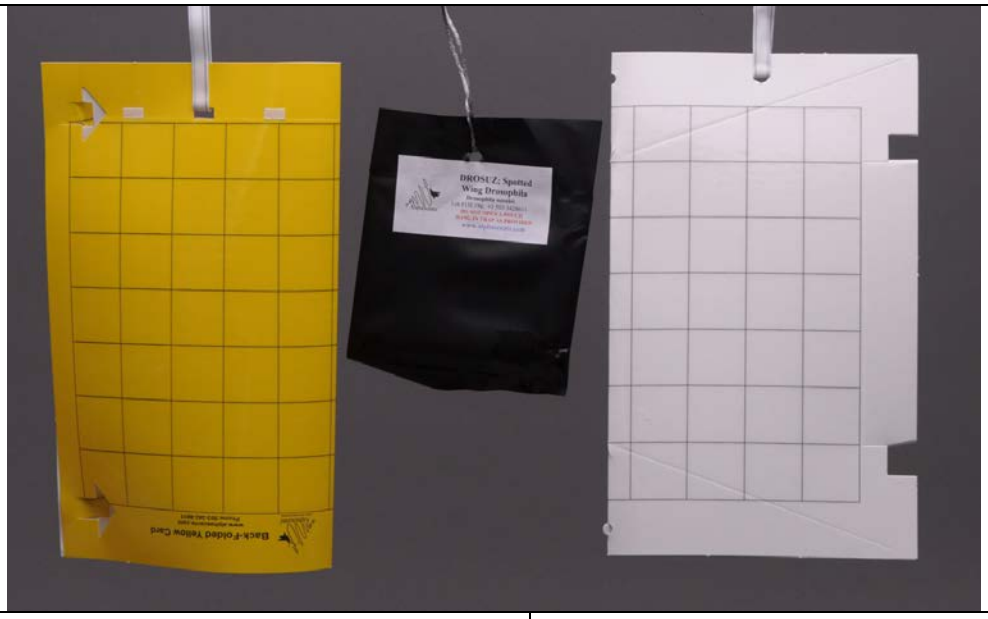
build a predictive regression line. This procedure will be repeated with multiple cultivars, representing the range from early to late-maturing.

	
Fruit texture analyzer (flesh firmness)	TAxT2i Texture Analyzer used to measure surface penetration

2. The first synthetic lure was available for testing in 2013, based on the Cha-Landolt blend of acetic acid, ethanol, methionol, and acetoin. In 2015, three commercial lures were available. All provided higher capture rates of SWD than apple cider vinegar. Two seasons of tests indicate that the Scentry lure consistently captures more SWD, thus the best opportunity for early detection of adult activity in an orchard, and to base a spray threshold on trap capture.

The use of traps for spray thresholds will be tested in six stone fruit orchards. Traps will be deployed and checked twice weekly beginning about 3 weeks before the anticipated first harvest. Three traps per block will be deployed in the center and two borders of the block. These traps will be the Scentry lure/Scentry trap combination. A second set of three traps will be deployed using the Scentry lure and the AlphaScents trap. The drowning fluid (water+sodium benzoate) in the Scentry traps will collected and changed at each visit, and the contents counted in the laboratory. The AlphaScents traps will be counted in situ, scanning only for males. A provisional threshold of 5 SWD in any of the six traps per block, individually or collectively, will trigger a pesticide application, which will continue at 7-10 day intervals through harvest.

The success of the threshold will be determined by collecting a sample of fruit at each harvest, examining it for SWD damage, and incubating fruit to determine if adult SWD emerge from the fruit.



AlphaScents traps and lure

Scentry trap

Scentry lure

3. Little is known about the source of SWD occurring in blocks, specifically whether the major source comes from habitat surrounding the block, or from within the block itself. This makes the number and position of traps used for action thresholds difficult to determine. Observations to date indicate that the older ACV traps have a limited range of attraction, but newer lures are untested.

To address this question, the blocks used in Objective 2 will be used, dividing the blocks into 2 parts. Half of the orchard will have a low trap density (Obj. 2), and the other half will have a high trap density, using only the Scentry lure/AlphaScents trap combination. Traps will be laid out in a grid pattern throughout the block, using 3-4x the trap density as Obj. 2. Traps will be checked twice weekly *in situ*, without changing the lure, and changing the trap only when it approaches saturation. The same threshold of 5 SWD (collectively) used in Obj. 2 will be used, as well as the same method of determining success of the threshold.

Literature Review:

Spotted wing drosophila (SWD) was first detected in western Washington in 2009, and in eastern Washington in 2010. Initial literature indicated an extremely broad host range, causing widespread concern among producers and crop consultants (Dreves et al. 2009, Walsh et al. 2011). Since that time, greater attention has been paid to determination of crop risk as opposed to host range; like any member of the genus *Drosophila*, this species can develop in senescent or decaying fruit, but this does not constitute a risk to crops harvested at normal commercial timing. Blueberries, caneberries, and cherries have been confirmed as high-risk crops, while non-cherry stone fruits (peaches, nectarines, apricots, plums and pluots), wine grapes, juice grapes, and table grapes all report low risk from SWD. However, low risk is not zero risk, and there scattered reports of damage under specific conditions (Anonymous 2009).

SWD has of has been a pest of the commercial cherry industry in Oregon and eastern Washington since its first detection in 2010 (Beers 2011, Beers et al. 2011). This species is Asian in origin (Hauser 2011), described by Kanzawa in 1939 (Kanzawa 1939). It was detected in Hawaii in 1980, but apparently without any harm to commercial crops. Its first detection in California in 2008 was followed by explosion of new records from the west coast, the eastern seaboard, and the Midwestern US (Isaacs et al. 2010) in rapid succession. Spread was occurring at the same time and rate in Europe, and South America (Deprá 2014) has now reported detections of this species. The original notion that distribution of this species would be limited by climate (Kimura 2004, Damus 2010) has proven to be erroneous; new finds appear to be more related to the amount of effort put into detection.

The invasion of SWD in eastern Washington coincided with the relatively early stages of implementation of a selective program for western cherry fruit fly (WCFF) based on a bait spray, GF-120. Before SWD, WCFF was the key direct pest of cherries, although black cherry aphid, grape mealybug, and spider mites were also considered in the overall spray program. Spider mites were likely induced by a combination of the previous WCFF program and the cherry mildew program. Reducing the need for secondary pest control, and short preharvest intervals for WCFF control were two of the distinct improvements allowed by GF-120 use.

The advent of SWD changed this scenario abruptly. Early anecdotal reports indicated that damage by SWD occurred despite GF-120 applications, and producers were not to rely on this technique for control of the new pest. As a result, full-canopy sprays returned to the program during the main part of the fruit maturation period. Provisional advice (Lee et al. 2011, Beers 2014) indicated that fruit were likely susceptible to SWD when red color first appears, which was later confirmed by more extensive field research (Beers, unpublished).

One issue that quickly became of concern for cherry growers wishing to export their fruit was that of Maximum Residue Levels (MRLs) of pesticides on fruit (Haviland and Beers 2012). The need for protection from attack by SWD necessitated spray coverage up to, and through, the harvest period. The difference in MRLs between the US and target export countries meant that the use pattern specified by the US pesticide label might produce a residue level that was unacceptable in another country. Pesticide

choice was also guided by considerations such as the preharvest interval and the length of residual control. The combination of these factors tended to drive Washington cherry growers to focus on a single mode of action for controlling this pest, the spinosyns (spinosad [Success or Entrust]; and spinetoram [Delegate]).

Although the early focus was simply on adequate control and damage prevention by this previously unknown pest, IPM efforts must eventually consider a longer-term horizon. One of the first issues that must be considered is that of pesticide resistance, and a long-term plan for preventing or managing it. In order to be meaningful, baseline susceptibility must be established before, or shortly after a pest is exposed to a new pesticide. In the case of SWD, it is the pest rather than the pesticide(s) that are new, but the principle is still the same. Subsequent efforts can then be aimed at screening populations for early detection of resistance, and implementing rotation strategies if they are not already in place.

Another long-term IPM goal is to develop and implement thresholds that can be used to prevent unnecessary sprays, or conversely, ensure that adequate measures for fruit protection are taken in a timely manner. Thresholds are dependent on some means of monitoring to determine when the threshold has been reached. Although other methods may be explored in the future, food odor attractants in traps have been the most widely and successfully used to date (Lee et al. 2012, Lee et al. 2013). Various baits have been tested, and while most are functional to a greater or lesser extent, they are still in the developmental phase. Because the development of thresholds is time-consuming and laborious, lure/trap optimization should precede it. Electroantennegram testing has revealed that four components of wine and vinegar are the most important in attraction (Cha et al. 2013), and these components have been used to develop synthetic chemical lures in pouches. Dry lures would have the advantage of longer replacement cycles, although they are currently used with a collection fluid (e.g., water plus adjuvants), so a weekly trap checking cycle is still indicated. If first capture, rather than maximum capture is the goal, then even more frequent checks of the traps contents would be necessary.

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FINAL PROJECT REPORT

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

PI: Mark Mazzola
Organization: USDA-ARS
Telephone: 509-664-2280
Email: mark.mazzola@ars.usda.gov
Address: 1104 N Western Ave.
Address 2:
City: Wenatchee
State/Zip: WA 98801

Co-PI(2): Chang Lin Xiao
Organization: USDA-ARS
Telephone: 559-596-2722
Email: chang-lin.xiao@ars.usda.gov
Address: 9611 S Riverbend Ave.
Address 2:
City: Parlier
State/Zip: CA 93648

Co-PI(3): Christian Aguilar
Organization: WSU-TFREC
Telephone: 509-664-2280 ext. 243
Email: cgaguilar@wsu.edu
Address: 1104 N Western Ave.
Address 2:
City: Wenatchee
State/Zip: WA 98801

Other funding sources: None

Total Project Funding: $39,700

Budget History:

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

Footnotes: *Funding is requested to support a Research Assistant

OBJECTIVES

(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*.

(2) Determine the effectiveness of fungicide applications in the control of early versus late season apple fruit infection by *N. perennans* and *C. kienholzii* occurring in the field.

SIGNIFICANT FINDINGS

During both annual trials of this experiment, the only pre-harvest fungicide treatment providing statistically significant pre-harvest control of bull's eye rot caused by *Neofabraea perennans* or *Cryptosporiopsis kieholzii* was thiophanate-methyl (Topsin-M).

During both annual trials of this experiment, the only postharvest fungicide treatments providing statistically significant control of bull's eye rot caused by *N. perennans* or *C. kienholzii* were thiabendazole (Mertect) and pyrimethanil (Penbotec).

Inoculations conducted later in the growing season (at two weeks before harvest) resulted in higher incidences of bull's eye rot compared to early and mid-season inoculation periods (eighteen and five weeks before harvest, respectively). Regardless of inoculation timing, the pre-harvest fungicide thiophanate-methyl, and postharvest fungicides thiabendazole and pyrimethanil were most effective for control of *N. perennans* and *C. kienholzii* infections.

RESULTS

Efficacy of pre-harvest fungicide applications

Only two fungal inoculation time-points were explored during the first year of this study, (five and two weeks prior to harvest). Infection was significantly greater when inoculations were conducted at two weeks prior to harvest compared to inoculations at five weeks before harvest ($P < 0.0001$). Overall the proportion of apples with bull's eye rot decay due to *N. perennans* infection was not significantly different from infections attributed to *C. kienholzii* (P=0.0960). Average bull's eye rot recovery for fruit treated with zinc (Ziram), and pyraclostrobin plus boscalid (Pristine) was not significantly different from average disease incidence recorded in the no fungicide control treatment. Only fruit treated with thiophanate-methyl (Topsin-M) exhibited a statistically significant lower disease incidence (P<0.0001; Table 1).

An additional inoculation time-point meant to simulate early season fruit infection was conducted during the second year of this experiment, (eighteen weeks before harvest). A significantly greater proportion of fruit were infected when inoculations were conducted at two weeks before harvest compared to five and eighteen weeks before harvest (P<0.0001). A statistically greater incidence of infection attributed to *N. perennans* was observed compared to inoculations with *C. kienholzii* (P=0.0193). Similar to the previous year, the only pre-harvest fungicide that effectively reduced *N. perennans* and *C. kienholzii* infections was thiophanate-methyl (P<0.0001; Table 1).

Efficacy of postharvest fungicide drenches

Inoculations conducted during the first year of this study yielded a greater incidence of bull's eye rot when *N. perennans* and *C. kienholzii* were applied at two weeks prior to harvest rather than five weeks (P<0.0001). In general, greater incidence of disease was attained when fruit inoculations were conducted using a spore suspension of *N. perennans* instead of *C. kienholzii* (P=0.0002). Bull's eye rot recovery for fruit treated with fludioxonil (Scholar) was not statistically different from recovery rates observed for the

no fungicide control. Applications of thiabendazole (Mertect) and pyrimethanil (Penbotec) to *N. perennans* and *C. kienholzii* inoculated fruit prior to storage resulted in significantly less bull's eye rot ($P<0.0001$), with thiabendazole treated fruit exhibiting less disease on average compared to fruit treated with pyrimethanil (Table 2).

During the second year of this study, bull's eye rot incidence was significantly higher when pathogen inoculum was applied to fruit two weeks prior to harvest rather than five and eighteen weeks before harvest ($P<0.0001$). Inoculations using a spore suspension of *N. perennans* conidia yielded significantly more bull's eye decayed fruit compared to *C. kienholzii* inoculations ($P<0.0001$). Bull's eye rot incidence for fruit treated with fludioxonil prior to storage was proportionally as high as fruit left untreated. Application of difenoconazole plus fludioxonil (Academy) to pathogen inoculated fruit only slightly reduced bull's eye rot incidence compared to the no fungicide control. Only fruit treated with thiabendazole or pyrimethanil demonstrated a significant reduction in bull's eye rot due to *N. perennans* and *C. kienholzii* inoculations ($P<0.0001$; Table 2).

DISCUSSION

In light of the temporary suspension of shipment of Washington grown apples to the Chinese market and subsequent strict phytosanitary regulations established in response to postharvest decay pathogens (Warner, 2014), bull's eye rot has become an economically important disease for pome fruit growers and packers of the Pacific Northwest region. Once considered a minor disease in Washington State, bull's eye rot outbreaks have become increasingly more common over the past decade. In effort to curtail the occurrence of bull's eye rot in this area, various fungicides registered for use on pome fruit were selected and their efficacy against bull's eye rot was tested. Results from this study indicate that among the pre-harvest and postharvest fungicides evaluated, only thiophanate-methyl (Topsin-M), pyrimethanil (Penbotec) and thiabendazole (Mertect) were effective in providing adequate control of bull's eye rot in stored apple relative to a no treatment control. While results from this study obviously provide growers and packers with invaluable information pertaining to bull's eye management, it also highlights the complexities encountered by members of the apple industry in dealing with postharvest decay issues.

As benzimidazole fungicides, thiophanate-methyl and thiabendazole are classified as having moderate to high risk of resistance developing in pathogen populations (Fungicide Resistance Action Committee). This fact has already been demonstrated by the appearance of thiabendazole-resistant strains of *Penicillium expansum* (Li and Xiao, 2008) and *Botrytis cinerea* (Zhao et al., 2010) throughout cold storage facilities in Washington State. The short life cycle and high sporulation capacity of *P. expansum* make it a likely candidate for developing resistance to high risk fungicides. *Neofabraea* species, however, are comparatively slow growing, and in requiring water splash for spore dissemination, are less capable of spreading at a high rate thus posing less of a risk for developing fungicide resistance. Nevertheless, thiophanate-methyl-resistant strains of *N. perennans* and *N. alba* have already appeared in pathogen populations originating from Northern Germany, partially in response to excessive use of this fungicide (Weber and Palm, 2010). The fact that two of the most effective fungicides available to pome fruit growers for bull's eye rot control share a common mode of action, should be of concern for the Washington apple industry. In order to minimize the potential for resistance, chemistries with differing modes of action should be alternated regularly. As a fungicide belonging to the anilinopyrimidine class, pyrimethanil seemingly presents a useful alternate fungicide as part of a bull's eye rot management program. Unfortunately, pyrimethanil resistance has also appeared in *P. expansum* populations originating in Washington State (Xiao et al., 2011), further confounding bull's eye rot management. While the primary aim of this project was to provide growers with information concerning fungicides that can be used to successfully manage bull's eye rot, the results from this work further highlight the resounding need for additional fungicides registered for use in pome fruit production systems.

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Table 1. Average recovery of bull's eye rot from cv. Fuji apples inoculated with spores of either *Neofabraea perennans* or *Crytosporiopsis kienholzii* at various pre-harvest periods and treated with select pre-harvest applied fungicides.

Year	Pathogen	Inoculation period (weeks before harvest)	Pre-harvest fungicide	Average bull's eye rot recovered (%)
2014	*Neofabraea perennans*	5 wbh	No fungicide control	63.75%
			Zinc (Ziram)	62.50%
			Pyraclostrobin + boscalid (Pristine)	58.75%
			Thiophanate-methyl (Topsin-M)	15.00%
		2 wbh	No fungicide control	78.75%
			Zinc (Ziram)	81.25%
			Pyraclostrobin + boscalid (Pristine)	81.25%
			Thiophanate-methyl (Topsin-M)	27.50%
	Cryptosporiopsis kienholzii	5 wbh	No fungicide control	49.25%
			Zinc (Ziram)	37.50%
			Pyraclostrobin + boscalid (Pristine)	60.00%
			Thiophanate-methyl (Topsin-M)	7.50%
		2 wbh	No fungicide control	88.75%
			Zinc (Ziram)	65.00%
			Pyraclostrobin + boscalid (Pristine)	90.00%
			Thiophanate-methyl (Topsin-M)	17.50%

Table 1 (continued). Average recovery of bull's eye rot from cv. Fuji apples inoculated with spores of either *Neofabraea perennans* or *Crytosporiopsis kienholzii* at various pre-harvest periods and treated with select pre-harvest applied fungicides.

Year	Pathogen	Inoculation period (weeks before harvest)	Pre-harvest fungicide	Average bull's eye rot recovered (%)
2015	*Neofabraea perennans*	18 wbh	No fungicide control	17.50%
			Zinc (Ziram)	22.50%
			Pyraclostrobin + boscalid (Pristine)	23.75%
			Thiophanate-methyl (Topsin-M)	8.75%
		5 wbh	No fungicide control	31.25%
			Zinc (Ziram)	22.50%
			Pyraclostrobin + boscalid (Pristine)	32.50%
			Thiophanate-methyl (Topsin-M)	14.00%
		2 wbh	No fungicide control	69.75%
			Zinc (Ziram)	38.75%
			Pyraclostrobin + boscalid (Pristine)	57.50%
			Thiophanate-methyl (Topsin-M)	16.25%
	Cryptosporiopsis kienholzii	18 wbh	No fungicide control	36.25%
			Zinc (Ziram)	10.00%
			Pyraclostrobin + boscalid (Pristine)	21.25%
			Thiophanate-methyl (Topsin-M)	12.50%
		5 wbh	No fungicide control	12.50%
			Zinc (Ziram)	17.50%
			Pyraclostrobin + boscalid (Pristine)	17.50%
			Thiophanate-methyl (Topsin-M)	11.00%
		2 wbh	No fungicide control	52.50%
			Zinc (Ziram)	37.50%
			Pyraclostrobin + boscalid (Pristine)	43.75%
			Thiophanate-methyl (Topsin-M)	10.00%

Table 2. Average recovery of bull's eye rot from cv. Fuji apples inoculated with spores of either *N. perennans* or *C. kienholzii* in the field at various pre-harvest periods and treated with select postharvest applied fungicides.

Year	Pathogen	Inoculation period (weeks before harvest)	Postharvest fungicide	Average bull's eye rot recovered (%)
2014	*Neofabraea perennans*	5 wbh	No fungicide control	61.25%
			Fludioxonil (Scholar)	52.50%
			Pyrimethanil (Penbotec)	17.50%
			Thiabendazole (Mertect)	8.75%
		2 wbh	No fungicide control	60.00%
			Fludioxonil (Scholar)	73.75%
			Pyrimethanil (Penbotec)	61.25%
			Thiabendazole (Mertect)	20.00%
	Cryptosporiopsis kienholzii	5 wbh	No fungicide control	51.25%
			Fludioxonil (Scholar)	62.50%
			Pyrimethanil (Penbotec)	2.50%
			Thiabendazole (Mertect)	1.25%
		2 wbh	No fungicide control	89.00%
			Fludioxonil (Scholar)	86.25%
			Pyrimethanil (Penbotec)	1.25%
			Thiabendazole (Mertect)	2.50%

Table 2 (continued). Average recovery of bull's eye rot from cv. Fuji apples inoculated with spores of either *N. perennans* or *C. kienholzii* in the field at various pre-harvest periods and treated with select postharvest applied fungicides.

Year	Pathogen	Inoculation period (weeks before harvest)	Postharvest fungicide	Average bull's eye rot recovered (%)
2015	*Neofabraea perennans*	18 wbh	No fungicide control	17.50%
			Fludioxonil (Scholar)	27.50%
			Pyrimethanil (Penbotec)	6.25%
			Thiabendazole (Mertect)	10.00%
			Difenoconazole + Fludioxonil (Academy)	20.00%
		5 wbh	No fungicide control	48.75%
			Fludioxonil (Scholar)	36.25%
			Pyrimethanil (Penbotec)	17.50%
			Thiabendazole (Mertect)	12.50%
			Difenoconazole + Fludioxonil (Academy)	25.00%
		2 wbh	No fungicide control	68.75%
			Fludioxonil (Scholar)	41.25%
			Pyrimethanil (Penbotec)	14.00%
			Thiabendazole (Mertect)	28.00%
			Difenoconazole + Fludioxonil (Academy)	52.50%
	Cryptosporiopsis kienholzii	18 wbh	No fungicide control	26.25%
			Fludioxonil (Scholar)	26.25%
			Pyrimethanil (Penbotec)	1.25%
			Thiabendazole (Mertect)	5.00%
			Difenoconazole + Fludioxonil (Academy)	18.25%
		5 wbh	No fungicide control	21.25%
			Fludioxonil (Scholar)	15.00%
			Pyrimethanil (Penbotec)	1.00%
			Thiabendazole (Mertect)	0.00%
			Difenoconazole + Fludioxonil (Academy)	10.00%
		2 wbh	No fungicide control	60.00%
			Fludioxonil (Scholar)	52.50%
			Pyrimethanil (Penbotec)	1.00%
			Thiabendazole (Mertect)	6.00%
			Difenoconazole + Fludioxonil (Academy)	37.50%

EXECUTIVE SUMMARY

The primary objectives of this research were to evaluate the efficacy of various pre-harvest and postharvest applied fungicides for control of bull's eye rot caused by *Neofabraea perennans* and *Cryptosporiopsis kienholzii*. To accomplish this goal, fungicide evaluations were conducted in the orchard at multiple pathogen inoculation intervals and the trials were replicated across two years. Data from both years consistently indicated that among the fungicides tested, thiophanate-methyl is the only pre-harvest fungicide capable of adequate bull's eye rot control while pyrimethanil and thiabendazole were the only two postharvest chemistries providing acceptable control of these two pathogens.

These data come at a pivotal time as bull's eye rot and other postharvest diseases native to the Pacific Northwest now present quarantine concerns for international trade. During the year prior to China's temporary trade closure of Washington grown apples, the value of Washington apples shipped to China was estimated at $6.5 million. Interception of Sphaeropsis rot, speck rot and bull's eye rot on apples exported from Washington resulted in a two year shut down that potentially cost the Washington apple industry $13 million. The need to identify fungicides that can effectively manage bull's eye rot is high, and while this research accomplishes this need, it also emphasizes the need for additional research in this area.

The three fungicides identified as effective against bull's eye rot only provide temporary relief for this complex situation. Issues regarding fungicide resistance are a major concern that can only exacerbate the future of bull's eye rot management. Newly registered fungicides for use in pome fruit production have become available during the course of this study. These new fungicides provide a great opportunity for additional work to be completed in this research area.

Currently, *in vitro* spore germination and mycelial growth assays using fungicide amended media are being conducted in the Mazzola laboratory for control of bull's eye rot and other fungi causing economically important postharvest disease. The outcome of this work appears promising, and should contribute much needed information to strengthen bull's eye management.

FINAL PROJECT REPORT **YEAR**: 2 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:** Russ Karow
Telephone: (541) 737-4066 **Email address:** Russell.Karow@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.

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

SIGNIFICANT FINDINGS

- Blossom Protect applied once at 70% bloom continued to provide significant fire blight control in apple and pear.
- In 2015, integrated fire blight control programs that began with Blossom Protect and followed by Serenade Opti, Cueva soluble copper or combinations of these materials showed enhanced suppression compared to Bloom Protect alone.
- In multi-location trials, Blossom Protect (comprised of two strains of *Aureobasidium pullulans*) showed a slight potential to increase fruit russeting at a wet location (Corvallis) and on sensitive cultivars (Comice and Golden Delicious), but did not induce russeting in Braeburn apple or Bartlett pear grown in semi-arid climates (Medford and Lakeport).
- Compared to Blossom Protect, the soluble copper Cueva showed a higher potential to russet fruit. Copper-induced russeting was observed on Bartlett pear fruit and Golden Delicious apple in a wet climate (Corvallis) and on Comice pear fruit in a semi-arid climate (Medford), but compared to treatment with water, was not increased significantly on Braeburn apple or Bartlett pear grown in a semi-arid climates (Medford and Lakeport).
- 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.
- Molecular methods to identify Blossom Protect strains of *A. pullulans* were verified and used to demonstrate that about half of *A. pullulans* isolates detected on trees treated with Blossom Protect were the strains from the biocontrol product, but that on trees not treated with Blossom Protect, the detected *A. pullulans* isolates were likely from a source within the orchard.
- Several additional materials – oxidizing agents (Oxidate and R42014), soluble copper (Previsto), alum (potassium aluminum sulfate), and E. amylovora-specific phage – showed potential to contribute to non-antibiotic fire blight control programs.

RESULTS & DISCUSSION

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

1.a. Fire blight control. Integrated fire blight control programs that began with Blossom Protect and followed by Serenade Opti, Cueva soluble copper or combinations of these materials were conducted during 2014 and 2015 in apple and pear orchards located in Corvallis, OR (**Fig. 1 & 2**).

Bartlett pear, 2014. 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 (Fig. 1A). 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 all program combinations 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 2014. 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 (**Fig. 1B**). Compared to the water-treated control, each of the treatments reduced significantly ($P < 0.05$) incidence of disease; the exception treatment was 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).

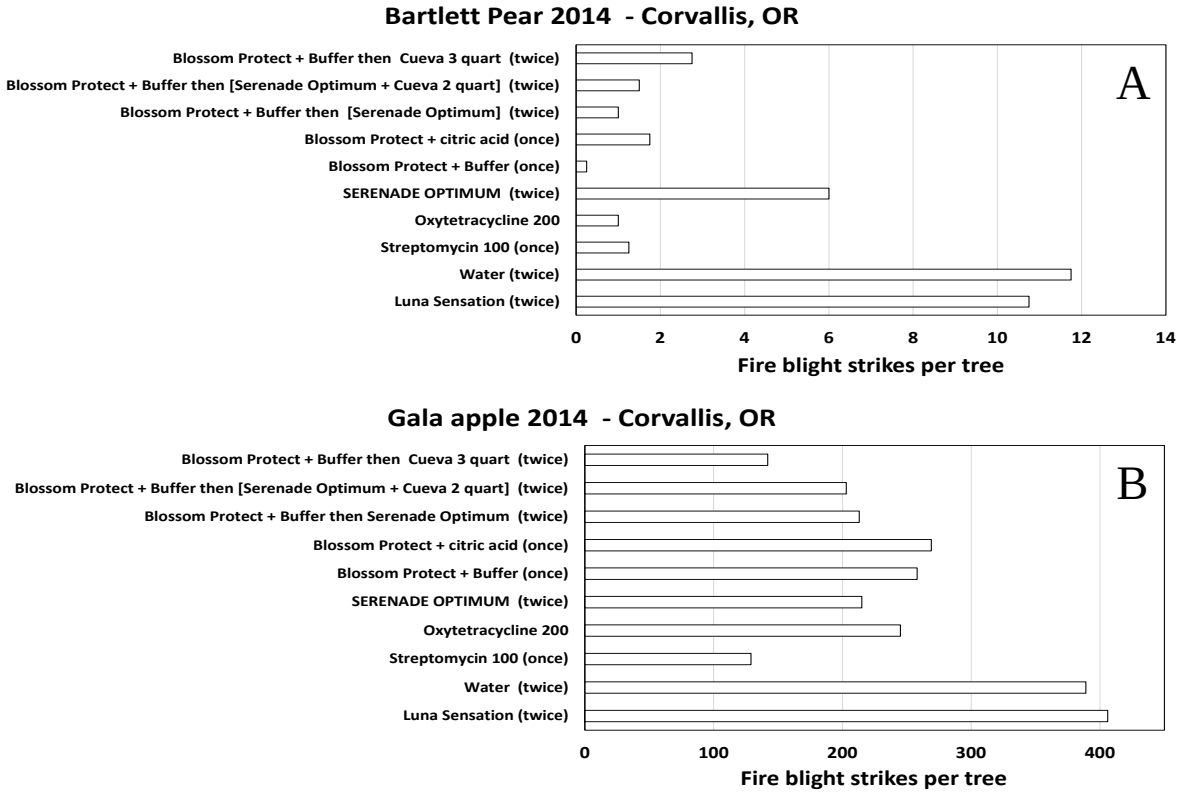


Fig. 1. Results of inoculated fire blight trials conducted near Corvallis, OR in 2014. Bars represent means of four replicate trees. Blossom Protect treatments were applied at 70% bloom; other materials were applied at full bloom and petal fall if applied twice. *Erwinia amylovora* strain Ea153N (streptomycin-sensitive) was inoculated onto the trees on an evening 1 to 2 days before the full bloom treatment applications; inoculum concentration was 1 x 10^6 CFU/ml.

Bartlett pear, 2015. Trees used in the study averaged 319 flower clusters per tree. Fire blight risk as determined by the heat unit risk model, COUGARBLIGHT, was low during the bloom period. For a pathogen-inoculated trial, disease intensity was low with fire blight infections on water-treated trees averaging 12.5 strikes per tree (**Fig. 2A**). Compared to the water-treated control, all non-antibiotic programs that began with one treatment of Blossom Protect reduced significantly ($P < 0.05$) total strikes per tree and incidence of disease. Treatment programs where Cueva, R40214 (called 'Oxycom Ca' in the chart) and potassium aluminum phosphate (called 'Alum' in the chart) followed Blossom Protect resulted in significantly ($P < 0.05$) fewer blighted flower clusters than trees-treated with Blossom Protect only. Numerically, the fungicide Luna Sensation performed worse than the water treated control, which may be

due to its ability to suppress secondary, non-target colonization of flowers by the yeast, *A. pullulans*. The antibiotic standards, streptomycin and oxytetracycline, did not provide significant fire blight suppression.

Golden Delicious apple, 2015. Trees used in the study averaged 288 flower clusters per tree. Fire blight risk as determined by the heat unit risk model, COUGARBLIGHT, was low in early bloom but high near petal fall. For a pathogen-inoculated trial, disease intensity was moderate with fire blight infections on water-treated trees averaging 78 strikes per tree (**Fig. 2B**). Compared to the water-treated control, all non-antibiotic programs that began with one treatment of Blossom Protect reduced significantly ($P < 0.05$) total strikes per tree and incidence of disease. Based on total number of blighted flower clusters per tree, the program where Previsto followed Blossom Protect resulted in significantly ($P < 0.05$) fewer blighted flower clusters than trees-treated with Blossom Protect only. The fungicide Luna Sensation performed similar to the water treated control.

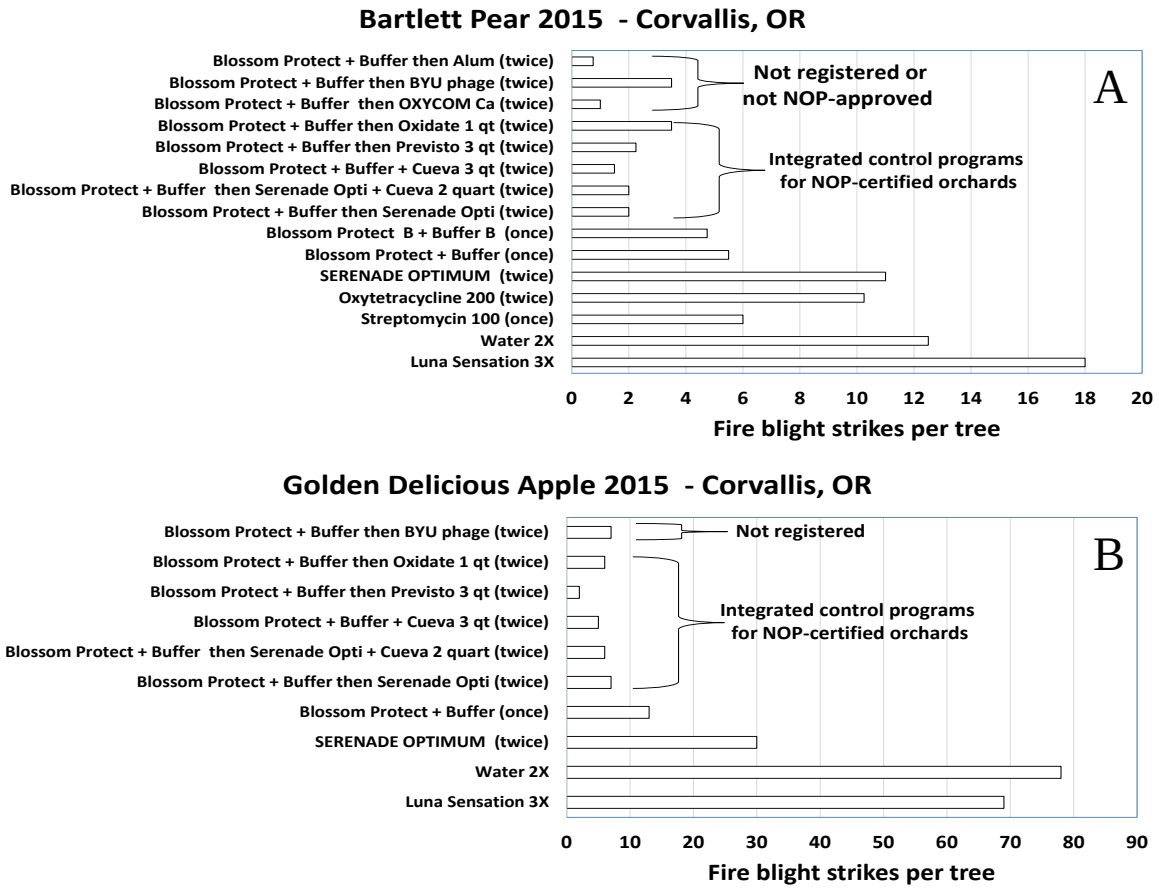


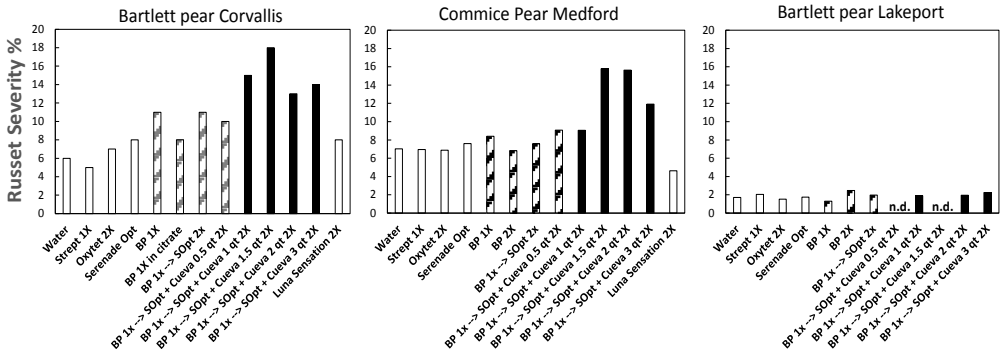
Fig. 2. Results of inoculated fire blight trials conducted near Corvallis, OR in 2015. Bars represent means of four replicate trees. Blossom Protect treatments were applied at 70% bloom; other materials were applied at full bloom and petal fall if applied twice. *Erwinia amylovora* strain Ea153N (streptomycin-sensitive) on an evening 1 to 2 days before the full bloom treatment applications; inoculum concentration was 1 x 10^6 CFU/ml.

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.

In 2015, fire blight severity ranged from light (pear) to intermediate (Golden Delicious apple) but overall results were similar. Blossom Protect once provided 60 to 80% of the observed disease suppression. Following Blossom Protect with Serenade Opti, Cueva soluble copper or combinations of these materials showed enhanced suppression compared to Bloom Protect alone. In the pear trial, integrated non-antibiotic control was superior to the antibiotic treatments.

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

In 2014, fruit russet data was collected from pear trials: Bartlett-Corvallis, Bartlett-Lakeport, Comice-Medford. In 2015, russet trials were established in Corvallis (Bartlett pear and Golden Delicious apple), Medford (Braeburn apple) and Lakeport (Bartlett pear). Among locations, Corvallis showed the most fruit russet independent of treatment. At this location, in both years, trees that received soluble copper after Blossom Protect had russet severites that exceeded 10%; in contrast,



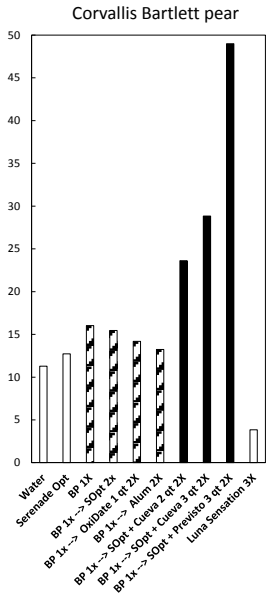
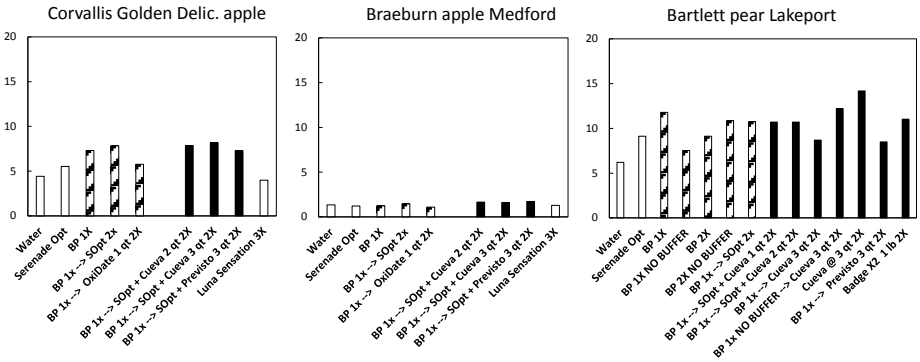


Fig. 3. Mean fruit russet severity (%) on pear and apple fruit treated with integrated non-antibiotic fire blight control programs. Open bar: fruit from trees that received a low russet potential treatment (water, Serenade Opti or oxytetracycline); hatched bar: fruit from trees treated with Blossom Protect only or Blossom Protect followed by non-russeting material; black bar: fruit from trees treated with Blossom Protect followed by soluble copper (Cueva or Previsto). Upper panel: 2014 trials; lower panel: 2015 trials.



trees that received Blossom Protect treatments (either alone or or followed by non-russeting material) showed intermdiate levels of fruit russuting compared to treatments that received water (low russeting) or copper (high russeting). Also at Corvallis, in 2015 the Golden Delicious apple fruit showed less rutteting than pear fruit but Blossom Protect or Blossom Protect then copper increased the level of fruit russting compared to the water treated control. Similary, at Medford in 2014, 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 russet severity in 2014 was low (< 2% severity) and not effected by any of the treatments, but russet was increased across all treatments in 2015. At several locations, the Luna Sensation treatment showed the lowest level of russet, which may indicate suppression of non-target spread of Blossom Protect strains of *A. pullulans* and/or suppression of indigenous russet-inducing yeast populations (see next section).

Discussion of A. pullulans populations and fruit russetting potential. Initially, pear cultivars 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 2015, we also added apple cultivars: Golden Delicious (russet sensitive) in Corvallis and Braeburn (russet tolerant) in Medford. 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 and Golden Delicious apple 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 Braeburn apple at the same location or Bartlett pear in Lakeport in the same season. In the semi-arid climates, Blossom Protect showed very little potential to enhance russet. In contrast, Cueva showed more potential to induce russetting; although based on Bartlett pear at Lakeport in 2014 and Braeburn apple at Medford in 2015, 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).

Implementation of non-antibiotic fire blight control is now 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 <u>Blossom Protect</u>:
  - 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:
  - <u>Serenade Optimum</u> every 2 to 5 days (<u>most fruit safe</u>)
  - Improved control: Mix Serenade Opt with Cueva (2 to 3 qt./A)
  - <u>Cueva</u> every 3 to 6 days (3 to 4 qt./A) (good blight control but <u>least fruit safe</u>)

1.c. 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 one to two sampling dates between full bloom and petal fall. Trials included those inoculated with the pathogen (Corvallis) and fruit russet evaluation trials in southern Oregon (Braeburn apple, Medford) and northern California (Bartlett pear, Lakeport). Over all trials, *A. pullulans* was detected on nearly every flower (> 99%) from trees treated with Blossom Protect, and was detected on a majority of flowers (> 50%) sampled from trees not treated with this material (**Fig. 4**). In general, the measured population sizes of *A. pullulans* on non-treated trees was 0.5 to 2 log units smaller than the population size of this organism on trees treated with Blossom Protect only. In 2015, oversprays of Serenade Optimum and soluble coppers 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. This result was in contrast to 2014 results where mixing Serenade Optimum with 2 or 3 quarts of Cueva significantly suppressed *A. pullulans* populations ($P \leq 0.05$) compared to Blossom Protect only (see 2014 progress report).

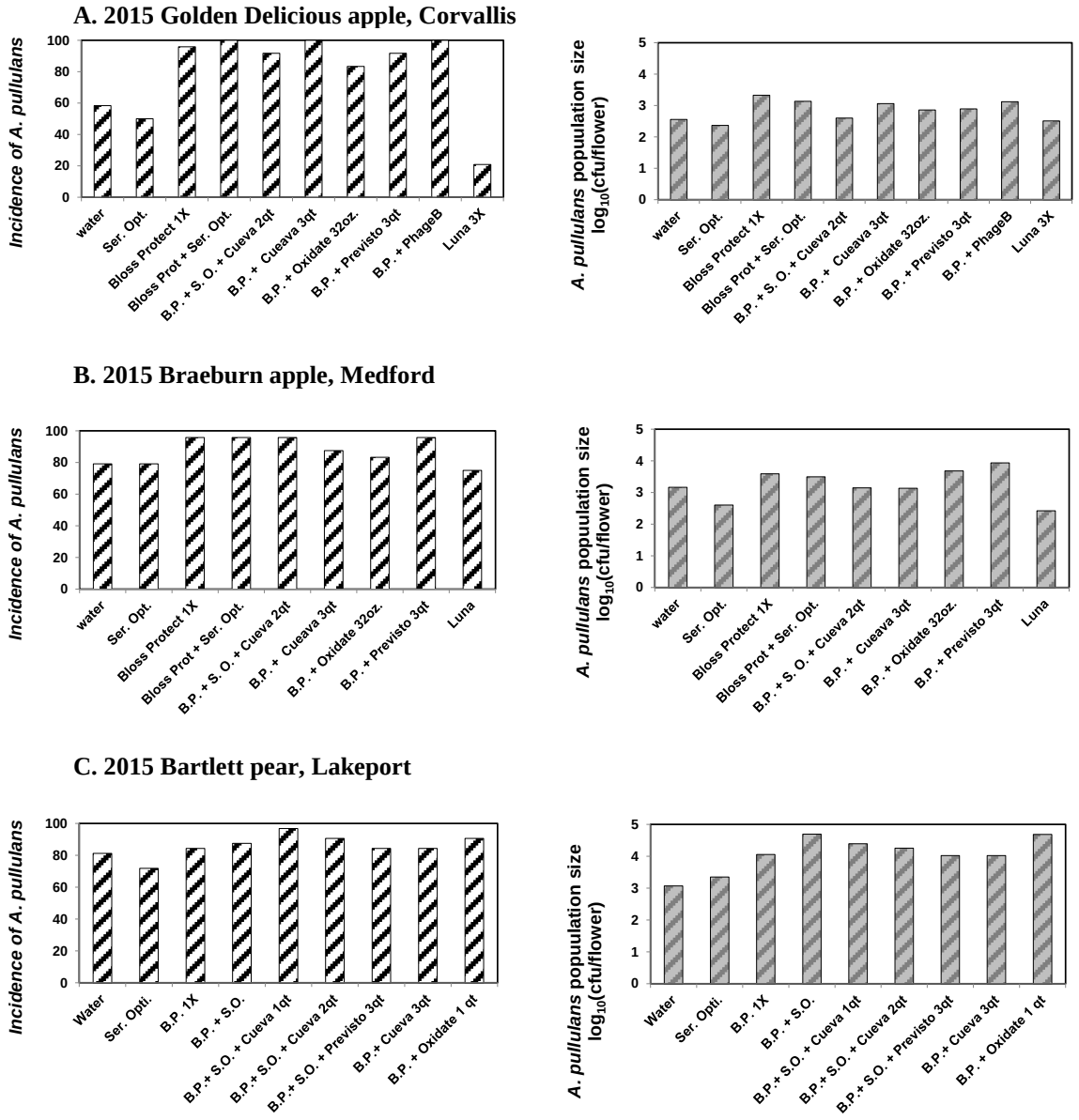


Fig. 4. Incidence (left panels) and population size (right panels) on *Aureobasidium pullulans* on pome fruit flower treated with integrated non-antibiotic fire blight control programs.

d. Molecular identification of *Aureobasidium pullulans* isolated from pome fruit flowers.

The fire blight biocontrol product, Blossom Protect, consists of strains CF10 and CF40 of *A. pullulans*, which are produced separately then mixed together in the bag. 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. With a PCR analyses that utilized general *A. pullulans* and strain-specific primers, we confirmed *A. pullulans* but also found the *A. pullulans* isolates were not Blossom Protect strains (see 2014 report).

These observations on *A. pullulans* led us to further investigate published molecular PCR protocols for specific identification of the Blossom Protect strains of *A. pullulans*. DNA extracted from CF10 and CF40 (positive controls) yielded their respective PCR products (**Table 1**).

Table 1. PCR products from *Aureobasidum pullulans*		**PCR Primer set**		
Isolate:	**Source:**	**AP ITS**	**SCAR6**	**SCH3RAPD**
CF10	Blossom protect	100 bp	307 bp	-
CF40	Blossom protect	100 bp	-	962 bp
Non-Blossom Protect isolates of *A. pullulans*	orchards & fruit lots	100 bp	-	-

From each of three bags of Blossom Protect, we made 12 single spore isolations of *A. pullulans* (36 isolates in total). To verify the stability of the PCR markers in each isolate, we made ten sequential transfers of the single spore isolates (approximately 50 days to complete the 10 sequential transfers). We subjected the single spore isolates to PCR analysis after the 1st, 5th and 10th transfers. The general *A. pullulans* primer set (AP ITS) identified all isolates and stability of the strain-specific *A. pullulans* PCR markers was confirmed (**Fig. 5**).

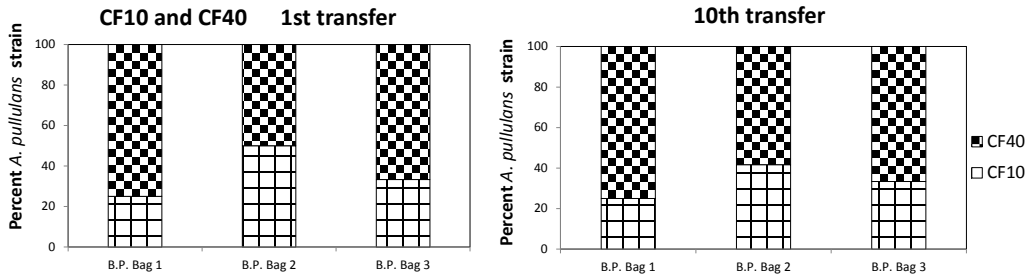


Fig. 5. Molecular identification of 36 *Aureobasidium pullulans* isolates from 3 bags of Blossom Protect (12 isolates per bag). After 1 and 10 sequential transfers of each isolate, all isolates were subjected to PCR analysis with primers specific to *A. pullulans* biocontrol strains CF10 and CF40.

From the fire blight and fruit russet trials in Corvallis, Medford and Lakeport, the individual floral wash samples used to measure *A. pullulans* population size by dilution plating (see above) were saved and stored frozen. Beginning in late summer, *A. pullulans* was re-isolated from each floral wash and subject to PCR analysis with the *A. pullulans* primer sets described in Table 1.

A. pullulans was readily re-isolated from the frozen floral wash samples, and colonies that we had visually identified as this organism were verified with the general *A. pullulans* primer set (AP ITS). In contrast, the primer sets specific for Blossom Protect strains of *A. pullulans* (CF10 and CF40) provided results with variation that could be attributed to the treatment that each tree received. In general, if a flower was from a tree treated with Blossom Protect, then ~50% of the floral washes yielded a positive reaction with primer sets specific to strains CF10 and CF40 (**Fig. 6**). If a tree did not receive Blossom Protect as a treatment (e.g., water or Serenade Opti), then the proportion of *A. pullulans* isolates identified as Blossom Protect strains was only 8%.

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. We used these tools to investigate strain identity of *A. pullulans* isolates from pome fruit flower treated with integrated non-antibiotic fire blight control programs. Based on isolation onto dilution plates and the general AP-ITS primer set, we conclude ate *A. pullulans* is a very common organism on pear and apple flowers. Surprisingly, however, Blossom Protect strains of *A. pullulans* were not as large of proportion of the total population as we expected. Blossom Protect strains of *A. pullulans* were only about half of the strains detected on flowers from trees treated with this product, and < 10% of strains on trees not-treated with this product. We still consider these results preliminary and will investigate further in 2016. If these observations remain consistent, it means we don't completely understand why Blossom Protect is

highly effective for fire blight control. Perhaps the low pH buffer (Buffer protect) is playing a role larger than solely aiding the establishment of Blossom Protect strains of *A. pullulans* on floral surfaces. If so, then perhaps other materials used for fire blight could be enhanced with pH adjustment.

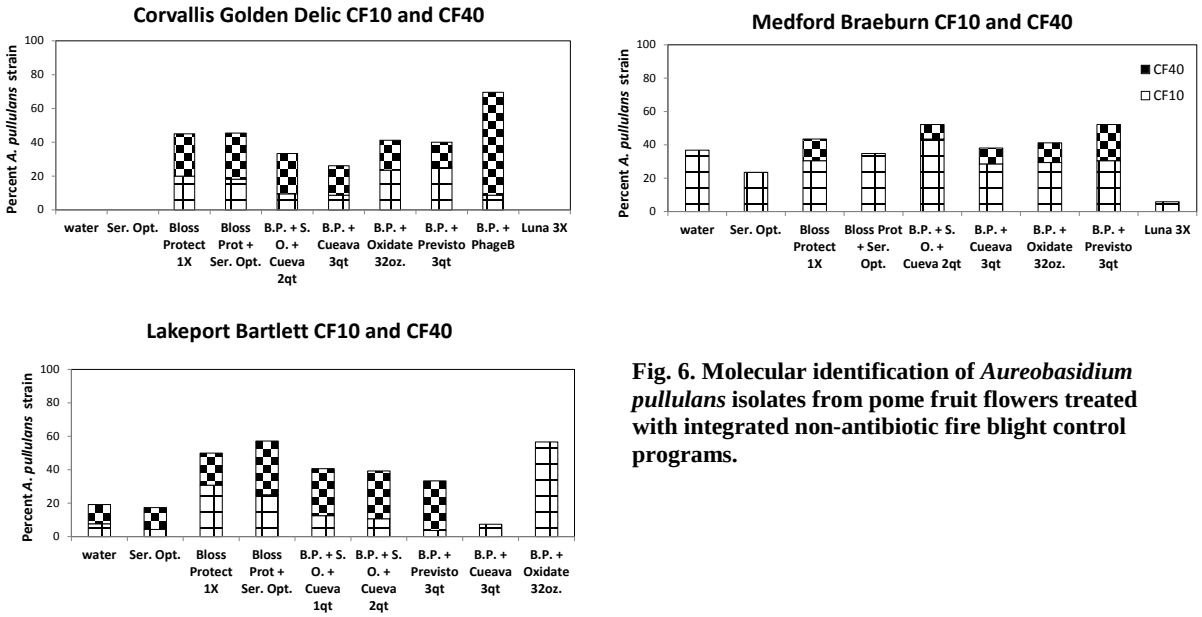


Fig. 6. Molecular identification of *Aureobasidium pullulans* isolates from pome fruit flowers treated with integrated non-antibiotic fire blight control programs.

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 16 -yr-old 'Gala' orchard near Corvallis, OR. COUGARBLIGHT, was low in early bloom but high near petal fall. For an inoculated trial, disease intensity was moderate with fire blight infections on water-treated trees averaging 42 strikes per tree (Table 1). Compared to the water-treated control, several materials by themselves reduced significantly ($P \leq 0.05$) incidence (%) of blighted flower clusters: the mineral potassium aluminum sulfate, the soluble coppers Cueva and Previsto, OxiDate (with HOLDit sticker), FireWall (streptomycin) and FireLine (oxytetracycline). Serenade Opti (with BioLink spreader) and FireQuencher A by themselves provided an insignificant level of suppression (23 to 28%); in contrast, the combination of Serenade Opti (with BioLink) and FireQuencher A provided very good control (70%), which was significantly ($P \leq 0.05$) better than either material alone. The combination of Double Nickel and Cueva also provided good control (58%), which was significantly ($P \leq 0.05$) better the Double Nickel alone. *P. agglomerans* strain C9-1 and the mixture of this bacterium with Fire Quencher both provided intermediate but insignificant levels of control (29 to 34%, respectively).

Discussion. Blossom Protect was excluded from this trial to determine how some of the materials we have been recommending for integrated, non-antibiotic control would perform without the pretreatment of the yeast. In this regard, Serenade, Cueva, and Oxidate were all intermediate performers, which we believe indicates the importance of Blossom Protect in the control program. From the perspective of certified organic production, Previsto is expected to be registered in 2016 and

Oxidate is expected to have a label modification to clarify that it can be used at the rate shown above. The mineral material, potassium aluminum sulfate, is being used for fire blight control in Europe, and a petition has been made to the National Organic Standards Board to allow for its use in organic agriculture (as a treatment for animal waste). More research on alum is needed to better understand its properties and to determine optimal rates. Fire Quencher A and B are experimental *E. amylovora*-specific phages (bacterial viruses) from the Dept. of Microbiology, Brigham Young University. Results of most phage treatments for fire blight control (and for plant disease control, in general) have been disappointing. Nonetheless, in the above table, the treatment of Fire Quencher A mixed with Serenade Opti and an ultraviolet protectant (sunscreen) provided outstanding control. We have proposed to look more closely at phages (two sources) and alum over the 2016-17 seasons.

Table 1. Gala apple, alternative materials fire blight trial, Corvallis, 2015.

Treatment	Rate per 100 gallons water	Date treatment applied* 9 Apr 70% bloom	15 Apr Full bloom	20 Apr Petal Fall	Number of blighted clusters per tree**		Percent blighted floral clusters***	
Water	-	**X**[§]	**X**	---	**42**	**a**[#]	**10.3**	**a**[#]
Double Nickel	64 fl. oz.	X	X	---	46	a	12.8	a
Fire Quencher B	16 fl. oz.	X	X	---	37	ab	10.7	ab
Fire Quencher A	16 fl. oz.	X	X	---	37	ab	7.9	ab
Serenade Opti and	20 oz.	X	X	---	23	abc	7.4	abc
BioLink spreader	4 fl. oz.	X	X	---				
Pantoea vagans C9-1	10^8 cfu/ml	X	X	---	27	abc	7.4	abc
Pantoea vagans C9-1	10^8 cfu/ml	X	X	---	23	abc	6.8	abc
plus Fire Quencher A	16 fl. oz.	X	X	---				
Cueva (3 quarts)	96 fl. oz.	X	X	---	20	abc	6.2	abcd
Cueva (2 quarts)	64 fl. oz.	X	X	---	22	abc	5.8	bcd
OxiDate 2.0	128 fl. oz.	---	X	X	25	abc	5.8	bcd
plus HOLDit sticker	32 fl. oz	---	X	X				
FireLine 200 ppm	16 oz.	X	X	---	21	abc	5.8	bcd
Double Nickel	32 fl. oz.	X	X	---	15	bc	4.3	cde
plus Cueva (2 quarts)	64 fl. oz.	X	X	---				
GWN 10073 (Previsto)	96 fl. oz.	X	X	---	14	c	3.4	de
Serenade Opti and	20 oz.	X	X	---	5	c	3.2	de
BioLink spreader	4 fl. oz.	X	X	---				
plus Fire Quencher A	16 fl. oz.	X	X	---				
FireWall	8 oz.	X	X	---	9	c	2.7	de
Alum 2%	16.6 lb.	---	X	X	7	c	1.6	e

* Trees inoculated on 12 April with 1 x 10^6 CFU/ml *Erwinia amylovora* strain Ea153N (streptomycin-sensitive and nalidixic acid resistant fire blight pathogen strain).
** 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 same letter do not differ significantly (P = 0.05) based on Fischer's protected least significance difference.

EXECUTIVE SUMMARY

Significant findings:

- Blossom Protect applied once at 70% bloom continued to provide significant fire blight control in apple and pear.
- In 2015, integrated fire blight control programs that began with Blossom Protect and followed by Serenade Opti, Cueva soluble copper or combinations of these materials showed enhanced suppression compared to Bloom Protect alone.
- In multi-location trials, Blossom Protect (comprised of two strains of Aureobasidium pullulans) showed a slight potential to increase fruit russeting at a wet location (Corvallis) and on sensitive cultivars (Comice and Golden Delicious), but did not induce russeting in Braeburn apple or Bartlett pear grown in semi-arid climates (Medford and Lakeport).
- Compared to Blossom Protect, the soluble copper, Cueva, showed a higher potential to russet fruit. Copper-induced russeting was observed on Bartlett pear fruit and Golden Delicious apple in a wet climate (Corvallis) and on Comice pear fruit in a semi-arid climate (Medford), but compared to treatment with water, was not increased significantly on Braeburn apple or Bartlett pear grown in a semi-arid climates (Medford and Lakeport).
- After full bloom, *Aureobasidium pullulans* was detected on nearly 100% of flowers sampled from trees treated with Blossom Protect, and on most flowers sampled from non-treated trees.
- Molecular methods to identify Blossom Protect strains of *A. pullulans* were verified and used to demonstrate that about half of *A. pullulans* isolates detected on trees treated with Blossom Protect were the strains from the biocontrol product, but that on trees not treated with Blossom Protect, the detected *A. pullulans* isolates were likely from a source within the orchard.
- Several additional materials – oxidizing agents (Oxidate and R42014), soluble copper (Previsto), alum (potassium aluminum sulfate), and E. amylovora-specific phage – showed potential to contribute to non-antibiotic fire blight control programs.

Industry implications: Owing to the antibiotic phase-out in National Organic Program-certified production, the fire blight control programs developed from this research project have been implemented by the organic tree fruit producers in Washington State. Compared to antibiotic programs, the non-antibiotic spray programs are effective for fire blight suppression but carry somewhat higher risks of fruit russeting. These risks are influenced by orchard climate and cultivar. For example, a russet-tolerant apple cultivar grown in an arid environment is much less russet-prone than a sensitive pear cultivar produced in a wet climate. We view the yeast material Blossom Protect (*Aureobasidum pullulans*) as an essential component to non-antibiotic fire blight control programs. Blossom Protect is produced to an excellent quality standard, and in most of central Washington, the risk of fruit russeting from this material is negligible. Soluble coppers (Cueva and Previsto) are also effective for disease suppression but carry a risk of fruit russet that is higher than observed for Blossom Protect. Consequently, their use during the bloom period needs to be limited to tolerant cultivars in dry environments, and for russet sensitive cultivars, to periods of fruit development when risk of russet has diminished (summer). Results of this project lead to three recommendations for further research: i) continued search for effective materials that can substitute for copper in late bloom treatments, and ii) obtain a better understanding of the effects of the Buffer Protect companion to Blossom Protect on the floral microbiome, and iii) investigate the ecology of *A. pullulans* strains resident in orchards, which could perhaps lead to cultural manipulation of their populations.

FINAL PROJECT REPORT

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

PI:	Yanmin Zhu	**Co-PI:**	Mark Mazzola
Organization:	TFRL-ARS-USDA	**Organization:**	TFRL-ARS-USDA
Telephone:	(509) 664-2280	**Telephone**	(509) 664-2280
Email:	yanmin.zhu@ars.usda.gov	**Email:**	mark.mazzola@ars.usda.gov
Address:	1104 N. Western Ave Wenatchee, WA 98801	Address:	1104 N. Western Ave Wenatchee, WA 98801

Cooperators: Gennaro Fazio, apple rootstock breeder, USDA ARS

Agency Name: USDA ARS Tree Fruit Research Lab
Amount awarded: **Year 1: $**54,000 **Year 2: $**55,000

Budget history:

Contract Administrator: Charles Myers, Extramural Agreements Specialist		
Email:cwmyers@pw.ars.usda.gov		
Item	**Year 1**	**Year 2**
Salaries *	38,790	38,790
Benefits	13,577	13,577
Wages		
Benefits		
Equipment		
Supplies	1,633	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 candidate apple genes based on the results of two previous genomic studies.
2. Screen these genes based on their expression behaviors between resistant and susceptible apple rootstocks.
3. Validate gene-trait associations within an expanded germplasm collection of a rootstock breeding population.

SIGNIFICANT FINDINGS

1. A working phenotyping protocols was established to characterize the biological basis between field observed ARD tolerant G.935 and susceptible B.9 apple rootstocks at the biological and microscopic levels.
2. A total of 43 apple genes were selected to test their expression behavior during *P. ultimum* infection among different rootstock genotypes.
3. Several apple genes related to plant secondary metabolism showed differential patterns of expression among rootstock cultivars with contrasting resistance responses.
4. Preliminary phenotyping data indicated a wider spectrum of resistance responses to infection by *P. ultimum* among O3 x R5 progeny.

RESULTS AND DISCUSSION

1. Phenotypic characterization of apple root resistance response using a streamlined protocol

Establishing the phenotyping protocol. Reliable phenotypes are essential for any molecular genetics study. Considerable effort resulted in the establishment of a streamlined protocol for characterizing the detailed apple root responses to pathogen infection. In the current study, *P. ultimum* was used as a primary ARD model pathogen, although a preliminary experiment was also carried out using other ARD pathogens. Taking the tolerant G.935 and the susceptible B.9 rootstock cultivars as examples, the resistance responses in apple root tissues were carefully examined at both biological and microscopic levels. In brief, the synchronized tissue culture processes were used to propagate both B.9 and G.935 plants with defined genetic background and equivalent ages for *P. ultimum* inoculation assay. As shown in **Figure 1**, similar root masses and root generating patterns were observed between B.9 and G.935 by this tissue culture procedure. A one-week "in-soil" acclimation period to condition the root tissues in the soil environment is necessary to ensure reliable resistance phenotypes for all these tissue-culture generated plants. Plants of both genotypes were inoculated with the same preparation of pathogen inoculum and under identical greenhouse management. For susceptible B.9, the wilt symptoms on the above-ground tissue were observable at 3 dpi. The necrosis patterns in root tissue inoculated with *P. ultimum* were observed at 1, 2, 3, 7 and 14 days post inoculation (dpi); plant survival rate, root biomass, shoot biomass and maximum root length for surviving plants were scored at 30 dpi. The same established procedure was used to assay the individual accessions within the O3 x R5 population.

Genotype-specific survival rate and biomass in response to *P. ultimum* infection. As shown in **Figure 2**, all inoculated G.935 plants except one survived; in contrast, only about 25% of B.9 plants were still alive at 14 dpi. Based on the repeats of inoculation assay, 97% of inoculated G.935 survived compared to 25 % for B.9 plants (**Figure 3**). Noticeably, a few inoculated G.935 plants (less than 5% of total tested) demonstrated light wilt symptoms around 7 dpi, but symptoms disappeared at 14 dpi. Differential responses in terms of root biomass fresh weight, maximum root length and shoot biomass fresh weight were observed among surviving G.935 and B.9 plants at 30 dpi (**Table 1**). Between mock inoculated and *P. ultimum* inoculated G.935 plants, no statistically significant differences were observed in root biomass and root maximum length; although a 30% reduction in shoot biomass was observed. The observed

reduction in shoot biomass suggested that the intrinsic functionality of the root system was partially compromised, even though there was no statistic difference in overall plant survival rate, root biomass and maximum root length. In contrast, shoot biomass for the surviving B.9 plants decreased 68% at 30 dpi compared to the mock inoculated B.9 plants. Similarly, total root biomass and maximum root length for surviving B.9 plants demonstrated a 70% and 40% reduction compared to those of mock inoculated B.9 plants. These observations represent the first evidence of a genotype-specific response among apple rootstocks to challenge by a representative ARD pathogen under controlled conditions.

Figure 1. Images of root system in culture medium and plants for in-soil acclimation. Left panels: Top images: G.935; bottom images: B.9. Images showed the comparable root system between B.9 and G.935 after three-week root induction in tissue culture medium immediately before in-soil acclimation. Right panels: images of plants after one-week in-soil acclimation and before *P. ultimum* inoculation. Top images: G.935; bottom images: B.9.

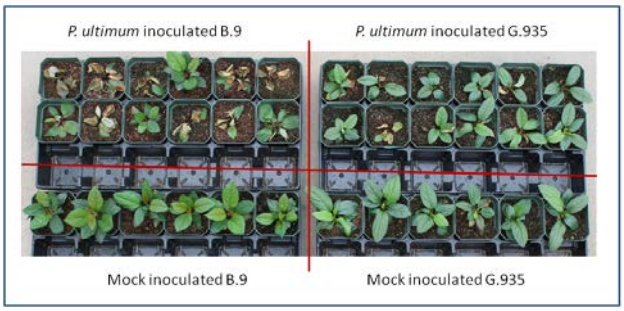


Figure 2. Images of a typical inoculation assay at 14 dpi. At the lower sections are the images of mock-inoculated plants for both B.9 (left) and G.935 (right); the upper panels are the images for *P. ultimum* inoculated plants, similarly B.9 plant on the left and G.935 on the right. The control plants are larger size of compared to *P. ultimum* inoculated and survived plants.

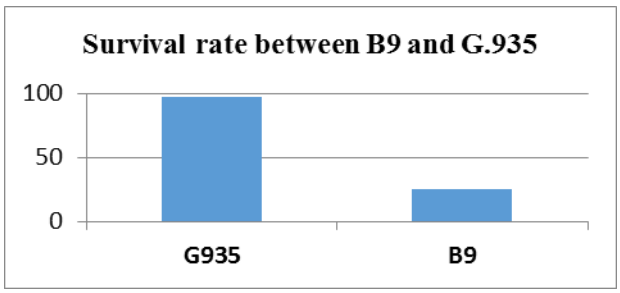


Figure 3. Survival of *Pythium ultimum* inoculated plants a percentage of the mock-inoculated control plants (y-axis is percent). The values were based on at least 155 plants for each cultivar in two independent infection assays.

Table 1. Root biomass, shoot biomass and maximum root length of mock inoculated and *Pythium ultimum* inoculated G.935 and B.9 plants.

	G.935		B.9	
	Mock inoculation	*P. ultimum* inoculation	Mock inoculation	*P. ultimum* inoculation
Root biomass (Fresh weight in g)	1.23 ± 0.31[a]	1.21 ± 0.42[a]	1.12 ± 0.43[a]	0.34 ± 0.10[b]
Maximum Root length (cm)	12.5 ± 3.90[a]	12.3 ± 3.32[a]	11.97 ± 4.91[a]	7.44 ± 4.5[b]
Shoot biomass (Fresh weight in g)	1.40 ± 0.33[a]	0.98 ± 0.26[b]	1.38 ± 0.51[a]	0.43 ± 0.33[b]

The means and standard deviations were based on the values of at least 155 survived plants for mock inoculated G.935, mock inoculated B.9 and *P. ultimum* inoculated G.935 in two independent inoculation assays. At least 40 survived B.9 plants were included from *P. ultimum* inoculated treatment. Means in a row designated with the same letter do not differ according to *t* test, with $P < 0.05$.

Differential root necrosis patterns between B.9 and G.935. No identifiable symptoms were observed on roots of G.935 or B.9 at one day after *P. ultimum* inoculation. At 2 dpi, the majority of G.935 root system remained healthy, showing characteristic white-color root tissues, except localized discolored areas along the root (left panels in **Figure 4A**, arrows). In contrast, symptoms were easily identified on inoculated roots of B.9 (right panels in **Figure 4A**, arrows). The typical symptoms were brown-colored root sections with collapsed or compressed tissues. Uninfected root sections were visible, though rare among B.9 roots. Minor wilt symptoms were observed on leaves for some inoculated B.9 plants. At 7 dpi, larger section of necrosis was easy to spot (left panel in **Figure 4B)**, yet most part of the root system were still healthy as indicated by the typical white color of G.935 roots. Noticeably, the relatively well defined zones which separate the healthy and infected root sections were easily identifiable (left panels in **Figure 4B**) as indicated by arrow. On the other hand, almost the entire B.9 root system was necrotic and brown colored at 7 dpi (right panels in **Figure 4B**). Upon closer examination, hyphae of *P. ultimum* were emerging profusely from infected root tissue (arrow on the images at the right bottom).

Figure 4A. Root necrosis patterns between B.9 and G.935 at 2 dpi

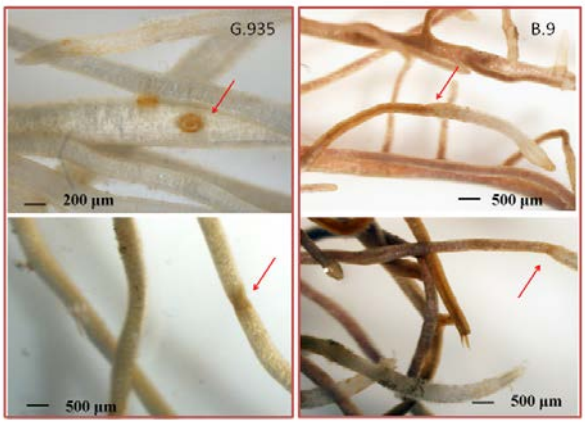


Figure 4B. Root necrosis patterns between B.9 and G.935 at 7 dpi

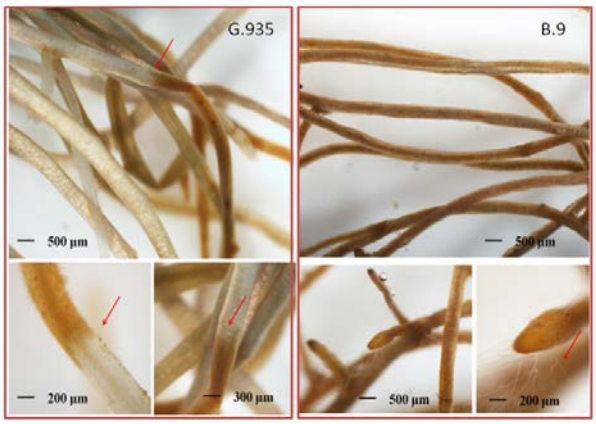


Figure 4. Microscopic images of infected roots for both tolerant G.935 and susceptible B.9. A. the box at left side, root images for both G.935 and B.9 at 2 dpi; B. The box at right, root images for both G.935 and B.9 at 7 dpi. Images show typical symptoms observed using three plants for each of two independent infection assays.

In summary, differential dynamics in necrosis development were observed along the roots of G.935 and B.9. The roots of G.935 exhibited an effective deterrence to pathogen progression, as indicated by limited or localized root necrosis at 2 dpi and a defined separation zone between healthy and necrotic tissue at 7

dpi. In contrast, the B.9 root demonstrated an inability to limit pathogen progression, resulting in rapid development of necrosis and discoloration across the entire root system at 7 dpi.

Field evaluation has indicated that apple rootstock cultivar G.935 is more tolerant to ARD compared to B.9, but the underlying biological bases and molecular mechanisms behind variation in field performance are unknown. Results from this study indicate that the disparity between the G.935 and B.9 resistance responses, in terms of plant survival rate, shoot and root biomass and patterns of root tissue necrosis, is the result of effective functional resistance mechanisms that exist in the roots of tolerant G.935, but not in B.9.

2. Selection of the apple candidate genes

Forty three (43) apple candidate genes, as shown in **Table 2**, were selected for initial analysis of their expression patterns during the infection process among core rootstock varieties with different responses to ARD. The selection of these genes was based on a previous transcriptome analysis, which identified the global gene expression network in apple roots in response to *P. ultimum* infection (1-96 hours after inoculation). Several groups (or families) of genes with annotated function in or regulation of plant secondary metabolism were the primary targets during the candidate gene selection. Many of these genes have multiple closely related members in the apple genome, and therefore initial screenings were performed.

Table 2. Selected apple genes for expression pattern analysis among apple rootstock germplasm

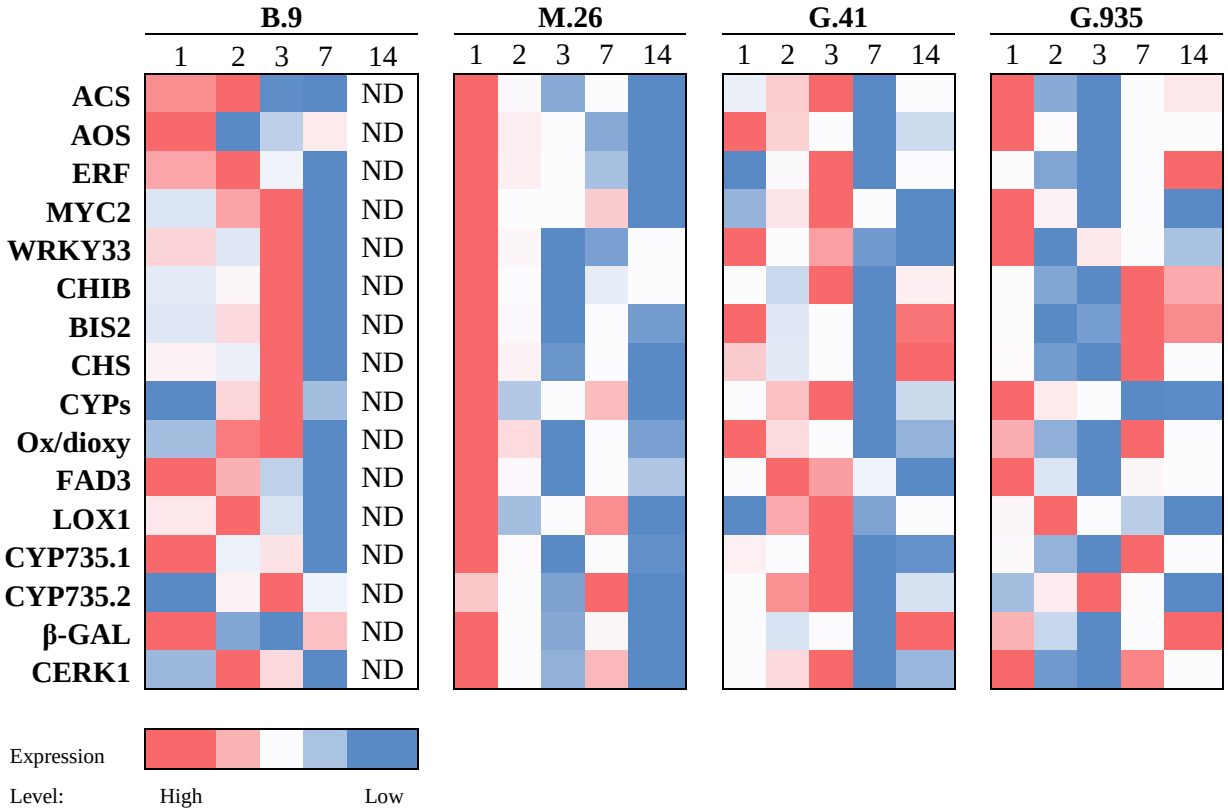
Gene name	Annotated functions	Apple gene identifiers
Chitin Elicitor Receptor Kinase	Pathogen detection	MDP0000136494
Aminocyclopropane-1-carboxylate synthase (ACS)	Ethylene biosynthesis	MDP0000435100, MDP0000130748, MDP0000262827
Allene oxide synthase (AOS)	JA biosynthesis	MDP0000132456, MDP0000195885, MDP0000230092
lipoxygenase (LOX)	JA biosynthesis	MDP0000312397, MDP0000423544
Ethylene response factor (ERF)	ET/JA signaling	MDP0000235313, MDP0000127134, MDP0000880063
Endochitinase (PR-4) CHIB	Pathogenesis-related protein	MDP0000430546, MDP0000655939
MYC2	Transcription factors	MDP0000029168
WRKY33	Transcription factors	MDP0000708692, MDP0000935996
NahG	Pathogenesis-related protein	MDP0000188175
Chalcone synthase (CHS)	Phenylpropanoid biosynthesis pathway	MDP0000431621, MDP0000686666, MDP0000067565, MDP0000302905
Beta-glucosidase (Beta-gluc)	Phenylpropanoid biosynthesis pathway	MDP0000175949, MDP0000315857
Flavonol synthase/flavanone 3-hydroxylase	Phenylpropanoid biosynthesis pathway	MDP0000218810
Biphenyl synthase 3 (BIS3)	Phenolics biosynthesis	MDP0000287919
Biphenyl synthase 2 (BIS2)	Phenolics biosynthesis	MDP0000208899, MDP0000432621, MDP0000716308, MDP0000168735
Spermidine synthase (SPDS)	Secondary metabolisms	MDP0000198590, MDP0000294813
Cytochrome P450	Secondary metabolisms	MDP0000678795
2-Oxoglutarate/Fe (II)-dependent dioxygenase	Secondary metabolisms	MDP0000128879
NAD(P)-linked oxidoreductase	Secondary metabolisms	MDP0000857724
Omega 3-fatty acid desaturase	Secondary metabolisms	MDP0000127630, MDP0000156530
Mandelonitrile lyase	Hydrogen cyanide generation	MDP0000318256
Cyanogenic beta-glucosidase	Hydrogen cyanide generation	MDP0000047586, MDP0000805281
Squalene monooxygenase	Terpenoid biosynthesis	MDP0000168437
Cytokinin hydroxylase	Cytokinin biosynthesis	MDP0000305091, MDP0000747755

In other pathosystems, the activation of these genes is known to play a role in deterring pathogen progression and proliferation in plant tissues. The induced production of anti-microbial metabolites, such as those from the phenylpropanoid biosynthesis pathways, at the localized infection site is believed to be one of the major mechanisms implicated in disease resistance. The hypothesis to be tested in this study is that the differential expression patterns of these genes contribute to genotype-specific apple root resistance responses to *P. ultimum* infection. All these genes were subjected to multi-sequence alignment analysis and gene-specific primer design before characterizing their expression patterns using quantitative reverse transcription polymerase chain reaction (qRT-PCR) method.

3. Differential expression patterns for selected candidate genes among rootstock genotypes

The overall gene expression data identified several apple candidate genes with variable expression patterns between tolerant and susceptible apple rootstocks. There was an elevated expression level for most of the tested genes in susceptible cultivars B.9 and M.26 in the early stage of infection; however, the induced expression quickly faded away for most of the tested genes. In contrast, there were a few genes that exhibited sustained activation in the tolerant cultivars of G.41 and G.935.

Table 3. Heat map representation of the activation levels of apple candidate genes among rootstock cultivars during the infection processes.



The level of induction was expressed as the relative fold change in infected tissues compared with the level in the mock inoculated tissues at the same time points. The darker red color represents the higher fold increase of gene activity in infected root tissue in relation to mock inoculation control; on the other hand, the darker blue color represents the lower fold change.

MdCHS (MDP0000431621), which catalyzes the early step of phenylpropanoid biosynthesis pathway, was highly induced in G.935 at 7 dpi, and a ß-endochitinase (or pathogenesis related protein 4) encoding

gene (*MdCHIB*: MDP0000430546) was induced at a higher level in the root tissue of G.935 and G.41, as compared to B.9 and M.26. In other pathosystems, it has been established that the key transcription factors of WRKY33 directly regulate the secondary metabolism in the process of generating antimicrobial metabolites. The gene expression patterns of *MdWRKY33* (MDP0000708692) showed different activation patterns between tolerant and susceptible cultivars. A gene encoding a ß-glucosidase (*MdGluc*: MDP0000175949), which is mapped to later phenylpropanoid biosynthesis pathway, exhibited a stronger induction toward the later stage in roots of tolerant cultivars G.41 and G.935, compared to those in B.9 and M.26. A gene encoding a homolog protein of chitin elicitor receptor kinase (or CERK), functions in detection of the pathogen presence and early activation of defense response. The CERK encoding gene (MDP0000136494) showed an earlier and stronger activation in the root of G.935, although it is also activated early in M.26. Therefore, it is possible that the activation of CERK is an essential part for the early stage of the defense activation; however, the coordinated activation of later-steps in secondary metabolism could also be critical.

Variation in the innate immunity responses for individual apple rootstock genotypes ultimately determines the outcome when challenged by ARD pathogens, in this case *P. ultimum*. The capability in early detection of pathogen presence, the strong activation of defense pathways and the production of antimicrobial metabolites are possibly the key to limit pathogen progression in the roots of G.935 and G.41. Similarly, the inability to foil "attacks" from pathogens, such as detoxification of damaging pathogen-derived chemicals, could also terminate effective defense response in B.9 and M.26. To our knowledge this is the first attempt to associate specific apple genes with a role in defending against attack by ARD pathogens. It will require additional experiments to exclusively and definitively identify the functional apple disease defense response genes including comparative transcriptome analysis using apple germplasm with well-defined resistance phenotypes.

4. Validating the selected genes using phenotyped accessions from O3 x R5 population

Figure 5A. A representing images showing variable resistance responses to infection by *P. ultimum* among O3 x R5 accessions

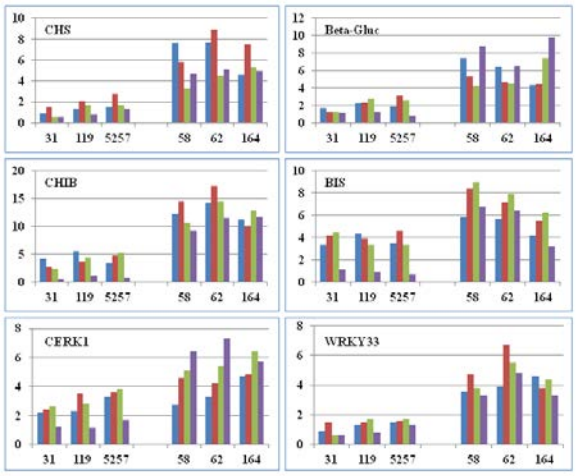


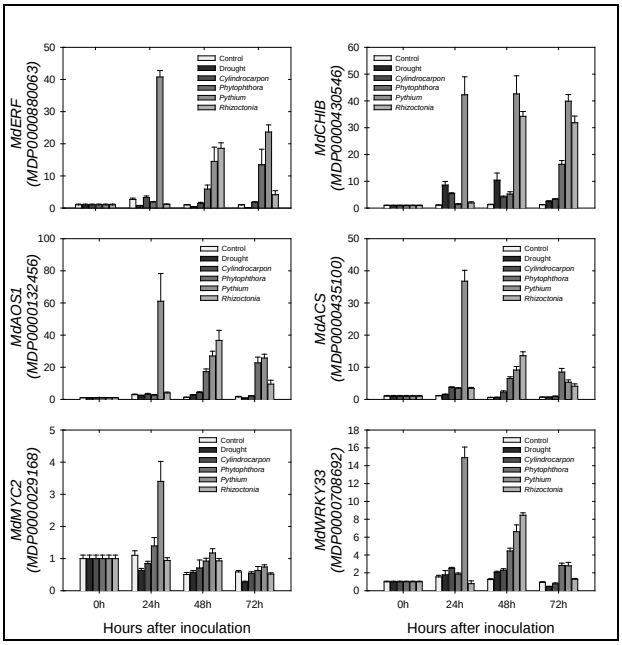
Figure 5B. Expression patterns for selected genes among O3 x R5 accessions

Figure 5. The expression patterns of six selected genes in response to *P. ultimum* infection between two groups (highly resistant and susceptible) of apple rootstock genotypes from O3 x R5 accessions. A: a representing image showing different resistance responses among individual accessions in O3 x R5 cross population. B: distinguishable gene expression patterns for selected six candidate genes between the more susceptible accessions (31, 119 and 5257) and the more resistant accessions (58, 62 and 164). Values on the Y axis are the fold changes after calibrating with the value from 0 dpi, i.e. the root sample before

inoculation assay, and normalized to the expression level in mock inoculated tissue at the same time point. The blue, red, green and purple bars (or from left to right) in each group of bars represent the time point of 1, 2, 3 and 7 dpi.

Six phenotyped accessions from O3 x R5 population showed more extreme resistance or susceptibility compared to the observed responses between B.9 and G.935. Among these accessions, no.58, 62 and 164 exhibited higher level of resistance as indicated by survival rates at the end of 30 days, while accession no.31, 119 and 5257 showed the extreme susceptible phenotypes such as earlier and more severe wilt symptoms. Four out of six tested genes, i.e. CHS, Beta-Gluc, CHIB and WRKY33, showed distinct expression profiles between two groups of rootstocks. These gene expression data for the selected candidate genes are in general aligned with those data using core collection of apple rootstocks, i.e. B.9 and M.26 versus G.41 and G.935.

5. Differential activation of selected candidate genes in response to infection by various ARD pathogens



Most of the selected genes were known to be induced by *P. ultimum* infection based upon a previous study. However, their expression patterns as challenged by other ARD pathogens have not been examined. As shown in **Figure 6**, the preliminary data suggested that, at least to these selected genes, their activation seemed to be more responsive to infection by *P. ultimum*, compared to other ARD pathogens. In particular, the highest expression levels were observed at 24 hpi for these tested genes. As controls, mock inoculation or the drought treatment did not activate the expression of these genes.

Figure 6. The expression patterns of selected candidate apple genes in response to the infection to *P. ultimum* and other ARD pathogens as well as drought treatment. Plants used in this experiment were those from apple (cv Gala) seed germination. Each group of bars represents a time point as indicated on X axis. Within each group of bars, from left, were control, drought treatment, inoculated by *Cylindrocarbon*, *Phytophthora*, *Pythium ultimum* and *Rhizoctonia solani* AG5. The values of expression level at 0 hr, i.e. before inoculation, were arbitrarily set as 1 for comparison of various treatments.

EXECUTIVE SUMMARY

The differential responses of apple rootstock genotypes to infection by ARD pathogens have been observed from both field evaluations and greenhouse based investigations. Nevertheless, the genetics behind such variations are unknown. Accurate and reliable phenotyping of apple rootstock germplasm in response to ARD pathogen infection is a critical prerequisite to identifying the underlying genetic components. Therefore, establishing the detailed and quantified phenotyping protocol was an important part of this study. Due to the complex etiology of ARD, *P. ultimum* was selected as a representative ARD pathogen in the current study. Defined plant materials that were generated by tissue culture procedures were utilized to characterize the resistance responses under controlled environment conditions. Distinct resistance responses were observed between the susceptible B.9 and the tolerant G.935. The accessions in O3 x R5 population exhibited even more extreme susceptible or resistant phenotypes. Building on the observed resistance phenotypes, apple candidate genes were selected based on the results of a previous transcriptome analysis. Specifically, forty three (43) candidate apple genes were screened for their differential expression patterns between tolerant G.41 and G.935, and susceptible M.26 and B.9. More distinguishable expression patterns were observed for several candidate genes among the phenotyped accessions from O3 x R5 population. Our data indicated that the differential resistance responses at the biological, microscopic and gene expression levels may ultimately contribute to the contrasting survival rate between B.9 and G.935 as they were challenged by the same preparation of *P. ultimum*. This dataset provided a foundation for more definitive and conclusive association between specific apple genes and different levels of root resistance among apple rootstock germplasm. Our phenotyping work will expand to other members within the ARD pathogen complex, and extend to the evaluation under field conditions.

Plant-pathogen interactions can be described as a “chemical warfare” between two partners. The capability in timely and efficient production of antimicrobial compounds (phytoalexins), releasing preformed metabolites (phytoanticipins), or detoxifying the pathogen-originated toxin have been shown to contribute to disease resistance in other pathosystems (Ahuja et al., 2012; Chizzali et al., 2012; Grayer and Kokubun, 2001; VanEtten et al., 1994). The observed strong deterrence of the fast-growing *Pythium* in the root of G.935 may reflect the effective defense mechanism by efficient accumulation of antimicrobial metabolites. On the other hand, inadequate production of antimicrobial metabolites in the root of B.9 could have allowed rapid necrosis along the root of susceptible B.9. The observed variations of their survival rate could largely attribute to the difference in defense reaction. Applying other advanced approaches should further narrow down the candidate genes and/or metabolites which are responsible for the observed necrosis development in G.935. For example, the comparative transcriptome analysis between G.935 and B.9 should assist enormously in attempt to pinpoint the specific genes robustly associated with observed apple root resistance or susceptibility to *P. ultimum* infection. Identification of apple genes associated with resistance phenotypes will be the basis for developing molecular tools for efficient and accurate incorporation of the resistance traits into next generation apple rootstock. Maximized utilization of plant innate immunity will reduce, even eliminate, the need of chemical fumigants in managing ARD in the future, and improve the soil quality for sustainable production system. Therefore, results from current study are directly related to the sustainability of Washington State apple industry.

FINAL PROJECT REPORT

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

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

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

Other funding sources: *None*

Total Project Funding: $126,000

Budget History:

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

ORIGINAL 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.

SIGNIFICANT FINDINGS (ACCOMPLISHMENTS)

- Additional detoxification enzymes/heat shock proteins were identified from a transcriptome
  - 50 transcripts encoding putative esterases
  - 80 transcripts encoding putative cytochrome P450 monooxygenases
  - 22 transcripts encoding putative glutathione *S*-transferases
  - 45 transcripts encoding putative heat shock proteins
- Protein targets for most classes of insecticides, past and present, were identified
  - Nicotinic acetylcholine receptor subunits (targets of neonicotinoids and spinosads)
  - Ryanodine receptor (target of rynaxypyr – Altacor)
  - Acetylcholinesterases (targets of Carbamates and Organophosphates)
  - GABA-gated chloride channels (targets of Organochlorines and phenylpyrazoles)
  - Sodium channels (targets of Pyrethroids and Indoxacarb)
  - Glutamate-gated chloride channels (targets of Avermectins and Milbemycins)
- A putative PBAN receptor has been cloned

- 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)
- Preliminary quantitative real-time PCR analyses completed
    - Determined relative expression levels of 3 heat shock proteins in various life stages
    - Determined relative expression levels of 11 Glutathione *S*-Transferases
    - Analyzed Nicotinic Acetylcholine, Ryanodine and PBAN receptors

RESULTS AND DISCUSSION

Analysis of Nicotinic Acetylcholine and Ryanodine Receptor mediated resistance

Neonicotinoid (Calypso) Resistance

Nicotinic acetylcholine receptors (nAChR) are the protein targets of neonicotinoids (Calypso). nAChRs are nerve membrane proteins composed of a combination of 5 alpha and beta subunits, and the subunits can be all the same (homomeric) or any combination of multiple subunits (heteromeric). Resistance to neonicotinoids has been observed in the field, mainly through mutations in particular nAChR subunits. For the brown leafhopper, a single amino acid change in either nAChR subunit α1 or nAChR subunit α3 has been associated with neonicotinoid resistance (Fig. 1A). We have cloned and sequenced nAChR subunits α1 and α3 from our codling moth colony and have not seen any clones carrying the point mutation which leads to neonicotinoid resistance (Fig. 1A). Because our colony was derived from wild codling moth populations collected from various regions in eastern WA, it is likely that the point mutations in nAChR subunits α1 and α3 are not present at this time. However, it is possible that these mutations could occur in the future and we have developed a PCR assay to monitor for this occurrence. There is also another nAChR point mutation that can lead to target site resistance. For the green peach aphid, a single amino acid change in nAChR subunit β1 has been associated with neonicotinoid resistance in the field (Fig. 1A). We have cloned and sequenced nAChR subunit β1 from our codling moth colony and have not seen any clones carrying the point mutation which leads to neonicotinoid resistance (Fig. 1A). We have developed a PCR assay to also monitor the nAChR subunit β1 for occurrence of point mutations.

Spinetoram (Delegate) Resistance

Nicotinic acetylcholine receptors are also the protein targets of spinosads (Delegate). Two different types of mutations in nAChR subunits have been found to confer resistance to spinosads. For the Western flower thrip, a point mutation in nAChR subunit α6 has been associated with spinosad resistance (Fig. 1A). We have cloned and sequenced nAChR subunit α6 from our codling moth colony and have not seen any clones carrying the point mutation which leads to spinosad resistance (Fig. 1A). Another type of mutation in nAChR subunit α6 has been associated with spinosad resistance in the silkworm, *Bombyx mori*. A deletion of 15 amino acids in the silkworm nAChR subunit α6 was determined to be the cause of spinosad resistance (Fig. 1A). We did not detect this deletion in our sequenced codling moth nAChR subunit α6 clones (Fig.1A). We have also developed a PCR assay so that we can monitor for the occurrence of this mutation in codling moth.

Anthranilic diamide (Altacor) Resistance

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. For the diamondback moth, a point mutation causing a single amino acid change has been associated with field resistance to chlorantraniliprole (Fig. 1B). We identified the ryanodine receptor from our codling moth transcriptome and cloned and sequenced the region containing the point mutation found in diamondback moth. We have not detected any clones of the ryanodine receptor from our codling moth colony that carry the point mutation that could lead to Altacor resistance (Fig. 1B). We are in the process of developing a PCR assay that we can use to monitor for the occurrence of a point mutation in codling moth that could lead to Altacor resistance.

A) Nicotinic Acetylcholine Receptor Subunits

Amino acids associated with susceptibility and resistance to neonicotinoids

```
Brown planthopper α1 (sus)     ...CEIDVRYFPFDQQKCFMKFGSWTYDGNHVDLRHM...
Brown planthopper α1 (res)     ...CEIDVRYFPFDQQKCFMKFGSWTSDGNHVDLRHM...
Codling moth      α1 (sus)     ...CEIDVEYFPFDEQTCFMKFGSWSYDGYTVDLRHL...

Brown planthopper α3 (sus)     ...CEIDVEYFPFDEQKCVMKFGSWTYNGAQVDLLKH...
Brown planthopper α3 (res)     ...CEIDVEYFPFDEQKCVMKFGSWTSNGAQVDLLKH...
Codling moth      α3 (sus)     ...CEIDVEYFPFDQQTCVMKFGSWTYDGFQVDLRHI...

Green peach aphid β1 (sus)     ...GLAFVQLINVNEKSQIMKSNVWLRLVWRDYQLQW...
Green peach aphid β1 (res)     ...GLAFVQLINVNEKSQIMKSNVWLTLVWRDYQLQW...
Codling moth      β1 (sus)     ...GLAFVQLINVNEKNQIMKSNVWLRLVWMDYQLMW...
```

Amino acids associated with susceptibility and resistance to spinosads

```
Western flower thrip α6 (sus)     ...TILLSLTVFLNMVAESMPTTSDAVPLIGTYFNCI...
Western flower thrip α6 (res)     ...TILLSLTVFLNMVAESMPTTSDAVPLIETYFNCI...
Codling moth         α6 (sus)     ...TILLSLTVFLNLVAETLPQVSDAIPLLGTYFNCI...

Silkworm     α6 (sus)   ...QIIDVDEKNQLLITNIWLSLEWNDY...
Silkworm     α6 (res)   ...QIIDV---------------EWNDY...
Codling moth α6 (sus)   ...QIIDVDEKNQILTTNVWLNLEWNDY...
```

B) Ryanodine Receptor

Amino acids associated with susceptibility and resistance to chlorantraniliprole

```
Diamondback moth (sus)   ...FFFAAHLLDVAVGFKTLRTILQSVT...
Diamondback moth (res)   ...FFFAAHLLDVAVEFKTLRTILQSVT...
Codling moth     (sus)   ...FFFAAHLLDVAVGFKTLRTILQSVT...
```

Figure 1. Target-site amino acids associated with insecticide susceptibility and resistance. Insects susceptible to the indicated insecticide are labeled with (sus). Resistant insects are labeled with (res). Amino acids indicated in the relevant mutations are high-lighted in gray.

Relative Quantification of Heat Shock Proteins and Glutathione* S*-Transferases

Glutathione S*-Transferase Expression*

An increase in glutathione S-transferases (GST) expression has been implicated in insect resistance to spinosads and rynaxypyr. To monitor codling moth for potential resistance to these compounds, we wanted to develop a quantitative real time PCR (qPCR) assay to look at transcript expression levels of individual GST enzymes. Basal GST expression levels relative to Actin (CpAct), a gene that is expressed at similar levels in all tissue types, for 11 GSTs, CpGST1, CpGST3, CpGST5, CpGST6, CpGST9, CpGST11, CpGST12, CpGST14, CpGST17, CpGST18 and CpGST19 were determined for neonates, adult male heads, adult female heads and a pool of RNA from all codling moth life stages (eggs through adults) using qPCR. In the RNA sample derived from all life stages, basal transcript expression of all 11 CpGSTs was detected, and ranged from 1.34×10^{-4} times that of Actin (CpAct) for CpGST6 to the highest level of 0.22 times that of CpAct for CpGST18 (Fig 2). Similarly, expression of all CpGSTs was detected in neonates, with levels ranging from 3.3×10^{-5} times that of CpAct for CpGST6 to 4.55×10^{-2} times that of CpAct for CpGST11 (Fig 2). No expression for CpGST3 was detected in adult male and female heads, and CpGST17 expression was also not detected in male heads (Fig 2). We observed that some GSTs display biased expression. For example, expression levels of CpGST3, CpGST12, CpGST14, CpGST17 and CpGST18 were higher in neonates than adult males and females while CpGST5, CpGST6, CpGST9 and CpGST11 were expressed higher in male and female heads than in neonates (Fig 2). Three GSTs, CpGST17, CpGST18 and CpGST19, displayed sex-biased expression with higher transcript levels detected in female heads compared to males. In the next two months, we will be determining the effects of sublethal doses of Altacor, Delegate and Calypso on GST expression levels. Additionally, we will finalize our development of a qPCR assay to monitor expression levels of cytochrome P-450 monooxygenases, another class of detoxification enzymes involved in insecticide resistance.

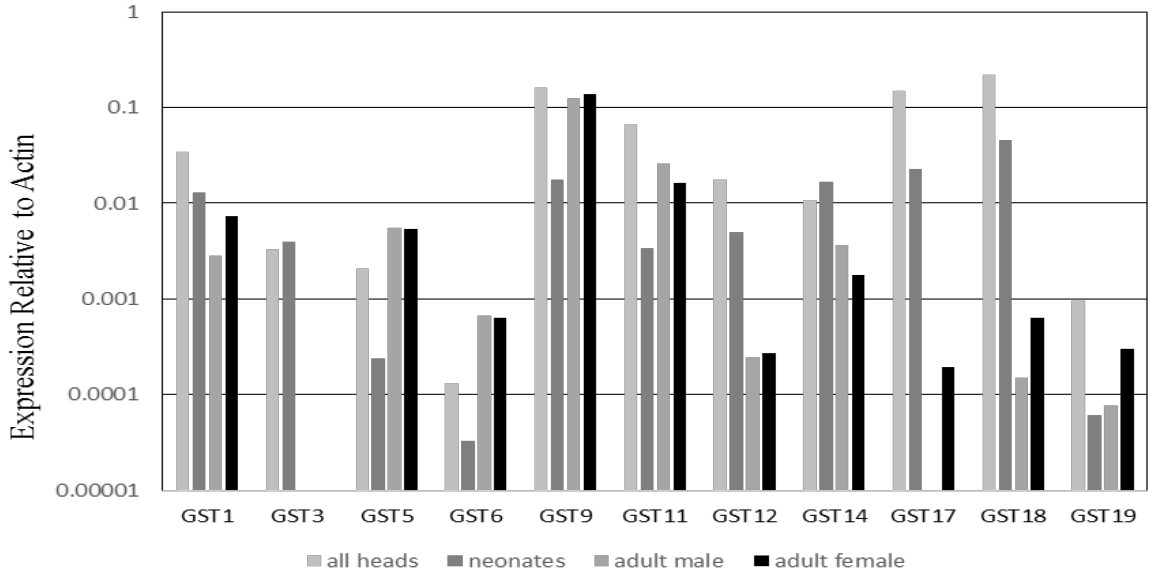


Figure 2. Relative expression of glutathione *S*-transferase transcripts in various life stages of codling moth as determined by quantitative real-time PCR. Expression levels of 11 CpGSTs relative to the Actin control.

Determining the basal expression levels for detoxification enzymes such as GSTs is an important first step in identifying potential roles of these enzymes in insecticide resistance. Detoxification enzyme mediated insecticide resistance usually occurs when the expression levels of one enzyme is substantially increased. Traditionally, microplate enzyme assays have been used in efforts to detect detoxification enzyme mediated resistance. These assays have often been found to be unreliable because they measure the activity of all isoforms of an enzyme in a single reading, even though resistance is usually the result of the overexpression of only one isoform. For example, when we perform a GST enzyme assay on codling moth extracts, we are measuring the activity of at least 26 unique GST proteins. The overexpression of just one of the GSTs may not be detected in a microplate assay. However, determining the expression levels of transcripts for individual GSTs (or other classes of detoxification enzymes) is more likely to provide us with the identity of the one enzyme isoform that is responsible for resistance.

Heat Shock Protein Expression

Insecticide resistance has also been correlated with expression levels of heat shock proteins. We have developed a qPCR assay to determine relative expression levels of three small heat shock proteins in codling moth. Relative expression levels of CpHsp19.8, CpHsp19.9, and CpHsp22.2, were determined for several developmental stages of codling moth (Fig. 3). In each stage, basal (without heat shock or insecticide treatments) transcript expression of all three CpHsps was detected, and ranged from 0.13 times that of CpAct for CpHsp19.8 in female pupae to 1.9×10^{-6} times that of CpAct for CpHsp22.2 in 3rd instar larvae (Fig.3). For CpHsp19.8, expression levels were significant between stages, with the highest expression found in pupal and adult females (Fig. 3), CpHsp19.9 expression was highest in 5th instars and adult males (Fig. 3). Significantly, very low expression of CpHsp22.2 was found in 3rd instars. These results provide us with an assay that can be used when trying to determine if heat shock proteins are involved in insecticide resistance mechanisms in codling moth.

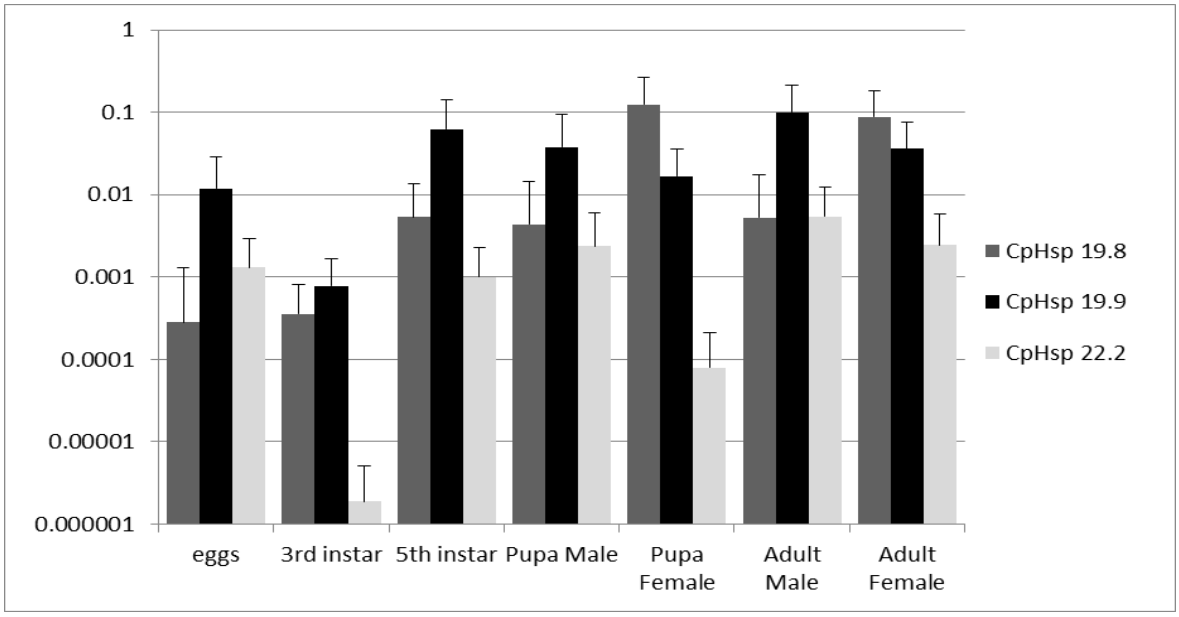


Figure 3. Relative expression of heat shock transcripts in various life stages of codling moth as determined by quantitative real-time PCR. Expression levels of CpHsp19.8, CpHsp19.9, and CpHsp22.2 relative to the Actin control.

EXECUTIVE SUMMARY

The use of neonicotinoids (Calypso), spinosyns (Delegate) and anthranilic diamides (Alatacor) have been effective in codling moth control programs. The possibility of codling moth becoming resistant to these control agents is of major concern to orchardists in the State of Washington. Insecticide resistance can occur by a couple of different mechanisms, target site resistance, and enzyme mediated detoxification. Therefore, the main goal of this project was to identify and characterize the protein targets of each of these insecticides, and to identify and characterize potential detoxification enzymes that could lead to resistance.

In previous WTFRC-funded projects, we prepared several transcriptomes from codling moth heads collected from all life stages. For this project, we used the transcriptome information to identify the protein targets of Calypso, Delegate and Altacor. Calypso and Delegate both target nicotinic acetylcholine receptors disrupting nerve function. Nicotinic acetylcholine receptors (nAChR) are nerve membrane proteins composed of five subunits. In this project, we identified, cloned and characterized 12 nAChR subunits (9 α, 3 β) expressed in codling moth heads. Point mutations and deletions in four of these subunits (α1, α3, α6 and β1) have been associated with field resistance to neonicotinoids and spinosyns. Analysis of clones of codling moth orthologs of nAChR subunits α1, α3, α6 and β1 revealed that there are currently no mutations present that would lead to target site resistance. We have developed PCR assays to monitor these subunits so that in the future if resistance occurs we can quickly determine if this resistance is caused by known target site alterations. The protein target of Altacor is the ryanodine receptor. From our transcriptome we were able to identify the codling moth ryanodine receptor. In diamondback moth, a point mutation has been associated with field resistance to anthranilic diamides. We cloned and sequenced the region associated with Altacor resistance and found that there are currently no mutations present that would lead to target site resistance. We are currently developing a PCR based assay to monitor codling moth ryanodine receptor so that in the future we can quickly determine if a point mutation is responsible for any increase in resistance.

Another potential resistance mechanism is through enzyme mediated detoxification of chemical control agents. The major classes of detoxification enzymes are carboxyl esterases, glutathione *S*-transferases and cytochrome P450 monooxygenases. From our codling moth transcriptomes, we have identified 50 transcripts encoding putative esterases, 80 transcripts encoding putative cytochrome P450s and 22 transcripts encoding putative glutathione S-transferases. We have cloned and sequenced 10 cytochrome P450 transcripts and 26 glutathione *S*-transferase transcripts. Using the sequence information from our cloned glutathione S-transferases, we have developed quantitative real time PCR assays to determine basal expression levels for these transcripts. We are currently treating insects with sublethal doses of Calypso, Altacor and Delegate so that we can determine if treatment with these chemicals has any effect on expression levels of the glutathione S-transferases. In the future, our qPCR assays will be used to quickly determine if resistant insects are using these enzymes to detoxify insecticides.

The results produced from this project have provided us with sequence information of protein targets and detoxification enzymes expressed by codling moth. Using this information, we have developed PCR based assays that will allow us to quickly determine potential insecticide resistance mechanisms in codling moth. Control of codling moth and other insect pests is critical for the production of clean fruit. We will continue to pursue this line of research so that in the future, should the need arise, we will be positioned to assist in analysis of codling moth that become resistant to chemical control agents.

CONTINUING PROJECT REPORT **YEAR**: 1 of 3
WTFRC Project Number: CP-15-100 (A, B and C)

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

PI: Jay Norelli
Organization: USDA-ARS Appalachian Fruit Research Station
Telephone: 304-725-3451 x264
Email: jay.norelli@ars.usda.gov
Address: 2217 Wiltshire Road
City/State/Zip: Kearneysville, WV 25430

Co-PI (2): Kate Evans
Organization: WSU Tree Fruit Research and Extension Center
Telephone: 509-663-8181 x245
Email: kate_evans@wsu.edu
Address: 1100 N. Western Ave
City/State/Zip: Wenatchee, WA 98801

Co-PI(3): Cameron Peace
Organization: WSU, Pullman
Telephone: (509) 335-6899
Email: cpeace@wsu.edu
Address: 149 Johnson Hall
Address 2: Department of Horticulture, Washington State University
City/State/Zip: Pullman, WA 99164-6414

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

Other funding sources

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

WTFRC Collaborative expenses: None

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

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

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

Budget 2
Organization Name: WSU-TFREC **Contract Administrator:** Carrie Johnson & Joni Cartwright
Telephone: 509 335 7667,509 663 8181 **Email address:** carriej@wsu.edu; joni.cartwright@wsu.edu

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

Footnotes:
[1]Wages for time-slip labor for orchard management and trait phenotyping
[2]Travel to research plots.

Budget 3
Organization Name: Washington State University **Contract Administrator:** Carrie Johnson
Telephone: (509) 335 4564 **Email address:** carriej@wsu.edu

Item	(type current year here)	(type additional year if relevant)	(type additional year if relevant)
Salaries			
Benefits			
Wages			
Benefits			
Equipment			
Supplies			
Travel			
Plot Fees			
Miscellaneous	$7,500[1]	$3,000[1]	$14,000[1]
Total	**$7,500**	**$3,000**	**$14,000**

Footnotes:1: genotyping

OBJECTIVES:

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

1. Determine the best sources of fire blight resistance in *Malus sieversii* to incorporate strong resistance into the **W**SU **a**pple **b**reeding **p**rogram (**WABP**).

- goal of Objective 1 is to incorporate strong fire blight resistance from *M. sieversii* into the WABP
- previous WTFRC project identified 12 *M. sieversii* accessions that are highly resistant to fire blight shoot infection and this project originally proposed evaluating only these 12, however we decide to expand the evaluation to 21 accessions of potential utility
- 17 of the 21 selected accessions were evaluated for their fruit quality last year (data not available at time of report preparation)
- the original 12 accessions were evaluated for their resistance to blossom blight infection (see Results and Discussion)
- all 21 of the candidate accessions will be evaluated for blossom blight resistance in spring 2016 and the 4 accessions not evaluated for fruit quality in 2015 will be evaluated in 2016 for fruit quality
- based on fruit quality and fire blight resistance the best 1-3 accessions will be used as parents to incorporate strong fire blight resistance into WABP in 2017

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

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

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

- goal of Objective 3 is to develop and evaluate DNA tests for known FBL among select fire blight tolerant parents that have been used in the WABP, specifically ‘Enterprise’, ‘Crimson Crisp’, ‘GoldRush’ and ‘Splendour’
- these DNA tests will be employed in the WABP at the completion of the current project (2018) to identify elite selections of the WABP carrying fire blight resistance genes and for the selection of fire blight tolerant seedlings

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

SIGNIFICANT FINDINGS:

- When 12 *M. sieversii* accessions that are highly resistant to fire blight **shoot** infection were evaluated for their resistance to **blossom** blight infection, 9 were found to have acceptable levels of blossom blight resistance for use in the WABP (Obj. 1)
- An orchard of 1,150 elite cultivars and their seedlings (3replicates of each, total = 3,500) was established at WSU's Columbia View Orchard for evaluation of fire blight resistance in 2016/17. (Obj. 2)
- 20 DNA markers for fire blight resistance were designed and are currently being evaluated for their ability to predict fire blight resistance in seedling (Obj. 3)

METHODS:

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

- Orchards of 194 *M. sieversii* accessions previously established in a 2012-2014 WTFRC project (PIs Norelli and Evans) at WSU's Columbia View Orchard and USDA-ARS, Kearneysville, WV are being used to evaluate the fruit quality and fire blight blossom susceptibility of 21 accessions of interest.

<u>Evaluation of fruit quality</u>:

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

<u>Evaluation of resistance to fire blight blossom infection</u>:

- Two-4 branches containing 75-100 blossom clusters will be identified on replicate trees and flagged prior to bloom.
- When at least 50 clusters contain at least 1 freshly opened blossom, the branches will be spray inoculated with a suspension of the fire blight bacteria using a back-pack sprayer.
- Two-three weeks after inoculation trees will be qualitatively evaluated for blossom infection (severe, moderate, light, none).
- Resistant and susceptible controls will be inoculated at the same time.
- Because all of 12 accessions will not bloom at the same time, inoculations will be staggered at 3 to 5 day intervals.
- Some accessions will be inoculated on multiple days to provide reference points to bridge comparison between accessions evaluated on different days.

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

- Because fire blight is a sporadic disease from year to year and in its distribution within the orchard, reliable evaluation of fire blight resistance requires controlled challenge of test plants with the fire blight bacteria.
- Because existing orchards of the RosBREED reference germplasm that were established for the evaluation of fruit quality could not be appropriately challenged with fire blight without risking destructions of many lines and the future establishment of fire blight, a distinct planting of the reference germplasm needed to be established.
- Vigorously growing shoots will be challenged by dipping a pair of scissors in a suspension of the fire blight bacteria and then cutting the youngest leaves of the shoot tip.
- Resistance will be determined by measuring the percent of the current seasons shoot length that becomes infected and measuring total lesion length.
- Results will be analyzed using FlexQTL software to identify and predict fire blight resistance-influencing loci (**FBL**). This software was developed and used in the previously funded "RosBREED: Enabling MAB" utilizing high-resolution genome scans already completed by the project and our analysis will draw upon expertise from the RosBREED project.

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

- DNA markers for 'Enterprise' FBL and related varieties 'GoldRush' and 'Crimson Crisp' will be designed based upon FBL recently described in the literature.
- Because parents of 'Goldrush' were used to previously identify FLB in the literature, the markers will initially be evaluated in a 'Goldrush' (resistant) X Pinata™ (susceptible) population. This cross was made by Norelli in 2013 and ca. 350 progeny are currently growing in the greenhouse.
- To evaluate the DNA markers, the 'Goldrush' progeny will be screened with the DNA markers and evaluated for their resistance to fire blight by controlled pathogen challenge. Association between the presence of specific DNA markers and resistance will be evaluated and potential markers identified.
- The DNA markers will then be evaluated in a 'Enterprise' X Pinata™ cross made by Norelli in 2014 that will be grown and evaluated in the greenhouse in 2015/2016 and in a 'Crimson Crisp' X 'Pinata™ cross in 2016/2017.
- Resistant progeny will be evaluated with DNA markers for fruit quality loci and resistant progeny containing desirable fruit quality alleles will be turned over to the WABP following the establishment of appropriate material transfer agreements between USDA-ARS and WSU.

- A similar approach will be used to identify DNA markers for 'Splendour' FBL, except that the FBL will be identified in the current RosBREED2 project.
- A 'Gala' X 'Splendour' population of 250 seedlings was established in 2011 and has been evaluated for fire blight resistance in the greenhouse in 2012-2014.
- The population was planted in the field at the USDA-ARS, Kearneysville in fall 2014 and will be further evaluated for fire blight resistance in 2016/17.

- An additional ‘Splendour’ X ‘Cripps Pink’ seedling population will be used to evaluate the DNAmarkers.

RESULTS & DISCUSSSION:

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

A previous WTFRC project identified several *M. sieversii* (wild apple) accessions that are highly resistant to fire blight shoot infection and could serve as sources of strong fire blight resistance for breeding. To identify the best 1-3 accessions to use in the WABP we are now evaluating these shoot blight resistant accessions for their fruit quality and resistance to blossom blight. Our original project plan proposed evaluating the 12 most resistant accessions, however we decide to expand the evaluation to 21 accessions of potential utility.

Evaluation of fruit quality: Fruit of 17 accessions were harvested from the research plot at USDA-ARS Kearneysville, WV and express shipped to Kate Evans at WSU-TFREC where it was run through the WABP’s standard fruit quality evaluation (data not available at time of report preparation). Due to the biennial bearing of some *M. sieversii* accessions, fruit was not available for 4 accessions which will be evaluated in 2016.

Evaluation of blossom blight susceptibility: Twelve accessions were evaluated for their resistance to fire blight blossom infection. Of these, 3 (Table 1: GMAL4211.a, PI657085, GMAL4211.d) were found to have unacceptably high levels of blossom blight susceptibility in either the West Virginia or Washington trial and will not be considered for use in the WABP. These evaluations will be repeated in 2016 on as many of the 21 accessions as possible.

Table 1. Evaluation of *M. sieversii* accessions for their resistance to fire blight blossom infection.

Accession	Type	WV Rating	WA Rating
Robusata 5	Control	nt	Highly Resistant
Delicious'	Control	Intermediate	nt
Empire	Control	Intermediate	nt
PI657115	Treatment	Resistant	Highly Resistant
GMAL3688.c	Treatment	Resistant	Highly Resistant
GMAL3975.c	Treatment	Resistant	Resistant
GMAL4002.k	Treatment	Resistant	Resistant
PI657116	Treatment	Intermediate	Highly Resistant
GMAL3616.o	Treatment	Intermediate	Highly Resistant
GMAL3989.c	Treatment	Intermediate	Resistant
GMAL4002.m	Treatment	Intermediate	Resistant
PI657054	Treatment	Intermediate	Intermediate
GMAL4211.a	Treatment	Susceptible	Intermediate
PI657085	Treatment	Highly Susceptible	Resistant
GMAL4211.d	Treatment	Susceptible	Highly Susceptible

Robusta 5 = a source of fire blight resistance used in Geneva apple rootstock breeding program; nt = not tested

The most devastating economic losses from fire blight are caused by the spread of the disease within the tree which can kill young trees outright or result in the loss of major structural scaffolds in the tree. These losses are compounded over many years due to both lost investment and reduced future productivity of the orchard, making them far more costly than annual or single year yield reductions. For this reason, susceptibility to the severity of shoot infection is considered more important than the incidence of blossom infection when evaluating material as a potential source of fire blight resistance. Growers can tolerate losing a large amount of bloom to fire blight if those infections do not run through the tree, while only a few blighted blossoms can be devastating if the disease spreads and kills the tree. Therefore, initial evaluation of almost 200 *M. sieversii* accessions as possible sources of fire blight resistance was based upon their resistance to the severity of shoot blight infection. In selecting among the accessions most resistant to shoot blight, fruit quality will be the primary selection factor. A secondary factor to be considered will be the accession's susceptibility to blossom blight, especially if the accession shows a high level of blossom blight susceptibility.

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

Although Objective 1 will identify the best sources of fire blight resistance to be used in future crosses, it will not facilitate selection of fire blight resistance among the existing seedlings and selections of the WABP. The goal of Objective 2 is to leverage resources developed in the first RosBREED project to identify genetic factors which are already present within the WABP that affect the severity of fire blight. Although the material currently in the WABP may not be immune to fire blight, we know there is a large gradation of susceptibility from "tolerance" to fire blight to high susceptibility. An example of a tolerant variety is 'Delicious', which may occasionally get some fire blight but devastating financial losses due to fire blight rarely occur in 'Delicious'. This type of resistance is very useful, however most of the genetic factors controlling this "tolerance" are currently not known. To identify these **f**ire **b**light resistance-influencing **l**oci (**FBL**) we will determine the fire blight resistance of the RosBREED reference germplasm. In depth genetic analysis of these elite lines and their seedlings has already been conducted in the RosBREED project and software has been developed to identify genetic loci controlling traits within the germplasm. Because existing orchards of the RosBREED reference germplasm established for the evaluation of fruit quality could not be appropriately challenged with fire blight without risking destructions of many lines and the establishment of fire blight within the orchard, a distinct planting of the reference germplasm needed to be established.

With financial support of the WTFRC the RosBREED reference germplasm was propagated at Willow Drive Nursery and was establish this past spring at the WSU Columbia View Orchard. The planting contains three replicate trees of 1,150 elite cultivars and their seedlings. Deer fencing surrounds the planting to prevent deer damage. This material will be evaluated for its fire blight resistance in 2016 and 2017. Results will be analyzed in 2017 using the software developed in RosBREED to identify FBL within the WABP. This will be a significant accomplishment because it will allow us to develop DNA tests to identify elite lines and seedlings already present in the WABP with a high probability of having "tolerance" to fire blight. However, the development of the DNA tests for the identified FBL will require further research before they could be used in the WABP.

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

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

Recent research in the USA and Europe has identified genomic regions, or "loci", influencing the fire blight tolerance of 'Enterprise', 'GoldRush' and 'CrimsonCrisp'. We have used this information to design 20 potential DNA markers, or tests, for the FBL present in these cultivars. These markers are currently being evaluated in approximately 300 seedling of an existing population derived from a cross of 'GoldRush' X Pinata™. The seedlings are evaluated for their fire blight resistance by challenging them with the fire blight pathogen in the greenhouse and DNA isolated from the seedling is run through the DNA test. The markers will then be evaluated based upon the association between the observed resistance of the seedling after pathogen challenge and predicted resistance from the DNA test. Approximately 300 seedlings derived from a cross of 'Enterprise' X Pinata™ will be similarly evaluated in 2016/17, and seedlings from a cross made this past season of 'CrimsonCrisp' X Pinata™ will be evaluated in 2017.

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

Incorporating fire blight resistance into the WABP will lead to the release of new cultivars with fire blight resistance similar to or greater than that of 'Delicious' and thereby greatly reduce the occurrence of fire blight and reduce the need for high-priced applications of antibiotics. This proposed research is partially supported by the RosBREED2 project aimed at combining disease resistance with horticultural quality in new fruit varieties. The support from the WTFRC for this project combined with the opportunity for Co-PIs to work together with other leaders in the field of fruit breeding in the RosBREED project is accelerating the progress of the project.

CONTINUING PROJECT REPORT **YEAR**: Extension
WTFRC Project Number: CP-14-102

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

PI:	Walter S. Sheppard	**Co-PI**:	Brandon Hopkins
Organization:	Washington State University	**Organization**:	Washington State University
Telephone:	509-335-0481	**Telephone**:	509-335-8598
Email:	shepp@wsu.edu	**Email**:	bhopkins@wsu.edu
Address:	Department of Entomology	**Address**:	Department of Entomology
City/State/Zip:	Pullman, WA 99164-6382	**City/State/Zip**:	Pullman, WA 99164-6382

Cooperators: Dr. Roman Jashenko, Institute of Zoology, 93 Al-Farabi Ave, Almaty, Kazakhstan

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

Project was originally funded in 2014 for 3 years.
No report submitted

CONTINUING PROJECT REPORT **YEAR**: 1 of 2 (No-cost extension)
WTFRC Project Number: CP-14-104

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

PI:	David Crowder	**Co-PI**:	Vincent Jones
Organization:	WSU-Pullman	**Organization**:	WSU–TFREC
Telephone:	509-335-7965	**Telephone**:	509-663-8181 ext. 291
Email:	dcrowder@wsu.edu	**Email**:	vpjones@wsu.edu
Co-PI:	John Reganold	**Co-PI:**	Elizabeth Beers
Organization:	WSU–Pullman	**Organization:**	WSU-TFREC
Telephone:	509-335-8856	**Telephone:**	509-663-8181 ext. 234
Email:	reganold@wsu.edu	**Email:**	ebeers@wsu.edu

Cooperators: Apple growers throughout Washington State

Total Project Request: **Year 1:** $56,279 **Year 2:** $57,669 **Year 3:** $0

Other funding sources

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

Amount Awarded: $194,910

Notes: This award expands our project to include an analysis of how apple growers make management decisions for woolly apple aphid, other pests, and soil quality based on interviews. We will also include an economic analysis of how woolly apple aphids affect apple production.

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

Item	2014	2015	2016
Salaries[1]	$27,099	$28,183	
Benefits[2]	$2,453	$2,552	
Wages[3]	$11,133	$11,322	
Benefits[4]	$594	$612	
Supplies[5]	$9,000	$9,000	
Travel[6]	$6,000	$6,000	
Total	$56,279	$57,669	0

Footnotes:
Continuation report submitted for a no cost time extension

Objectives:

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

The goal of our project is to determine the factors affecting woolly apple aphid populations on conventional and organic orchards. To accomplish this, we sampled paired conventional and organic orchards throughout Washington. In each orchard we sampled populations of aphids and natural enemies and collected data on soil quality, plant nitrogen, and incidence of perennial canker. We were able to accomplish all of our research objectives for the 2015 field season. This work will be finalized in 2016. Combining data across three years will allow us to assess factors influencing woolly apple aphids on both conventional and organic orchards.

Significant Findings from 2015:

- Sampled 20 orchards (10 conventional, 10 organic for woolly apply aphids). Densities of aphids in organic orchards were higher than densities in conventional orchards
- Organic and conventional orchards had similar number of earwigs.
- The parasitoid *Aphelinus mali* and earwigs appear to be significant contributors to aphid biological control. Parasitism rates and earwigs typically peaked in late July and August, during which time aphid colonies crashed. Growers interviewed rarely think of earwigs as natural enemies, and the importance of this species may have been overlooked.
- Organic and conventional orchards did not differ significantly in soil nutrient content, and overall soil quality did not appear to significantly affect woolly apple aphids. .
- We found no relationship between the incidence of perennial canker on trees and woolly apple aphid abundance. However, it is clear that aphids use wounds caused by perennial canker as sites of infestation. This correlation may have led to the faulty conclusion that aphids are associated with transmission of this fungal pathogen.
- The percentage of perennial cankers infested with woolly apple aphids was much higher than the percentage of other feeding sites on tree trunks, but perennial cankers were rare.
- We worked with collaborating growers to obtain pesticide usage records from 2014. We are still collecting 2015 data. Based on 2014 data organic farms had lower pesticide usage compared to conventional farms. Our hypothesis is that less intensive pesticide practices lower aphid densities, which we will test when our dataset is complete in 2016.

Objective 1 - Sample populations of woolly apple aphids and natural enemies in organic and conventional apple orchards

Methods. We measured woolly apple aphid densities in 21 orchards (9 organic, 3 transitioning, 8 conventional). The number of woolly apple aphid colonies was counted on a total of 10 one-foot long twigs on each of 16 trees per orchard. We made 4 to 5 visits to each location from May to August. At each orchard we also collected lacewings as *A. mali* wasps with sticky cards combined with pheromone lures. Earwigs were monitored using rolled corrugated cardboard shelters, which earwigs hide in during the day, where they can easily be shaken out and counted in the field. Lacewings and *A. mali* wasps from 2015 are still being counted in the laboratory, but earwigs have been analyzed.

Results and Discussion

Woolly Apple Aphids. Organic orchards had 30% more woolly apple aphids compared with conventional orchards (Fig. 1), and transitional orchards had the highest aphid counts. However, these trends were not significant, suggesting that organic vs. conventional management may not be the most important factor for woolly apple aphids. Rather, our results to date suggest that pesticide intensity and natural enemy populations may be the most significant contributors to woolly apple aphid densities. We hope to clarify these relationships in 2016. It is important to note that woolly apple aphid counts were lowest in the period from late June to late July, when very hot temperatures (detrimental to woolly apple aphid growth and survival) occurred (Fig. 2).

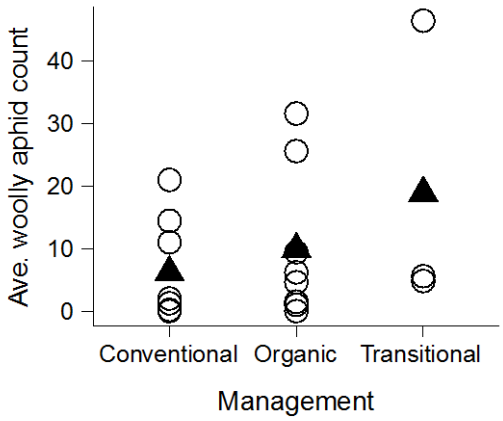


Fig. 1. Woolly apple aphid counts in 2015 at different orchards (open circles). Means for each management style are shown as triangles.

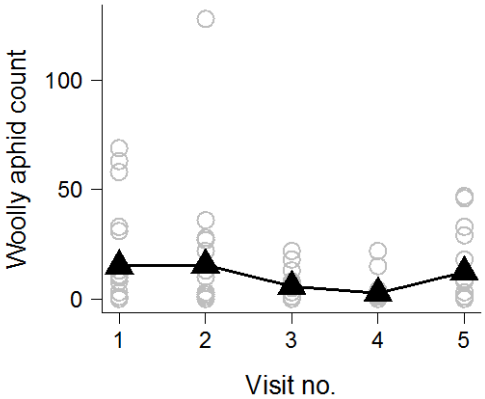


Fig 2. Aphid densities over time. Orchards are shown as open circles and the line following triangles represents means. Visit 1: 5/19-6/1; visit 2: 6/2-6/12; visit 3: 6/22-7/8; visit 4: 8/6-8/19; visit 5: 8/16-9/10

Natural Enemies. Conventional, transitioning, and organic orchards had similar mean counts of earwigs (Fig. 3). This result is similar to 2014, where earwigs did not differ between our different management styles. We are still processing the lacewing and parasitoid data, although in 2014 these species were more common in organic compared with conventional orchards.

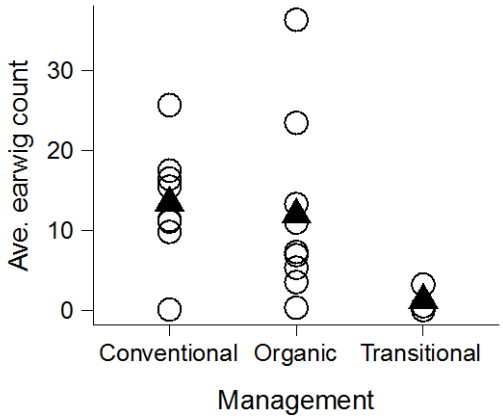


Fig 3. Average earwigs per trap. Circles show individual orchards while triangles show mean for an orchard type.

Aphids and Natural Enemies. The population dynamics of aphids is affected by natural enemies (Fig. 4). In this case, as was typical, aphid populations peaked in the summer. As *A. mali* and earwigs began to increase in late July/August), populations of aphids crashed. Aphid management depends on healthy populations of natural enemies in orchards. Continuing work will attempt to delineate the effects of each natural enemy species as well as the impact of orchard management practices and pesticide-use intensity on natural enemies.

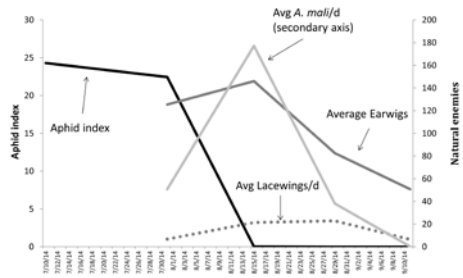


Fig. 4. Relationship between aphid densities and natural enemies in a typical orchard

Work this Coming Year. In the coming year we will continue to analyze data on lacewings and *A. mali* from our fields. This will be supplemented with natural enemy and woolly apple aphid sampling in 2016. We plan to more thoroughly investigate the role of earwigs using molecular gut-content analysis and mark-recapture studies in 2016. Moreover, once we have collected all the data on pesticides to supplement our biological data we will be able to build more complete models to explain the suite of factors affecting aphids and natural enemies in orchards.

Objective 2 - Collect information on soil quality, plant nitrogen content, and perennial canker

Methods. We measured soil nutrient content, texture, and plant nutrient content on all 20 orchards. Soil quality at the 20 orchards was quantified using an innovative soil quality index for Washington orchards, where a score of 1 is the best soil and a score of 0 is the worst. To measure plant nitrogen content, leaf samples were taken from the 16 trees where woolly apple aphid colonies were counted on each of four separate visits to each orchard. We used visual symptoms to identify cankers on trees in consultation with field men at each orchard.

Results and Discussion

Plant Nitrogen Content. Though conventional orchards had higher mean nitrogen than the organically managed orchards, the difference was not significant (two-tailed t-tests; P = 0.40) (Fig. 5). Within orchards, leaf nitrogen did not vary much over time (Fig. 6). This suggest leaf nitrogen is not likely a major factor affect variation in woolly apple aphids.

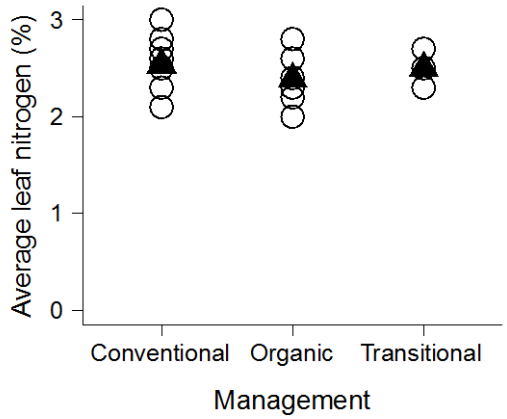


Fig. 5. Leaf nitrogen (%) at conventional, organic, and transitioning orchards. Results were averaged over 4 visits in 2015. Open circles represent individual orchards, and means are shown in closed triangles.

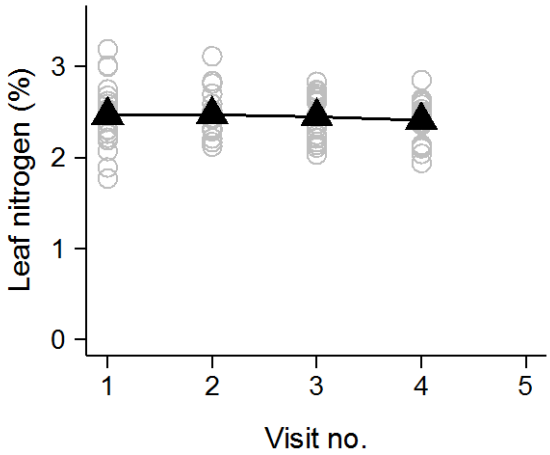


Fig. 6. Leaf nitrogen (%) averaged across orchards of all types at four visits in 2015. Open circles represent individual orchards, with triangles representing the mean values.

Soil Quality. Interestingly, although there was variation in soil quality scores in the 20 orchards, organic orchards did not have superior soil quality on average (Fig. 7). This contrasts with previous results that have shown organic orchards often have greater soil quality.

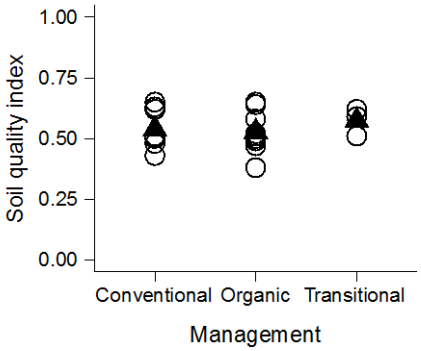
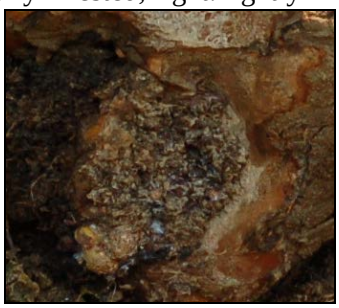


Fig. 7. Soil quality at 20 orchards in 2015. Orchards are represented by circles and mean values for each management type by triangles. Ideal soil quality receives a score of 1, and the lowest possible soil quality receives a score of 0.

Perennial Canker. Only one orchard we sampled had a noticeable incidence of perennial canker. Out of approximately 4,500 trees inspected at this site, perennial cankers were found on 32 trees (0.7%). Of those cankers, 30 (94%) were infested with woolly apple aphid. This percentage is much higher than the percentage infested of other potential feeding sites on trunks on a random sample of 60 trees. The number of structures and percentage infested with woolly apple aphid found in this survey were: burr knot: 35 (46% infested); pruning wound: 7 (0% infested); and misc. cuts, knicks, and unidentified tree wounds: 13 (18%). Of the 60 trees, 75% had infestations of woolly apple aphid on their trunks. These data suggest a strong association between perennial cankers and woolly apple aphids. However, the rarity of perennial cankers at the study orchards indicates a negligible role for cankers in affecting woolly apple aphid population dynamics.

Photos of woolly apple aphid feeding sites (all photos by Robert J Orpet)
An infested perennial canker (see aphids at bottom of canker in photo on the left). Also see aphids from under the trunk on this same tree after the bark was peeled back

Infested burr knots (left: heavily infested; right: lightly infested with few aphids).



Work this Coming Year. Data from both 2014 and 2015 indicate that soil quality and nitrogen content did not vary much between orchards, or contribute significantly to woolly apple aphid dynamics. However, there is potential that other management factors such as mulching techniques might impact aphids. This will be investigated in our no-cost extension year. In the coming year we will continue to explore the potential role of perennial canker in tree use by aphids. While it does not appear that aphids are associated with canker, they can be opportunistic and will quickly infest canker sites if they appear on trees.

Objective 3 - Analyze linkages between soil quality, plant nitrogen content, pesticide-use intensity, natural enemies, and woolly apple aphid populations in organic and conventional orchards.
Methods. As described above, using data collected in Objectives 1 and 2, we determined if woolly apple aphid and natural enemy densities, soil nutrient content, and plant nitrogen content differed across orchard farming systems. Once data is collected on soil quality and pesticide use on each orchard we will develop a model to evaluate the combined impacts of farming system, pesticide-use intensity, geographic location, natural enemy density, soil quality, and plant nitrogen content on woolly apple aphid densities. Our analysis will provide the first systems-wide examination of the effects of management and environmental factors on woolly apple aphids. We will also be able to isolate effects of management practices and geographic location on key aphid natural enemies. Importantly, we will be able to distinguish factors that affect woolly apple aphid and natural enemy population trends in both organic and conventional orchards and in two major apple growing regions (Wenatchee and Yakima valleys). This will provide important information that will be used to develop integrated pest management (IPM) programs for woolly apple aphids in organic and conventional orchards throughout the state.

Results and discussion
We were only able so far to obtain pesticide records for 2014, and observed there was variation in management intensity in the 20 orchards. Organically managed orchards tended to have fewer scored sprays than conventional orchards (Fig. 9), and transitional orchards had the least.

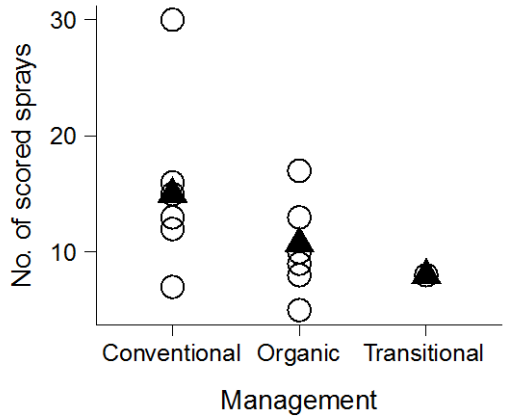


Fig. 9. Number of scored pesticide sprays at the 20 orchards. Each orchard is represented by a circle and mean values by management are by triangles.

Work this Coming Year. Objective 3 can only be completed once we have the complete dataset collected. This will require us to continue to collect and analyze data for one additional field season, using our no-cost extension. Once this entire dataset is completed we will complete this Objective in 2016.

CONTINUING PROJECT REPORT **YEAR**: 3 of 3 (No cost extension)
WTFRC Project Number: CP-13-102A

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

PI: Peter Landolt
Organization: USDA, ARS
Telephone: (509) 454-6570
Email: peter.landolt@ars.usda.gov
Address: 5230 Konnowac Pass Road
Address 2:
City/State/Zip: Wapato, WA 98951

Co-PI (2): Peter Lo
Organization: Plant and Food Research
Telephone: 64 6 975 8920
Email: peter.lo@plantandfood.co.nz
Address: Cnr Crosses and St. Georges Roads,
Address 2: Havelock North
City/State/Zip: Hawkes Bay, New Zealand 4130

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. Work to develop the sachet requires analytical chemistry supplies, to include solvents, GC gases, SuperQ traps, and replacement glassware. 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 (CM) in orchard plots using the attract-and-kill approach (A & K). Our prior research led to the use of a sticky trap as killing station, with a 3-chemical kairomone attractant as the lure. The technical objectives of the work are to:

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

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

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

METHODS (for 2016 field season).

1. Additional four-acre field plot tests will be conducted to determine consistency of the impact of our traps on codling moth populations and reductions or prevention of codling moth infestation of apple fruit. Treated plots will receive 50 A & K traps per acre along with 2 pheromone-, and 2 kairomone-baited monitoring traps. Control plots will receive pheromone- and kairomone-baited 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.

2. Additional field testing will be conducted to assess and validate laboratory work on the development of the sachet dispenser. A two-sachet system will be compared with our standard method of vials for AA and NBS and a septum for PE. These systems will be

tested both in the Delta trap and in a tube trap design. We expect at the end of the project to have a combination sachet dispenser in a tube trap that is as effective as the system used at this time, but is significantly less expensive.

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.

We concluded 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 and 2014, we obtained preliminary evidence that a density of 50 A & K traps per acre significantly reduces numbers of adult codling moths in the orchard, which we referred to as "knockdown". Also, in 2014, we expanded plot size from one to four 4 acres to buffer the plot from effects of immigration of moths from outside the plot. However, the increase in plot size increased the cost of running replicates and the difficulty of finding orchard sites with suitable codling moth populations.

Much of our effort in 2015 was focused on obtaining evidence of both knockdown of CM in treated orchard plots, but more importantly of reduced CM infestation of apples

<u>One acre plot moth knockdown</u>. 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 $\pm$ 16.3 female and 35.2 $\pm$ 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.

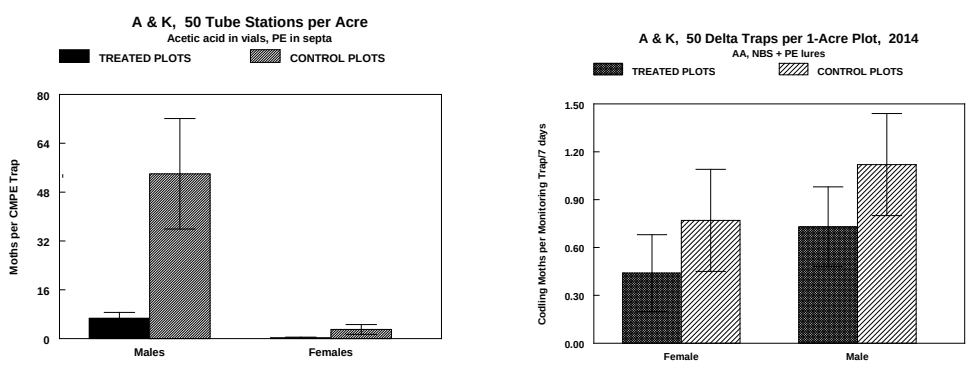


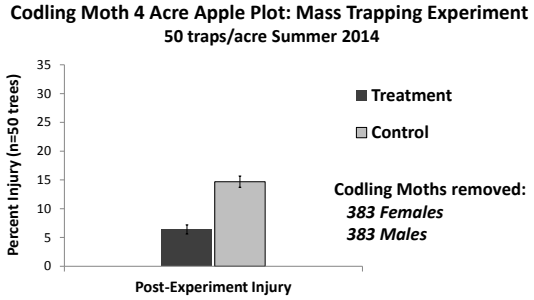
Figure 1. Mean numbers of codling moths captured in monitoring traps in one-acre plots. On the left, in 2013, numbers of moths were greatly reduced in kairomone-baited monitoring traps in plots with tube shaped A & K traps. On the right, in 2014, numbers of moths in kairomone-baited monitoring traps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

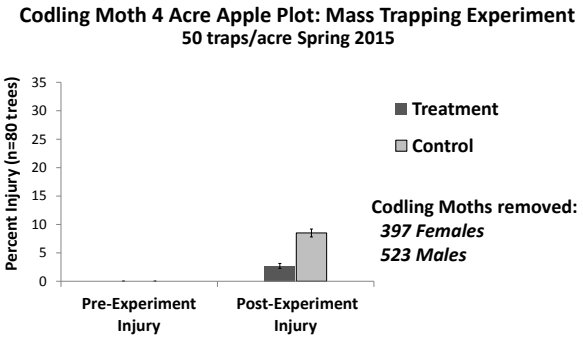
Four-acre plot infestation reduction. Pairs of 4-acre plots were set up in August of 2014, June of 2015 and August of 2015, to compare CM infestation rates in apples with and without deployment of attract-and-kill traps. A & K traps baited with acetic acid + pear ester + N-butyl sulfide, were evenly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

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

Results of the field plot tests consistently indicate an impact of the A & K traps on CM; both reducing the numbers of moths in plots, and reducing the codling moth damage to fruit. However, we will seek to conduct additional replicates of the larger scale plot tests, so that conclusions reached are rigorous by the end of the project.

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





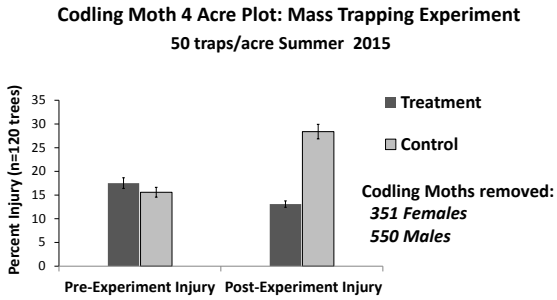


Figure 2. Mean percentages of apple fruit damaged by codling moth, in 4 acre apple blocks that were untreated controls, or were treated with 200 attract and kill traps at a rate of 50 traps/acre.

Development of inexpensive technology**.** We have also been working to develop less expensive alternatives to the trap and to the lure that we are using in the field plot tests. Work under a prior WTFRC project and in the first year of this project indicated that we could use a simpler disposable trap that is a tube with adhesive on the inside. The current attractant is dispensed from polypropylene vials and a rubber septum, which we aim to replace with sachets (little bags). We originally considered mixing all three chemicals together in one polyethylene bag, but this system failed in field tests. In 2015, we determined release rates of pear ester from vials, sachets, and septa, and found that pear ester was best released from its own sachet. These results will be validated in field tests.

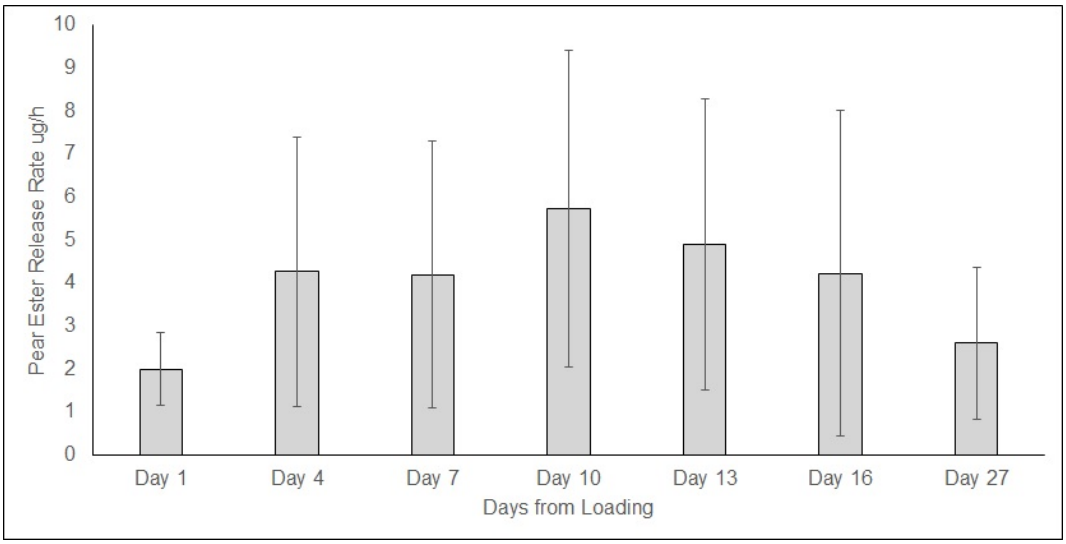


Figure 3. Amounts of pear ester released from polyester sachet over a 4 week period of time.

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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**: 3 of 4 (No cost extension)
WTFRC Project Number: CP-13-100A

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

PI: Peter Landolt
Organization: USDA, ARS
Telephone: (509) 454-6570
Email: peter.landolt@ars.usda.gov
Address: 5230 Konnowac Pass Road
Address 2:
City/State/Zip: Wapato, WA 98951

Co-PI (2): Jocelyn Millar
Organization: University of California
Telephone: (951) 827-5821
Email: Jocelyn.millar@ucr.edu
Address: Department of Entomology
Address 2: 3401 Watkins Drive
City/State/Zip: Riverside, CA 92521

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 2016)

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.

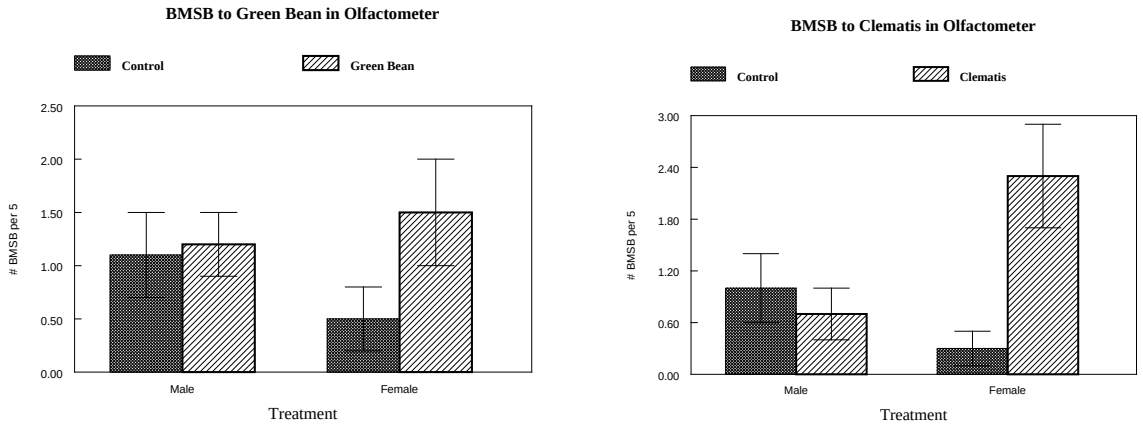


Figure 1. Numbers of female BMSB responding to airflow from over plant material in a Y-tube or choice olfactometer. N = 60, as 12 groups of 5. Control is airflow through an empty jar.

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.

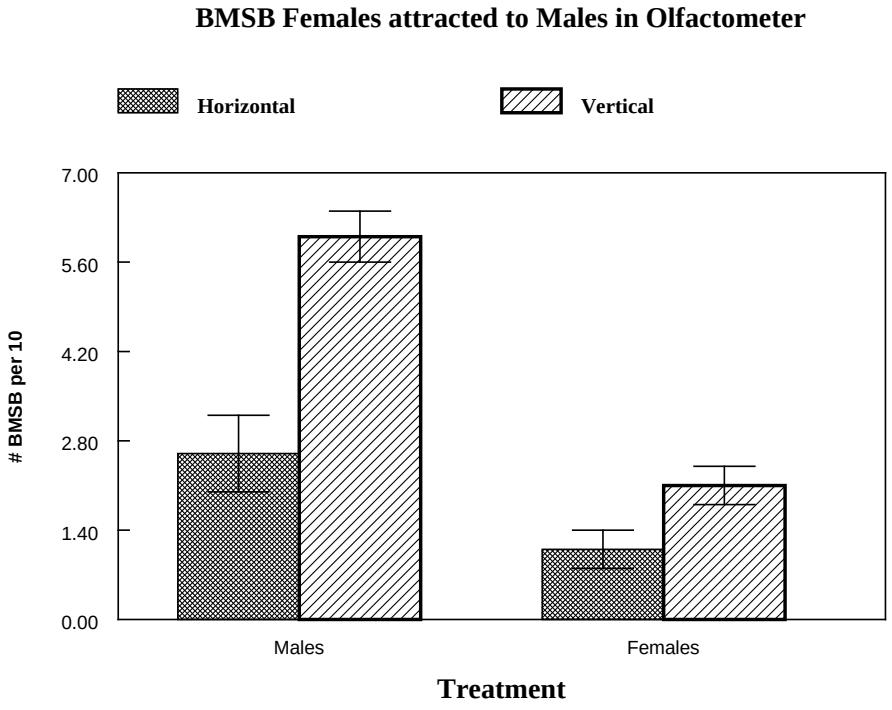


Figure 2. Numbers of female BMSB responding to airflow from over a male occupied shelter versus a female occupied shelter in a Y-tube or choice olfactometer. N = 60, as 12 groups of 5. The experiment was conducted twice; once with the olfactometer tubing horizontal, and again with the tubing vertical.

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.

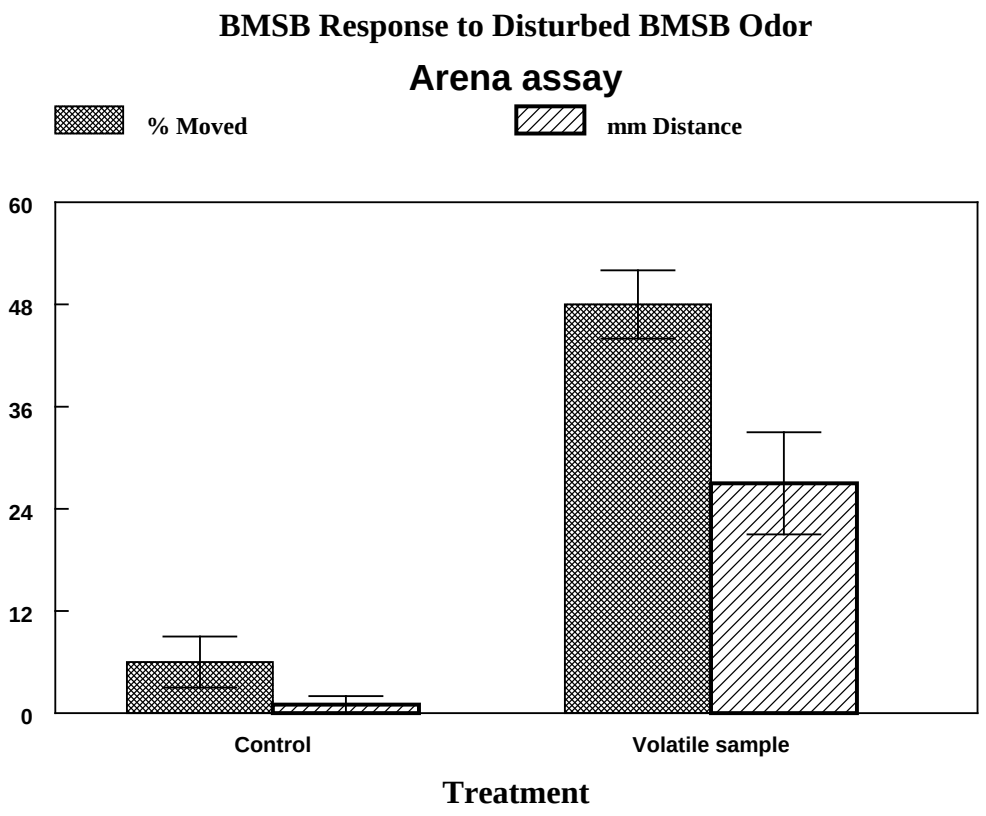
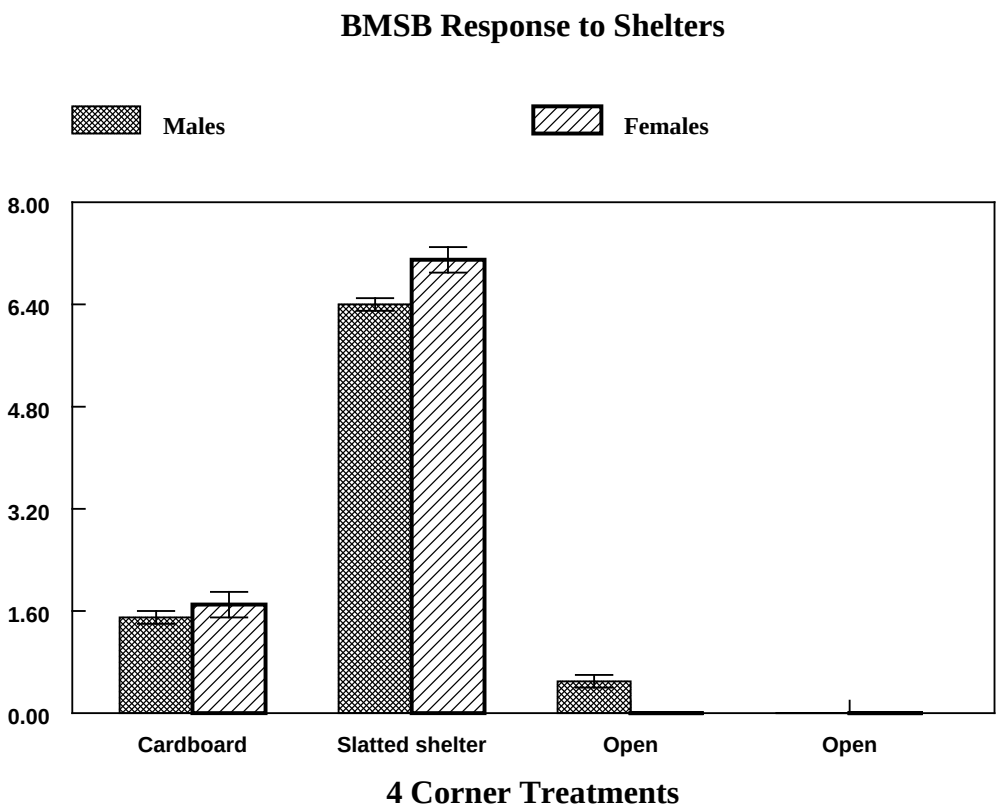


Figure 3. Numbers of male and female BMSB (combined) responding to airflow from a single disturbed BMSB. Movement was noted, as well as the distance moved in the 20 second long test. N = 100 as 20 groups of 5. Control is air from an empty jar.

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.

No-cost extension of the project. We were unable to hire temporary personnel to assist with insect rearing and field work in 2016, and requested and obtained an extension to the project so that field tests and laboratory assays of sex pheromones can be completed in the coming year.

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

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

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

Co-PI (2): Gary Judd
Organization: Agriculture and Agri-Food Canada
Telephone: 250-494-6372
Email: gary.judd@agr.gc.ca
Address: Box 5000, 4200 Hwy 97
City/State/Zip: Summerland, British Columbia, Canada V0H 1Z0

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

Other funding sources

Agency Name: Agriculture and Agri-Food Canada**,** Science and Technology Branch, Annual Call for Research, Development and Technology Transfer Proposals
Amt. requested: $90,000 USD total over three years (2016-2019)
Notes: The status of this grant will be announced by 1 April 2016. If this grant is received we will request the transfer of some of the funds for Dr. Judd to Dr. Knight for the remaining two years of this project.

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

Item	2015	2016	2017
Wages	17,500	20,000	20,500
Benefits	1,500	2,000	3,500
Supplies	6,000	5,000	6,000
Travel	1,000	1,000	1,000
Plot Fees	3,000	2,000	3,000
Total	29,000	**30,000**	34,000

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

Item	2015	2016	2017
Wages[1]	18,000	20,500	21,200
Benefits	2,500	2,500	2,800
Supplies	4,000	4,500	5,000
Travel[2]	1,000	1,000	1,500
Miscellaneous	500	500	500
Total	26,000	**29,000**	31,000

OBJECTIVES:

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

SIGNIFICANT FINDINGS

- Volatile analyses of leafroller and budmoth infested apple shoots identified six specific compounds released only following herbivore damage.
- Kairomones were identified from this list as being the most attractive to both male and female leafrollers and ESBM.
- A ternary blend and several binary ratios of three of these compounds were found not to significantly increase moth catches compared with the single most attractive compounds.
- The kairomone lures for OBLR and PLR were equal or superior to sex pheromone lures.
- Traps baited with the respective kairomone lures were highly effective in monitoring pest species in orchards treated with MD.
- Membrane lures loaded with the kairomones provide a consistent long-lasting emission rate.

Goals and activities for next year: We are establishing relationships with several insect chemical ecology laboratories and commercial semiochemical entities to speed the identification of active compounds and to build prototypes for grower testing in 2016. While specific active compounds have been identified and tested, new discoveries are likely to result, and will require another year of evaluation. PhD candidate Valentino Giacomuzzi with Dr. Sergio Angeli from the Free University of Bozen-Bolzano will be working with us beginning in April and has his own university funding. Dr. Esteban Basoalto from University of Chile in Valdivia, Chile will be working with us for a period of time this coming year also with independent funding. Analytical support will be provided through collaborations with several groups. Dr. James Mathesis with ARS in Wenatchee has offered to allow us to work in his laboratory. Dr. Lukasz Stelinski with the University of Florida will run analytical samples for us. Dr. Gerhard Gries at Simon Frazier University and Dr. Maya Evenden at University of Alberta will have one or more graduate students working on aspects of this project with Dr. Judd. Dr. El-Sayed, whom made the initial discovery of this group of compounds in New Zealand, has dropped out of the project.

Schedule of Activities:

1. Flight tunnel tests are ongoing at YARL to study the adult behavior of OBLR and PLR with both identified and suspected kairomones. These tests will likely continue until the start of the field season and then will start back up in October.
2. Volatile analysis of lures is beginning in February (Stelinski).
3. Volatile analysis of host plants will begin in April with V. Giaforri and Dr. Basoalto working with Dr. Mattheis and the Canadian group including students of Drs. Gries and Evenden.
4. Field testing of new lures, new kairomones, and new kairomone blends will begin in May in both Washington State and British Columbia and will continue through the summer.
5. Seasonal monitoring of ESBM, OBLR, and PLR in conventional and sex pheromone-disrupted orchards with our most advanced kairomone lures will begin in May.

6. Testing and refinement of novel trapping methods will run the entire season.

METHODS

Volatile collection. Headspace of host plants (apple, pear, and cherry) will continue to be sampled from shoots with feeding larvae of various species in the field and laboratory. The specific method was outlined in our initial proposal and no major modifications of this protocol are expected. However, studies will be expanded to include larvae feeding on both pear and cherry which are additional hosts of these tortricid pests. Also, beginning in 2016 the project will be coordinating with the internationally-recognized chemically ecologist, Dr. Gerhard Gries, Simon Frazier University, Delta, British Columbia and one or more students working with Dr. Maya Evenden at the University of Alberta through Dr. Judd's collaborative project with these universities. Also, Dr. Lukasz Stelinski who is very capable and has worked for years in tree fruits at Michigan State, but whom now works in citrus will be collaborating with us and will provide technical and analytical work. The availability of state-of-the-art analytical laboratory equipment plus the combined decades of research identifying new behavioral-active compounds and developing applications such as monitoring and lure-and-kill technologies by our new expanded team pushes our project to a new level of creative development.

Flight tunnel studies. Various flight tunnel bioassays will be conducted at both the Agriculture and Agri-Food Canada (2 research tunnels) and USDA laboratories (3 research tunnels) to study moth behaviors to the candidate attractants. Males and females of each species will be individually tested for their response to the following: a) different kairomones, b) different kairomone blends, c) and formulations with different evaporation rates. Flight tunnel tests will also be conducted to evaluate traps' relative catch efficiency of both moth sexes and to develop lure-and-kill technologies. These studies are currently in progress and will be conducted primarily from October to April.

*Field trials. S*tudies will continue to evaluate and compare the use of different kairomone and kairomone blends in traps for the major tortricid pest species. At this point we are particularly interested to evaluate the use of a single lure for all species compared with specific optimized lures for each. We will continue to evaluate additional kairomones, blends, and ratios as the project develops during the field season.

Formulation development. With commercial collaboration we will be able to fine-tune the development of membrane lures for use with these kairomones. Studies will be completed to measure the evaporation rate, longevity, and chemical stability of active materials in several prototypes. Similarly, we will be working with a commercial entity to evaluate the activity of the kairomones in several of their gels and pastes for use in 'attract-and-kill' applications.

Monitoring populations. We are very excited to expand the testing of the kairomone lures to monitor OBLR/PLR and ESBM under conventional and MD programs. This will be conducted with cooperation and help from one or more large growers and related Ag companies providing monitoring services for both conventional and organic orchards. Data will be collected on the phenology of both male and female catches in traps, and these data will be compared with the current phenology models for OBLR and PLR. Studies will be conducted to evaluate the kairomone lures with one to several types of smart traps that can remotely monitor multiple pest species.

Development of mass trapping. We are interacting with the Powder Trap Co. (Burnaby, Canada) and KaMin Performance Minerals (Valdosta, GA) a major manufacturer of kaolin powders, to develop a non-saturating trap that does not require any toxicant to retain and kill moths. Similar work was conducted with Commission support in the 1990's for codling moth and this should allow these high-capacity traps to be used in certified-organic orchards. Various studies will be run to compare the performance of several trap designs with these materials. Also, we will evaluate the interaction

(positive, negative, or additive) of the kairomone and sex pheromone attractants on moth catch in these traps.
Development of 'Lure and Kill'. Flight tunnel bioassays will be conducted to evaluate male and female contact with kairomone-laced drops and then both direct and sublethal (subsequent mating success and resulting fecundity) effects will be measured.

RESULTS & DISCUSSION
Volatile collection. Headspace samples (N = 126) of apple shoots infested with either OBLR or PLR were collected with help from Michele Preti from the University of Bologna, Italy from 24 August to 21 September. Samples were frozen at -80 ºC and then shipped to New Zealand for analysis (Table 1). Fifteen compounds were identified in the infested shoots and six of these were not found in the uninfested shoots. These six volatiles comprised 6.5 and 4.8% of the total volatile capture for ESBM and OBLR-infested shoots, respectively. Due to continuing legal issues we do not identify these compounds here.

Flight tunnel studies. Bioassays were initiated at YARL in November to study the response of male and female OBLR adults to several candidate attractants. The effect of mating status and prior adult feeding on adult response to these attractants is being evaluated. Preliminary data shows that both virgin and mated males and females are attracted to the primary kairomone lure for OBLR. However, a significant increase in the proportional moth catch occurs with mated versus virgin moths, particularly with female moths. Moths starved for 24 h are caught at a significantly higher rate than fed moths regardless of their mating status. Flight tunnel tests are ongoing and will include PLR beginning in late January.

Field trials. An extensive number of field trials were conducted in both countries to evaluate a number of kairomones and various multi-component blends. Dr. El-Sayed spent several months running these trials in Canada and Dr. Esteban Basoalto from Chile helped for one month with field trials near Yakima. Two different kairomone attractants (#7 and #3) were found to catch the greatest numbers of ESBM and OBLR, respectively (Tables 2, 3). However, the use of several blend ratios (1:3, 1:1, and 3:1) of these two kairomones did not significantly increase the moth catches achieved compared with either compound alone (Table 4). Also, binary and ternary blends of compounds 2, 3, and 7 did not significantly increase the catches of either OBLR or ESBM compared with compounds 3 or 7 alone, respectively (Tables 3, 4).

Formulation development. Several candidate formulations were evaluated as lure substrates following our initial work with sachet plastic bags with cellulose substrates. These included both open-vial and membrane lures. The sachet lures lasted only a short period and both of the other lures had much greater longevity. Thus we have discontinued further testing with sachet lures and we are currently evaluating the evaporation rate from several membrane lures in preparation for field testing in 2016. Discussions will be held in January with a commercial partner to discuss lure development for field testing.

Monitoring populations. Replicated studies were conducted primarily for ESBM and OBLR in orchards with and without sex pheromones for mating disruption. Several lure formulations were included in these studies and two kairomones were also included. Data showed that the kairomone lures catch both moth sexes and the total number caught was much higher for PLR and similar for OBLR as the male catch with sex pheromone lures (Fig. 1). Within orchards treated with MD the new kairomones were vastly more effective than sex pheromone lures (Table 5). The kairomone lures performed similarly across plots treated with or without sex pheromones for mating disruption.
Development of mass trapping. Several preliminary studies were conducted with the aim to develop mass trapping techniques using the kairomone lures; such as developing longer lasting lures,

establishing that the kairomone lures catch both mated and virgin females, and comparing the performance of several non-saturating bucket trap designs with the standard delta traps. The bucket traps were comparable to delta traps over a short time interval and will not get saturated with target or nontargets catches over time and thus are less labor intensive and likely less expensive. Additional studies still need to be conducted prior to field testing of mass trapping as a control strategy. These include the testing of the egg shell and kaolin powders and season-long trapping studies using the non-saturating traps.

Development of 'Lure and Kill'. No specific studies were conducted to develop this technology during 2015. Obviously, we first needed to identify the most attractive kairomones. Now that this has been partially accomplished we will obtain and begin to test several known gel and paste formulations

Table 1. Volatile capture analysis of uninfested and infested apple shoots with either ESBM or OBLR, Yakima, WA.

Compound	Mean (SE) % relative amount			
	ESBM		*OBLR*	
	Uninfested	Infested	Uninfested	Infested
1	7.44(1.26)	9.10(3.10)	6.40 (1.47)	3.15 (1.22)
2	nd	1.20(0.42)	nd	0.89 (0.26)
3	nd	0.93(0.31)	nd	1.36 (0.46)
4	2.47(0.64)	1.93(0.31)	1.93 (0.28)	2.32 (0.82)
5	5.77(0.94)	3.92(0.96)	3.7 (1.34)	7.42 (1.89)
6	3.32(0.69)	1.92(0.21)	8.43 (4.20)	2.65 (0.44)
7	nd	0.89(0.40)	nd	0.53 (0.32)
8	3.74(1.78)	1.92(0.14)	5.91 (1.33)	2.68 (0.95)
9	nd	0.93(0.37)	nd	0.79 (0.52)
10	2.14(0.74)	0.75(0.50)	2.25 (0.95)	2.81 (0.46)
11	5.29(1.46)	0.82(0.19)	6.10 (1.39)	4.90 (0.77)
12	9.88(2.87)	4.78(1.77)	4.65 (1.39)	4.78 (1.92)
13	59.95(5.82)	69.04(6.22)	60.46 (6.71	64.51 (3.38)
14	nd	1.30(0.44)	nd	0.56 (0.19)
15	nd	1.25(0.34)	nd	0.67 (0.31)

'nd' not detected

Table 2. Comparison of moth catches with kairomone lures, 2015

Compound	Mean (SE) moth catch			
	ESBM		*OBLR*	
	Total	Females	Total	Females
2	0.8 (0.5)b	0.8 (0.5)b	0.3 (0.2)b	0.2 (0.2)c
3	3.0 (0.7)b	1.6 (0.7)b	**15.3 (2.3)a**	**8.8 (1.8)a**
7	**13.6 (1.8)a**	**8.8 (1.5)a**	5.5 (0.6)b	3.4 (0.8)b
9	1.6 (0.7)b	0.6 (0.2)b	1.5 (0.7)b	0.6 (0.4)c
14	0.6 (0.4)b	0.6 (0.4)b	0.2 (0.2)b	0.0 (0.0)c
ANOVAs	$F_{4, 16} = 28.55$ $P < 0.001$	$F_{4, 16} = 16.94$ $P < 0.001$	$F_{4, 16} = 51.99$ $P < 0.001$	$F_{4, 16} = 19.31$ $P < 0.001$

Table 3. Comparison of a kairomone blend with individual components, 2015.

Compound	Mean (SE) moth catch					
	ESBM		*OBLR*		*PLR*	
	Total	Females	Total	Females	Total	Females
2	5.6 (2.4)b	4.4 (2.0)b	3.3 (0.5)a	2.7 (0.4)a	1.0 (0.6)c	0.2 (0.2)a
3	9.4 (2.0)b	6.0 (1.6)b	3.3 (0.9)a	3.0 (0.7)a	**4.8 (1.4)ab**	**1.4 (0.4)a**
7	**27.4 (4.6)a**	**14.8 (3.3)a**	2.7 (0.7)a	2.0 (0.5)a	1.6 (0.5)bc	0.8 (0.2)a
2+3+7	24.0 (6.2)a	14.0 (3.0)a	3.6 (0.9)a	2.4 (0.8)a	4.6 (0.8)ab	0.8 (0.4)a
ANOVAs	$F_{3, 12} = 5.79$ $P < 0.01$	$F_{3, 12} = 3.64$ $P < 0.05$	$F_{3, 12} = 1.84$ $P > 0.05$	$F_{3, 12} = 1.03$ $P > 0.05$	$F_{3, 12} = 6.24$ $P < 0.008$	$F_{3, 12} = 2.09$ $P > 0.05$

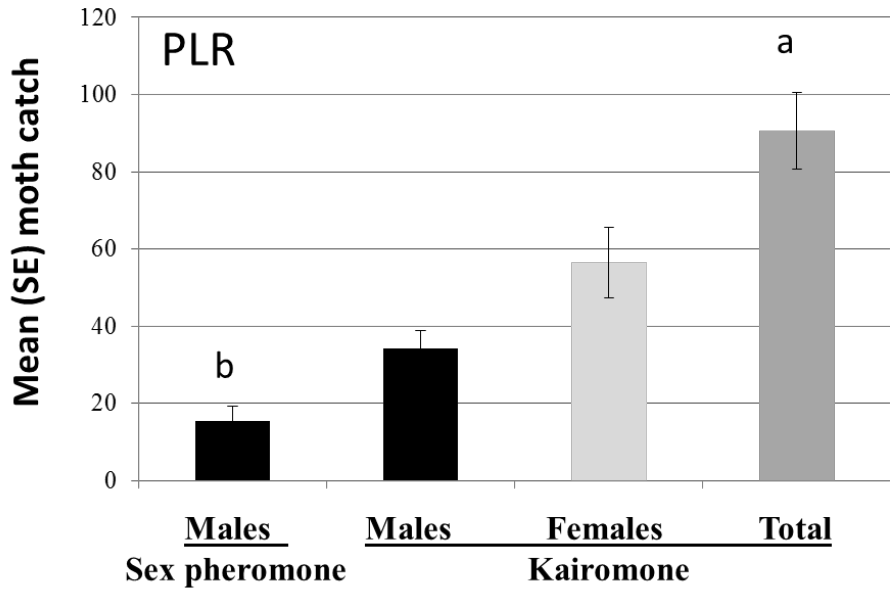
Table 4. Comparison of kairomone blends, 2015.

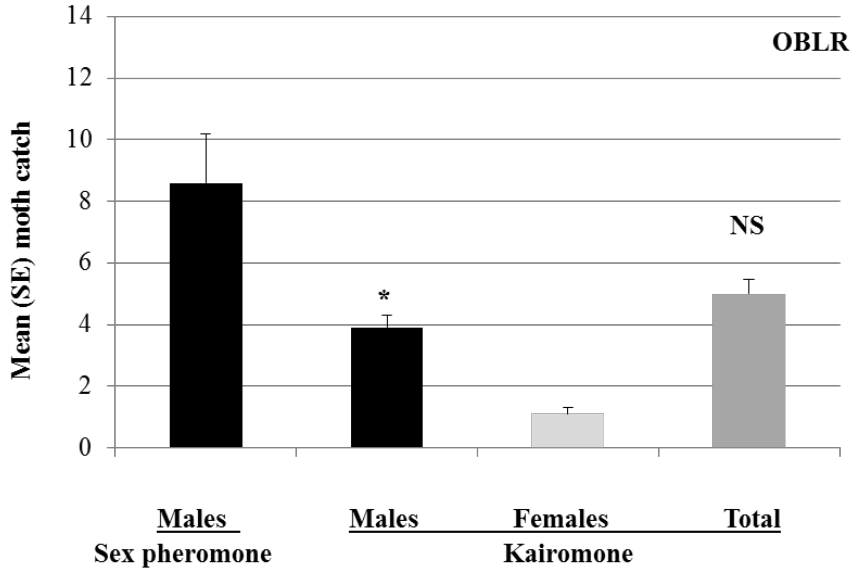
Compound	Ratio	Mean (SE) moth catch		Compound	Ratio		
		ESBM				*OBLR*	
		Total	Females			Total	Females
				3	**1:0**	**5.0 (2.1)abc**	**0.8 (0.3)**
				3 + 7	3:1	4.0 (1.0)abcd	1.2 (0.2)
				3 + 7	1:1	1.8 (0.9)de	0.2 (0.2)
				3 + 7	1:3	5.4 (0.8)ab	0.6 (0.2)
				7	0:1	2.8 (0.7)bcde	0.6 (0.4)
				3+ 2	3:1	5.6 (1.3)a	0.8 (0.4)
				3 + 2	1:1	3.6 (0.7)abcde	0.2 (0.2)
7	**1:0**	**27.2 (9.1)a**	**13.0 (4.1)a**	3 + 2	1:3	5.6 (0.7)a	0.4 (0.2)
7 + 2	3:1	17.4 (4.1)a	8.8(2.6)a	2	0:1	2.4 (0.2)cde	0.0 (0.0)
7 + 2	1:1	20.0 (5.7)a	9.4(2.1)a	7 + 2	3:1	1.8 (0.8)de	0.5 (0.3)
7 + 2	1:3	37.2 (5.9)a	20.4(5.0)a	7 + 2	1:1	1.6 (0.2)e	0.0 (0.0)
2	0:1	15.0 (1.9)a	8.2 (0.2)a	7 + 2	1:3	1.6 (0.6)e	0.4 (0.2)
ANOVA (Total) : $F_{4, 16} = 2.29$, $P > 0.05$				ANOVA (Total) : $F_{11, 46} = 3.45$, $P < 0.01$			
ANOVA (Females): $F_{4, 16} = 2.03$, $P > 0.05$							

Table 5. Season-long trials conducted in apple blocks treated with and without sex pheromone mating disruption (MD) from three sites in Washington State, 2015.

Sites	Treatment	Mean (SE) catch per trap		
		Pheromone	Kairomone	
		Total	Total	Females
1	None	49.3 (8.1)	13.3 (3.2)	4.0 (0.6)
	OBLR MD	0.3 (0.3)	13.7 (2.6)	4.0 (2.0)
2	None	303.5 (84.5)	136.0 (51.0)	58.0 (23.0)
	ESBM MD	0.0 (0.0)	126.0 (86.0)	62.0 (38.0)
3	None	53.2 (10.1)	13.3 (3.0)	4.5 (1.4)
	OBLR MD	0.0 (0.0)	10.0 (2.2)	2.3 (0.7)

Fig. 1 Comparison of PLR and OBLR moth catches in traps baited with either a sex pheromone or kairomone lure, 2014-2015.





CONTINUING PROJECT REPORT **YEAR**: 1 of 2
WTFRC Project Number: CP-15-101

Project Title: Evaluating plant volatiles for augmenting biological control

PI: Vincent P. Jones
Organization: WSU-TFREC
Telephone: 509-663-8181 x291
Email: vpjones@wsu.edu
Address: 1100 N. Western Ave.
City/State/Zip: Wenatchee, WA 98801

Co-PI (2): Conor O'Leary
Organization: WSU-TFREC
Telephone: 509-663-8181 x288
Email: conor.oleary@wsu.edu
Address: 1100 N. Western Ave.
City/State/Zip: Wenatchee, WA 98801

Cooperators: Jay Brunner, TFREC

Total Project Request: **Year 1:** $54,573 **Year 2: $55,493**

Other funding sources

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

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

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

Objectives:

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

Significant Findings:

- Daily activity of the two lacewing species was highest around dusk and dawn, with very little activity during the rest of the day.
- Distance of attraction from both the squalene and the AMP lures was <10' for both species of lacewings. This suggests large-scale disruption of natural enemy searching will not cause pest outbreaks in other areas of the orchard and that the lures can be used to manipulate lacewing adults on a small scale.
- Trees with either the squalene or AMP lures had slower population recoveries than those without lures indicating biological control activity was greater on lured trees.

Methods:

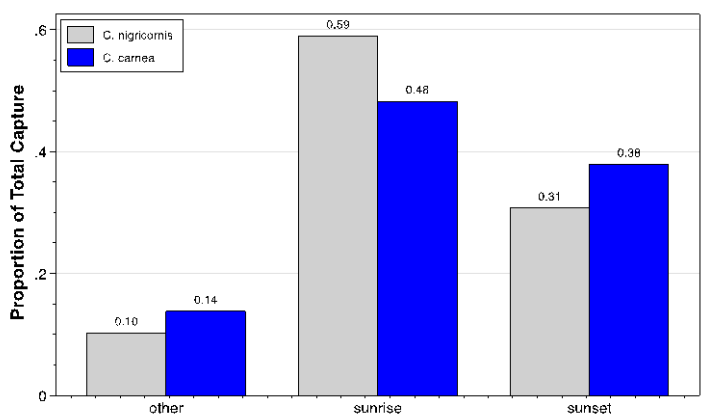
Objective 1. Determine the area of attraction of lures for the lacewings C. nigricornis and C. carnea.

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

Results:

Daily Activity Patterns: Video analysis of the time of day showed that peak activity of *both C. nigricornis* and *C. carnea* occurred around sunrise, with the second peak around sunset. Flight activity at other times of the day or night was very limited, with only 10-15% of the total flights observed (Fig. 1).

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



Distance of attraction:
C. nigricornis was most abundant on the video on the lured tree, with the activity five or 10 feet way not significantly different from the 150-foot control camera (Fig. 2). We were surprised to see *C. carnea* in the vicinity of the squalene lure, but the distance drop off was similar to *C. nigricornis* with the numbers significantly higher on the

lured tree, but no significant difference between the longer distances. It is possible that squalene affects *C. carnea* much like it does female *C. nigricornis* which are attracted near squalene lures, but will not enter traps.

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

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

Work next year: We will repeat this experiment again this next year to verify our results and concentrate our efforts on evaluating behavior around the lures. The current experimental design did not allow us to observe behavior around the lure because we had the sticky material on the video observation platform so that we could see which species responded (vs just the generic lacewing designation). We would like to make sure that the lure affects on behavior are restricted to the period around sunrise and sunset and do not affect mating and feeding at other times of the day.

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

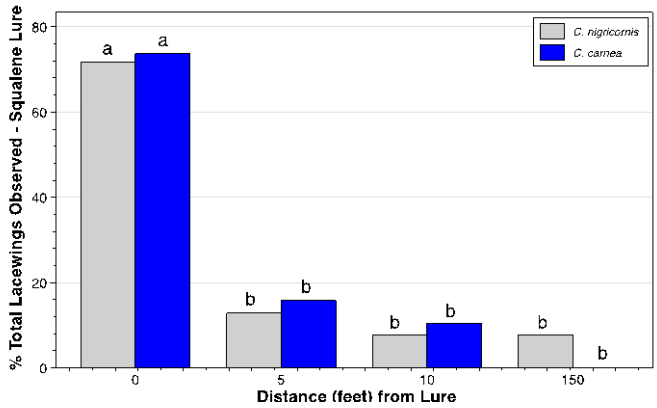
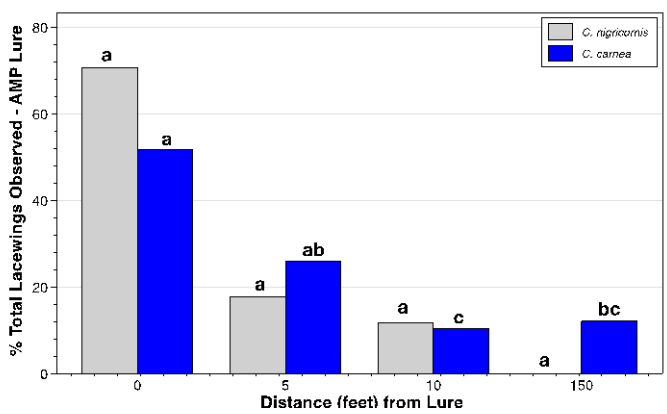


Fig. 3. Percent of total activity observed at each distance from an AMP lure. Bars for each species with the same letter are not significantly different at $p = 0.05$.



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

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

Methods:
These two objectives use the same experiments to evaluate the effects of the lures. We set up 24 trees in a modern fruiting wall orchard that were separated from each other by 200 feet, and randomly assigned each of the trees to one of the three treatments: a squalene lure placed on the tree, an AMP (Acetic Acid, Methyl Salicylate, and 2-phenylethanol) lure, or no lure at all. Each week, lacewing eggs were counted using a 3-minute visual count on the central tree and the two adjacent trees within the row. We also evaluated WAA population levels at weekly intervals by evaluating 10 randomly

selected shoots and determining the average percentage infestation. The evaluations were made over a six-week period using the original trees.

Results:

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

Work next year: The results this year were somewhat surprising, so we would like to set up multiple 10 day experiments where we count all the egg masses on a set of trees, mark any found, and then put the lures up and evaluate how much change in egg-laying patterns occurs during that period. The 10-day period should be plenty long to evaluate response to the lures. These experiments can be blocked over time, so that we don't have to use the same trees each time which removes some of the potential issues with keeping the lures on the same tree.

Suppression of WAA populations on lured trees. We found that the initial set up of the experiment did not account for the extraordinarily warm conditions. We had initially hoped to sample for a week or two, then put out the lures and see how the population changed after the lures were placed. However, the warm temperatures completely flat-lined the population so that from 6 July to 21 July no infested shoots were found. Fortuitously, this allowed us to follow the population rebounding after the temperatures started to drop over the period from 27 July to 20 August. Overall, the percentage infested shoots increased from 0% (21 July) to nearly 50% in the control by 20 August, with the lure treated trees having significantly lower infestation levels compared to the control trees (Fig. 4).

Work next year: Our studies next year need to be set up earlier to make sure that we can evaluate the effect of adding the lures when the population is at a high level and to evaluate how long it takes for population suppression to occur. We also want to focus on the larval lacewing populations to evaluate whether we are getting increased predation. Because the AMP lure also attracts syrphids, we also need to partition out the effects of syrphids versus lacewing contributions to population suppression. The results of the objective 1, where trap attraction is quite limited (<10') should allow us to set up a better experiment because we will not have to separate the treatments so much, which will allow us to set up more replicates in a given area.

Fig. 4. % Shoot infestation with WAA over time.