Apple Crop Protection Research Review

CONTINUING PROJECT REPORT **YEAR**: 2 of 3
WTFRC Project Number: CP-08-805 (WSU Budget #13C-3643-5095)

Project Title: Integrated biological control of woolly apple aphid

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Total project funding request: **Year 1**: $38,238 **Year 2**: $33,962 **Year 3**: **$36,435**

Other funding sources
Agency Name: Washington State Commission on Pesticide Registration
Amount requested/awarded: $14,338 (for 2010)
Notes: To be presented at the December 2009 meeting. Funded in 2009 ($14,792)

Budget 1
Organization Name: WSU-TFREC **Contract Administrator:** Kevin Larson; ML Bricker
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Item	2008	2009	2010
Salaries	11,007	11,448	11,448
Benefits	941	954	955
Wages	19,432	15,560	20,180
Benefits	1,858	2,178	1,917
Equipment	0	0	0
Supplies	2,000	1,000	1,000
Travel	3,000	2,822	935
Miscellaneous	0	0	0
Total	$38,238	$33,962	**$36,435**

Objectives

1. Identify and quantify the predators and parasitoids of woolly apple aphid in eastern Washington (year 1 - completed).
2. Determine the relative impact of the predators and parasitoids (years 2 and 3).
3. Determine the effect of orchard pesticides on the key natural enemies (years 2 and 3).

Significant Findings

- Delegate caused 100% mortality of *Aphelinus mali*; Rimon and Warrior were intermediate (43%), while Altacor and Kumulus were nontoxic.
- Both predators (syrphid larvae) and the parasitoid *A. mali* successfully reduced woolly apple aphid densities in a potted tree experiment.
- Both Rimon and Delegate appear to exacerbate woolly apple aphid populations in large-scale trials when used during the 1st codling moth generation; the effect is worsened when the two were used together. The effect occurred both in a block with a history of woolly apple aphid problems, and one with no prior history.
- Delegate and Rimon suppressed earwigs, one of the predators of woolly apple aphid.
- Rimon, and to a lesser extent Delegate, were associated with increased spider mite levels. Rimon suppressed apple rust mites, which may contribute to disruption of integrated mite control.

Methods

Objective 1. (Completed in 2008)

Objective 2. This experiment was conducted in the greenhouse facilities at the Tree Fruit Research and Extension Center in Wenatchee, WA. The experiment had three treatments and five replicates in a complete randomized design (CRD) design. The experimental unit was a caged, potted apple tree ('Morning Mist'/M9.337') about 30 inches in height. The trees were infested with 20 crawlers (1st instar nymphs) of woolly apple aphid collected from a research orchard, and the population was allowed to expand during a 34-d period. After the establishment period, the trees were assigned to one of three treatments: 1) parasitoids (four adult female *A. mali*); 2) predators (four 2nd-3rd instar syrphid larvae) and 3) check (no natural enemies). Natural enemies were allowed to attack the aphid population for a period of 21 d, at which time the aphid and natural enemy densities were evaluated. The mean number of aphids was analyzed using ANOVA and means separated using Tukey's HSD.

Objective 3a. Orchard pesticides were screened for toxicity to adult *Aphelinus mali* in laboratory bioassays. Adults were exposed to pesticides in a plastic Petri dish (100 x 15 mm) lined with filter paper. Thirty adult parasitoids were transferred from the emergence cages to a Petri dish and held at -5°C for 3-5 min to immobilize them. The Petri dish with adults was then placed in a Potter Spray Tower and sprayed with the various concentrations of pesticides. There were three treatments (dosages) for each pesticide: 1) 1x, the maximum label rate of the pesticide when used on apple (calculated by using the maximum amount per acre per application in 100 gallons of water); 0.1x (a 1:10 dilution of trt. 1), and 3) a distilled water check. Each concentration was applied at 2 ml at a pressure of 7 psi. After treatment, the adults were moved to a smaller Petri dish (one adult per dish) containing a cotton ball imbibed with a 1:1 solution of honey and water. The lid was perforated with a small hole covered with micropore tape to allow air exchange. Mortality was evaluated 24, 48, and 72 h after treatment. Data were analyzed using PROC FREQ (SAS 1988) with a binary response specification and the Pearson χ^2 test statistic.

Objective 3b. Large-scale tests of pesticide programs were conducted in two commercial orchards in central Washington, near Bridgeport ('Cameo') and Othello ('Delicious'). The blocks were 20-25 acres in size, with four treatments and four replicates of 1-1.5 acres in size. The treatments consisted of a petal fall (100 degree-days), delayed 1st and 2nd cover codling moth sprays (Table 1).

Table 1. Codling moth programs tested for effects on secondary pests and natural enemies

Trt. #	Treatment[1] (PF, 1st cover, 2nd cover)	Rate/acre
1	Rimon-Delegate-Delegate	32 fl oz, 7 oz, 7 oz
2	Intrepid-Delegate-Delegate	16 fl oz, 7 oz, 7 oz
3	Rimon-Altacor-Altacor	32 fl oz, 4.5 oz, 4.5 oz
4	Intrepid-Altacor-Altacor	16 fl oz, 4.5 oz, 4.5 oz

[1]Applications were made with an airblast sprayer calibrated to deliver 100 gpa.

Woolly apple aphid, green apple aphid, spider mites were monitored from April through November, 2009, along with a broad range of natural enemies (lady beetles, syrphids, earwigs, spiders, and predatory mites). Samples were taken at 1-3 week intervals. Sample types included timed counts (both aphid species), leaf samples (mites and their predators), tap samples (various natural enemies), cardboard band traps (earwigs and spiders), and HIPV (herbivore-induced plant volatiles) attractant traps (lacewings, syrphids). Cumulative mite days (CMD) were calculated for spider (tetranychid), predatory, and rust mites, giving an estimate of population densities integrated over the course of the test. CMDs are the sums of the average density of mites on two dates multiplied by the number of intervening days [$CMD = \Sigma 0.5(P_a+P_b)D_{a-b}$] where P_a is the population density (mean mites/leaf at time a), P_b is the population density at time b, and D_{a-b} is the number of days between time a and time b. Other sample data were summed or averaged over the season to provide a single number for comparison. Data were analyzed using the Statistical Analysis System (SAS 1988). PROC GLM was used to conduct an analysis of variance, and treatment means were separated using the Waller-Duncan *k*-ratio *t*-test.

Results and Discussion

Objective 2. Aphids established rapidly on the potted trees and high densities were present at the time of natural enemy introduction. Even after exposure to natural enemies, high numbers were found at the time of evaluation, with over 15,000 aphids in the check. Both syrphids and *A. mali* reduced the number of aphids relative to the check in the 21-d exposure period (Fig. 1).

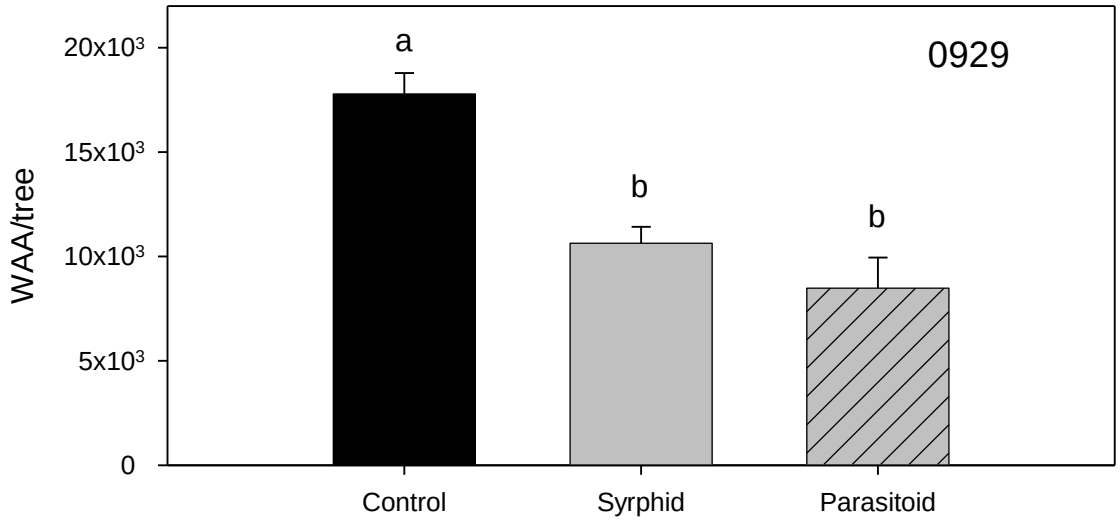

Fig. 1. Woolly apple aphid densities after exposure to syrphid larvae or the parasitoid *Aphelinus mali.*

Objective 3a. Delegate was highly toxic to *A. mali* at both the 1x and 0.1x rates. Warrior and Rimon were intermediate, causing about 43% mortality after 72 h (Fig. 2). Altacor and Kumulus were nontoxic. The preliminary data in these bioassays indicate Delegate should be avoided when adults are present in the field, as high mortality would be expected. Further experimentation is needed to determine if residues on plant surfaces are toxic, and the length of toxic activity under field conditions. Absent information on sublethal effects, Rimon and Warrior could be used sparingly. However, sublethal effects are more common with IGRs, and this aspect needs to be investigated further. Altacor and Kumulus appear to be the least toxic choices at this point, although sublethal and large-plot tests are necessary to verify this.

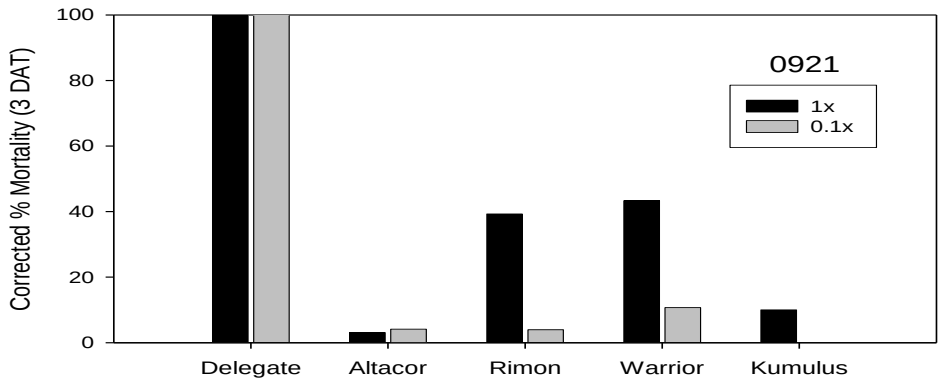

Fig. 2. Corrected percentage mortality of *A. mali* in response to five orchard pesticides, 2009.

Objective 3b.

Bridgeport. Woolly apple aphid pressure was high in the Bridgeport plot, peaking in late July, and again in October (Fig. 3a). Significant treatment differences appeared by late June, with the Intrepid-Altacor treatment having consistently the lowest aphid densities. The highest densities occurred in the Rimon-Delegate plot, followed by the Rimon-Altacor treatment and the Intrepid-Delegate. Rimon appears to have the stronger disruptive effect on woolly apple aphid, despite the fact that it was only applied a single time at petal fall (PF). Delegate also appears to be disruptive, but somewhat less so

than Rimon; when paired with Intrepid at PF, the seasonal mean aphid densities were significantly higher than when Intrepid was paired with Altacor (Fig. 3b).

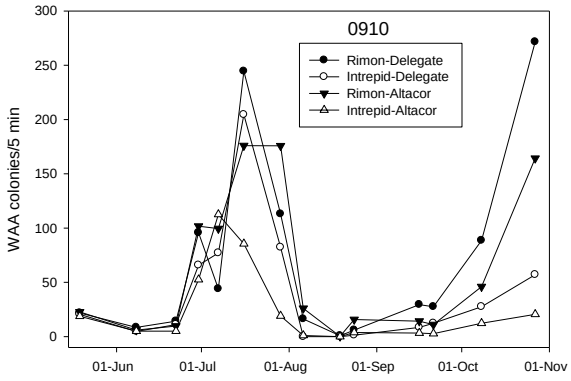

Fig. 3a. Woolly apple aphid populations, Bridgeport, 2009.

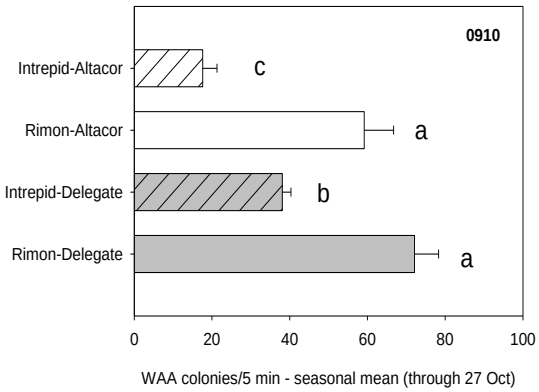

Fig. 3b. Seasonal means of woolly apple aphid timed colony counts, Bridgeport, 2009.

Spider mite populations were moderately high in this block, peaking in late July-early August (Fig. 4a). While even the lowest treatment (Intrepid-Altacor) experienced some elevation of spider mites (ca. 11 mites/leaf), the maximum populations in the two treatments containing Rimon were significantly higher than the Intrepid-Altacor treatment on 28 July, with the Intrepid-Delegate treatment intermediate. However, this trend was reversed on the secondary peak in late August, although this may have been due to poorer leaf quality from prior mite damage. Because of this reversal, no significant differences were found in the seasonal cumulative mite days (Fig. 4b).

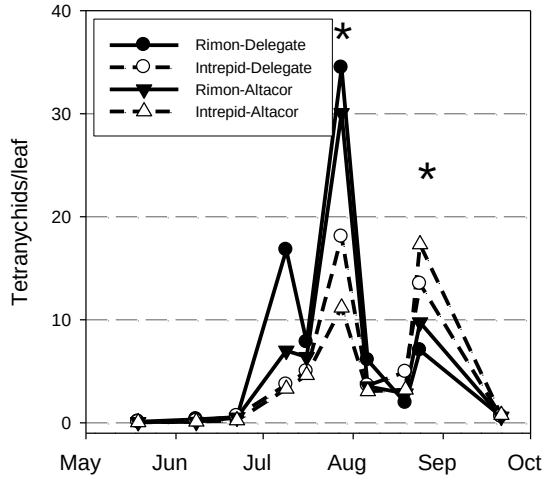

Fig. 4a. Spider mite populations, Bridgeport, 2009

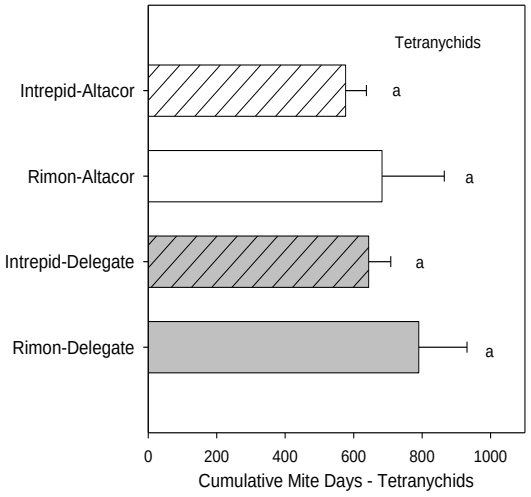

Fig. 4b. Seasonal cumulative spider mite days, Bridgeport, 2009.

Earwigs were abundant in the Bridgeport plot, with densities caught in traps rising throughout the growing season, peaking in mid-September (Fig. 5a). There were clear differences among treatments by mid-June, and these differences persisted into fall. The Altacor-Intrepid treatment had the highest numbers of earwigs, while the Rimon-Delegate treatment had the fewest. Parallel to the woolly apple

aphid results, Rimon and Delegate appear to be detrimental to earwigs, while Intrepid and Altacor are not (Fig. 5b). While these results are only correlations, they raise interesting questions about the role of earwigs as woolly apple aphid predators.

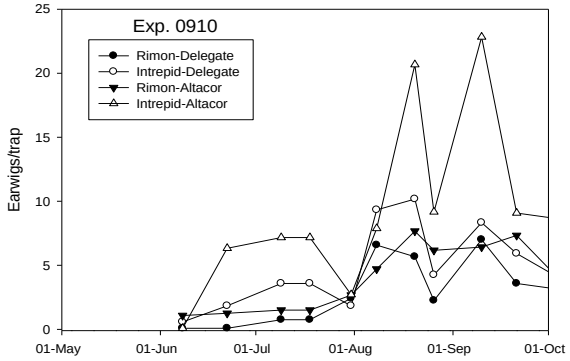

Fig. 5a. Earwig densities over time, Bridgeport, 2009.

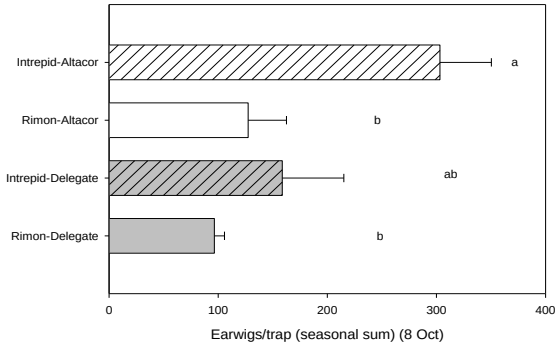

Fig. 5b. Seasonal sums of earwigs caught in traps, Bridgeport, 2009.

The HIPV traps caught large numbers of lacewings (methyl salycilate+benzaldehyde) and syrphids (geraniol) throughout the season (data not shown). There were two distinct peaks in lacewing activity, one in early July, and the second in mid-August (Fig. 6a). There was also a treatment effect in lacewing captures, with an indication that Altacor may suppress lacewing populations (Fig. 6b). Although these insects are also important woolly apple aphid predators, there was no indication that this was the primary mean of disruption.

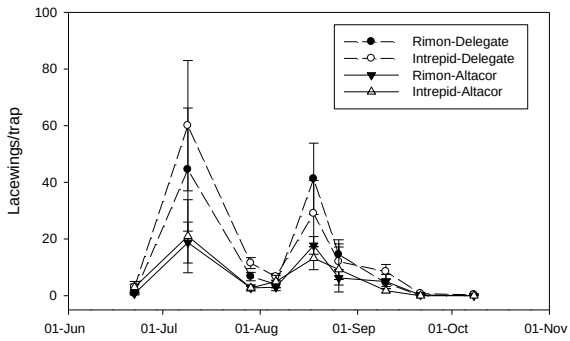

Fig. 6a. Lacewings caught in HIPV-baited Delta traps throughout the season, Bridgeport, 2009.

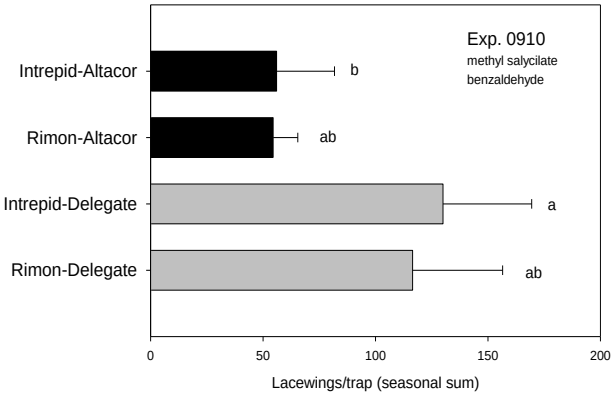

Fig. 6b. Seasonal sum of lacewing captures in Delta traps, Bridgeport, 2009.

Othello. Woolly apple aphid densities were relatively low throughout the summer, but began increasing in early September, and peaking in October (Fig. 7a). Treatment effects were similar to the Bridgeport plot, in that the Rimon-Delegate treatment had significantly higher aphid densities than the other treatments. The Intrepid-Altacor treatment had the lowest levels, with the the other two treatments intermediate. In this block, Delegate appeared to be slightly more disruptive than Rimon (based on the Rimon-Altacor treatment) (Fig. 7b).

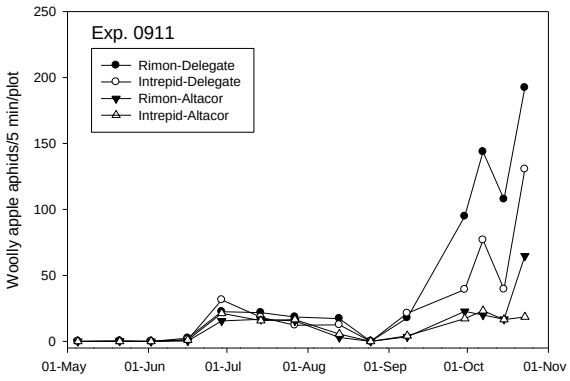

Fig. 7a. Woolly apple aphid densities, Othello, 2009.

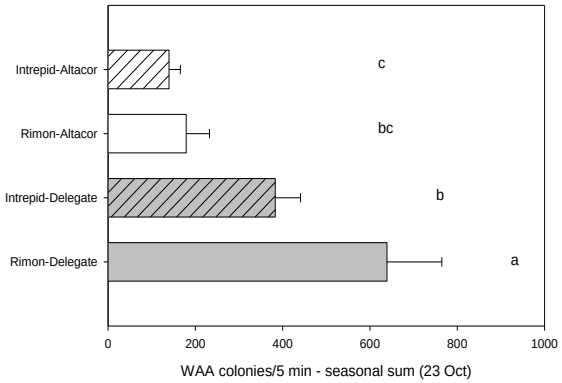

Fig. 7b. Seasonal sums of woolly apple aphid colonies, Othello, 2009.

Earwigs and spider mite densities were very low in this plot throughout the season (data not shown); thus it is difficult to ascribe strong integrated mite control disruption to any of the products tested. Similarly, since the treatment effects on woolly apple aphid were the same in the absence of earwigs, it suggests that other mechanisms may be as much, if not more, important, in the outbreak of woolly apple aphid. The other notable result from the Othello site was the trend in rust mite populations (data not shown). Both of the treatments containing Rimon had significantly depressed rust mite counts, which may contribute to disruption of integrated mite control.

CONTINUING PROJECT (Extension) **DURATION**: 2 years

Project Title: Wooly apple aphid resistance in advanced rootstock selections

PI:	Gennaro Fazio	**Co-PI:**	Betsy Beers
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Address:	630 W. North Street	**Address:**	1100 N. Western
City:	Geneva, NY 14456	**City:**	Wenatchee, WA 98801

Total Project Request: **Year 1:** $12,000

Other funding sources: None

WTFRC Collaborative Expenses: None

Budget 1
Organization Name: USDA ARS PGRU **Contract Administrator:** Dianne Emerson
Telephone: 315 787 2329 **Email address:** Dianne.emerson@ars.usda.gov

Item	2009	2010	
Salaries			
Benefits			
Wages			
Benefits			
Equipment			
Supplies **	2,000		
Travel			
Miscellaneous			
Total	2,000	**0**	

Footnotes: ** DNA genotyping supplies.

Budget 2
Organization Name: WSU TFREC Contract Administrator:
Telephone: **Email address:**

Item	2009	2010	
Salaries			
Benefits			
ension)Wages **	7,000		
Benefits	1,500		
Equipment			
Supplies***	1,500		
Travel			
Miscellaneous			
Total	10,000	**0**	

Footnotes: **Temporary labor help for greenhouse inoculations and data collection * Greenhouse supplies, fees.**

OBJECTIVES:
1. Test an array of Elite Geneva rootstocks and other commercially available rootstocks in a replicated greenhouse test for resistance to Wooly Apple Aphid (WAA) and disseminate knowledge to growers for planting recommendations.
2. Test the same array and as well as parents and other apple rootstocks for the presence of markers linked to wooly apple aphid the resistance genes.

SIGNIFICANT FINDINGS

- We have requested a no cost extension for this project because the plant material to be tested in Obj. 1 was damaged during shipping. Therefore activities regarding Obj. 1 cannot be reported and the correlation between molecular markers and WAA resistance was not performed.
- Objective 2 was completed and the published molecular markers listed in Table 2 were tested on an extended array that included 59 Geneva elite apple rootstocks, and 37 commercial rootstocks and wild apple species that have been and may be utilized as potential parents in the breeding program.
- The published molecular markers developed in New Zealand were difficult to amplify and cannot be used for Marker Assisted Selection (MAS) by the program. The microsatellite marker NZms_EB145764 linked to the *Er2*resistance gene is not useful because the difference between the resistant allele and the susceptible allele in some cases is only one base pair. We are searching for better markers for all of these genes.

METHODS

Objective 1.

Apple rootstock liners of appropriate cultivars (See table 1) will be grown in 6-in plastic pots using standard potting mix in the greenhouse. Ten replicates of each cultivar will be planted. Seedling rootstocks and M.9 liners will be used as a susceptible check. After planting, the trees will be grown for 4-6 wk to allow them to fully leaf out and the root systems to become established. Trees will be arranged in a randomized block design on the greenhouse bench.

Trees will be infested from a woolly apple aphid colony growing on seedling rootstocks. Stem sections 4-6 cm in length containing a colony of woolly apple aphid will be placed at the base of the experimental trees. Aphid crawlers will be allowed to transfer to the experimental trees, and establish new colonies. Trees will be checked for colony establishment after 5 d, and any trees without signs of new colonies will be re-infested. Aphid densities will be evaluated after 4 wk, or when colonies are well established on the susceptible checks. Greenhouse temperatures will be kept between 75 to 80°F to provide optimal development conditions for the aphid colonies.

Resulting aphid aerial densities will be evaluated in three ways: 1) a numerical rating system; 2) a digital estimation procedure, and 3) an *in situ* count of all aphids on the tree. The rating system consists of a subjective evaluation by the observer using a scale of 0 to 4, where 0=no colonies; 1=few, small colonies; 2=few, normal colonies; 3 = heavily infested; 4 = very heavily infested with a large volume of waxy filaments. For the digital estimation, the trees will be photographed against a black background, and the number of white pixels counted using a color-based selection tool in image editing software. Total aphid densities will be counted *in situ* using 5× magnification visors.

Root systems will be evaluated destructively after aerial colony evaluations are finished. Roots will be rated as infested or not infested, and with or without galls. Results are given as a percentage of the replicates which were positive for either infestation or galls.

Data will be analyzed using analysis of variance (SAS 1988). Data will be tested prior to analysis for homogeneity of variance using Levene's (1960) test. Variances found to be non-homogeneous will transformed [ln(y+0.5)] before analysis. ANOVA will be conducted using PROC GLM, and treatment means were separated using the Waller-Duncan *k*-ratio *t*-test.

Table 1. Potential list of rootstocks to be tested in greenhouse.

Source	Rootstocks	Parents
Geneva	CG2034, CG3001, CG5890, CG4214, CG4011, CG5087, CG5222, CG4013, CG3007, CG4814, CG2022, CG6001, CG4049, CG4291, CG2006 and others.	M.27, P2, P22, O.3, M.9, B.9, MM111, M. robusta, M. floribunda, M. sargentii, M. baccata, M. Prunifolia, M. micromalus

Progress report for Objective 2

We have synthesized DNA markers listed on table 2 and assessed their presence/absence in an array of 96 apple rootstocks DNA that includes genotypes in Table 1 as well as commercial apple rootstocks and parents in the breeding program. We have discovered that several of the SCAR markers in Table 2 were very hard to amplify by PCR and therefore not suitable for large scale Marker Assisted Selection. A sample of one of the many DNA marker gel images generated during this study is in Figure 1. We are waiting for the phenotypic assessment to be performed at WSU to make a detailed correlation between the marker status and the resistance to wooly apple aphids.

Table 2. A list compiled by Bus et al. (2008) represents some of the markers that we will use to tag resistance to WAA. We will add additional linked polymorphic markers as needed.

Marker name	Marker type	Original RAPD/EST	WAA gene	Forward primer	Reverse primer	Product size (bp)	PCR conditions[a]	Linkage group
NZsc_C20	SCAR	OPC20	*Er1*	TCTCTAACTCAATAACT CCCAAGAC	ACTTCGCCACCATTAT CACTCCTGA	2,000	Td 70–60	8
NZsc_GS327	SCAR	GS327	*Er1*	GCCAAGCTTCAATGTC GGAGTAGAT	CAAGCTTCCCCTAAGG CTATTGCCA	1,600	Ta 60	8
NZsc_O05	SCAR	OPO05	*Er1*	CCCAGTCACTAACATA ATTGGCACA	CCCAGTCACTGGCAAG AGAAATTAC	1,700	Ta 60	8
NZsn_O05	SNP	OPO05	*Er1*/*Er3*	AACGTCATGTCAATAT	CCCAGTCACTGGCAAG AGAAATTAC	880	Td 70–60	8
NZsc_E01	SCAR	OPE01	*Er3*	CCCAAGGTCCGAACAC AAATGAGAG	CCCAAGGTCCAAAACT ATCCCGAAG	1,350	Ta 60	8

NZsc_A01	SCAR	OPA01	*Er3*	CAGGCCCTTCAGCAAA GAGGTGTCT	CAGGCCCTTCACTACT AATAAGAAC	1,250	Ta 60	8
NZms_EB10 6753	SSR	EB106753	*Er1*/*Er3*	TCTGAGGCTCCCAAGT CC	TAGGAGCAGAAGAGGT GACG	175	Td 65–60	8
NZms_EB14 5764	SSR	EB145764	*Er2*	TTCCAGCGATCCAAAA CAAT	GCTCAGGAACACCTCG TTCT	198	Td 65–60	17

The SCAR and SNP primers were derived from RAPD markers (Operon Technologies, Alameda, CA), and the SSR primers were designed from ESTs of apple.

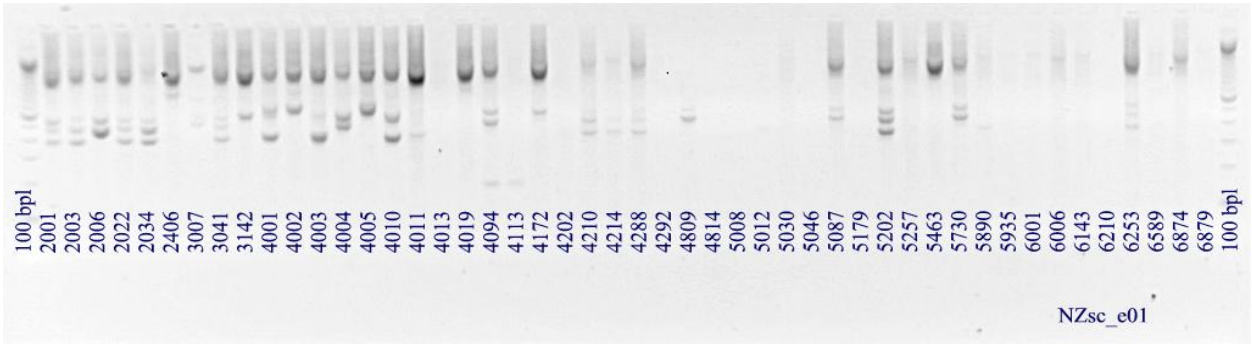

Figure 1. An example of a gel image of SCAR marker NZsc_E01 where the array of Geneva elites are tested for the presence of the resistance gene *Er3*.

References

V. G. M. Bus, D. Chagné, H. C. M. Bassett, D. Bowatte, F. Calenge, J.-M. Celton, C.-E. Durel, M. T. Malone, A. Patocchi, A. C. Ranatunga, E. H. A. Rikkerink, D. S. Tustin, J. Zhou and S. E. Gardiner, 2008. Genome mapping of three major resistance genes to woolly apple aphid (Eriosoma lanigerum Hausm.). Tree Genetics and Genomes 4:233-236

Previous work:

Resistance of Rootstock Selections to a Washington Strain of Woolly Apple Aphid

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[2]USDA-ARS, NY State Agric. Res. Station, Geneva, NY

Keywords: woolly apple aphid, *Eriosoma lanigerum*, host plant resistance, Robusta 5 gene

Abstract: Ten rootstock selections were tested for their ability to host woolly apple aphid aerial colonies. Differences among the various rootstocks were apparent within a few weeks of artificial infestation. After 4 wk, the susceptible rootstocks (including M.9, M.26, Bud 9, Bud 118, and seedlings from New York and Washington) were heavily infested. On MM.111 (whose resistance is derived from 'Northern Spy'), colonies established successfully, but were small and poorly developed. The majority of the replicates of the Geneva 'Robusta 5'

derived resistant rootstocks (G.202, G41, and 4210) were free from infestation; but some replicates had a few very small colonies.

Host plant resistance is a little used tactic in tree fruit pest management, and the long-known resistance of certain rootstocks to woolly apple aphid is one of the few examples. This resistance is based on a naturally occurring resistance in the apple cultivar 'Northern Spy'. The characteristic was incorporated into the Malling-Merton 100 series of rootstocks in the 1920s, and these stocks were widely planted for this reason. Two phenomena occurred to effectively curtail their use. First, new rootstocks with more favorable characteristics in terms of precocity, productivity and size control were introduced. These included the Malling series (of which M.9 and M.26 have been widely planted in Washington), and the Budagovsky series. Both these series are susceptible to woolly apple aphid. The second phenomenon was that biotypes capable of overcoming the 'Northern Spy' based resistance were discovered in three areas of the world (Gilliomee 1968, Sen Gupta and Miles 1975, Klimstra and Rock 1985).

More recently, a new line of woolly apple aphid-resistant rootstocks have been introduced from the apple rootstock breeding program at Cornell's Geneva Experiment Station (Robinson et al. 2003). This resistance is based on a *Malus × robusta* selection known as 'Robusta 5'. This parent also confers a degree of fireblight resistance, and has been widely used in the Geneva program.

The objectives of this test were twofold: 1) to determine if a Washington strain of woolly apple aphid had overcome the 'Northern Spy'-based resistance, and 2) to confirm the 'Robusta 5' based resistance in our area.

Materials and Methods

Apple rootstock liners, from ¼ to ⅜ inch diameter, were planted in a soil mixture of equal parts peat, perlite, and vermiculite on 21 April. Ten rootstock types were used: The Geneva line 4210, Geneva 41, Geneva 202, Bud 9, Bud 118, M.9, M.26, MM.111, seedlings from Washington (Willow Drive Nursery), and seedlings from New York. Ten of each type were planted. Trees were infested about one month after planting, when new shoot growth was approximately 6 cm.

Parentage of rootstocks

Rootstock	Parentage
G.41	M.27 × Robusta 5
G.202	M.27 × Robusta 5
4210	O.3 × Robusta 5
MM.111	N. Spy × Merton 793
M.9	Juane de Metz clones
M.26	M.16 × M.9
B.9	M.8 × Red Standard
B.118	Moscow pear × M.9 or M.8

Insects were collected from Mountain View Orchard, East Wenatchee. Stem sections 4-6 cm long, each with 50-200 aphids, were placed at the base of each tree on 19 May (Plate 0625.1). First instars were seen on the trees the next day. Fresh stem sections were collected on 22 May and placed on any trees that appeared to have a low number of first instars. This included all of Geneva 41, Geneva 202, 4210, and about half of the other trees. Trees were arranged on a greenhouse bench in a randomized complete block design (10 types × 10 reps) (Plate 0625.2).

Aphids had matured by 8 June and had begun to produce new first instars. Aphid densities on aerial parts of the tree were evaluated on 16 June. Two types of evaluations were performed: 1) a numerical rating system and 2) digital estimation.

Rating system: Tree were rated on scale of 0 to 4, where 0=no colonies; 1=few, small colonies; 2=few, normal colonies; 3 = heavily infested; 4 = very heavily infested with a large volume of waxy filaments.

Root evaluation. Evaluations of the root systems were performed on 14 July. Root systems were rated as infested or not infested, and with or without galls. Results are given as a percentage of the replicates which were positive for either infestation or galls.

Results and Discussion

All of the Geneva rootstocks (G.41, G.202 and 4210) were virtually immune to woolly apple aphid (Table 0625.1, Fig. 0625.1; Plate 0625.3). Only a few of the replicates had any colonies established, and those consisted of only a few aphids. By comparison, at the same point in time, the Malling, Budagovsky, and seedling rootstocks were highly infested (Plates 0625.4. 7). The MM.111 was intermediate between the two extremes (compare Plates 0625.5 and 6), however, colonies were able to establish on most replicates, but grew very slowly by comparison, never reaching the level of infestation of the susceptible rootstocks. Apparently, this is typical of the MM rootstocks, in that the resistance was never complete, but rather expressed as a marked degree of antixenosis. Based on this, there is no evidence that the Washington strain of woolly apple aphid has changed its ability to infest this resistant series over time.

Because the rootstocks in the trial were either clonally propagated or seedlings, their phenotypic expression of resistance was throughout the tree (roots and shoots). Not surprisingly, then the degree of infestation and galling on the roots mirrored that of the shoots. Neither the Geneva rootstocks nor MM.111 had any root infestation or evidence of galls by the end of the test (56 d after initial inoculation). The susceptible rootstocks had 60-100% of the replicates with root infestation, and 10-70% had evidence of gall formation. Given the high pressure of this test, the percentages of both categories would have been higher given sufficient time. However, the trees were nearly dead at the time of root evaluation, so continuing it was not feasible.

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Robinson, T., H. Aldwinckle, G. Fazio, and T. Holleran. 2003. The Geneva series of apple rootstocks from Cornell: performance, disease resistance and commercialization. Acta Hortic. 622: 512-520.

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CONTINUING PROJECT REPORT **YEAR: 2 of 3**
WTFRC Project Number: CP-08-800 (WSU Project 13C-3643-6368)

Project Title: Defining natural enemy biology and phenology to improve IPM

PI:	Vince Jones	**Co-PI(2):**	Dave Horton
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City:	Wapato	**State/Zip:**	Canada V0H 1Z0
State/Zip:	WA 98951		

Cooperators: Jay Brunner, WSU-TFREC; Qing-He Zhang, Sterling International, Inc., Spokane

Total Project Request: Year 1: $132,478 **Year 2:** $137,978 **Year 3: $135,378**

Other funding sources

Agency Name: USDA-CSREES SCRI Grant $2.244M

WTFRC Collaborative Expenses: None

Budget 1:
Organization: WSU-TFREC **Contract Administrator**: ML Bricker, Kevin Larson
Telephone: 509-335-7667, 509-663-8181x221 **Email**: mdesros@wsu.edu, kevin_larson@wsu.edu

Item	2008	2009	**2010**
Salaries[1]	43,604	45,176	47,162
Benefits[2]	7,203	8,004	7,792
Wages	8,000	8,320	8,653
Benefits	1,256	1,498	1,359
Equipment	0	0	0
Supplies	3,500	3,640	3,786
Travel[3]	3,500	3,640	3,786
Total	$67,063	$70,278	**$72,538**

[1]Half-time project manager (Nik Wiman); 0.33FTE Associate in Research (Callie Baker).
[2]Wiman (7%); Baker (34%).
[3]Within state travel for surveys

Budget 2:

Organization: USDA-ARS **Contract Administrator**: Janet Tsukahira

Telephone: 510-559-6019 **Email**: jtsukahira@pw.ars.suda.gov

Item	2008	2009	**2010**
Salaries*	38,782	37,231	43,638
Benefits	11,635	11,169	18,702
Wages	0	0	0
Benefits	0	0	0
Equipment	1,450	0	0
Supplies	500	500	500
Travel	0	0	0
Miscellaneous	1,200	1,200	0
Total	$53,567	$50,100	**$62,840**

* Salaries are for a term appointment with 13, 12, and 11 months in 1st, 2nd, & 3rd years, respectively

Budget 3:

Organization: Agriculture & Agri-Food Canada **Contract Administrator**: Karen St. Martin, Goewin Demmon

Telephone: 250-494-7711 **Email**: KSM stmartink@agr.gc.ca, GD demmong@agr.gc.ca

Item	2008	2009	2010
Salaries	0	0	0
Benefits	0	0	0
Wages[1]	0	10,500	0
Benefits	0	2,100	0
Equipment	0	0	0
Supplies[2]	0	3,500	0
Travel[3]	0	1,000	0
Miscellaneous	0	500	0
Total	0	$17,600	0

[1] Summer student wages with 20% benefits plus inflation in year 2.

[2] Supplies in year 2 include cost for synthesizing several grams of Ascogaster pheromone for group.

[3] Travel costs are for local travel within Okanogan Valley

Objectives:

1. Characterize the phenology of key natural enemies using banding, beat-sampling, and attractant-trapping.
2. Evaluate various semiochemicals as a method of monitoring natural enemy abundance / phenology and impacts of control treatments.
3. Use video monitoring to identify predator species attacking codling moth and develop a polyclonal antibody for expanded predator gut content analysis of codling moth.
4. Further investigate the life history of tachinid parasitoids of leafrollers and their potential for enhancing management of leafrollers.
5. Integrate the information on natural enemy phenology and abundance into the WSU-Decision Aid System to help users gauge the impact of pesticide sprays at different times of the season.

Significant Findings:

- Beating trays are poor for estimating phenology and abundance of many natural enemy populations. For example, caught 33,948 lacewing adults using attractant-baited traps in five WA apple orchards: beating tray samples conducted concurrently collected 12.
- A preliminary model from three Washington apple orchards (Jones' data) shows the phenology of our key lacewing species, *C. nigricornis*, was very similar across multiple sites and to data from walnut orchards.
- We now have powerful lures for *C. nigricornis*, several other lacewing species, parasitic wasps, predatory Hemiptera (*Deraeocoris*, *Campylomma*, *Orius*), and syrphids.
- We completed testing of 14 lures for longevity and release rate over time.
- We test three new promising lures that will be integrated into 2010 trapping programs, taking the place of less active lures.
- Using the molecular protocol developed last year in Unruh's lab, we were able to detect codling moth remains within the bodies of spider predators up to 96 hrs post-ingestion.
- Tachinid flies exposed to field rate or 10% field rate residues of Esteem for 24 hours suffered greatly accelerated mortality which would kill newly emerging flies before they could reproduce.
- Intrepid had a more moderate effect on the tachinid *Nilea erecta*, mostly eliminating survival in the latter half of adult life.

Significant Progress:

Objective 1. Our sampling produced phenology data from three data sources: (1) overwintering bands that were placed in orchards last fall and brought to an outdoor shaded shelter by early December; (2) beating tray samples collected in eight orchards in the Wenatchee and Yakima areas from early spring to fall; and (3) attractant-baited trapping in the same eight orchards.

The banding data suggest that by early April, adult parasitic wasps (6-Apr-09) and syrphids (13-Apr-09) began to emerge. While the adult syrphid numbers peaked by late April, wasp numbers rose sharply in late-May/early-June, which corresponded closely to the egg-laying period of the codling moth. Many of the wasps captured were *Ascogaster*, an important parasitoid of codling moth.

We caught 33,948 lacewing adults using four attractant traps in five orchards. In contrast, beat-samples conducted concurrently in the same orchards captured 12 lacewings. This is the second year in which beating trays have severely underestimated natural enemy abundance and/or activity (see Table 1).

Table 1. Comparison of the abundance and phenology of the lacewing *Chrysopa nigricornis* measured in Washington apple orchards using beat-tray samples and attractant traps during 2009.

	Initial Capture		Last Capture		Total Captured	
Orchard	Beat Tray	Attractant	Beat Tray	Attractant	Beat Tray	Attractant
1	22-Jul	24-Apr	22-Jul	15-Oct	1	7,158
2	24-Jul	13-May	4-Sep	6-Oct	4	13,770
3	none	18-May	none	6-Oct	0	9,108
4	11-Aug	21-May	8 Sept	19-Oct	5	3,195
5	18-Aug	4-Jun	10 Sept	30-Sep	2	717
Difference	75.4 days		42.6 days		12	33,948

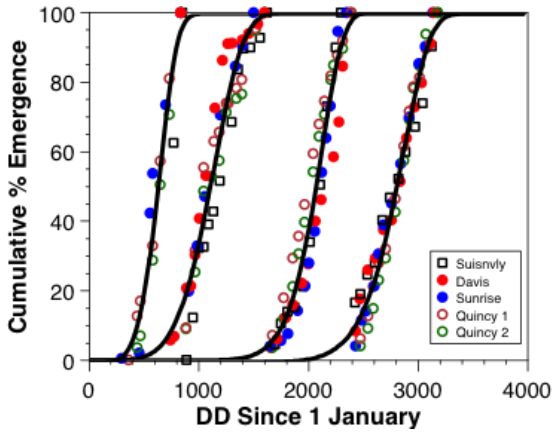
At each site, the attractant baited traps caught lacewings earlier and later in the season, and in dramatically higher numbers (Table 1). This information shows that adult lacewings emerge earlier in the spring (≈75 days earlier) and that they continue to fly later in the fall than expected based on beat samples (≈43 days late). Our data show that while beat-samples may have its place for estimating populations of certain natural enemies, it is clear that are unsuitable for monitoring many natural enemies. For example, looking at the *C. nigricornis* data from beat-samples, it would appear that this predator is rare, when it is actually extremely abundant.

A preliminary model from three Washington apple orchards (Jones' data) shows that the phenology of the key lacewing species, *C. nigricornis*, was very similar across multiple sites and when combined with data from California walnuts, demonstrate the potential for a robust phenology model for this species (Fig. 1). Data from Horton's work in Yakima and from Hood River pears (Shearer's data from the SCRI grant) will be added when the data are available later in the winter.

Plan for next year: The beating tray data will be taken from the same orchards and emergence data from overwintering sites will again be available from 13 orchards. This winter, modeling studies on *C. nigricornis* will continue with validation being performed using data collected next year.

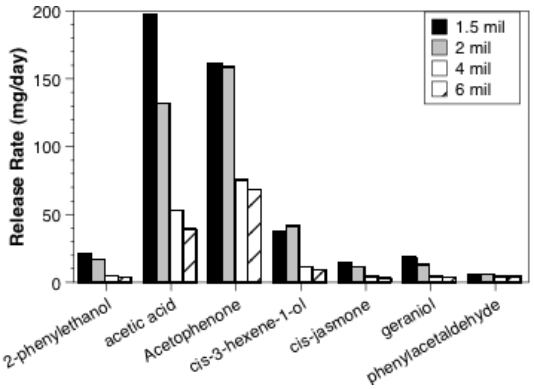
Objective 2A. *Determine release rates of semiochemicals.* We investigated a total of 14 lures for longevity and release rate over time in both lab and field settings at WSU-TFREC.

Fig. 1. Preliminary phenology model for *Chrysopa nigricornis* from 3 Washington apple orchards and 2 California walnut orchards.



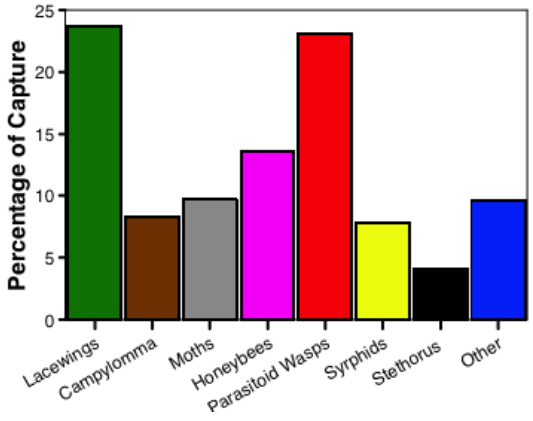
The polyethylene tubing was generally acceptable as a release device (Fig. 2); our studies showed that the

Fig. 2. Release rate of seven different attractants through four different thicknesses of lures



release rate for each compound is a function of membrane thickness and temperature. We did need to modify the dispensers for six compounds that released too quickly. In four of the cases, we were able to just increase the lure load, in the other two cases we modified the lure to decrease the size of the window through which the attractant was released. All the lures last at least 28 days in the field at biologically relevant rates.

Fig. 3. Diversity of insect groups captured in the top three compounds in the long term-trapping.



Objective 2B. *Evaluate field effectiveness and spectrum of activity of the different attractants*. This sub-objective had five different experiments run this year (1) long-term testing at eight apple sites in Washington (four in the Wenatchee area, four in Yakima); (2) an in-depth study of the independent and interactive effects of three particularly attractive compounds (geraniol, methyl salicylate and 2-phenylethanol); (3) the investigation of new materials for next year's studies, (4) measuring insecticide impacts by using our attractant lures to accurately assess natural enemy abundances before and after sprays, (5) using trap type to enhance trap capture of natural enemies and reduce capture of non-targets such as honey bees.

Long-term trapping: This past year, we examined ten lure blends as potential tools for monitoring natural enemy diversity, abundance and phenology. We looked at 1) differences in attractiveness among blends of seven different attractant compounds, 2) arthropod diversity and the attraction of particular taxonomic groups to certain lure types, and 3) phenological trends in the four most abundant insect groups. At four commercially managed apple orchards (WSU Sunrise and 3 orchards in the Quincy area of WA), we deployed a total of 160 traps (40 traps/orchard × 4 orchards) from late May to early October, all of which were checked weekly.

The lures attract a broad diversity of natural enemies and in some cases, herbivorous taxa (*e.g.*, moths, western flower thrips) (Fig. 3). In general, our lures bring in large numbers of lacewings and parasitic Hymenoptera, with other groups also well represented in some of our blends. Our lures can be used to develop a better understanding of the phenology of target natural enemy groups (Fig. 4). However, they also demonstrate that the reliance in the past on primarily one method of sampling (the beating trays) has grossly underestimated numbers and distorted our understanding of natural enemy importance and phenology. For example, we found that beat-tray sampling suggested that parasitoid densities spiked early and then late in the season, while our attractant-trapping indicated that they remain abundant throughout the growing season. It is clear that designing management plans without knowing the phenology of these organisms cannot be successful.

"Best blend" experiment: Our long-term trapping data in NC WA showed the blend of geraniol, 2-phenylethanol, and methyl salicylate consistently captured more lacewings (five species), syrphids (12 species), and parasitoids (19 different families) than any other blend. The question was which components were the different taxa responding to? To answer the question, a factorial experiment was designed to allow us to look at all possible combinations of attractants. We found that green lacewings responded to any lure with 2-phenylethanol, syrphids responded to geraniol containing lures, and parasitoids responded best to lures containing 2-phenylethanol and to a lesser extent methyl salicylate (Fig. 5).

New attractants: Large numbers of certain parasitoid families (Scelionidae, Eulophidae, Pteromalidae, Trichogrammatidae, and Eucoilidae, were caught with phenyl-acetaldehyde (PAA) lures. The compound cis-jasmonate (CJ) was attractive to two taxonomic groups: syrphids and *Campylomma* (predatory bugs). Acetophenone (AP) consistently caught a particular large

Fig. 4. Phenology of lacewings (A.), syrphids (B.), and *Campylomma* and *Deraeocoris* (C.) compared to codling moth larval phenology at different sites in Quincy.

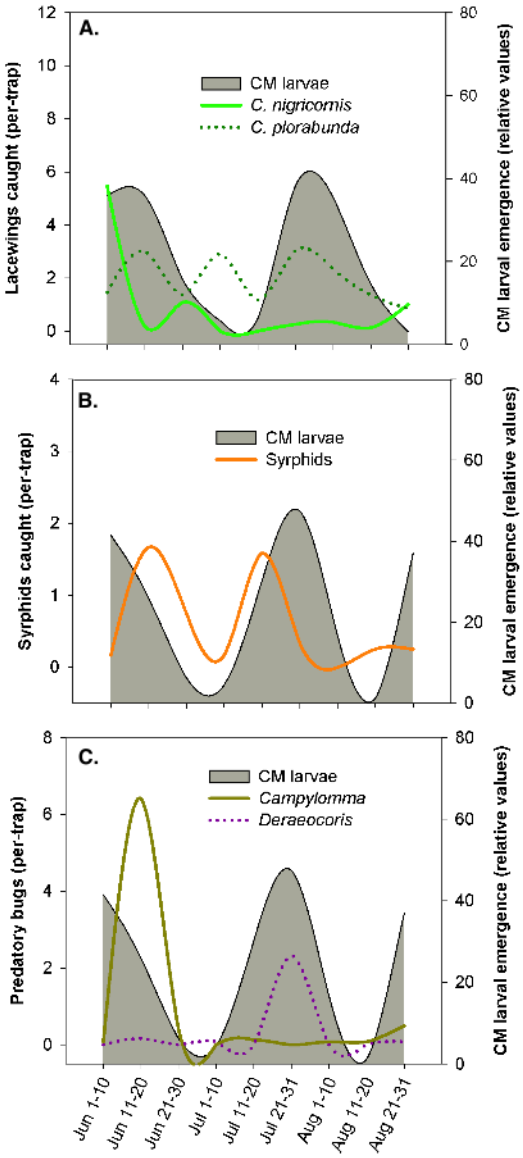
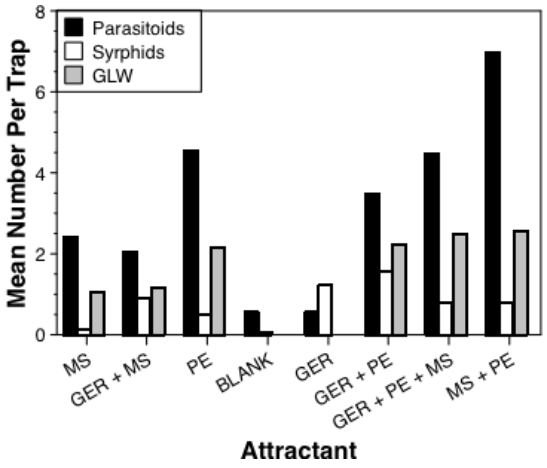
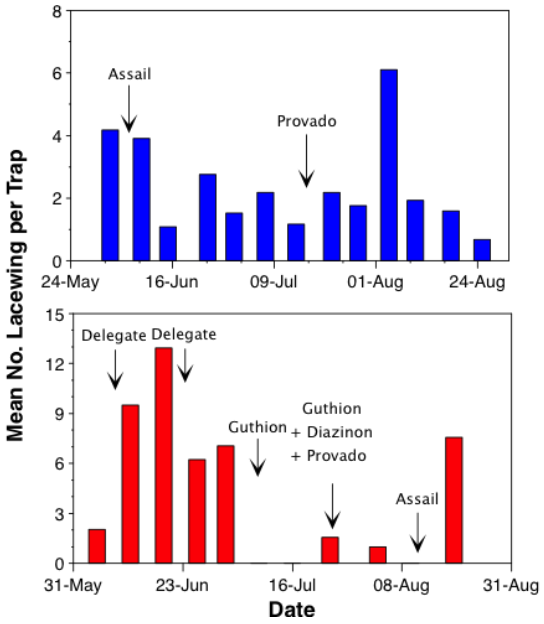


Fig. 5. Experiment to determine which attractant in our blend of geraniol, 2-phenylethanol, and methyl salicylate is responsible for captures of green lacewings, parasitoids, and syrphid flies.



ichneumonid wasp. Ichneumonids are known to be important enemies of OBLR and CM, suggesting that we may have serendipitously discovered an effective lure for this wasp species. AP was also very attractive to western flower thrips. AP is very promising: in an experiment conducted earlier in the season, a blend of AP, PE, and acetic acid was tested against our "best blend" (GER +PE+MS), and the AP blend caught as many lacewings, syrphids, and parasitic wasps as the GER blend.

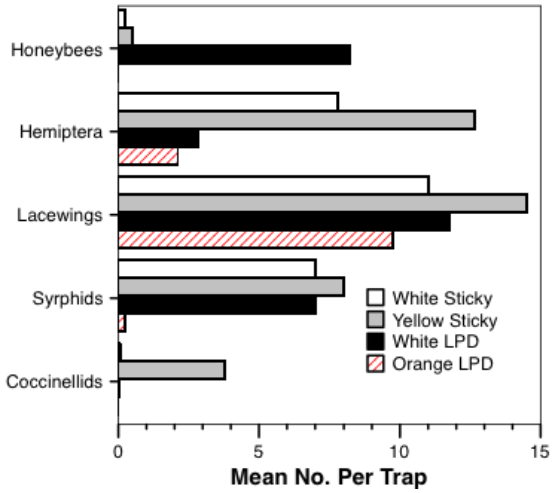
Fig. 6. Comparison of the trap catch of *Chrysopa nigricornis* in an orchard with minimal pesticide program (top) and heavy pesticide program (bottom).



Evaluations of insecticide impacts: The efficacy of our attractants permits us to document how natural enemy populations are influenced by insecticide sprays. Focusing on lacewing populations, our data this year show that reduced-risk insecticides had less impact on the lacewings, while organophosphates appear to decimate lacewing populations (Fig. 6). In one orchard near Quincy with a minimal pesticide program, we found that lacewing populations experienced typical oscillations but never "crashed" (Fig. 6 top). A different orchard in the same area saw its lacewing population decline

sharply and remain low for approximately four weeks, during which two applications of azinphos-methyl (Guthion) were applied (Fig. 6 bottom). During this same four week period at other Quincy orchards, our trapping indicated that lacewing populations were rising. The trapping data show that the attractant lures can be used to evaluate pesticide applications.

Fig. 7. Effect of trap type on capture of several predaceous groups.



Trap type effects: The effect of trap type was evaluated with a combination of three lures and an untreated check. We evaluated the standard white delta trap, an orange delta trap, a white sticky card, and a yellow sticky card (Fig. 7). We chose lures to evaluate that had shown a broad spectrum of activity so that we could evaluate trap type for a number of taxa. The lures used were: (1) geraniol + 2-phenylethanol, (2) 2-phenylethanol + methyl salicylate, and (3) acetophenone. We present data for a trap type using only the most active lures for a given taxa.

The yellow card was consistently one of the most sensitive for all the natural enemies, edging out all the other traps types for all natural enemies (Fig. 7). The effects were most marked for the parasitic hymenoptera (data not shown because we caught >360 per trap on the yellow cards), but the trends were mostly consistent for the other taxa as well. The honeybees did not respond to orange delta traps, white sticky traps, or yellow sticky traps, allowing us to use any of those trap types to minimize undesirable honeybee captures.

Plans for next year: Promising compounds, such as PAA, AP, and CJ, will be incorporated into the general pool of attractants to be tested in the 2010 growing season. These new compounds will also be integrated into investigations of trap type and dose response effects. We will also change some of the lures in the season-long phenology of natural enemies (Obj. 2B) based on data collected this year. We are also still looking for a lure that attracts ladybird beetles.

Objective 3A. Video monitoring equipment proved unreliable in the field for both technical and biological reasons. Using motion detection functions, recorder memory was conserved, but the effect of moving shadows and blowing leaves effectively reduced the efficiency of this function. Bird and rodent predators were responsible for virtually all predation events observed (all but 2 examples of ant predation). We are uncertain if the lack of observed insect predators was a result of the artificial observation arenas and set up or truly reflects normal situations in those orchards. We did attempt to use a cover to reduce bird predation and a screen fence to reduce rodents. The cover eliminated birds, but the screen fence (3 feet high) did not prevent rodents (*Peromyscus* spp.) from entering the arena.

Objective 3B. *Gut content analysis methods to replace PCR:* Using the loop-mediated DNA amplification (LAMP) protocol, we were able to detect codling moth remains within the bodies of predators. In fact, the LAMP technique was able to detect codling moth DNA in whole-body homogenates of crab spiders, up to 96 hrs post-ingestion.

Plans for next year: We will continue sampling with pitfall traps and will also use beat trays to collect predators for gut content analysis. Video cameras will be used in the lab to observe ground predator behavior with cocooned versus active larvae to determine if their behavioral repertoire supports attack of the cocooned larval stage. Predators collected in pitfall traps will be analyzed for codling moth in gut contents.

Objective 4. *Determining the impact of IGR's used for leafroller control on tachinid adults.* This year

we evaluated the effect of adult contact with Esteem (pyriproxyfen), and Intrepid (methoxyfenozide) residues to help determine if these compounds negatively affect biological control in orchards. Compounds were applied at 10% increments from 100% to 10% of field rates to plastic deli cups and air-dried. Cups were then provisioned with 10% honey-water solution. Cohorts of male and female *Nilea erecta* and *Nemorilla pyste* were placed in the treated cups individually upon the day of emergence to the adult stage, with 10% of the cohort reserved for untreated (control) cups. Cups were monitored daily to determine the day of death. Results of this study demonstrate that adult parasitoids are sensitive to IGR residues, even at very low rates (Fig. 8). The experiment needs additional replication, and completion of the first experiment with *N. pyste* and Intrepid is pending. Although 335 flies have been tested, experiments proceed slowly at times because colony production of adult flies determines the amount of flies that can be tested. However, looking specifically at results for *N. erecta,* it is apparent from the position of mortality curves of the flies exposed to residues relative to the controls, that Esteem is more toxic to *N. erecta* than Intrepid (Fig. 8), with even 10% field rate resulting in only 20% survival by three days. The Esteem treatment differed from the control primarily by decreased survival during the latter half of adult life, with control flies living out to 24 day after treatment, versus Intrepid flies all dying by 10 days.

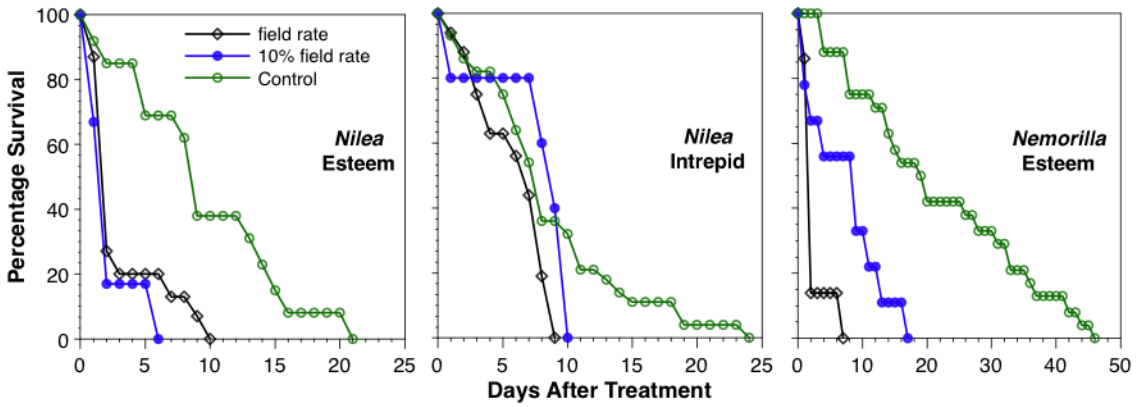
The second tachinid species (*Nemorilla pyste*) is harder to rear and fewer assays were performed with this species. Our data show that it is longer lived, with our control flies lasting up to 45 days (Fig. 8). However, exposure to the residue of Esteem was highly toxic to this species, with survival time shortened dramatically at the field rate to only 6 days and at 10% field rate to 18 days (Fig. 8).

Early mortality caused by the IGR residues is of most concern given that this is the period during which mating occurs, and because early reproduction is the most important factor in terms of population increase. Esteem clearly increased early mortality at most concentrations and thus can be expected to strongly reduce population growth, likely leading to extinction of the tachinid populations. Intrepid seemed to contribute mostly to later mortality with *N. erecta* (Fig. 8), and thus is likely more compatible with biological control. However, it is important to note that physiological effects may prevent successful reproduction with exposure to any of these residue concentrations.

Plans for next year. Most of the work next year will focus on getting more replications and evaluating Intrepid against *N. pyste*.

Objective 5. This objective is scheduled to occur in the last year of the grant as more details on natural enemy phenology become available. Our preliminary phenology models for *Campylomma* and *Chrysopa nigricornis* will be validated this next year, and more models will be developed and implemented, as the work gets further along.

Fig. 8. Results of IGR residue tests on survival of the tachinid parasitoids *Nilea erecta* and *Nemorilla pyste*



CONTINUING PROJECT REPORT **YEAR**: 1 of 3
WTFRC Project Number: CP–09-904

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

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

Research Tech: Esteban Gutierrez

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

Total Project Request: **Year 1:** $18,294 **Year 2:** $18,760 **Year 3:** $19,071

Other funding sources

Trident provided in-kind support (fumigation) $9000 value, and a grant of $4000. Other financial support of $18,500 was received from companies supplying products to be tested for effect on fire blight or orchard replant disease.

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

	2009	**2010**	**2011**
Salaries	11,493	11,951	12,429
Benefits	5,401	5,617	5,842
Wages			
Benefits			
Equipment	100		
Supplies	100		
Travel	1,200	1,200	800
Miscellaneous			
Total	$18,294	**$18,760**	$19,071

Footnotes: Salaries and benefits are in support of 0.34 FTE of a full time technician. Travel is to plot sites.

SUMMARY OF SIGNIFICANT FINDINGS

- For the second season in apple and pear fire blight control material trials, a biological organism provided blossom protection similar or superior to antibiotics.
- The antibiotic kasugamycin, which recently was granted a special label for use in Michigan, protected apple blossoms from a streptomycin susceptible strain of blight bacteria to a degree similar to the protection provided by streptomycin (AgriStrep, etc.) and both were superior to oxytetracycline (Mycoshield, etc.).
- A specific proprietary copper compound formulation provided blossom protection equal or superior to all antibiotics in both trials this season. The standard (Kocide) copper compound used as a comparison in the trials did not adequately protect the flowers from infection, a result common in past trial copper treatments. The new tested copper compound did not appear to russet D'Anjou pears, though it burned apple flowers, so much more extensive fruit safety tests will be done in 2010.
- In the newly established "Radar Hill" fumigation trial, tree growth in all fumigation treatments was remarkably equal, but all treatments were significantly superior to the untreated check.

FIRE BLIGHT PROJECT (75% of effort in 2009)

OBJECTIVES- ***Fire blight of apple and pear, as stated in the proposal:***

1. We will continue to assess efficacy of new or inadequately tested sprayed fire blight control products in the orchard, on both apple and pear.
2. To increase confidence in the biological organism that appeared promising in the 2008 trials, we will significantly expand our testing in 2009 and 2010 to include a range of alternative spray timings and rates.
3. We will further study the relationship of temperatures to fire blight infection risk, with the intention of changing the temperature assumptions used in the Cougarblight model, if these studies show that changes are necessary.

METHODS:

Two fire blight control material efficacy trials were carried out, one on Red Delicious apples at WSU TFREC, and the other on D'Anjou pears at the WSU Smith Tract research unit. These sites and cultivars were chosen due to their low sensitivity to the necessary fire blight exposure and infection. About 400 blossom clusters, 100 per replicate, were treated by back-pack mist sprayer at various timings and rates relative to stage of blossom development, then inoculated by spraying a known concentration of a streptomycin-susceptible laboratory strain of *Erwinia amylovora* to assure a high degree of infection, dependant on degree of protection by the tested substance. Efficacy was evaluated by counting the number of blighted vs. unblighted fruit clusters on the inoculated area on the tree. This method skews the data to indicate a higher percentage of blossom infection than would ever be likely in an orchard, but is very useful when comparing the relative efficacy of one product to another. The reader should not extrapolate the percent control achieved in these trials to the percent control likely in the orchard under natural infection conditions.

The methods used are standardized under the European and Mediterranean Plant Protection Organization protocol on efficacy evaluation of bactericides, 2002, OEPP/EPPO, Bulletin 32, 341-345.

RESULTS & DISCUSSION: FIRE BLIGHT CONTROL PRODUCT EFFICACY
Note: Some of the products reported below are not yet registered for use in orchards. They are reported only to illustrate the results of research. Check the label prior to use.

Product	Rate	Timing	% Infection	% Control
Copper Product TS (Trade Secret)	4 qt./A	80, 100% bloom & 1 day post inoculation	1.0	98.4 a
*Streptomycin 17%	1 lb/A, 200 ppm	100% bloom	4.0*	93.4 ab
Copper Product TS	2 qt./A	80, 100% bloom & 1 day post inoculation	8.0	87.6 bc
Specific A.p. Yeast + Acid Buffer	1.34 lb/100gal/A 9.35 lb/100/A	20, 40, 70 & 100% bloom	12.4	80.8 cd
Kasugamycin	200 ppm	100% bloom	13.2	79.5 cde
Specific A.p. Yeast + Acid Buffer	1.34 lb/100gal/A 9.35 lb/100/A	40 & 80% bloom	17.4	73.0 def
Oxytet. 17%	1 lb/A, 200 ppm	100% bloom	19.0	70.5 efg
Specific A.p. Yeast + Acid Buffer, Then oxytet. 17%	1.34 lb/100gal/A 9.35 lb/100/A 1lb/100/A	20 & 50% bloom then oxytet @ 100% bloom	19.1	70.4 fg
Specific A.p. Yeast, NO Acid Buffer	1.34 lb/100gal/A	20, 40, 70 & 100% bloom	19.7	69.5 fgh
Specific A.p. Yeast, NO Acid Buffer Then oxytet. 17%	1.34 lb/100gal/A 1 lb/100/A	20 & 50% bloom then oxytet @ 100% bloom	20.0	69.0 fgh
Serenade Max	1 lb/100/A	20, 50 & 100% bloom	21.9	66.0 fgh
Kocide 3000	0.5 lb/100/A	80& 100% bloom	23.5	61.1 hi
Copper Product TS	1 qt./A	80, 100% bloom & 1 day post inoculation	30.1	53.3 i
Serenade QRD 146	0.5 lb/100/A	20, 50 & 100% bloom	36.0	44.2 j
No treatment, inoculated check	**0**	**NA**	**64.4**	**0 k**
No treatment No inoculation	0	NA	0.0	NA

Table 1. ***Apples***: Summary of data. Values followed by the same letter should not be considered different. Least Significant Difference in percent control = 9
*Streptomycin was effective in this trial because a streptomycin susceptible lab strain of the blight bacteria, *Erwinia amylovora*, was used to inoculate the flowers. The author does not recommend this product for use in Washington orchards due to high streptomycin resistance in wild E. amylovora.

Two non-antibiotic materials performed very well in the 2009 trials. The copper compound, which I'm calling "copper product TS (Trade Secret)," reduced fire blight infection as well as, or better than, standard and test antibiotics, and to a far greater degree than any other copper material or copper/fungicide combination tested by the author in this or previous trials. The company that has the marketing rights for this product requested that the product remain unnamed at this time, a request that the author did not believe to be of much consequence when he agreed to it. There was a strong correlation between efficacy of the copper product and rate per acre. This product caused obvious rapid phytotoxicity of apple flower petals, especially at the 4 quart per acre rate, but there was no russet on the fruit skin observed at harvest, even on the usually russet-prone D'Anjou pears. This concern about potential russet induction will be addressed in detail next season.

The biological product, a powder of dried organism, which the sponsoring company admitted was classified as a yeast, was also provided for test with limited information as to its identity. So, the author cannot say for certain what this yeast is. However, after the grant sponsor company stated the product was a yeast of European origin, it was simple to search the web for the phrase "fire blight and yeast" to find out that there is a product called "Blossom-Protect" that is registered in some European countries. That product is made up of yeast called ***Aureobasidium pullulans*** with a mixture of two strains, ApCF10 and ApCF40, which is applied in combination with a pH 5 acid buffer. This genus and species of yeast is commonly found in the Pacific Northwest as a natural colonizer of apple and pear flowers so will probably thrive and spread to newly opened blossoms under PNW conditions. It is not likely that this organism is producing its own antibiotic to achieve antibiotic-like performance in inoculated trials, as this is not typical of yeasts. It is possible that another mechanism, such as successful competition for resources on the stigma surface or within the nectary, serves as a control process. In order for control to occur, it appears that this organism must be in place soon after each flower opens so as to become well-established on the flower before the introduction of ***Erwinia amylovora***, the fire blight pathogen.

The results detailed in table 1 indicate that there was a numerical, but not a statistically significant, advantage to four vs. two applications of the yeast prior to inoculation. This practical aspect of orchard use will be studied further in next season's trials.

The sponsor company recommended the addition of a large quantity of a specific acid buffering additive (9.35 lb. per 100 gal./A) to be applied along with the yeast. The addition of this pH 4 buffer significantly improved control (80.8% with the buffer, 69.5 without.) However, the author wonders if growers would appreciate mixing almost 40 pounds of a specific buffering product to each tank load of a product combination that was recommended at four sprays applied over a 7 to 10 day bloom period. It is likely that the buffer provided is a organically acceptable product marketed by the company having the yeast tested. Perhaps there is another easier-to-use acidifying buffer that would work as well. As Erwinia amylovora does not grow well in an acid environment, it is slightly possible that an acid buffer alone might provide some degree of control, and that possibility, as well as alternative buffers to be added to the yeast, will be tested next year.

ORCHARD REPLANT DISEASE PROJECT (25% of effort in 2009)

OBJECTIVES as stated on the proposal:
We will demonstrate the positive effect on soil fumigation on the productivity and quality of apples grown under a very modern production system.

1. We will determine apple tree growth and productivity over a range of chloropicrin and 1, 3-DCP rates.

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

METHODS, *The replant disease treatment trials:*
Establishment: In the fall of 2008, block of land south of Othello, Washington that had recently supported an apple orchard (with one fallow season) was selected as a site for the fumigant trial. The land was ripped thoroughly and smoothed prior to fumigation. On October 27, 2008 a replicated fumigation trial was established, with four treatments and untreated checks. Each replicate was approximately 0.8 to 1 acre, with a total of about three acres for each treatment. Fumigant application was by Trident Agricultural Products, Inc. Application depth was 16 inches. Shank spacing was 20 inches. At the base of each shank were 4 inch wings where the fumigant was emitted. Maximum spacing of fumigation outlets was 12 inches. The soil temperature and moisture were well within the optimum range. Treatments applied are as listed in table 2.

Treatment	Rate chloropicrin per acre	Rate 1,3 DCP per acre
Pic-Plus	10.9 gal. = 150 pounds	0
Pic-Clor 60, 20 gpa	10.5 gal. = 144 pounds	9.5 gallons = 94 lbs.
Telone C-35, 25 gpa	7.0 gal. = 97.5 pounds	18.0 gallons = 178 lbs.
Telone C-17, 30 gpa	3.7 gal. = 51 pounds	26.3 gallons = 259 lbs.
Untreated	0	0

Table 2. Soil fumigant rates applied in the 2009 Radar Hill soil fumigation/orchard replant disease trial. The rate range of current orchard replant site fumigation is 50-100 lbs. chloropicrin + 20-30 gal. per acre of 1,3 Dichloropropene.

This block was planted by Allen Brothers Fruit Company to Cripp's Pink "sleeping eyes" at 8.5 x 3 feet in the spring of 2009. These trees are being trained to a five wire upright trellis. At the proposed tree spacing, approximately 5000 trees were planted in each treatment, split into three replicates. The untreated checks are much smaller than the treatment areas in deference to the valid concerns of the orchard owners and manager. Approximately 40 – 50 trees will be growing well away from treated soil in the interior of each of the three untreated areas, which in the author's experience will be sufficient for valid statistical analysis.

Judging by adjacent and nearby blocks of orchard under similar management, the system promises to produce very well, reaching full production in five years or less. An orchard managed in this manner will offer great advantages as a replant trial for at least two reasons: 1. the rapid return to full production will reduce the number of necessary evaluation years from the seven to ten common in the past down to five, and 2. As this orchard system is very modern, it will help us determine if planting trees at high densities, drip irrigating and fertigating reduces the economic impact of this root-damaging disease complex.

Evaluation: The following data was/will be taken:

Year 1: Height of central leader. (2009 Data in Table 3, below)
Year 2: Cross sectional area of the trunk at 4 inches above the graft union, plus yield, if any (2010).
Year 3 (4 and 5): Cross sectional area of trunk, fruit per tree, fruit size, yield.

RESULTS & DISCUSSION: REPLANT TREATMENT TRIALS
Radar Hill Trial:

Treatment:	PicPlus (150 lbs./A Chloropicrin) 0 DCP	PicClor (144 lbs./A Chloropicrin) 94 lb/A DCP	Telone C-35 (25 GPA, 98 lb/A cloropic) 178 lb/A DCP	Telone C-17 (30 GPA, 51 lb/A cloropic) 260 lb/A DCP	Untreated
Average Height (in inches)	57.1a	58.8a	58.8a	58.6a	48.9b

Table 3. Average inches height of first season Cripp's Pink apple "sleeping eye" on M9 planted after fumigation on a site that had recently been an orchard.

The height of about 150 trees in each treatment was measured in October 2009 after first season growth stopped. All of the young trees, planted as a fall 2008 budded "sleeping eye," grew as a single upright shoot. Growth across the orchard and within the fumigated treatments was very uniform, averaging 58 inches, with most trees within a few inches of that average height. There are a few areas within the fumigated treatments where the young trees grew less well than the average, especially along the eastern edge where the soil texture appears sandier. In the unfumigated checks, the growth was much more variable and height averaged about 10 inches, or 16% less tall than the crop average. This degree of growth suppression is very similar to that seen in past replant treatment trials where significant yield differences were measured.

Brays Landing Trial:

Year	Telone C-35 at 30 gal./A	Telone C-35 at 39 gal./A	Methyl Br 200 lb.+ Pic 100 lb	Metam 75 gal./A	Telone C-17 at 30 gal./A	Untreated
2005	6,425	6,315	6,429	3,732	5,754	2,844
2006	13,917	14,544	14,265	13,155	14,097	9,496
2007	9,016	6,965	7,344	7,319	6,975	6,450
2008	15,657	16,028	15,042	13,788	14,688	8,088
2009	**19,641a**	**19,444a**	**18,411b**	**17,921b**	**15,439c**	**10,851d**
Total	64,656	63,296	61,491	55,915	56,953	38,073

Table 4. Average yield per acre in pounds in a large, replicated replant disease treatment soil fumigant trial. 7th leaf Golden Delicious, planted spring 2003, free standing central leader on MM106 rootstock, planted 9 x 14 ft., managed under organic methods from time of planting. Numbers followed by the same letter are not significantly different.

CONTINUING PROJECT REPORT **PROPOSED DURATION**: Year 2 of 2
WTFRC Project Number: CP08-802 (WSU Project #: 13J-3661-5365)

Project Title: Control of postharvest fruit rots in apple

PI:	Chang-Lin Xiao	**Co-PI (2):**	Bruce Campbell
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Address:	1100 N Western Ave	**Address:**	800 Buchanan St.
City:	Wenatchee	**City:**	Albany
State/Zip:	WA 98801	**State/Zip:**	CA 94710

Cooperators: Selected packinghouses across central Washington State

Total Project Request: Year 1: $89,289 Year 2: $93,406

Other funding Sources: N/A

WTFRC Collaborative expenses:

Item	2008	2009	2010 (extension)
Stemilt RCA room rental	6,368	6,368	
Crew labor	0	0	
Shipping	0	0	
Supplies	0	0	
Travel	0	0	
Total	$6,368.42	$6,368.42	**0**

Footnotes: The estimate of the RCA room rental cost was based on a projection of 40-bin space needed for this research.

Budget 1:
Organization: WA State University-TFREC **Contract Administrator:** ML Bricker; Kevin Larson
Telephone: 509-335-7667; 509-663-8181 x221 **Email:** mdesros@wsu.edu; kevin_larson@wsu.edu

Item	2008	2009	2010 (extension)
Salaries[1]	54,331	56,001	
Benefits	21,173	23,505	
Wages (time slip)	5,000	5,000	
Benefits	785	900	
Equipment	0	0	
Supplies[2]	6,000	6,000	
Travel[3]	2,000	2,000	
Miscellaneous	0	0	
Total	$89,289	$93,406	**0**

Objectives:

1. Develop preharvest fungicides and postharvest fungicides integrated programs for decay control.
2. Develop preharvest fungicides and postharvest biocontrol integrated programs for decay control.
3. Develop pre- and post-storage integrated programs for decay control.
4. Determine patterns of sensitivity or resistance of fludioxonil- and pyrimethanil-resistant *Penicillium expansum* and *Botrytis cinerea* to various pre- and postharvest fungicides and use the information for guiding fungicide use.
5. Establish an industry-coordinated program to monitor the shift in sensitivity of *P. expansum* to fludioxonil and pyrimethanil.
6. Collaborate with Bruce Campbell in evaluating natural compounds for management of fungicide resistance and decay control.

Significant findings:

This project deals with postharvest diseases. Some experiments on the 2009 crops are still in progress and will have results in late spring 2010. The final report of this project will be available for the next research review. Progress as of the end of 2009 is presented in this report.

A one-year extension of this project without additional funds has been approved by the WTFRC.

- For the first time we observed the occurrence of pyrimethanil (Penbotec) resistance among the isolates of *Penicillium expansum* from Penbotec-drenched fruit, but the frequency was low.
- The finding of the occurrence of pyrimethanil resistance in *P. expansum* suggests that further research is needed to monitor the frequency of pyrimethanil-resistant populations, understand the biological characteristics of pyrimethanil resistance in *P. expansum*, determine whether the level of pyrimethanil resistance results in the failure of blue mold control with Penbotec, and develop relevant measures to manage pyrimethanil resistance.
- Residual protection of apple fruit by preharvest Pristine was still evident 5 months after harvest but declined after the fruit were stored at room temperature for one week after CA storage.
- Preharvest Pristine plus postharvest BioSave further reduced blue mold incidence during cold storage. However, the effectiveness of these treatments declined after the fruit were stored at room temperature for one week after cold storage
- On Fuji and Red Delicious fruit, both Scholar and Penbotec on drenched fruit exhibited very good residual protection of fruit from infection by *P. expansum*. BioSave alone applied at packing reduced blue mold incidence, but the effectiveness declined when the fruit were stored for one additional week at room temperature after cold storage. Bio-Save did not provide additional protection for Scholar-drenched or Penbotec-drenched fruit.
- Octylgallate alone, or in combination with Scholar, did not control blue mold on apple fruit caused by fludioxonil-resistant *P. expansum*.

Methods:

Blue mold-decayed fruit were collected from grower lots that had been drenched with Penbotec or Scholar from packinghouses. Isolations of *P. expansum* from decayed fruit were attempted. Isolates of *Penicillium* spp. were identified to species. Isolates of *P. expansum* were screened for resistance to pyrimethanil, fludioxonil and thiabendazole (TBZ). A subset of isolates was also tested to determine EC_{50} values of the fungicides.

Pristine was applied to Fuji fruit one week before harvest. Fruit were stored in CA for 5 months. Fruit were removed from CA, washed and brushed through a research packing line, wounded and inoculated with *P. expansum*, and treated with either BioSave or a fungicide. Inoculated fruit were stored at 32°F in air for 8 weeks and one additional week at room temperature. Decay was evaluated.

Two trials (one on Fuji and one on Red Delicious) were conducted on the 2008 crops and completed in late spring 2009. Fruit from commercial orchards were drenched with either Scholar or Penbotec and stored in CA. The fruit were removed from CA 5 and 7 months after harvest, washed and brushed through a research packing line, wounded and inoculated with *P. expansum*, and treated with either BioSave or a fungicide. Inoculated fruit were stored at 32°F in air for 8 weeks and one additional week at room temperature. Decay was evaluated.

Identified natural compounds from Bruce Campbell's lab were tested for activity against fungicide-resistant strains of *P. expansum* in an agar medium and apple fruit.

Results & Discussion:

Monitoring resistance of P. expansum to pyrimethanil and fludioxonil

In 2009, 186 and 16 isolates of *P. expansum* were collected from Penbotec-drenched and Scholar-drenched apple fruit, respectively (Table 1). One isolate from Penbotec-drenched fruit showed significant resistance to pyrimethanil. EC_{50} values of pyrimethanil for a subset of 37 pyrimethanil-sensitive isolates ranged from 0.632 to 1.518 mg/L, with a mean of 1.07 mg/L, which is within the baseline sensitivity (Fig. 1). This was the first time we observed the occurrence of pyrimethanil resistance among the isolates of *P. expansum* from Penbotec-drenched fruit, but the frequency was low. Further research is needed to monitor the frequency of pyrimethanil-resistant populations, understand the biological characteristics of pyrimethanil resistance in *P. expansum*, determine whether the level of pyrimethanil resistance results in the failure of blue mold control with Penbotec, and develop relevant measures to manage pyrimethanil resistance.

All isolates were sensitive to fludioxonil. Of the 202 isolates tested, 35 were resistant to TBZ, indicating that TBZ-resistant strains remained in *P. expansum* populations even after TBZ was not used.

Table 1. Monitoring of resistance to postharvest fungicides in *Penicillium expansum* from apples

Source	**Drench Treatment**	**# isolates of *P. expansum***	**# isolates resistant to Penbotec**	**# isolates resistant to Scholar**	**# isolates resistant to TBZ**
Packinghouse 1	Penbotec	8	0	0	2
Packinghouse 2 –Lot 1	Penbotec	34	0	0	6
Packinghouse 3 –Lot 1	Penbotec	29	0	0	5
Packinghouse 3 –Lot 2	Penbotec	4	0	0	4
Packinghouse 3 –Lot 3	Penbotec	14	0	0	1
Packinghouse 3 –Lot 4	Penbotec	97	1	0	12
Packinghouse 2 –Lot 2	Scholar	16	0	0	5
Total isolates from Penbotec-drenched fruit		**186**	**1**	**0**	**30**

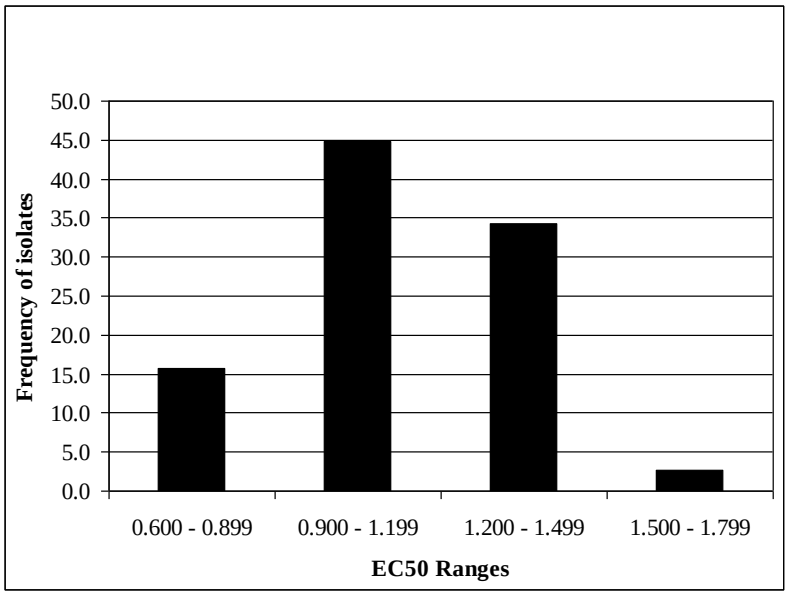

Fig. 1. Distribution of EC_{50} values of pyrimethanil for pyrimethanil-sensitive isolates of *Penicillium expansum* collected in 2009 from decayed apple fruit that were drenched with Penbotec prior to storage.

Preharvest Pristine in combination with postharvest biocontrol agent or fungicide for blue mold control.

Pristine was applied to Fuji apples 7 days before harvest. Fruit were removed from CA 5 months after harvest, washed and brushed through a research packing line, wounded and inoculated with *P. expansum*, and treated with either BioSave or a fungicide. Inoculated fruit were stored at 32°F in air for 8 weeks and one additional week at room temperature. Residual protection of apple fruit by Pristine was still evident 5 months after harvest but declined significantly after the fruit were stored at room temperature for one week after CA storage (Table 2). BioSave alone reduced blue mold incidence to 30% during the 8-week cold storage. Preharvest Pristine plus postharvest BioSave further reduced blue mold incidence to 2.5% during cold storage. However, the effectiveness of these treatments declined after the fruit were stored at room temperature for one week after cold storage.

Table 2. Preharvest Pristine in combination with postharvest BioSave for blue mold control on Fuji apples.

Preharvest Treatment	Fungicide applied 5 months after harvest	8 weeks at 32F post inoculation		1 week at room temp after cold storage	
		% decay	Lesion (mm)	% decay	Lesion (mm)
Nontreated	No Fungicide	98.8a[z]	27.5b	98.8ab	57.7a
	Scholar	0.0d	0.0e	1.3d	3.4e
	Penbotec	0.0d	0.0e	0.0d	0.0e
	TBZ	100.0a	31.3a	100.0a	61.6a
	BioSave	30.0c	11.1c	100.0a	27.7c
Pristine	No Fungicide	32.5c	10.7c	93.8b	24.6c
	Scholar	0.0d	0.0e	0.0d	0.0e
	Penbotec	0.0d	0.0e	0.0d	0.0e
	TBZ	88.8b	11.0c	100.0a	37.9b
	BioSave	2.5d	2.5d	77.5c	12.2d

[z] Values within the same column followed by the same letter are not significantly different according to the Waller-Duncan K-ratio *t* test at K ratio = 100 ($P = 0.05$).

Pre-storage fungicide drench in combination with postharvest biocontrol agent or fungicide for blue mold control.

Two trials (one on Fuji and one on Red Delicious) were conducted on the 2008 crops and completed in late spring 2009. On Fuji, both Scholar and Penbotec on drenched fruit exhibited very good residual protection of fruit from infection by *P. expansum* (Table 3). BioSave alone applied at packing reduced blue mold incidence to 33-43%, but the effectiveness lost when the fruit were stored for one additional week at room temperature after cold storage. Bio-Save did not provide additional protection for Scholar-drenched or Penbotec-drenched fruit. The results also suggest that Scholar and Penbotec have long lasting residual protections against *P. expansum* on drenched apple fruit.

Table 3. Postharvest drench in combination with Biosave applied at packing for blue mold control on Fuji apples

		5 months post drench treatments		7 months post drench treatments	
Drench treatment applied prior to storage	Fungicides applied at packing 5 or 7 months post drenching	% blue mold at 8 weeks at 0ºC post packing	% blue mold at one additional week at room temperature after storage	% blue mold at 8 weeks at 0ºC post packing	% blue mold at one additional week at room temperature after storage
Nontreated	No fungicide	96.3a[z]	100.0a	100.0a	100.0a
	Scholar	0.0c	0.0c	1.3c	5.0de
	Penbotec	0.0c	0.0c	0.0c	2.5e
	TBZ	1.3c	5.0bc	0.0c	3.8e
	Bio-Save	42.5b	96.3a	32.5b	93.6b
Scholar	No fungicide	0.0c	7.5b	0.0c	6.3de
	Bio-Save	0.0c	12.5b	1.3c	13.8cd
Penbotec	No fungicide	1.3c	5.0bc	0.0c	2.5e
	Bio-Save	0.0c	7.5b	0.0c	20.0c

[z] Values within the same column followed by the same letter are not significantly different according to the Waller-Duncan K-ratio *t* test at K ratio = 100 ($P = 0.05$).

On Red Delicious, both Scholar and Penbotec on drenched fruit also exhibited very good residual protection of fruit from infection by *P. expansum* during the 8-week cold storage, but Scholar's residual protection declined when the fruit were move to room temperature (Table 4). BioSave alone applied at packing reduced blue mold incidence to 43-70%, but the effectiveness lost when the fruit were stored for one additional week at room temperature after cold storage. Bio-Save did not provide additional protection for Scholar-drenched or Penbotec-drenched fruit. The results suggest that residues of Scholar and Penbotec on drenched Red Delicious apple fruit protect apple fruit for several months post-drenching.

Residual effects of Scholar and Penbotec on control of blue mold in Fuji apple fruit.

When Fuji fruit were drenched with Scholar or Penbotec, excellent residual protection against *P. expansum* was still evident 5 and 7 months after harvest (Table 5). These results are consistent with that we observed on Red Delicious. Taken these together, our research suggests that residues of fludioxonil and pyrimethanil on/in apple fruit are persistent during cold storage and that residual protection of apple fruit by the two fungicides can last for at least 7 months under apple-storage conditions.

Table 4. Postharvest fungicide drench in combination with Biosave applied at packing for blue mold control on Red Delicious apples

		5 months post drench treatments		7 months post drench treatments	
Drench treatment applied prior to storage	Fungicides applied at packing 5 or 7 months post drenching	% blue mold at 8 weeks at 0ºC post packing	% blue mold at one additional week at room temperature after storage	% blue mold at 8 weeks at 0ºC post packing	% blue mold at one additional week at room temperature after storage
Nontreated	No fungicide	100.0a[z]	100.0a	100.0a	100.0a
	Scholar	0.0c	0.0d	0.0e	0.0d
	Penbotec	0.0c	0.0d	0.0e	0.0d
	TBZ	1.3c	5.0c	0.0e	0.0d
	Bio-Save	70.0b	100.0a	42.5b	100.0a
Scholar	No fungicide	0.0c	13.8b	8.8c	23.8b
	Bio-Save	0.0c	21.3b	5.0cd	25.0b
Penbotec	No fungicide	0.0c	1.3cd	0.0e	2.5c
	Bio-Save	0.0c	0.0d	3.8d	18.8b

[z] Values within the same column followed by the same letter are not significantly different according to the Waller-Duncan K-ratio *t* test at K ratio = 100 ($P = 0.05$).

Table 5. Residual protection of Fuji apple fruit by fludioxonil and pyrimethanil

		5 months post drench treatments		7 months post drench treatments	
Drench treatment applied prior to storage	Fungicides applied at packing 5 or 7 months post drenching	% blue mold at 8 weeks at 0ºC post packing	% blue mold at one additional week at room temperature after storage	% blue mold at 8 weeks at 0ºC post packing	% blue mold at one additional week at room temperature after storage
Nontreated	No fungicide	100.0	100.0	97.5	98.8
	Penbotec	0.0	1.3	0.0	0.0
	Scholar	0.0	2.5	0.0	3.8
	TBZ	0.0	0.0	0.0	6.3
Penbotec	No fungicide	0.0	0.0	0.0	0.0
	Scholar	0.0	0.0	0.0	0.0
	TBZ	0.0	0.0	0.0	0.0
Scholar	No fungicide	0.0	10.0	1.3	6.3
	Penbotec	0.0	0.0	0.0	0.0
	TBZ	0.0	2.5	0.0	0.0
TBZ	No fungicide	33.8	36.3	3.8	15.0
	Penbotec	0.0	0.0	0.0	0.0
	Scholar	0.0	0.0	0.0	0.0

Evaluate natural compounds for controlling fludioxonil-resistant Penicillium expansum.

This research was done in collaboration with Bruce Campbell. On Petri dishes, octylgallate showed the potential as a promising chemosensitizing agent to overcome fludioxonil resistance in *P. expansum*. We further evaluated octylgallate in combination with Scholar (fludioxonil) for control of blue mold caused by two different fludioxonil-resistant strains (FR2: resistant to fludioxonil but sensitive to TBZ; FR3: resistant to both fludioxonil and TBZ) (Table 6). Octylgallate alone, or in combination with Scholar, did not control blue mold caused by fludioxonil-resistant *P. expansum*. It appears that in vitro and in vivo test results were not consistent. It is not known what causes the difference in results between the two tests, but compounds present in apple fruit flesh may affect the activity of octylgallate against fludioxonil-resistant *P. expansum*.

Table 6. Efficacy of octylgallate and Scholar for controlling blue mold caused by fludioxonil-resistant isolates (FR2 and FR3: fludioxonil resistant; W2: fludioxonil sensitive) of *Penicillium expansum*

Treatment	**% Blue mold 8 wk at 32 F**		**% Blue mold 1 week at room temperature**
	FR3	**W2**	**FR2**
CK	76.3	100	100
Octylgallate 0.15 mM	81.3	96.3	100
Octylgallate 1.0 mM	78.8	100	100
Octylgallate 0.15 mM + Scholar 230 SC	88.8	2.5	100
Octylgallate 1.0 mM + Scholar 230 SC	93.8	3.8	100
Scholar 230 SC	85	1.3	100

CONTINUING PROJECT REPORT **YEAR**: 2 OF 3
WTFRC Project Number: CP-09-900 (WSU Project #13C-3643-4676)

Project Title: Pheromone technology for management of codling moth and leafrollers

Co-PI(1):	Jay Brunner	**Co-PI(2):**	Larry Gut
Organization:	WSU-TFREC	**Organization:**	Michigan State University
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Co-PI(3):	Vince Hebert	**Co-PI(4):**	Peter Landolt
Organization:	WSU Tri-Cities	**Organization:**	USDA-ARS
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City:	Richland	**City:**	Wapato
State/Zip:	WA/99354	**State/Zip:**	WA/98951

Cooperators: Mike Doerr, WSU-TFREC; Peter McGhee, Michigan State University.

Total Project Request: Year 1: $120,797 **Year 2: $100,434** **Year 3:** $128,513

Other funding Sources
Agency Name: Pheromone Companies

- Shin-Etsu: $40,000 ($20K each to WSU and MSU) to help fund evaluation of new dispenser technology.
- Scentry Biologicals: $10,000 to WSU for assessment of dispenser release rates.
- Private Company: $30,000 ($15K each to MSU and WSU) for assessment of release rates, video recording of behavior, and attraction of dispensers to codling moth.
- Private Company: $25,000 to MSU for assessment of a novel attract-and-kill formulation.
- In 2009 there may be agreements with some of the companies listed above for continued work.
- There is also a possibility of two more companies interested in collaborating on discovery or development aspects associated with the objectives of this project.
- Potential SBIR grant ($75K) and private company investment ($10K) to help fund development of an automated delivery system for a pheromone dispenser.

The financial information provided in addition to sponsor support simply communicates research program support costs vs. specific project cost-share commitment.

Budget 1:
Organization: WSU-TFREC **Contract Administrator:** ML Bricker; Kevin Larson
Telephone: 509-335-7667; 663-8181 X221 **Email:** mdesros@wsu.edu; kevin_larson@wsu.edu

Item	2009	2010	2011
Salaries [1]			
Technical – M. Doerr (1 month)	5,278	5,489	5,709
Res. Analyst III	8,714	9,063	9,426
GRA – 9 mo appt @ 0.50FTE	22,014	0	23,811
Benefits			
Technical – M. Doerr (2 months)	1,689	1,757	1,827
Res. Analyst III	4,270	4,441	4,619
GRA – 9 mo appt @ 0.50FTE	1,881	0	2,035
Wages (temporary labor)	7,000	8,000	8,000
Benefits (18%)	1,260	1,440	1,440
Equipment	0	0	0
Supplies [2]	5,000	5,000	5,000
Travel [3]	2,085	2,085	2,085
Total	$59,191	**$37,275**	$63,952

Footnotes:
[1] Technical – Mike Doerr for 2 months; GRA – 9 months and salary for Jane LePage, Research Analyst III @ .02 FTE per year. Graduate student funding was not used in 2009 so is carried over into 2010 to fund a student.
[2] Temporary labor to assist with data collection and entry and student wages and benefits at $15 hr over a 10 week period (20 hours/week).
[3] Expenses of operating vehicles allocated to the project – fuel, maintenance, and repairs and travel at $.0385/mile x 1000 miles per field season (ten 100 mile trips).

Budget 2:
Organization: Michigan State Univ. **Contract Administrator:** Emily Flanner
Telephone: 517-355-5040 x256 **Email:** flanner@cga.msu.edu

Item	2009	2010	2011
Salaries	26,187	26,981	27,782
Benefits	12,847	13,506	14,107
Wages	3,000	3,000	3,000
Benefits	172	172	172
Equipment	0	0	0
Supplies	1,000	1,000	1,000
Travel	1,000	1,000	1,000
Total	$44,206	**$45,659**	$47,061

Budget 3:

Organization: USDA-ARS, Wapato **Contract Administrator:** Chuck Myers
Telephone: 510-559-5769 **Email:** Chuck.Myers@ars.usda.gov

Item	2009	2010	2011
Salaries	0	0	0
Benefits	0	0	0
Wages	14,000	14,000	14,000
Benefits (10% of labor)	1,400	1,400	1,400
Equipment	0	0	0
Supplies	1,000	1,000	1,000
Travel	1,000	1,000	1,000
Total	$17,500	**$17,500**	$17,500

Project objectives:

1. Improve hand-applied dispenser mating disruption systems for codling moth by determining an optimized dispenser release rate and density.
2. Characterize adult moth behavior that leads to optimization of an attract and kill (A&K) technology for codling moth and leafrollers.

Significant findings 2009

Optimization of hand-applied dispensers

- Various studies showed different levels of codling moth disruption in field cages and small field plot trials, attraction to, and activity around dispensers of different pheromone release rates.
- Research findings played a significant role in the development of a new, more efficient, dispenser that is scheduled for commercial release soon.
- Field-cage and small plot experiments demonstrated the importance of dispensers that limit the per night visits of males to pheromone sources.
- Video recording of behavior showed that the number and duration of visits to pheromone sources varied with pheromone release rate and showed a bimodal versus a linear form.
- Collaborations with three private companies allowed us to expand our research and to influence new product development.
- A new automated system for placing a pheromone dispenser in trees was tested and showed great promise for commercialization.
- Assessment of fresh and frozen pheromone dispensers demonstrated that pheromone release rates were not impacted when using the volatile capture system.

Attract and Kill (A&K) Research

- Either Warrior or Assail in different formulations were shown to be good candidates for toxicants for A&K.
- Sublethal concentrations of Warrior had a negative impact on male moth ability to orient to a pheromone source in the wind tunnel.
- Wind tunnel and video recordings demonstrated that the shape of an A&K device was an important factor influencing moth contact with a toxicant.
- Field-cage provided confirmation of the power of A&K for population control and provided valuable clues on effective designs and formulations.
- Small plot field trials confirmed observations of field-cage studies on different A&K formulations.
- Video recordings were valuable in showing the relative attraction of moths to A&K devices and the rate of moth contact.

Methods:

Methods used in this project were outlined in last year's new project proposal (2009) and have not changed significantly enough to warrant their repetition here. A basic discussion of methods is provided in each results and discussion section below.

Results and Discussion:

Optimization of hand-applied dispensers. The first objective of this project was to improve hand-applied mating disruption systems for codling moth. A team effort focused on understanding the impact of pheromone release rates from different devices on codling moth behavior. Behaviors were assessed with pheromone-baited traps, in small plot and field-cage studies, and with video recordings.

Pheromone traps. Based on previous research we anticipated that as the pheromone release rate of a dispenser increased moth capture would decrease. With the exception of the Flex25 tube-type dispenser, which captured more moths relative to lures loaded with 5, 10, or 20 mg, there was a

decline in moth captures with an increase in pheromone release rate. The release rate of the Flex25 dispenser was expected to be the same as the 20 mg dispenser but it proved to be highly attractive when placed in a trap, though not as attractive as the 0.1 or 1.0 mg lures (Fig. 1).

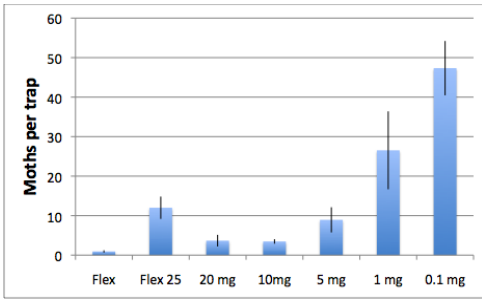

Fig. 1.

Interpretation. While data from this experiment generally confirm previous studies, the relative attraction of the Flex25 dispenser was unexpected. How this result informs interpretation of other studies and its impact on the design of dispensers is discussed below.

Field-cage studies. Field cages enclosing 12 large apple trees were used to assess pheromone treatments at dispenser densities approximating 200/acre. Conducting studies in large cages has the advantage of being able to replicate treatments while keeping the density of moths constant (76 males or 76 male and females per cage). When only males were released, all treatments reduced their ability to find a female mimic (trap baited with 0.1 mg lure) compared to the untreated control and there were no differences between treatments (Fig. 1 - white bars). When males and females were present a different pattern was observed. There was no difference between treatments with lowest pheromone release rate and the untreated control in the ability of male moths to locate a female mimic. As pheromone release rate increased there was greater impact on the male's ability to locate a female mimic (trap) (Fig 2 - dark bars).

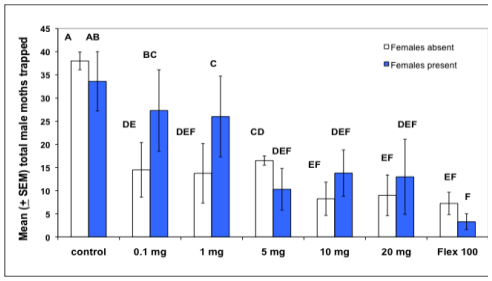

Fig. 2.

Interpretation. Dispensers releasing very small amounts of pheromone can result in reduction of a male moth's ability to locate a female. As pheromone release rate from a source increased there was only a slight increase in effect on male success in finding a female mimic. When females were present, males likely found a female, mated, and then continued searching in treatments with the lowest pheromone release. These results strongly suggest that an optimized pheromone release rate from a dispenser will be associated with the suppression of a male's ability to make multiple searches (matings) in the same night.

Small plot field trials. The same dispensers used in field-cage studies were also used in small plot field trials. Plots consisted of 20 trees (0.08 acres) separated by at least 30 meters. Treatments were applied at a rate of 200 dispensers/acre. Treatment effects were assessed by the ability of male moths to locate a female mimic (trap with a 0.1 mg lure in the center of each plot). The 10 and 20 mg dispenser treatments provided greater disruption (90%) than 0.1, 1.0 or 5 mg dispenser treatments in the first generation (Fig. 3 - top). However, even the 0.1 mg dispenser provided about 50% disruption. In the second generation a tube-type dispenser (Flex ≈ Isomate C plus) provided 88% disruption (Fig. 3 - bottom). All the other treatments, including a 1 mg dispenser, provided similar levels of disruption (65-80%).

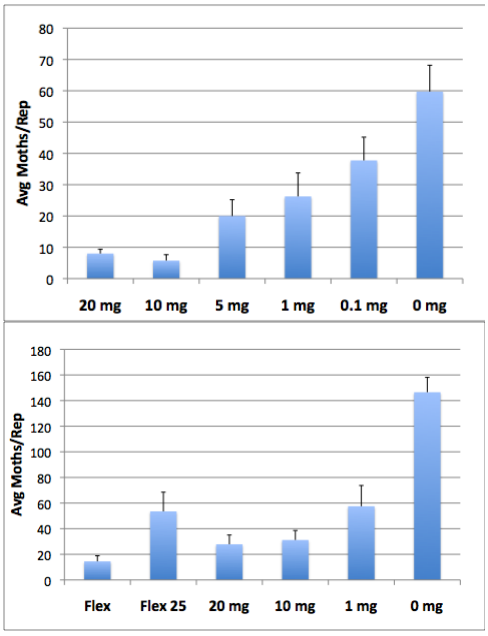

Fig. 3.

Interpretation. Very low level of pheromone release from a dispenser can exert a relatively large impact (50%) on a male moth's ability to locate a female. Increasing pheromone release rate provides greater disruption. In order to maximize behavioral disruption the pheromone release rate from a dispenser, and perhaps the plume structure, are more important that we previously thought. There were different impacts of treatments observed between generations. These differences are most likely due to the time moths can search due to temperatures (see video recording section).

Video recordings. A portable video camera system (see image at right) was used to record behavior of codling moth to pheromone dispenser treatments used in trapping, field-cage and small plot studies. Video recording of moth behavior around various devices used in field cage and small plot studies showed differences based on pheromone release rate and time of year recordings were made. For example, in May-June (1st generation) the number of visits was highest to dispensers with the lowest and highest release rates (Fig. 4-top), while the duration of visits was highest with the highest release rate device (Fig. 4-bottom. The percent of moths making source contact was small (10%) and only occurred with the 0.1 and 10 mg dispensers.

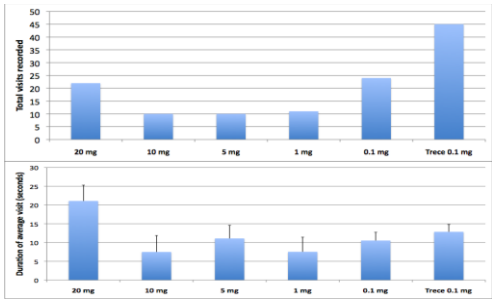

Fig. 4.

In July (2nd generation), the number of visits roughly doubled and a pattern similar to the first generation was observed; that is a higher number of visits occurred with the lowest and highest release rate dispensers. The average duration of visits was again highest to dispensers with the lowest and highest release rates (Fig. 5). The percent of source contacts was low (10-20%) and occurred only to the two lowest and two highest release rate dispensers.

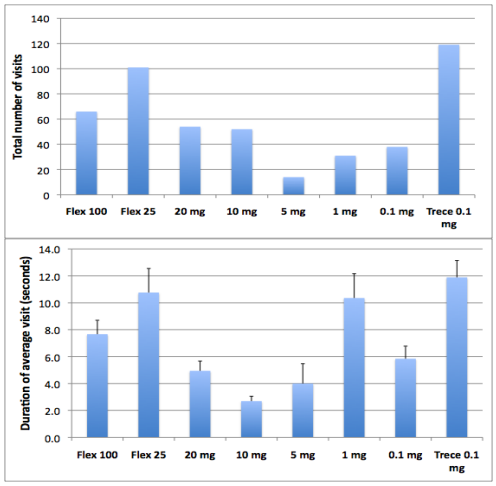

Fig 5.

An advantage associated with video recording is that we can see the effect of environmental conditions on behavior. For example, Fig. 6 shows that moth activity in June started before sunset and lasted only about one hour while activity in July started after sunset and lasted for almost two hours.

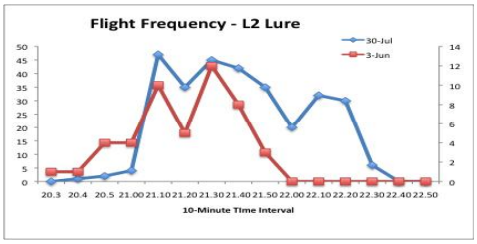

Fig. 6

Interpretation. Video recording helps us understand the relative activity of moths around different pheromone sources. The number of visits was expected to mimic the capture of moths in pheromone traps; however, there were more visits and of longer duration to the high release rate dispensers than we expected. This result shows that the higher release rate dispensers are attractive and that moths often approach within at least 12 inches. This is encouraging because we hypothesize that getting close to high release rate dispensers desensitizes the moths, thereby reducing the likelihood of them being able to search for females the same night. The fact that moths do not enter traps baited with high release rate dispensers suggests that certain behaviors are inhibited at close range.

Dispenser release rate studies. Collaborations with three private companies allowed us to expand our research and to influence new product development. Using the volatile capture system (VCS) developed by Dr. Hebert we showed that a new tube-type dispenser had a near zero order release profile and rates proportional to loading through 150 days – (Fig. 7). In field trials these dispensers provided similar levels of disruption under low pressure but under high pest pressure the Flex25 dispenser did not suppress moth captures as much as the others. No significant fruit injury was noted in any of the treatments.

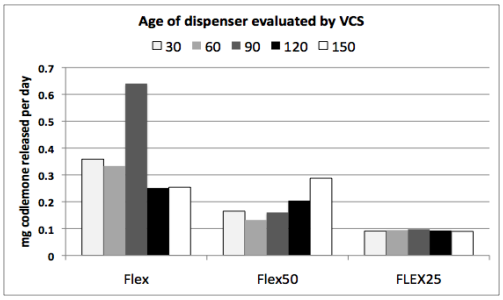

Fig. 7.

We also evaluated another hand-applied pheromone dispenser. As the dispenser aged its pheromone release rate declined dramatically based on VCS evaluations. In this cooperative project we showed that pheromone release rates measured by VCS provided the same results when dispensers were fresh (taken from the field) or frozen for a period of time. Comparing release rates and residual pheromone should help the company determine how to improve their dispenser.

Another experimental dispenser was shown to have a low pheromone release rate after only a few days of aging. Pheromone was tied up in the dispenser's matrix. Moths were attracted to dispensers in traps but attraction diminished dramatically with dispenser age. Video recordings showed moths approaching this dispenser, a potential A&K technology, but with only low levels of contact, which would compromise its effectiveness.

Collaborative work with a MI company has resulted in the development of an automated pheromone dispenser applicator. This device, the "tangler" (see image to right), was shown to have a release profile and efficacy similar to Isomate C plus. The tangler is shot into the tree canopy, wraps around a limb, and is retained over the entire season. This new technology will be shown at the review and evaluated further in 2010 if an SBIR grant is received to fund production of the dispensers and applicators.

Interpretation. By working with commercial pheromone companies this project is helping to improve dispensers in ways that should reduce costs to growers or at least maintain the current costs. Project findings played a role in the development of a modified, more efficient, dispenser that is scheduled for commercial release soon. In addition, new technologies are being developed as new companies become interested in entering the pheromone marketplace. This should provide for competition and eventually reduce costs for growers. As was anticipated, private companies are providing financial support to scientists working on this project, which supplements the core funding provided by the commission.

Attract and Kill Research. The second objective of this project was to characterize behavior of adult codling moth and leafroller in order to optimize development of A&K technologies. A team effort focused on assessing moth behaviors in different environments and to different A&K technologies.

Laboratory studies. At MSU several different devices were evaluated in the wind tunnel to determine relative ability to impact codling moth. When codling moth adults were exposed to lambda-cyhalothrin (Warrior) at 61.4 ug/cm^2, high levels of mortality occurred within 30 seconds. The LC_{50} for males and females were 30.1 and 27.1 ug/cm^2. Based on the above dosage response curve, a 15.0 ug/cm^2 concentration of Warrior caused about 40% mortality. This concentration was selected for flight tunnel assays. Male moths were exposed to Warrior for 4 h prior to the wind tunnel bioassay. Their response was directly compared to that of untreated males exposed to a rubber septum loaded with 0.1 mg of codlemone. Behaviors were measured: wing-fanning, upwind flight, upwind flight followed by landing or source contact, no response, and non-orientational flight. Results showed that male moths exposed to sublethal concentrations of Warrior were not successful in orienting in to a pheromone source in the wind tunnel (Fig. 8). Warrior was also mixed with Vaseline and aged in the field for up to 126 days in a prototype A&K device. Male moths were exposed to the aged Warrior residues in flight tunnel assays. Males that landed on the A&K device were recaptured and held to measure effects on survival. The toxicity lasted for over 4 months killing more than 90% of moths that contacted the toxicant mixture.

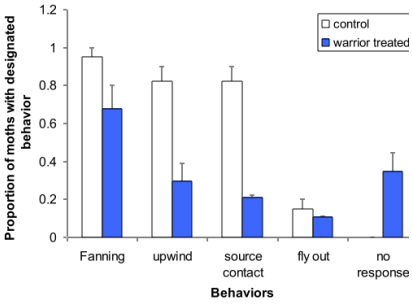

Fig. 8

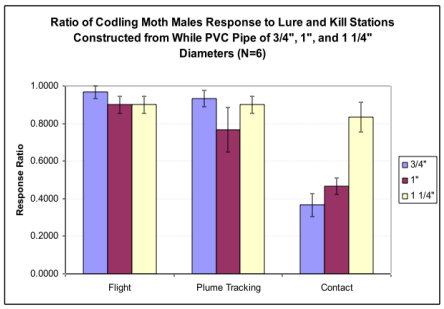

Fig. 9

At the USDA-ARS the attraction of male moths to an A&K device was evaluated in the wind tunnel. The A&K devices tested were a clear vinyl cylinder of different diameters or a 1¼ inch diameter section of white PVC pipe. The pesticide used in these studies was Assail mixed in a silicone grease at 0.5%, 1.0%, 2.0% or 4.0% (w/w). Fewer moths contacted the inside of the clear cylinder when small diameter tubes were used (Fig. 9). The 1¼ inch diameter cylinder showed the best ratio of moths contacting the A&K device. The clear cylinder was compared to white PVC of the same diameter with no significant differences in attraction or contact shown. There was no impact of Assail mixed with grease on moth orientation to, or contact with, the A&K device. The most effective concentration of Assail was 4% (w/w) causing 100% mortality in 24 hours.

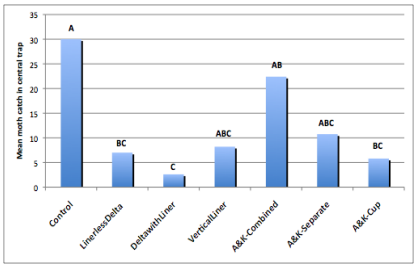

Fig. 10

Interpretation. There are two insecticides that quickly kill codling moth adults by contact. Exposure of moths to sublethal residues had a dramatic impact on their ability to orient to a pheromone plume. Different shapes of A&K devices, including the size of an opening, indicated that these were important in optimizing moth contact with a treated surface.

Field Trails - Cages. In MI several treatments were evaluated using the caged-tree design (see Field-cage studies). In a direct comparison of a male removal (delta trap with liner) and mating disruption (pheromone dispensers), male removal was shown to be 4 times more powerful than a full rate of pheromone dispensers in mate location. Several kinds of A&K devices showed promise, although the large delta trap with a liner proved superior (Fig. 10).

Interpretation. Field-cage trial results demonstrated that A&K was a more robust tactic than pheromone mating disruption when moth densities were controlled. The reason for the greater impact of A&K is because it reduces the number of visits per night to a pheromone source (female). A comparison of different types (deigns) of devices also support the potential for A&K as a robust codling moth management tool, but clearly shows that design has a great impact on efficacy.

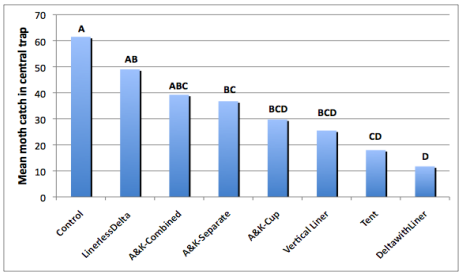

Fig. 11

Field Trials - small plots. In MI seven treatments, two experimental commercial formulations, two prototype A&K devices, a pheromone lure only, a large lined delta trap and untreated control were compared for male removal efficiency in replicated small plots (0.1 acre). Treatments were applied at a rate of 100 A&K devices/acre. There was a significant reduction in male captures in monitoring traps but only the large delta trap with liner showed a significant reduction compared to the lure only treatment (Fig. 11).

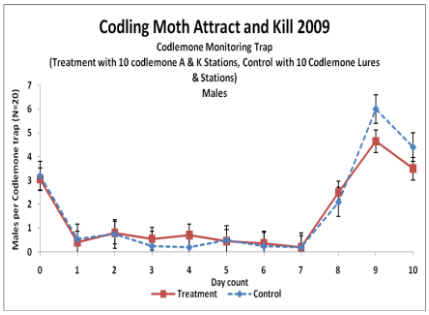

Fig. 13

A preliminary trial was conducted with A&K devices for the obliquebanded leafroller (OBLR) in MI in small (0.2 acre) replicated plots. All treatments reduced moth capture in a central monitoring trap but there were no differences between treatments. The observation that much lower captures of OBLR moths occurred in the center of the male removal treatment than on the edges suggested that larger plots are required to evaluate A&K treatments for this species.

In WA a PVC A&K device was evaluated in small plots at a rate of 10 or 20 per acre. The attractant used was either a 1 mg codlemone lure or an acetic acid/pear ester lure (AAPE). There was no difference in the number of moths in monitoring traps in treatment versus control plots (Fig. 13).

Interpretation. Moth contact of the A&K device is critical to its efficacy. Based on these results four important factors have been identified that can influence the efficacy of A&K technologies, density per area, shape, orifice size (if required) and attractant release rate. Video recordings provide some additional insights into factors that will be further evaluated in 2010.

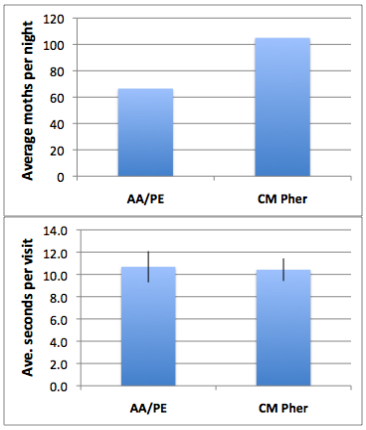

Fig. 14

Video Recording. Video monitoring of behavior around A&K devices was informative in identifying limitations of designs and the relative attraction of different lures. For example, a kairomone in an A&K device developed by the USDA-ARS was only half as attractive as the device baited with a pheromone lure (Fig. 14-top), however, the duration of visits was equal between the two attractants (Fig. 14-bottom). Contact with the A&K device was low, 3%, and was the same for both lure-types.

Video recordings to a private company's A&K device showed that there was no impact of attractant (pheromone) load rate on the number of visits but the duration increased slightly with load rate and was similar to a 0.1 mg pheromone lure. With this A&K device there was a consistent but low level of contact by moths, 10-25%.

Interpretation. Video monitoring was shown to be a powerful tool to demonstrate the relative attraction to different potential A&K devices and the actual level of contact with the source, which is

the most important factor. The lack of moth contact with the two devices tested in 2009 suggested that lure strength is important in enhancing contact with an A&K device. It also showed that the shape of the device is important in enhancing or limiting contact. Improved designs based on 2009 results and wind tunnel studies over the winter will be developed for field testing in 2010.

CONTINUING PROJECT REPORT **YEAR**: 1 of 2
WTFRC Project Number: CP-09-902

Project Title: Assay development to monitor insecticide resistance in codling moth

PI: Stephen F. Garczynski
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Total Project Request: Year 1: $24,150 **Year 2:** $24,960

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

Item	**2009**	**2010**	
Salaries	15,000	15,750	
Benefits	1150	1210	
Wages			
Benefits			
Equipment			
Supplies	8000	8000	
Travel			
Miscellaneous			
Total	$24,150	**$24,960**	

OBJECTIVES

The overall goal of this project is to develop methods to monitor insecticide resistance mechanisms in codling moth orchard populations. Successful completion of the objectives below will provide tools that can be used to guide orchardists in pesticide selection in the event of insecticide resistance.

1) Develop assays that determine the levels of enzymes that degrade pesticides. Pesticide degradation is the main cause of an insect's resistance to insecticides. Assays have been developed by other researchers that enable us to measure the activities of the different pesticide degrading enzymes. The goal of this objective is to develop and adapt these assays for use in determining the enzyme levels in all stages of codling moth.

2) Clone transcripts that encode known enzymes that confer insecticide resistance. Enzymes that degrade pesticides have been identified in other insects. Using the DNA and amino acids sequences of those enzymes, the homologous enzymes will be cloned from codling moth. The cloned codling moth DNA sequences will then be used as probes that can determine expression levels of the genes that encode detoxifying enzymes.

3) Clone transcripts that encode known targets of insecticides currently used in the orchard. A second form of insecticide resistance, although occurring less frequently than enzyme degradation, involves modification of the target proteins. Because most of these targets are known, DNA sequences encoding these proteins will be cloned from codling moth using information from homologous proteins identified in other insects. This will enable us to "build a library" of target genes that can later be used in assay development.

4) Develop assays to determine target mediated resistance. In the cases of target site resistance that have been previously reported, mutations in the DNA and amino acid sequences of the protein targets have been identified. When resistance is detected, and assuming it is not due to enzyme degradation, we can use our "target library" to identify the mutation in the insecticide resistant codling moth. We will develop PCR assays to make it easier to detect target mediated resistance in codling moth.

SIGNIFICANT FINDINGS (ACCOMPLISHMENTS)

- Optimized conditions for common assays used to measure enzymes involved in insecticide resistance.
- Used enzyme assays to determine initial differences between laboratory and field populations of codling moth.

METHODS

The methods for this project are fairly straightforward and have been used by many researchers who study insecticide resistance. Because the majority of researchers perform their assays on larvae, the challenge will be to develop these assays for use with codling moth eggs and adults. We routinely use microplate-based assays and PCR cloning in my laboratory so we possess the equipment and expertise necessary to complete this project.

1) Sample preparation: Insects for use in biochemical assays must be freshly killed for immediate use or frozen for future use. We will test the differences between freshly killed and frozen codling moth using insects obtained from the USDA-ARS insect rearing facility here in Wapato, WA.

2) Development of assays to determine levels of pesticide degrading enzymes. We will first try the standard assays developed by the Centers for Disease Control to determine degradation enzyme

levels for evaluating insecticide resistance in mosquitoes. Assays will be tested first on codling moth larvae to determine protein levels (which are used to correct for insect size differences), non-specific esterase levels (esterases mediate resistance to organophosphates, carbamates and pyrethroids), Mixed function oxidase levels (testing for altered activity of Cytochrome P450s, a broad spectrum detoxifying enzyme family), Glutathione-s-transferase levels (a class of enzymes that can mediate resistance to organophosphates, organochlorines and pyrethroids), and Insensitive acetylcholinesterase levels (which determines specifically if acetycholinesterase has been altered). We will compare the assays developed by the CDC with those reported elsewhere in the literature (Reyes et al., 2007 for example) to determine the assay best suited for the different stages of codling moth. The advantage of the CDC method is that they use equipment found in most laboratories, while Reyes et al (2007) method requires specialized equipment (which we have in my lab).

3) Cloning transcripts that encode known enzymes that confer insecticide resistance. We will be using standard cloning techniques using polymerase chain reaction (PCR) amplification with primers specifically designed against Cytochrome P450s (mixed function oxidases), Glutathione-s-Transferases, and non-specific esterases. We will also identify and clone genes encoding the enzymes above by identifying them in DNA libraries of genes expressed in codling moth neonate and fifth instar larvae and those from adult males and females are currently being sequenced by Dr. Amit Dhingra at Washington State University. When the sequences become available, we will determine the identity of these gene transcripts by homology with those already identified in other insect species. Once we identify potential targets, they will be cloned and analyzed for function using the microplate assays above.

4) Clone transcripts that encode known targets of insecticides currently used in the orchard. As above, we will be using standard cloning techniques using polymerase chain reaction (PCR) amplification with primers specifically designed against known targets of insecticides currently used in the orchard. We will also identify and clone genes encoding the enzymes above by identifying them in DNA libraries of genes expressed in codling moth neonate and fifth instar larvae and those from adult males and females are currently being sequenced by Dr. Amit Dhingra at Washington State University. When the sequences become available, we will determine the identity of these gene transcripts by homology with those already identified in other insect species. Targets to identify (insecticide class in parentheses) and include in our "library" are Acetylcholinesterase (organophosphates), Sodium channel (pyrethroids), Nicotinic acetylcholine receptor (neonicotinoids and spinosyns), Chloride channel (avermectins), Voltage dependent sodium channel (Indoxacarb) and Ryanodine receptor (Diamides).

RESULTS AND DISCUSSION

There are two broad mechanisms by which insect pests develop resistance to insecticides. They may produce large amounts of detoxifying enzymes which either break down the insecticide molecule or bind to it so tightly that it cannot function (a process known as sequestration). The second mechanism, and much less frequent, involves mutation of the insecticide target site, such as the acetylcholinesterase enzyme in the nervous system. This effectively blocks the action of the insecticide. Both types of mechanism have been studied in various species of insect.

Detoxification enzymes are a natural part of the insect defense system against foreign agents, such as toxic plant compounds. These enzymes also function to inactivate insecticides. There are three main classes of detoxification enzymes; cytochrome P450 monooxygenases (P450s), esterases, and glutathione-*S*-transferases (GST). P450s have broad substrate specificities so this class of enzyme can mediate resistance to all classes of insecticides. This broad substrate specificity and the fact that 600 genes encoding P450s have been identified in insects makes this family of enzymes a major contributor to insecticide resistance. Glutathion-*S*-transferases play a role in the defense by

attaching a glutathione molecule to a foreign molecule, an insecticide for example. Once the glutathione is attached, the foreign molecule with glutathione is sequestered by the insect, making it unable for the insecticide to reach its target site. Esterases are the third important group of detoxification enzymes. An esterase is an enzyme that splits ester bonds into an acid and an alcohol in a chemical reaction with water. Esterases have been well documented for their role in insecticide resistance either by a mutation in the enzyme that causes it to bind tightly to organophosphates or by over expression of the gene which is responsible for detoxification of carbamates and pyrethroids.

Enzyme assays to determine esterase, GST, and P450 levels in codling moth males and females were developed using the insects from lab colony at YARL. Once the assays were optimized, enzyme levels were determined for 30 – 50 individuals. Enzyme activity levels for males and females are listed in Table 1. Enzyme levels were different in males and females. This sex specific difference indicates the importance in treating males and females separately when determining a baseline level of enzyme activity. Determination of the baseline enzyme levels for the moths from lab colony gave us the ability to compare those to field collected insects. Dr. Alan Knight provided me with field collected codling moth, and presumably organophosphate and neonicotinyl resistant, from a highly sprayed orchard (LatA). Significant increases in esterase and GST enzyme activity was observed in the field collected insects (Table 1). The results of the individual enzyme assays are also presented in Figures 1, 2, and 3 located on the following pages. This initial study shows the utility of the enzyme assays developed this year. Dr. Knight and I are in the process of testing more insects and we hope that the assays will allow us to determine resistance using this procedure.

Table 1. Enzyme Activities for Laboratory Reared and Field Collected Adult Codling Moth

	Lab male	***Lab female***	***Lab M+F***	***Field male***	***Field female***	***Field M+F***
Esterase	612 $\pm$ 111	435 $\pm$ 137	525 $\pm$ 152	826 $\pm$ 424	852 $\pm$ 253	835 $\pm$ 369
P450s	12.2 $\pm$ 3.9	15.2 $\pm$ 3.1	13.7 $\pm$ 3.8	15.4 $\pm$ 6.3	6.6 $\pm$ 3.4	11.9 $\pm$ 7.2
GST	12.2 $\pm$ 5.1	8.7 $\pm$ 3.7	10.3 $\pm$ 4.8	31.9 $\pm$ 14.3	31.5 $\pm$ 8.0	31.8 $\pm$ 12.2

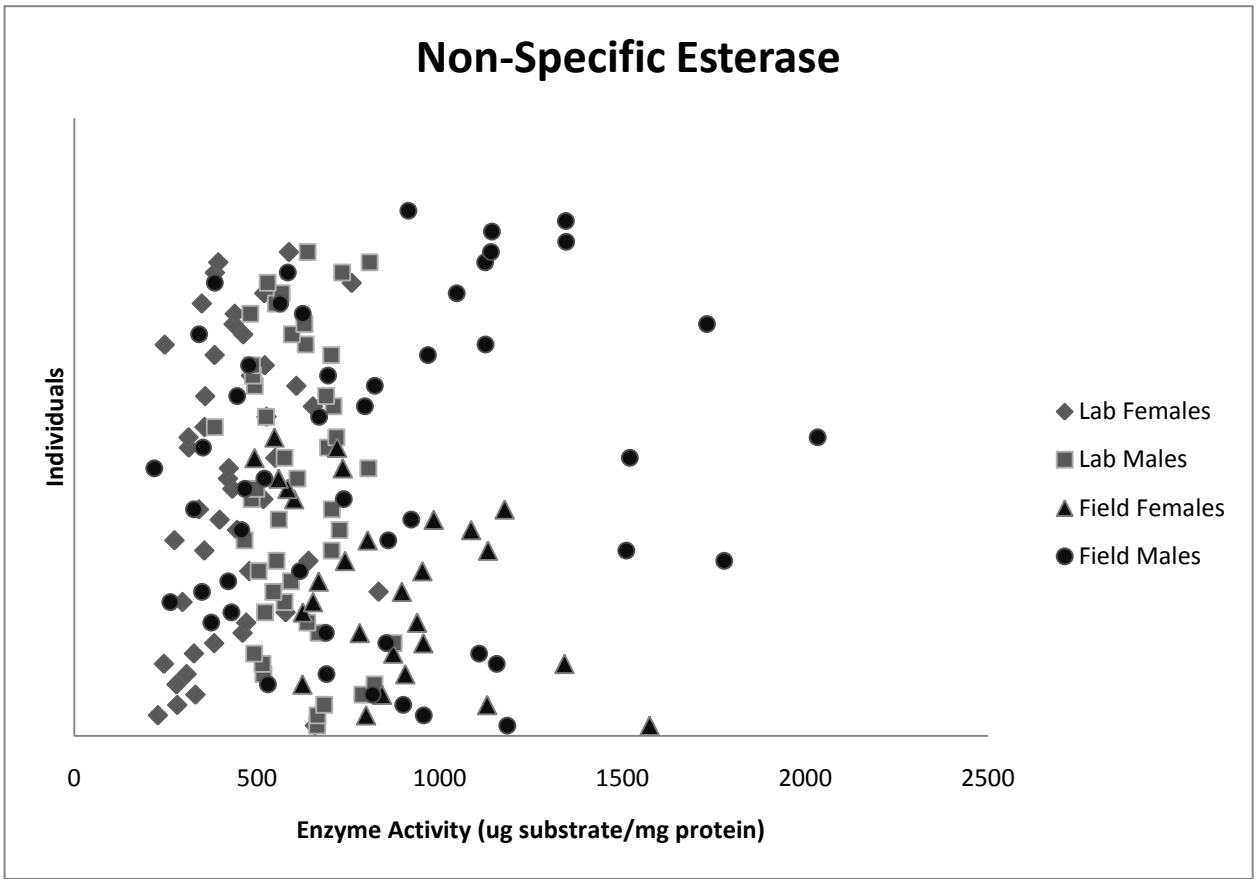

Figure 1. Graphical representation of esterase activity in individual codling moth adults from lab and field collected codling moth adults. Esterase levels for lab males and females (boxes and diamonds) cluster to the left of the graph (lower activity), while field males and females (circles and triangles) are skewed to the right of the graph (higher activity). To determine if this trend is significant, we will be testing hundreds of more insects in the upcoming year.

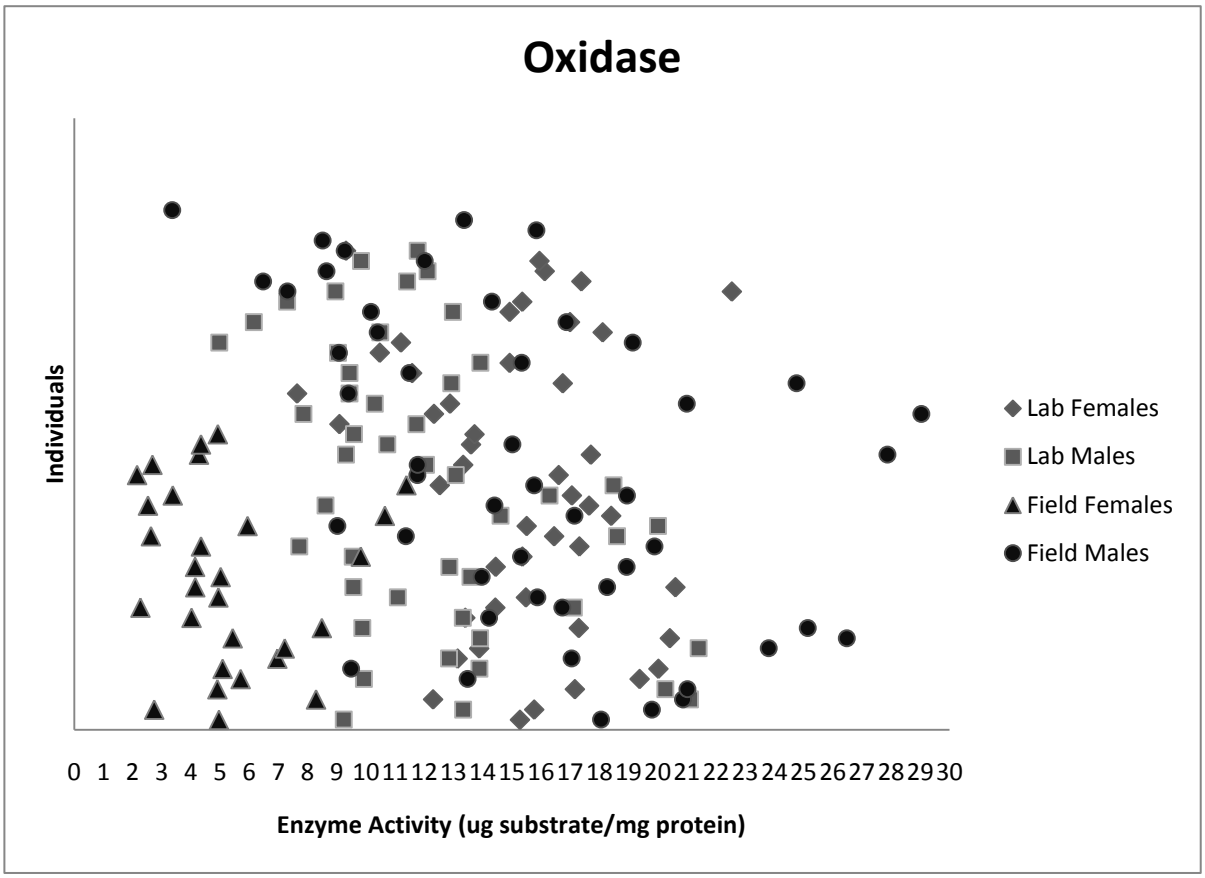

Figure 2. Graphical representation of oxidase (P450) activity in individual codling moth adults from lab and field collected codling moth adults. Oxidase levels for lab males and females (boxes and diamonds) and field collected males (circles) are equally distributed, while those for field collected females(triangles) are skewed to the left of the graph (lower activity). To determine if this trend is significant, we will be testing hundreds of more insects in the upcoming year.

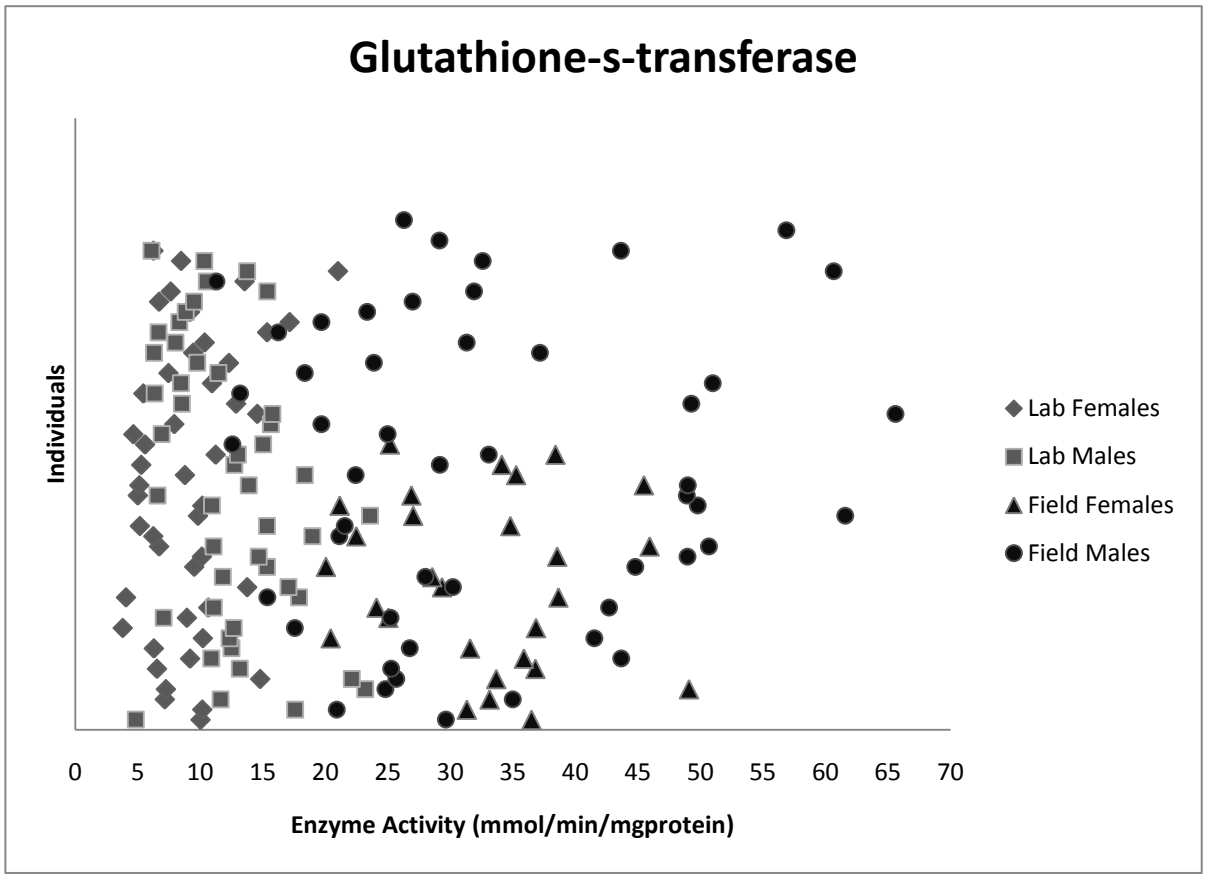

Figure 3. Graphical representation of glutathione-*S*-transferase activity in individual codling moth adults from lab and field collected codling moth adults. Glutathione-*S*-transferase levels for lab males and females (boxes and diamonds) cluster to the left of the graph (lower activity), while field males and females (circles and triangles) are skewed to the right of the graph (higher activity). To determine if this trend is significant, we will be testing hundreds of more insects in the upcoming year.

CONTINUING PROJECT REPORT **YEAR**: 1 of 3
WTFRC Project Number: CP-09-903A and CP-09-903B

Project Title: Identification of critical physiological targets in codling moth

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Cooperators: Dr. Amit Dhingra, WSU; Dr. Kevin Clark, University of Georgia; Dr. Kevin Wanner, Montana State University

Total Project Request: **Year 1:** $47,785 **Year 2:** $49,000 **Year 3:** $50,400

Other funding sources

Agency Name: USDA-CSREES National Research Initiative
Amount awarded: $365,042 (2008-2011)
Notes: This grant was awarded based on results generated from my 2006-2007 WTFRC funded research to identify codling moth chemosensory receptors.

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

Item	2009	2010	2011
Salaries[1]	14,000	15,000	16,000
Benefits	1019	1024	1204
Wages			
Benefits			
Equipment			
Supplies	12,000	12,000	12,000
Travel			
Miscellaneous [2]	5000	5000	5000
Total	$32,019	**$33,024**	$34,204

Footnotes:
[1]Salary will be used to support Ms. Jennifer Stout, a part time GS-7 Technician (½ year)
[2]The miscellaneous funds requested are to help defray the cost of equipment maintenance which includes autoclaves, yearly Laminar Flow hood (biosafety cabinet) certification and Pipette calibration, repair and certification.

Budget 2
Organization Name: Wash. State Univ. **Contract Administrator:** ML. Bricker / Barb Smith
Telephone: (509) 335-5504 **Email address:** mdesros@wsu.edu / niehoff@wsu.edu

Item	2009	2010	2011
Salaries			
Benefits			
Wages	4800	4992	5192
Benefits	466	484	504
Equipment			
Supplies	10,000	10,000	10,000
Travel	500	500	500
Miscellaneous			
Total	$15,766	**$15,976**	$16,196

Footnote:
Wages are requested to support an undergraduate hourly employee. Travel funds are requested for travel between Wapato and WSU Pullman. Supplies are standard molecular biology reagents, disposables, and sequencing costs needed to conduct the research.

OBJECTIVES:

The goal of this project is to provide fundamental biological information on codling moth physiology that can be used to identify new targets for enhanced pest control. Identification of critical physiological targets in codling moth will provide information and methods that will allow us and other researchers to develop new strategies and tools for control of this major pest of apple.

1) Characterize pheromone biosynthesis activating neuropeptide (PBAN) and its receptor (PBANR) in late 5th instar larvae and adult females. Pheromone biosynthesis activating neuropeptide (PBAN) is a polypeptide (short protein) hormone that stimulates the production of pheromones in insects by interacting with its receptor (PBANR). Both PBAN and PBANR are potential protein targets that if blocked could inhibit pheromone biosynthesis (including codlemone), thus having great potential to enhance the effectiveness of mating disruption for codling moth control.

2) Characterize diapause hormone (DH) and its receptor (DHR) in eggs, neonate and 5th instar larvae. Diapause hormone (DH) is encoded by the same gene as PBAN. DH, along with other physiological factors, in some moth species regulates diapause through signals sent by its receptor (DHR). Both DH and DHR are potential targets that if altered may disrupt the codling moth's ability to enter or leave diapause, and allow researchers to take advantage of this physiological pathway for codling moth control.

3) Identify potential targets in eggs and neonate larvae by analyzing the transcriptome, and determine those that may be critical for insect survival. Because of the codling moth's life cycle, eggs and neonate larvae are accessible to control measures in the orchard. Sequencing the transcriptome (a compilation of genes that are being actively expressed) of eggs and neonate larvae will allow us to identify potentially critical protein targets in these codling moth life stages. After identification, we will characterize potential protein targets to gain a further understanding of the basic physiology of eggs and neonate larvae and assess their usefulness for codling moth control.

4) Identify potential targets in adult males and females by analyzing the transcriptome, and determine those that may be critical for insect survival. Adult males and females are also accessible to control measures in the orchard. Sequencing transcriptomes made from chemosensory organs (mouthparts, antennae, and legs) will allow us to identify smell and taste receptors expressed in males and females. Further characterization of these receptors and their signal transduction pathways will help us to gain a further understanding of physiology as it is related to host and mate finding. Proteins important in these physiological pathways are potential targets for enhanced insect control measures.

SIGNIFICANT FINDINGS (ACCOMPLISHMENTS):

- A gene transcript encoding the codling moth PBAN receptor has been cloned.
- A gene transcript encoding the codling moth Insulin receptor has been cloned.
- A gene transcript encoding the codling moth short neuropeptide F receptor has been cloned.
- Transcriptomes from codling moth eggs, neonate larvae, and adult male and female chemosensory organs have been made.
- Full length transcripts of heat shock proteins (HSP) have been cloned and the expression profiles for the small heat shock proteins in various codling moth stages have been determined.

METHODS

1) Characterization of Pheromone Biosynthesis Activating Neuropeptide Receptor in Codling Moth
   a) PBANR will be expressed in a mammalian cell line using standard protocols.
   b) PBANR identity will be confirmed by monitoring second messenger formation in response to exogenously added PBAN.
   c) Initial experiments to "knock-out" the PBANR in female codling moths will be performed using RNA interference (RNAi) techniques.

2) Cloning and Characterization of the Diapause Hormone Receptor in Codling Moth
   a) DHR will be cloned using a degenerate primer based technique (identical to that which was used to clone PBANR).
   b) Expression patterns of DHR transcript will be determined to identify the codling moth stage(s) that use this hormone/receptor signal.

3) Identify potential targets in eggs, neonate larvae and adults by analyzing the transcriptome, and determine those that may be critical for insect survival.
   a) cDNA sequences were determined and assembled by Dr. Amit Dhingra (WSU).
   b) The final assembled sequences for each life stage will be annotated and targets of interest will be determined using bioinformatic approaches.
   c) Targets of interest will be cloned and characterized using appropriate techniques.

RESULTS AND DISCUSSION

The overall goal of this project is to identify and characterize targets in the codling moth that can potentially be used in the control of this insect pest. The main targets are proteins important in pathways that regulate critical physiological functions in the insect. Four physiological systems, endocrine (hormones), chemosensory (smell and taste), reproductive (egg formation and development), and digestive, are being examined in this study. In the past year, several gene transcripts encoding proteins involved in the above physiological pathways have been identified in the codling moth.

The Endocrine System

Neuropeptides and peptide hormones regulate most every physiological system in animals. In humans, deficiencies in the endocrine system are the causes of many diseases, and receptors for neuropeptides and peptide hormones are targets of many drug discovery efforts. For the codling moth, and other insect pests, disruption of the endocrine system as a target for insect control has been successful, leading to the development of the juvenile hormone and ecdysone mimics currently used in orchards today. In the past year, several neuropeptide and peptide hormone receptors have been cloned from the codling moth. These cloned receptors are thought to be involved in pheromone biosynthesis, regulation of feeding and digestion, regulation of growth and development, and regulation of egg maturation. Further characterization of these receptors should enable us to target these for future codling moth control methods or products.

Pheromone Biosynthesis

The production and regulation of pheromone biosynthesis in the codling moth is under hormonal control. The neuropeptide hormone system responsible for the regulation of pheromone biosynthesis is PBAN (Pheromone Biosynthesis Activating Neuropeptide) and its receptor (PBANR). PBAN is produced by a patch of nerves near the insect brain and is released into the hemolymph (insect blood) where it circulates until it interacts with its receptor. The interaction of PBAN with its receptor activates a signal transduction cascade that stimulates pheromone production. In the adult

female codling moth sex pheromone production is regulated by the interaction of PBAN with its receptor, which is located in the sex pheromone gland in the female abdomen. An understanding of this target system may lead to the ability to control sex pheromone production in codling moth females, or to provide information that will help others to increase the effectiveness of mating disruption in the orchard.

In the past year, gene transcripts encoding PBAN and PBANR have been cloned from codling moth females and identified based on homology of the determined DNA sequence compared to those previously reported in other moths. Over a 215 amino acid segment, the codling moth PBANR is 88% identical to that from *Manduca sexta*, a moth species whose PBANR has been previously characterized (refer to Figure 1). Now that the codling moth PBAN receptor is cloned, future work will allow for its functional characterization.

In the next two years, several lines of investigation will be undertaken to characterize the codling moth PBANR. First, expression patterns of PBAN and PBANR in different codling moth life stages will be determined. This information will provide key information as to the timing of the application of potential products targeting this neuropeptide hormone system. Second, a full length clone will be expressed in a mammalian cell line. This will provide a cell based system that can be used to show that the receptor is for PBAN, and will also provide a tool that can be used to screen for compounds that may be useful to block pheromone biosynthesis.

Reproduction and Egg Development

As with pheromone biosynthesis, development, digestion and reproduction are regulated by the endocrine system. The insulin signaling pathway is critical for regulating egg and larval development, and reproduction (egg maturation) in adult females. In the past year, a gene transcript encoding the insulin receptor has been cloned from codling moth and identified based on homology to those previously reported for other moth species. Over a 169 amino acid segment, the codling moth insulin receptor is 92% identical to previously identified from *Manduca sexta* (refer to Figure 2). In the next two years, lines of investigation will include cloning the full length Insulin receptor gene transcript, and characterizing its expression patterns during egg and larval developmental stages.

Transcriptome Generation and Analysis

The overall objective of this portion of the target ID project is to generate codling moth transcriptomes from various codling moth life stages (egg, neonates, 3rd and 5th instar larvae, and adult male and female chemosensory organs). A transcriptome is the set of all RNA molecules produced in one or a population of cells, or in an entire organism. Unlike the genome, the transcriptome can vary with external environmental conditions or with life stage. Because a transcriptome includes all mRNA transcripts in the cell or organism, the transcriptome reflects the genes that are being actively expressed at any given time. Completion of this objective will provide information that will enhance ongoing projects to identify novel protein targets for biorational design of tools and methods for codling moth control.

RNA was extracted from various codling moth life stages, and converted to double-stranded cDNA. The resultant cDNA pools were amplified using SMART primer technology. Unlike Sanger sequencing, the amplified cDNA was not cloned to prepare a library but sheared to be sequenced using Roche/454 pyrosequencing technology (performed by Dr. Amit Dhingra, WSU). After the cDNA sequences were determined, they were assembled, extraneous amplification primer sequence removed, and the resultant sequences converted to FASTA files. The final assembled cDNA sequences for each life stage are now available for annotation, which will be completed this year, and the annotated sequences will be used in this and other ongoing projects.

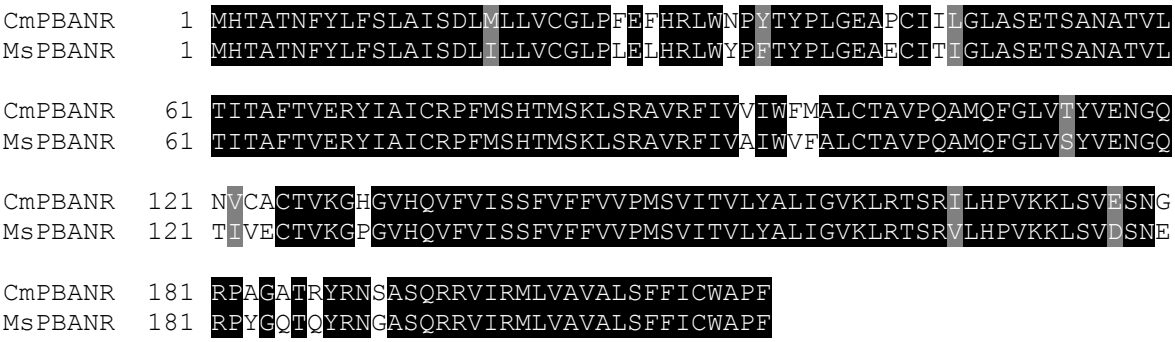

Figure 1. Boxshade of Clustal alignment of deduced amino acid sequence of Codling Moth Pheromone Biosynthesis Activating Neuropeptide Receptor (PBANR) with that previously identified in *Manduca sexta*. Black background indicates the amino acids at that position are identical between the two insects; grey background indicates the amino acids at that position are conserved.

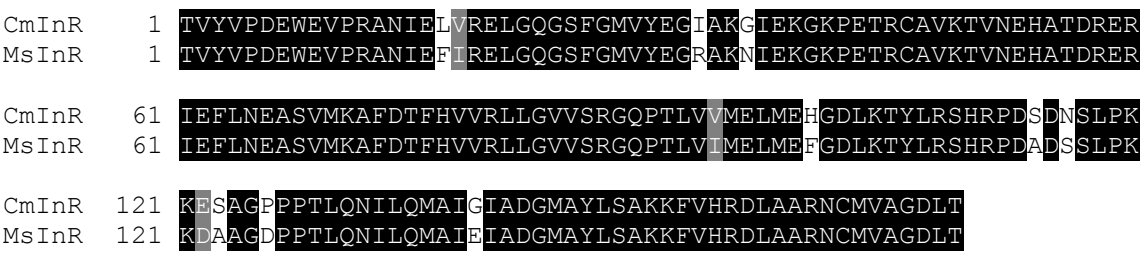

Figure 2. Boxshade of Clustal alignment of deduced amino acid sequence of Codling Moth Insulin Receptor (InR) with that previously identified in *Manduca sexta*. Black background indicates the amino acids at that position are identical between the two insects; grey background indicates the amino acids at that position are conserved.

FINAL PROJECT REPORT

Project Title: DNA and morphometric diagnostics for apple and snowberry maggot flies

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City: Wapato
State/Province/Zip WA/98951

Cooperators: David Sheets, professor, Dept. Physics, Canisius College, Buffalo, New York

Total Project Request: **Year 1:** $15,000

Other funding sources: None

Total Project Funding: $15,000

Budget History

Item	**2009**		
Salaries	0		
Benefits	0		
Wages	13,600		
Benefits	1,400		
Equipment	0		
Supplies	0		
Travel	0		
Miscellaneous	0		
Total	**$15,000**		

RECAP ORIGINAL OBJECTIVES

Objectives:
1. Increase sample sizes for diagnostics using wing shape for apple and snowberry maggot flies.
2. Analyze ovipositor shape and male clasper shape to separate apple from snowberry maggot flies.

SIGNIFICANT FINDINGS

- By increasing the sample size, use of wing shape in an assignments test identified female apple maggot with 98.5% and female snowberry maggot with 99.0% accuracy,
- It correctly identified 100% of flies whose identities were questionable based on ovipositor lengths.
- Using canonical variates analysis (CVA), an assignments test using a CVA-distance based classification method, and multivariate analysis of variance, we found that clasper shape of flies from multiple sites accurately classified 99.8% of males to species.
- We found that ovipositor shape accurately classified 85.3% of females to species.
- Ovipositor length was longer in apple maggot than snowberry maggot, and combining ovipositor length with shape increased classification accuracy to 94.5%.
- Combining clasper or ovipositor shape and wing shape along with size of other structures in a single analysis should result in nearly 100% discrimination between species. This may benefit regulatory agencies and apple growers that depend on accurate identifications for fly quarantine and management measures.

RESULTS AND DISCUSSION

Wing Shapes of Female Flies. The wing of female apple maggot is more tapered at the tip than that of female snowberry maggot, giving it an overall narrower appearance than the wing of snowberry maggot, which is broader at the terminus (Figs. 1A and 1B). Canonical variates analysis (CVA) separated the wing shapes of six populations of female apple maggot and the two populations of snowberry maggot, although there was overlap between the two species (Fig. 2); the overlap was due to a few central WA apple maggots from black hawthorn (C Wash pom black haw).

There were misclassifications among conspecific populations of apple maggot, and some incorrect conspecific assignments between the two snowberry maggot populations. However, overall, 98.5% of female apple maggot and 99.0% of snowberry maggot were correctly identified to species. In addition, 100% of 18 'unknown' female apple maggots that fell in the ovipositor overlap zone were correctly identified as apple maggot using wing shape. They were assigned to five groups of apple maggot, but not to the C Wash pom black haw group. The one 'unknown' female snowberry maggot was also correctly identified using wing shape.

Wing Shapes of Male Flies. Bookstein coordinates indicate that the wing of male apple maggot is more tapered near the tip of the wing and is narrower than that of male snowberry maggot (not shown). As with females, CVA separated the wing shapes of the six populations of male apple maggot and the two populations of male snowberry maggot, and a few flies from the C Wash pom black haw group again fell between the two major species clusters (not shown). There were misclassifications within species, but 98.8% of apple maggot and 96.4% of snowberry maggot were correctly identified to species. We show for the first time that wing shape is a good character to discriminate between apple maggot and snowberry maggot in Washington state, even when the problematic central Washington black hawthorn flies are included.

Clasper Shape. The clasper configurations of apple maggot and snowberry maggot flies differed (Fig. 3). However, while the configuration in apple maggot is parallel, the configuration in snowberry maggot can be either divergent or parallel (Fig. 3B and 3C). Clasper shape of the species provided a more accurate reflection of species differences (Fig. 4). The clasper of apple maggot, as viewed from the side, is shovel or scoop shaped, with the upper curve slightly concave or flat and the bottom curve convex. The clasper of snowberry maggot is flipper or fin shaped, with upper curve distinctly sloping at the apex (SLMs 39–43 and LM 2) and the bottom curve only slightly convex

(Fig. 4). The CVA axes plot (Fig. 5) show little separation within species, but strong separation between them. Overall correct assignments at the species level were very high (Table 1). Addition of two measures of size, centroid size (CS) and length, did not appreciably improvement species discrimination. In sum, the results indicate that we obtained very good discrimination between species using clasper shape, and no statistically significant differences between populations of the same species.

Ovipositor Shape. The ovipositor shapes differ between species, as the ovipositor of apple maggot is proportionately more slender than that of snowberry maggot (Fig. 6). However, there was overlap in ovipositor shapes, as shown in the CVA plot (Fig. 7). Overall correct assignment to species was 85.3% using shape alone.

Ovipositor Length and Shape Combined. Apple maggot flies had longer ovipositors than snowberry maggot flies. When ovipositor length and shape were included in an assignments test, better separation was obtained than when shape alone was used (Table 2). Use of CS was less effective than use of length when combined with shape (Table 2).

Our findings are important for the Washington apple industry because they indicate that use of wing shape provides a significant improvement over the use of ovipositor length alone for identifying female apple maggot and snowberry maggot, thus potentially reducing misidentifications. Ovipositor shape is different between species, but there is overlap and it appears to be less useful than ovipositor length alone.

Our findings also show that wing shape and clasper shapes are different in males, and nearly 100% of individuals can be classified based on them. Clasper shape is more reliable than clasper configuration for species identification.

In practice, when ovipositor lengths and clasper configurations are ambiguous, wing or clasper shape analysis should be used, followed by use of multiple body measurements if ambiguity remains. A wing and clasper shape data base from known flies can be maintained by a regulatory agency such as WSDA and used to classify problematic flies (our data can be provided upon request). Morphometric programs for analysis and classification of unknowns used in this study are available online (http://www.canisius.edu/~sheets/ morphsoft.html). With training, it takes <30 min to photograph a wing or clasper of a fly, digitize it, subject it to analysis, and classify the fly. One caveat for using the method is that apple maggot in central WA, at least in our sample sites in wild areas from black hawthorn, may not be identified as accurately as flies from western WA using our current data.

In this project, shapes of different structures were analyzed separately. The possibility of combining wing, ovipositor, and clasper shape along with body measurements to discriminate 100% of flies should be investigated. If all flies are accurately identified, apple orchards would never mistakenly be placed under threatened or quarantine status and unmanaged apple or hawthorn trees would never need to be treated unnecessarily for apple maggot flies.

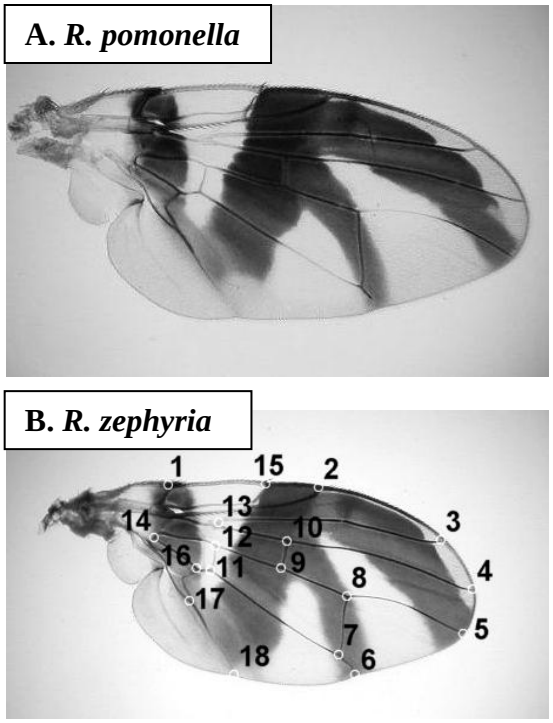

Fig. 1. Wings of (A) apple maggot and (B) snowberry maggot (with landmarks shown).

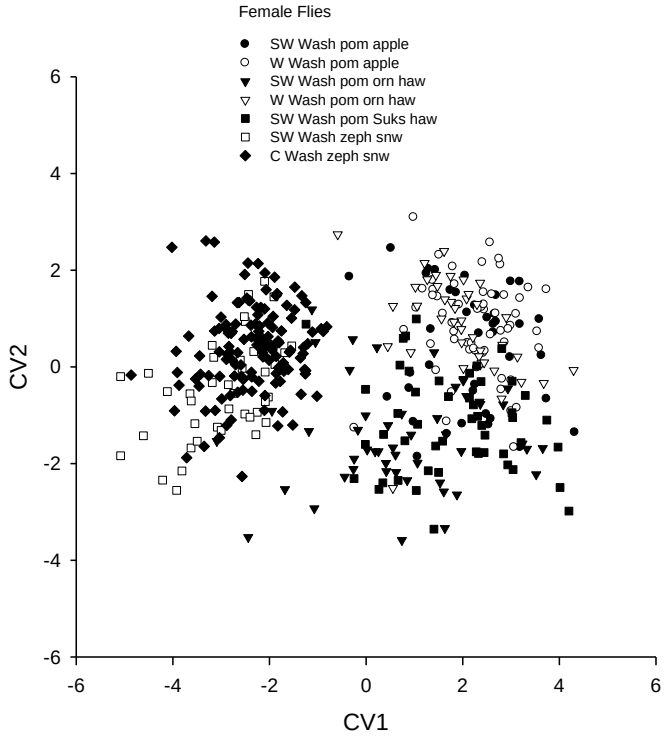

Fig. 2. Scatter plot from CVA of female apple maggot and snowberry maggot wings.

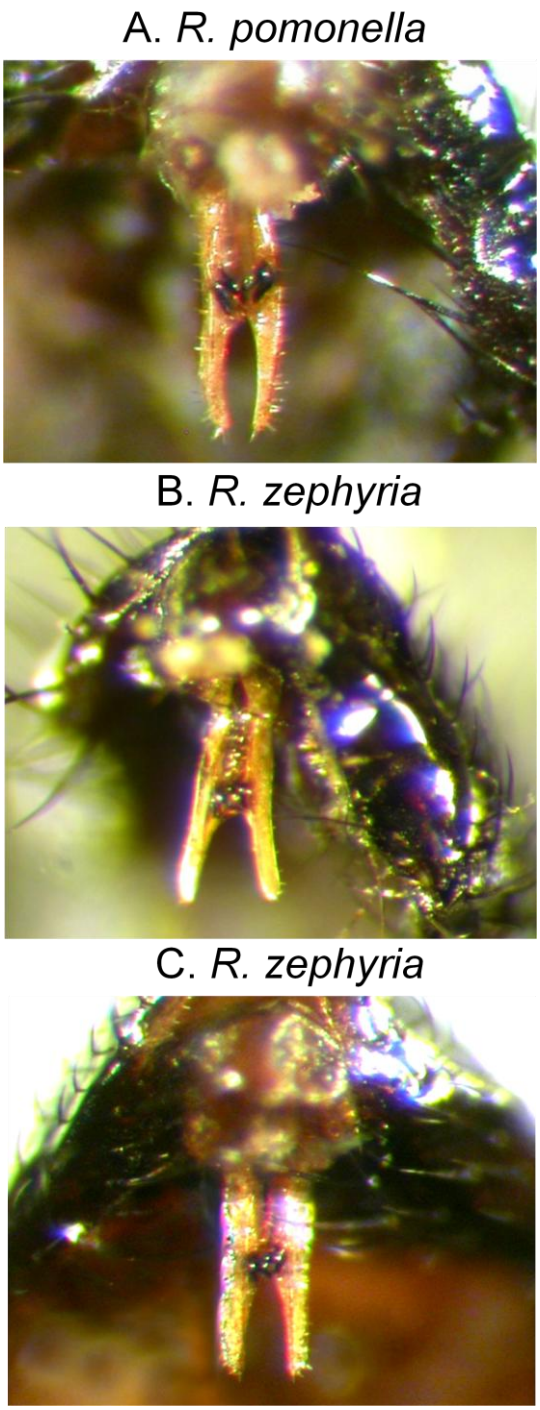

Fig. 3. Clasper configurations of (A) apple maggot and (B, C) snowberry maggot, showing variations in divergence.

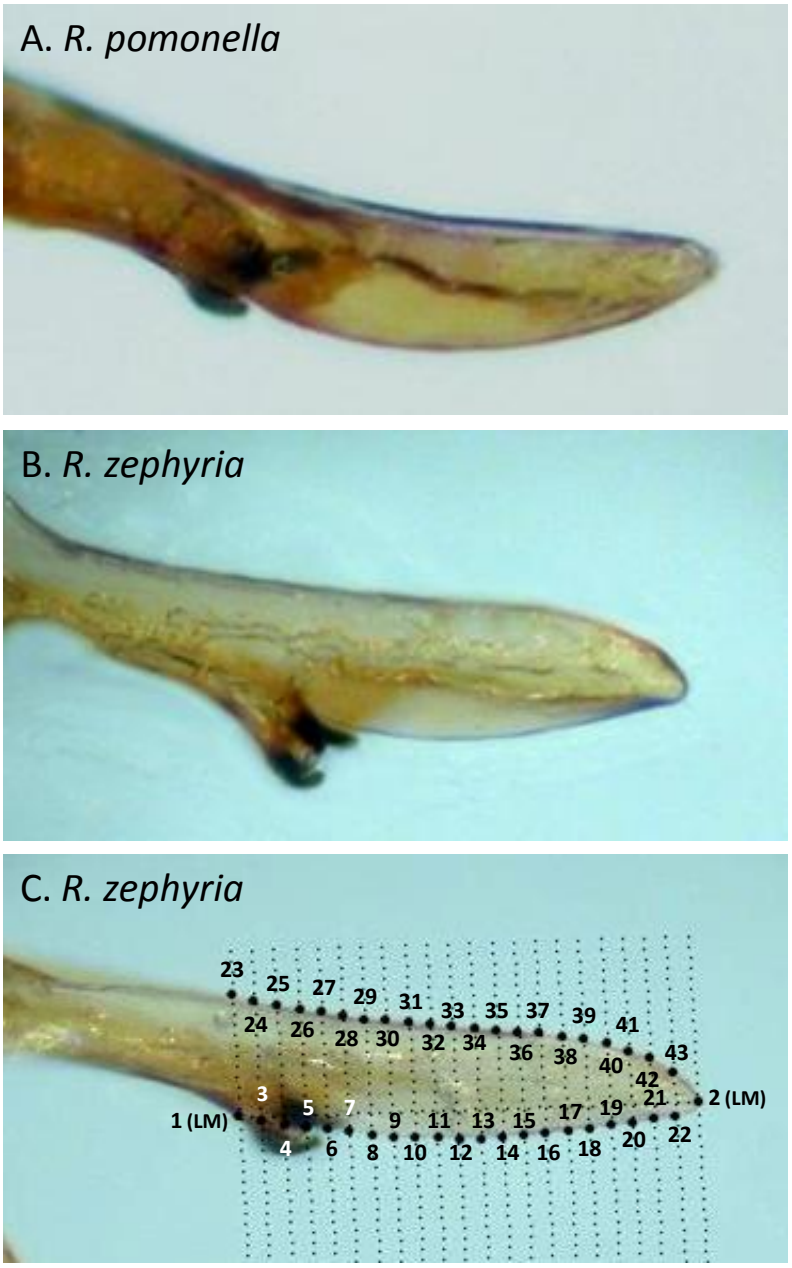

Fig. 4. Claspers of (A) apple maggot, (B) snowberry maggot, and (C) snowberry maggot showing landmarks and semi-landmarks.

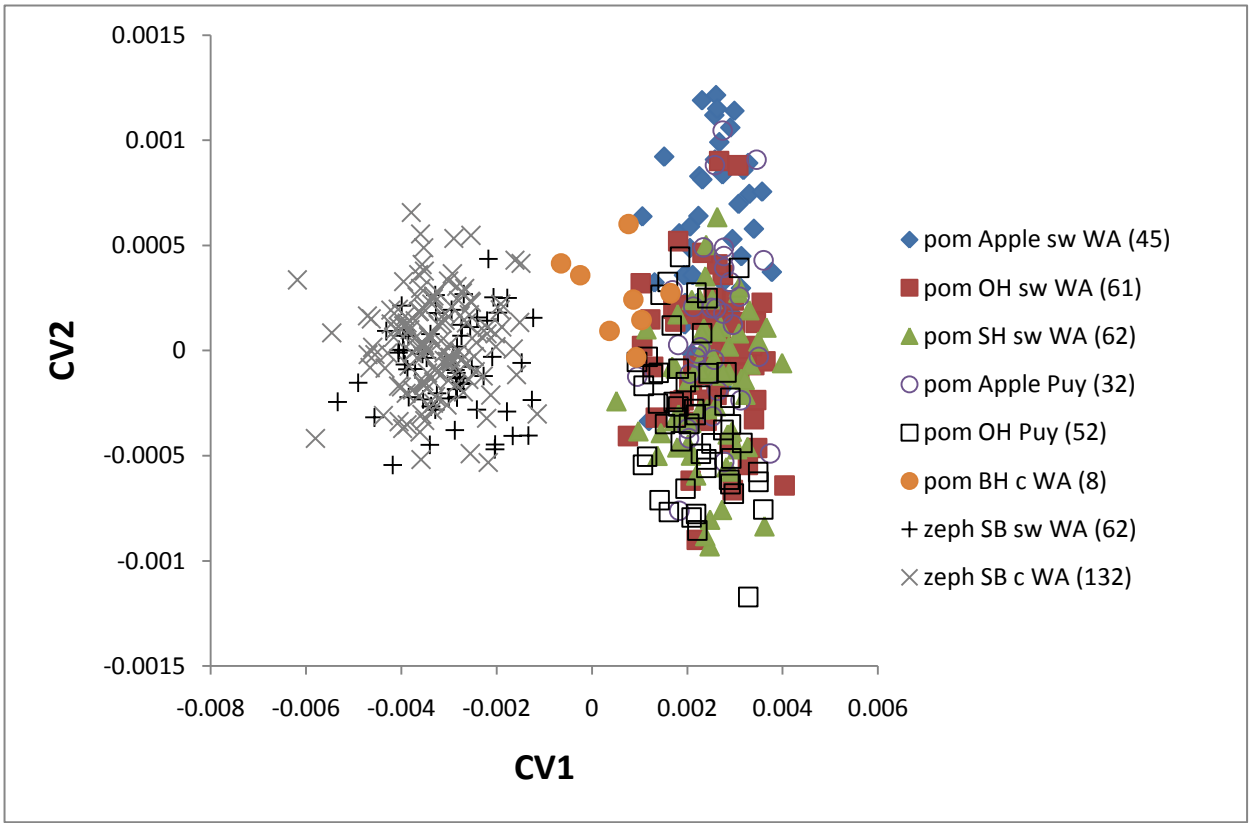

Fig. 5. Scatter plot from CVA of clasper shape of apple (pom) and snowberry maggot (zeph).

Table 1. Jackknifed groupings of 260 *R. pomonella* and 194 *R. zephyria* using surstylus shape and size from CVA-distance based method for assigning specimens to groups

Assignment Method	% correct, in 8 groups	% pom correct	% zeph correct	% species level correct
8 groups, shape only	41.2	100.0	95.9	98.2
2 groups, shape only	------	99.6	100.0	99.8
8 groups, shape + CS	40.3	100.0	96.9	98.7
2 groups, shape + CS	-----	99.6	100.0	99.8
8 groups, shape + length	40.3	100.0	96.9	98.7
2 groups, shape + length	-----	99.6	100.0	99.8

pom, *R. pomonella*; zeph, *R. zephyria*. CS, centroid size. 8 groups: six *R. pomonella* and two *R. zephyria*; 2 groups: all *R. pomonella* and all *R. zephyria*.

Table 2. Jackknifed groupings of 197 *R. pomonella* and 150 *R. zephyria* using aculeus shape and size from CVA-distance based method for assigning specimens to groups

Assignment Method	% correct, in 8 groups	% pom correct	% zeph correct	% species level correct
8 groups, shape only	37.2	90.4	78.7	85.3
2 groups, shape only	------	61.4	89.3	73.5
8 groups, shape + CS	43.2	86.3	94.7	89.9
2 groups, shape + CS	-----	71.1	97.3	82.4
8 groups, shape + length	43.2	94.4	94.7	94.5
2 groups, shape + length	-----	71.1	97.3	84.4

pom, *R. pomonella*; zeph, *R. zephyria*. CS, centroid size.
8 groups: six *R. pomonella* and two *R. zephyria*; 2 groups: all *R. pomonella* and all *R. zephyria*.

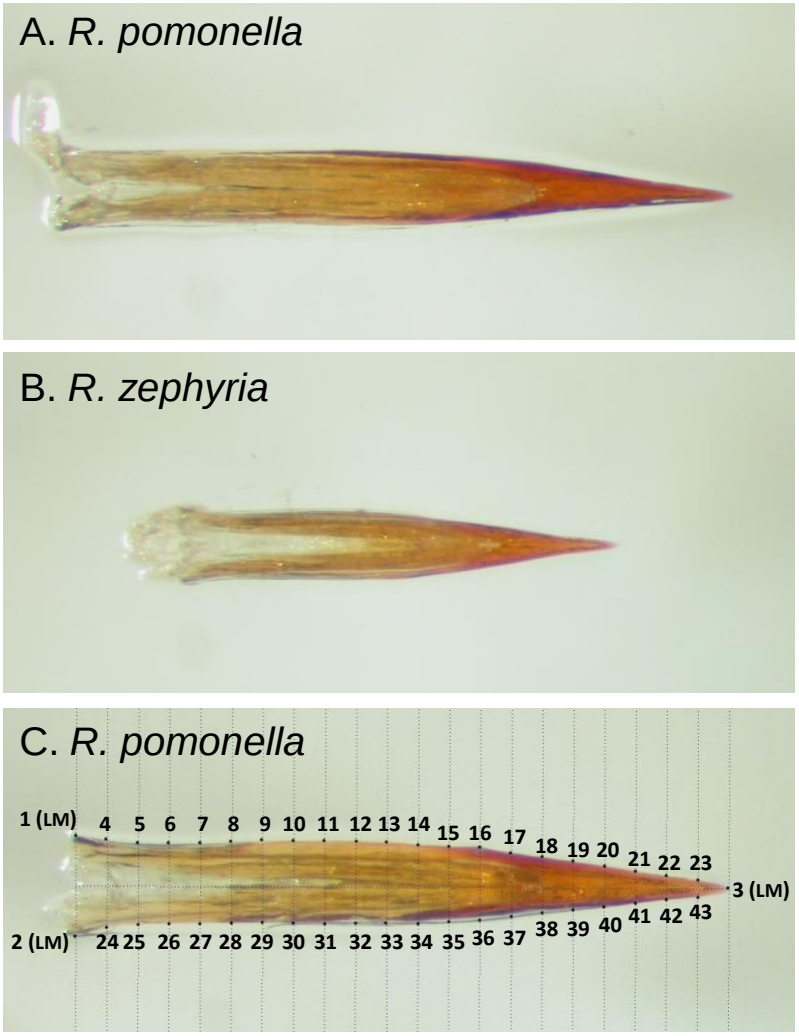

Fig. 6. Ovipositor of (A) apple maggot, (B) snowberry maggot, and (C) apple maggot, showing landmarks and semi-landmarks.

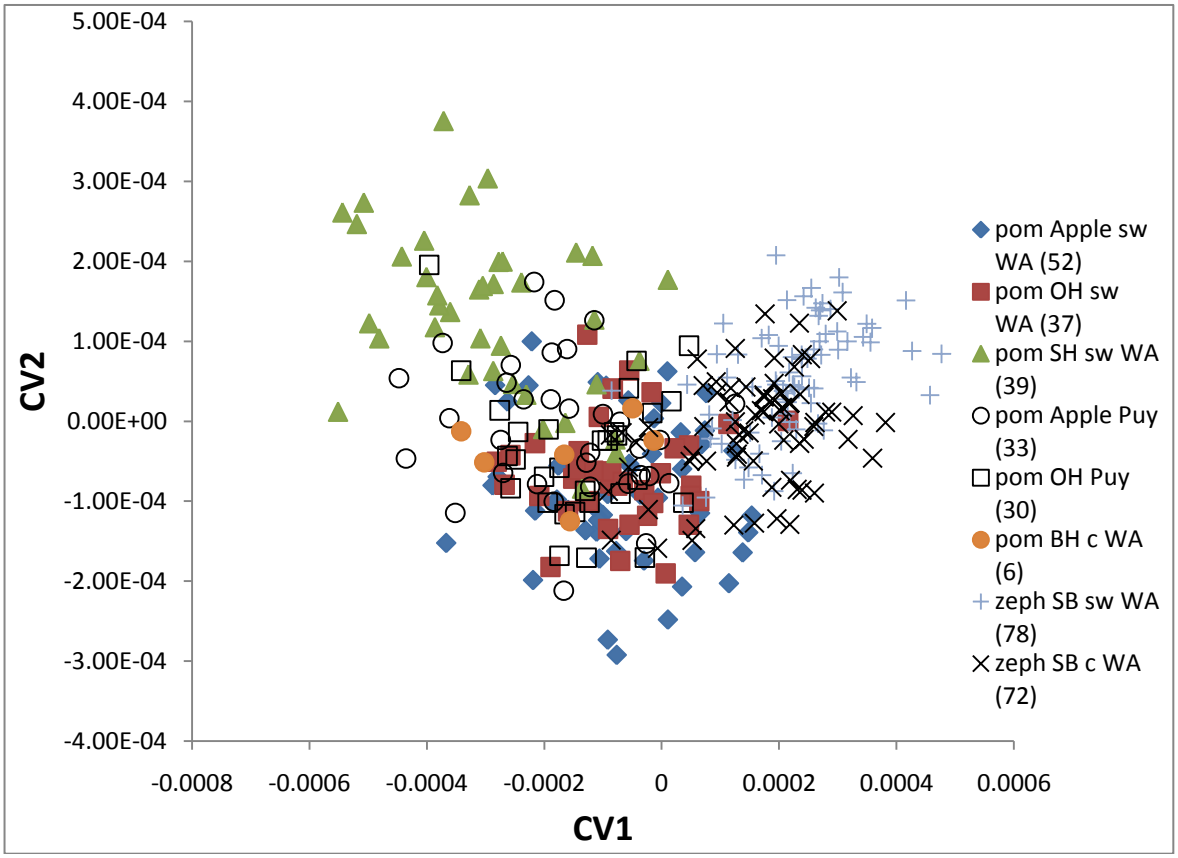

Fig. 7. Scatter plot from CVA of ovipositor shape of apple maggot (zeph) and snowberry (zeph) maggot.

EXECUTIVE SUMMARY
Apple maggot fly is a quarantine pest of apple in Washington state that is almost identical morphologically to snowberry maggot fly, a non-pest of apple. Historically, the longer ovipositor in apple maggot has been used to separate it from snowberry maggot, despite overlap in ovipositor lengths. Here, the objectives were to determine if use of wing shape, clasper shape, and ovipositor shape can be used to better discriminate of the species. Ovipositor lengths allowed 94.6% correct identification of female apple maggot but only 7.0% correct identification of snowberry maggot. Geometric morphometrics and CVA separated wing shapes between species in both sexes. Bookstein shape coordinates indicated that the wing of apple maggot is more tapered at the tip than that of snowberry maggot. Use of wing shape in an assignments test identified female apple maggot with 98.5% and female snowberry maggot with 99.0% accuracy, and it correctly identified 100% of flies whose identities were questionable based on ovipositor lengths. Results indicate that use of wing shape is an improvement over the use of ovipositor length alone for identifying female apple maggot and snowberry maggot in Washington state. Then we used geometric morphometrics to test the hypothesis that shapes of claspers and ovipositors of apple maggot and snowberry maggot differ. We found that all apple maggots had a nearly parallel surstyli configuration, but that many snowberry maggots had a parallel or divergent configuration. Using CVA, an assignments test using a CVA-distance based classification method, and multivariate analysis of variance, we found that clasper shape of flies from multiple sites accurately classified 99.8% of males to species. We found that ovipositor shape accurately classified 85.3% of females to species. Ovipositor length was longer in apple maggot than snowberry maggot, and combining ovipositor length with shape increased classification accuracy to 94.5%. Clasper and ovipositor shapes did not discriminate flies within species, regardless of host fruit and collection sites. Results suggest that use of clasper shape would benefit regulatory agencies and apple growers that depend on accurate identifications of apple maggot for quarantine and management measures. Combining clasper or ovipositor shape and shape and size of other structures in a single analysis should result in even greater discrimination (possibly 100%) between species. If all flies are accurately identified, apple orchards would never mistakenly be placed under threatened or quarantine status and unmanaged apple or hawthorn trees would never need to be treated unnecessarily for apple maggot.

FINAL REPORT **DURATION**: 2 years (2007-2008)

Project Title: Fate of codling moth in apples after harvest

PI: Lisa G. Neven
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lisa.neven@.ars.usda.gov
Address: 5230 Konnowac Pass Road
City: Wapato
State/Province/Zip WA, 98951

Cooperators: Michael Willett, NHC

Budget:

Year 1: $58,815 **Year 2:** $55,000 **Total Funding: $113,815**

Item	Year 1: 2007	Year 2: 2008
Salaries	$31,312	$32,250
Benefits	$9,393	$9,675
Wages		
Benefits		
Equipment		
Supplies	$18,110	$13,075
Travel		
Miscellaneous		
Total	$58,815	$55,000

Original Objectives:
The overall objective of this project is to develop information regarding the fate of codling moth in apples destined for export to Asian Pacific countries. The specific objectives were:

Objectives:
1) Determine the critical duration of chilling needed for diapause-destined larvae needed to break diapause.
2) Determine the fate of diapause-destined larvae under tropical environments (short photoperiod, elevated temperatures, high chilling temperatures, short chilling period).
3) Determine the proportion of field codling moth population entering diapause at each harvest date.
4) Determine the proportion of both field and laboratory codling moth diapause-destined larvae surviving cold storage.

Significant Findings:

1) During both the first and second years any larvae that were not subjected to a cold storage and did not receive a chilling period of at least 2 weeks at 10°C (50°F), did not emerge as moths when placed at 20°C (68°F) under a 12:12 L:D photoperiod (Figure 1, 0 days @ 33°F).
2) For larvae that were cold stored, but did not receive a chilling treatment of at least 2 weeks at 50°F, only 0.5% emerged as moths (Figure 1).
3) Of those moths that emerged from the non-chilling group, only 25% emerged within the 6 week window previously used by USDA-APHIS-PERAL in their risk assessment (Figure 2).
4) Only 17% of all moths emerging from all treatments in the first year emerged within the 6 week window. Only 34% of all moths emerging from all treatments in the second year emerged within the 6 week window (Figure 2).
5) There is only a 0.2% chance that any moths would emerge within the 6 week window stipulated by USDA-APHIS-PERAL. There is nearly a zero percent chance that any larvae resulting from a successful mating would ever be able to complete development and diapause to produce a second generation.

Results and Discussion:
We completed two years of the field experiments of codling moth infested apples over 7 harvest dates. We completed the collection of the second year emergence data in November 2009. We did collect a 3rd year of infested apples, but limited the study to only cold stored fruit and did not store any fruit at 10°C (50°F). We believe that these conditions more closely approximate the tropical conditions most likely to be experienced by any codling moth entering Taiwan. To date, we have not had any emergence in any sample held under the 12:12 L:D photoperiod at 68°F in this third year of samples. We have had significant emergence from samples held at 16:8 L:D photoperiod at 68°F.

There was no moth emergence from any harvest date over the two years from fruit that was not cold stored and did not receive chilling at 68°F of at least 2 weeks. This means that codling moth cannot develop and successfully complete diapause under a 12:12 L:D photoperiod at 68°F.

In fruit that was cold stored, but did not receive a chilling period of at least 2 weeks at 50°F, only 0.5% emerged as moths. In the first year a total of 6 moths emerged from the 864 larvae in the no-chill group. In the second year only 8 moths emerged from the 603 larvae in the no-chill group. Of those, only 25% emerged within the 6 week window previously (Figure 2) stipulated by APHIS in their risk assessment. This gives us an estimated risk of emergence for the zero chill group within the 6 week period of only 0.125%. This is greatly reduced from the 100% previously used by USDA-APHIS-PERAL.

For both years of the study when cardboard strips were examined following 6 months at 68°F 12:12 L:D, the proportion of larvae that remained in diapause for the zero chilling groups was 13.3%, and the number proportion of dead larvae in the strips was 6.3%. It was estimated that only 30% of the collected larvae were ever able to successfully exit the fruit and initiate diapause. This agrees with previous published research that only 30% of a codling moth population could survive from one season to the next. This number was not used in the APHIS model for early and middle season fruit, where they assumed 100% of all larvae entering the country would survive diapause and emerge and moths.

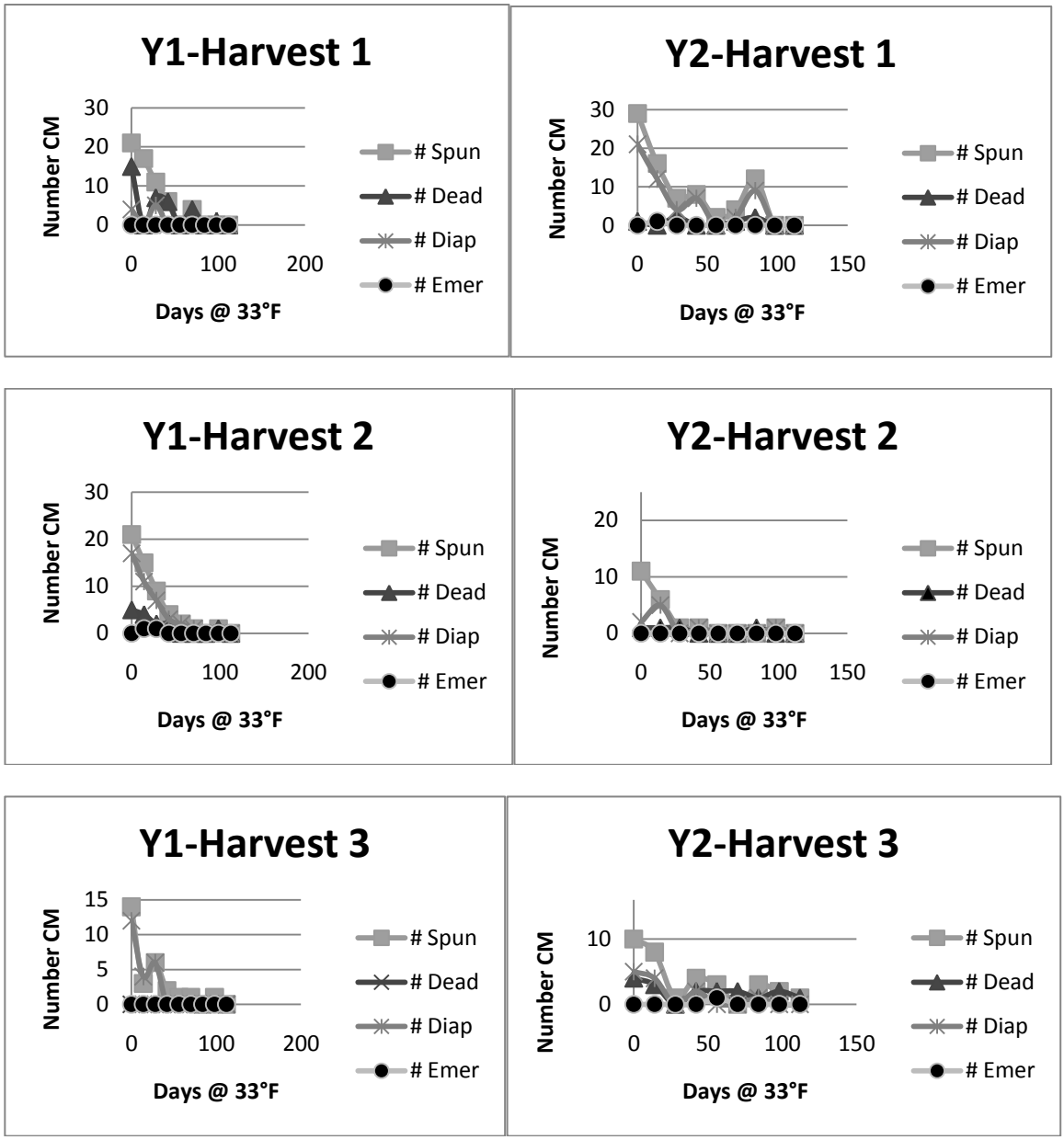
It is interesting to note that when the cardboard strips were examined following the 6 months of storage at 12:12 L:D photoperiod at 68°F, those larvae that were remaining in the strips were approximately 50 to 75% smaller than normally diapausing larvae held under a short photoperiod (8:16 L:D). This indicates that much of the energy reserves, predominately lipids, were utilized during the prolonged state of diapause. Loss of energy reserves would reduce future fecundity and the ability of adults to fly any significant distance. It is also interesting to note that a majority of the moths that emerged were females. This is most likely due to the additional lipid reserves in the ovaries. If lipid reserves were taken from the ovaries during the prolonged diapause period, as is the case for many diapausing insects, then overall fecundity of the females would be greatly reduced.

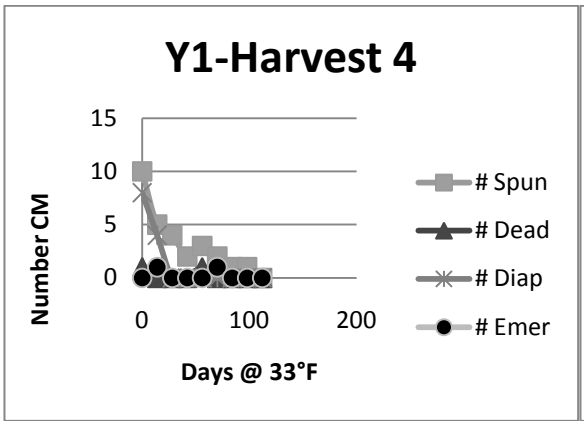
For the entire first year an estimated total of 8640 larvae were collected and only 125 moths emerged for a total for the entire experiment of 1.4%. For the entire second year an estimated total of 6030 larvae were collected and only 106 moths emerged for a total for the entire experiment of 1.7%. In addition, only 17% of the moths emerging from the first year and only 34% of the moths emerging from the second year emerged within the 6 week window. This brings the proportion of total moths emerging within the 6 week period down to 0.2% for the first year and 0.5% for the second year. These data include storage at 50°F for durations of 2 or more weeks, optimal conditions for meeting the requirement for diapause break. However, exposure of fruit/larvae to temperatures under 50°F for greater than 2 weeks in Taiwan is highly unlikely.

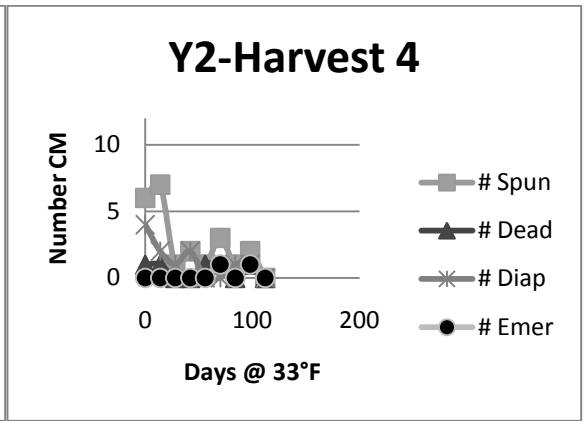
In studies with laboratory colony insects on thinning apples, no moths emerged from any larvae that successfully cocooned when reared under a 12:12 L:D photoperiod at 68°C with no cold storage or chilling. However, very few larvae successfully exited the thinning apples to reach cocooning strips, making this series of experiments difficult to analyze.

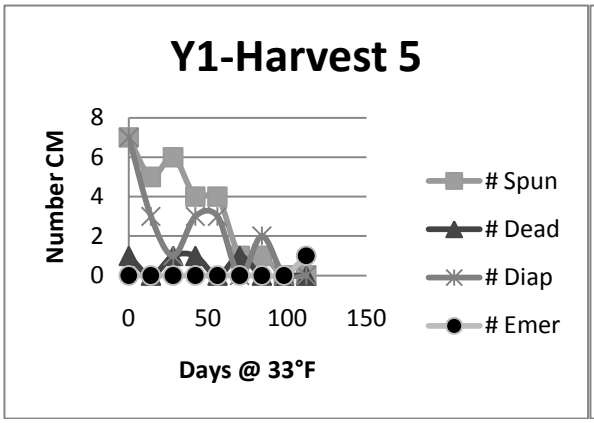
It is my understanding that USDA-APHIS-PERAL intends to publish their risk assessment of the potential of codling moth establishment in Taiwan this year. I will contact Dr. Robert Griffin director of the Plant Epidemiology and Risk Analysis Laboratory and provide his group with these new data so that they can re-assess the risk and incorporate those changes into a revised manuscript. In addition, we will complete our collection of data for the 3rd year in August 2010. When these data are complete and analyzed, a manuscript will be prepared for a peer reviewed scientific journal, most likely the Journal of Economic Entomology.

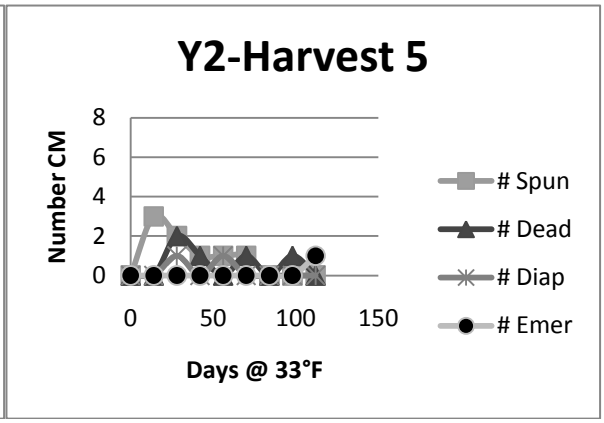
Figure 1. Comparison of years 1 & 2 moth emergence from codling moth infested apples stored at 33°F for 0 to 112 days then placed at 68°F under a 12:12 L:D photoperiod for up to 6 months.




Y1-Harvest 4
Number CM
15
10
5
0
0
100
200
Days @ 33°F
Spun
Dead
Diap
Emer


Y2-Harvest 4
Number CM
10
5
0
0
100
200
Days @ 33°F
Spun
Dead
Diap
Emer


Y1-Harvest 5
Number CM
8
6
4
2
0
0
50
100
150
Days @ 33°F
Spun
Dead
Diap
Emer


Y2-Harvest 5
Number CM
8
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4
2
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150
Days @ 33°F
Spun
Dead
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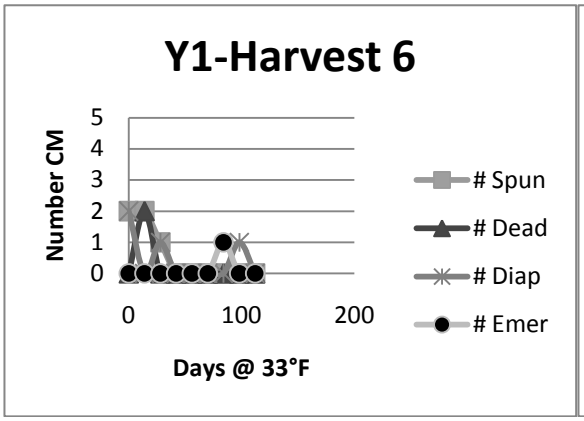
Emer


Y1-Harvest 6
Number CM
5
4
3
2
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Days @ 33°F
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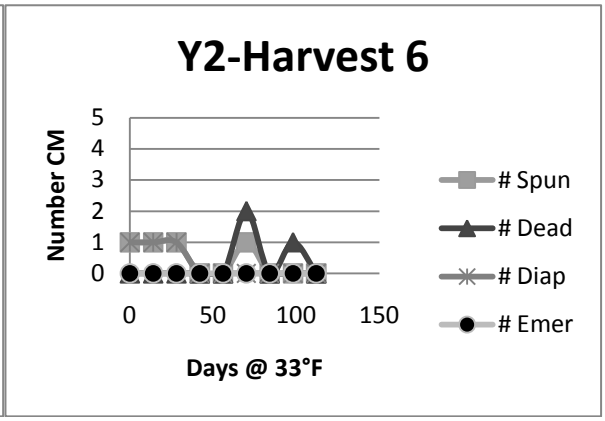
Y2-Harvest 6
Number CM
5
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Days @ 33°F
Spun
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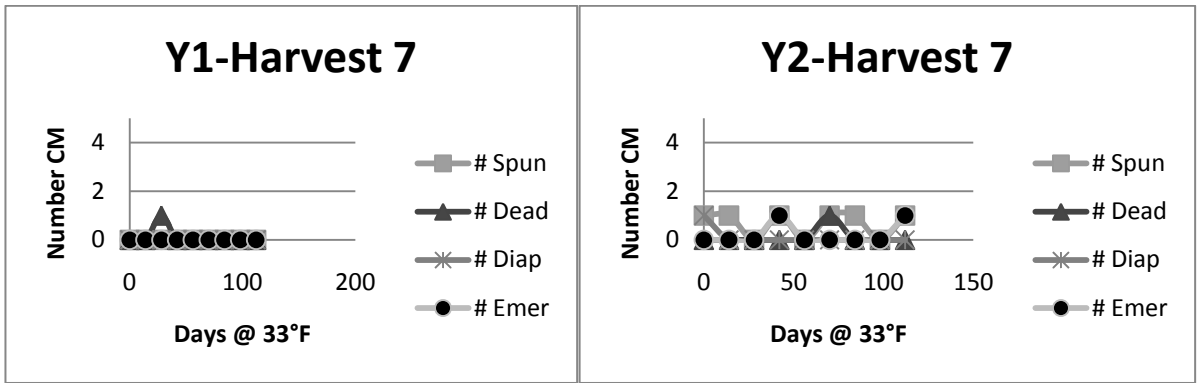
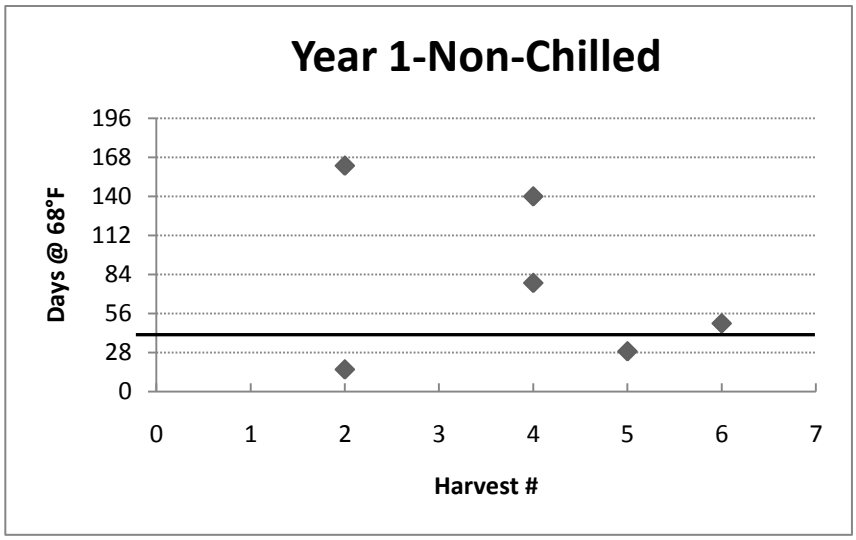
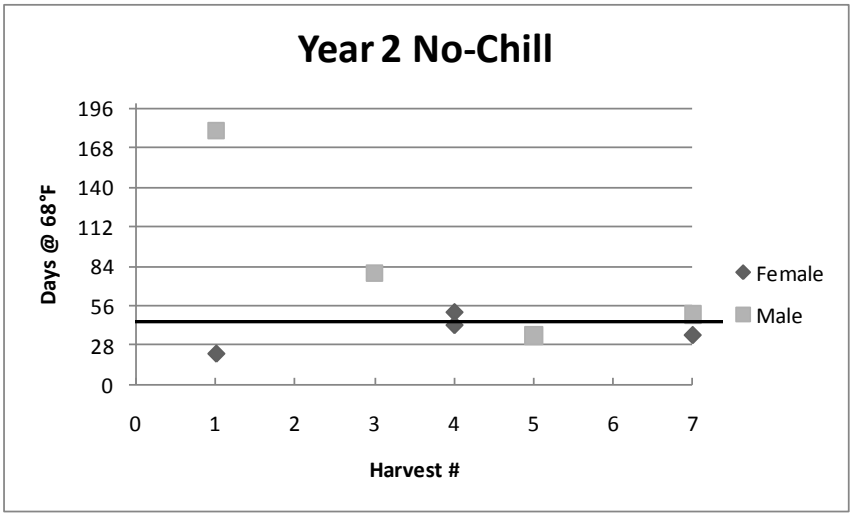


Figure 2. Days to emergence of codling moths from infested apples stored at 33°F for 0 to 112 days then placed at 68°F under a 12:12 L:D photoperiod for up to 6 months. Moths above the solid black line did not emerge within the 6 week window previously used by USDA-APHIS-PERAL in their risk assessment.





Executive Summary:

Project Title: Fate of codling moth in apples after harvest

The risk assessment performed by USDA-APHIS-PERAL manipulated the existing published data on codling moth presence in packed fruit, overall survivorship, and post-diapause emergence (not under tropical conditions). With this new information, we can provide USDA-APHIS-PERAL with new data that accurately describes the ability of codling moth to complete diapause and emerge under tropical conditions (i.e. photoperiods of 12:12 L:D) with no chilling at temperatures at or below 10°C (50°F). In the original assessment, they predicted that 100% of all larvae arriving in infested apples would successfully survive, complete diapause, and emerge as moths within a 6 week period. We now know that it is highly unlikely that any codling moths will be able to complete diapause and emerge as moths within this 6 week window. There is only a 0.125% chance that any moths from fruit that had been cold stored for any duration would emerge as moths. Taking the field and laboratory tests into account, it is highly unlikely that any progeny from a highly unlikely mating pair would ever complete diapause and emerge as moths. We will provide USDA-APHIS-PERAL with a report of the results of this study so that they can reassess the risk of codling moth establishment in Taiwan.

FINAL PROJECT REPORT

Project Title: Identification of Bt toxin targets in codling moth larvae

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State/Zip:	WA 98951	**State/Zip**:	WA 99164-6382

Total Project Funding:

Budget History:

Item	Year 1: 2007	Year 2: 2008	Year 3:
Salaries			
Benefits			
Wages	6,240		
Benefits	150		
Equipment			
Supplies	28,110		
Travel	500		
Miscellaneous	5000		
Total	$40,000	0	

ORIGINAL OBJECTIVES

The specific objectives of this proposal include:

1) Determine the potencies of 10 Bt toxins against codling moth larvae and cell line.
2) Determine the mode of action of the most potent Bt toxins.
3) Identify key molecules affected by the bioactive Bt toxins
4) Clone transcripts encoding the key molecules affected by Bt toxins
5) Develop a cell-based assay system to search for novel insecticides that alter key molecules affected by Bt toxins

Revised Objectives converting original objectives from a 3 year to 2 year time line as requested by Jim McFerson. Additionally, year 2 funds were not requested due to unexpected

Year 1

1) Determine the toxicity of Bt toxins against codling moth larvae and a codling moth cell line
2) Using the codling moth cell line as a model, determine Bt toxin effects on signal transduction pathways using established assays that monitor chemical signals and cell response.
3) Determine Bt toxin membrane receptors in codling moth larvae and cell line.

Year 2

1) Determine the effects of Bt toxins on signal transduction pathways in codling moth larvae.
2) Identify the key molecules affected by the bioactive Bt toxins.
3) Clone genes encoding key signal transduction proteins affected by the Bt toxins.

SIGNIFICANT FINDINGS (ACCOMPLISHMENTS)

- Growth conditions for bacteria expressing Bt toxins were optimized.
- Toxin purification methods were developed.
- Toxicity of 7 Bt toxins against codling moth was obtained.
- Effects of Bt resistance on sex pheromone perception in *Helicoverpa zea* was determined.
- A G-protein that potentially mediates signal transduction of odorant receptors in codling moth was cloned and sequenced. This G-protein is a potential Bt toxin target.

RESULTS AND DISCUSSION

The crystal (Cry) protein toxins produced by the bacterium *Bacillus thuringiensis* (Bt) are used to control pest insect species in the Orders Diptera (flies and mosquitoes), Coleoptera (beetles), and Lepidoptera (moths). The Bt toxins produced with lepidopteran activity mainly belong to the Cry1 family. Cry1 toxins have been used for years to control moth larvae either in formulations or in transgenic crops. Because of the life cycle of codling moth, Bt formulations have not been an effective means of control in the orchard. However, Cry1 proteins toxic to codling moth can be used as tools to discover new targets for insecticide development.

Cry1 toxin growth, purification and potency toward codling moth larvae

An initial hurdle facing this project was the preparation of Cry1 toxins. Bacterial strains expressing Cry1 proteins were obtained from the Bacillus stock center (Columbus, OH). In our hands, these bacterial strains did not produce the vast quantity of toxin needed for this study. Eventually, we determined optimal media and growth conditions to produce enough toxin for our studies (data not shown). Additionally, to obtain pure toxin required advanced chromatography conditions, which were also optimized for purification of large amounts of Cry1 proteins. Once these

hurdles were overcome, experiments determining Cry1 potency toward codling moth were able to proceed. Table 1 summarized the potency of Cry1 toxins toward codling moth larvae. The rank order of potency was Cry1Da > Cry1Ac > Cry1Aa > Cry1Ab > Cry1Fa > Cry1Ba >>>>Cry1Ca (see Table 1). Currently, we are determining Cry1 toxicity toward two insect derived cell lines, one from codling moth, and the other from *Trichoplusia ni*. Our goal is to use the cell lines as a model system to study Bt toxin mode of action.

Table1. Toxicity of Various Bt toxins against neonate Codling moth larvae

Toxin	***LC_{50} (95% fiducial limits)[a]***
Cry1Aa	23 (14–33)
Cry1Ab	25 (14–38)
Cry1Ac	20 (8–34)
Cry1Ba	35 (25–46)
Cry1Ca	>5000
Cry1Da	6 (4–8)
Cry1Fa	34 (4–74)

[a]Toxicity is indicated as LC_{50} in nanograms of toxin per gram of diet.

Resistance to Bt Affects Sex Pheromone Attraction to Males

A colleague who has a lab colony of *Helicoverpa zea* (corn earworm/cotton bollworm) that is resistant to the affects of Bt toxin observed that the males did not readily mate with females. Because our hypothesis is that Bt toxins exert their effects on signal transduction pathways, we started a collaborative effort explore the resistance mechanism of the Bt resistant *H. zea* colony. Males of the Bt resistant *H. zea* line do not recognize females. We have verified this observation using synthetic pheromones in the flight tunnel (Table 2). For control insects (a Bt susceptible lab colony), 41 of 50 males flew to and made contact with a synthetic sex pheromone lure in a flight tunnel (Table 2). However, only 9 of 34 males flew to and made contact with the same lure in the flight tunnel (Table 2). Because of our previously WTFRC funded project on pheromone receptors in codling moth, we were able to examine the chemosensory system in *H. zea* males. We have so far determined that a member of the pheromone receptor family expressed in Bt susceptible *H. zea* has not yet been detected in the Bt resistant colony (data not shown). Because a potential target of Bt toxins has been identified as a G-protein, we also tried to determine if it was being expressed in *H. zea*. The G-protein that potentially mediates signal transduction of odorant receptors in the antenna has not yet been detected in the Bt resistant colony of *H. zea*.

Table 2. Effects of Bt Resistance on Sex Pheromone Perception in *Helicoverpa zea* males

Colony	***# Flown***	***Wing Fan***	***Take off***	***Upwind***	***Midway***	***Close***	***Contact***
Bt Susc.	50	50	50	47	47	47	41
Bt res lab	34	31	26	25	25	18	9
Bt res field	19	15	13	11	11	11	9

Based on published results from *B. mori*, BmOR1 response to Bombykol and BmOR3 response to Bombykal are dependent on co-expression of BmOR2 (the ubiquitous receptor) and the olfactory specific G protein, BmGαq. Because of the potential importance of the G-protein in Bt toxin mode of action, I cloned the codling moth ortholog of the olfactory specific G protein, BmGαq. The deduced amino acid sequence from the cloned cDNA encoding the putative olfactory specific G

protein, CpGαq is shown in Fig. 1. The deduced amino acid sequence of the putative codling moth olfactory specific G protein is highly similar to those previously reported for *B. mori* and *Mamestra brassicae* with CpGαq sharing 97.3% identity and 98.2% similarity, and 96.4% identity and 97.6% similarity, respectively. A mammalian cell line expressing CpGαq has been generated and will be screened with Bt toxins to determine the effects.

Fig. 1. Boxshade alignment of deduced amino acid sequences of putative olfactory specific Gq-like protein alpha subunits.

Future Work

Examining Bt toxin mode of action with insect cell lines and codling moth is still an active component of research in my laboratory. As such, this final report is still a work in progress as I believe that a further understanding of how Cry1 toxins kill codling moth will potentially provide other targets for insect control.

EXECUTIVE SUMMARY

Commercial formulations containing *Bacillus thuringiensis* Cry proteins have been used for more than 40 years to control lepidopteran larvae and most recently, transgenic plants expressing these protein toxins are being used in insect control programs. Codling moth larvae are susceptible to Cry proteins, but these toxins must be ingested to be effective making their use in the orchard difficult because larvae rapidly bore into and are protected by the apple. However, if we take advantage of the fact that codling moth larvae are susceptible to Cry proteins, a full understanding of the mode of action of these toxins may yield targets for the development of novel insecticides for use in the orchard.

Progress was made in the development of procedures to grow and purify toxins for use in mode of action studies in cell line assays and with codling moth larvae. Based on studies with a Bt resistant colony of *Helicoverpa zea*, Cry toxins may affect the signal transduction pathway involved in the detection of sex pheromones. An olfactory specific G-protein was cloned from codling moth antennae and is now expressed in a mammalian cell line so that further examination of the effects of Bt toxins can be monitored.

Future directions for this project include the development of the cell based assay system to explore the mode of action of Bt toxins in further detail. Because there are over 50 different Bt toxins that kill lepidopteran larvae, there is the possibility that many new proteins can be identified as potential targets for novel insecticide development. We hope that potential advances in this line of investigation will help make codling moth a recognized model organism for the development of biorational means of pest control.

FINAL PROJECT REPORT
WTFRC Project Number: CP-08-804 (WSU Project #13C-3643-5092)

Project Title: Management of codling moth and leafrollers in apple orchards

PI: Jay F. Brunner
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Cooperators: Mike Doerr, WSU-TFREC; Steve Gacrzynski, USDA-ARS-Yakima; John Dunley, WSU-TFREC; Ashfaq Sial (graduate student), WSU-TFREC.

Other funding sources

Agency Name: Washington Commission on Pesticide Registration
Total amount awarded: $48,324

Agency Name: Private Chemical Companies
Total amount awarded: ≈ $85,000
The financial information provided in addition to sponsor support simply communicates research program support costs vs. specific project cost-share commitment.

WTFRC Collaborative expenses: None

Total Project Funding: $61,002

Budget History

Item	2008	2009
Salaries [1]	18,170	18,879
Benefits [1]	7,240	7,563
Wages [2]	3,000	1,500
Benefits	471	270
Equipment	0	0
Supplies [4]	1,000	1,000
Travel [3]	1,000	1,000
Miscellaneous	0	0
Total	30,881	30,212

Footnotes:
[1] Kathleen Pierre (3 months - Associate in Research) and Mike Doerr (2 months - Administrative Professional).
[2] Temporary or hourly workers.
[3] Pays for a vehicle used part-time on this project plus fuel and maintenance costs.
[4] Leafroller diet components, plastic Petri dishes, glassware.

Objectives:

1. Develop baseline toxicity bioassays for codling moth and leafroller of new insecticides under development.
2. Select populations of leafrollers (in the laboratory) to determine their inherent potential to develop resistance to selected insecticides.
3. Develop molecular markers to use as a tool for early detection of resistance development in leafrollers and codling moth.
4. Survey codling moth and leafroller populations using discriminating concentrations for key insecticides.
5. Characterize cross-resistance in leafrollers between old and new insecticides.
6. Evaluate new insecticides for control of codling moth and leafrollers in field tests.

Significant findings 2008

1. Field-aged bioassays and field trials confirmed previous laboratory results showing that Altacor (rynaxypyr) has activity against codling moth (CM) eggs, primarily when laid on residues. The same effects were not observed for Belt, a new insecticide in the same class, which at least partially explains why this product does not provide control of CM in field tests.
2. Laboratory selection for resistance in obliquebanded leafroller (*OBLR)* to Altacor (rynaxypyr) and Delegate (spinetoram), to insecticides registered for use in 2008, showed that after five or four generations, respectively, resistance to Altacor were seven times that of the susceptible laboratory colony while resistance to Delegate were only 3.5 times that of the laboratory colony.
3. Every population of *OBLR* collected in the field (6) showed significant levels of resistance to Altacor (rynaxypyr) relative to the laboratory colony. Resistance ratios ranged from two to five. There was some suggestion from the data that resistance to Altacor was correlated to resistance to the organophosphate insecticide azinphosmethyl (Guthion).
4. The field collected *OBLR* populations showed the same level of resistance or enhanced susceptibility to Delegate (spinetoram) as they did to Success (spinosad). These data demonstrate that resistance to Success will be conferred on Delegate as was expected.
5. Data from 2008 show *OBLR* populations resistance to Proclaim (emamectin benzoate) for the first time since it was registered in 2005. Previous data had not shown any sign of resistance in field-collected populations of leafrollers.
6. An international effort to characterize baseline resistance in CM to Altacor did not reveal any concerns for resistance, though there was considerable variation in the response of different populations to discriminating concentrations.
7. Field trials with Delegate and Altacor confirmed earlier studies, which showed them to be highly effective leafroller control products.

Significant findings 2009

1. Laboratory *OBLR* populations selected for resistance to Altacor and Delegate reverted to susceptibility in five and six generations, respectively, after selection pressure was removed.
2. The heritability of Altacor and Delegate selected *OBLR* populations declined over five of six generations, respectively, indicating that most of the genetic variation had been selected against.
3. Evaluation of Altacor and Delegate selected OBLR populations showed different biochemical mechanisms were at work. Esterases were elevated in Altacor selected populations while oxidases were elevated in Delegate selected populations.
4. A measure of the speed of resistance development suggested that the evolution of resistance would be slower in Delegate compared to Altacor.

5. Field trials confirmed that the application of residual ovicides at petal fall provided a delay in the onset of CM fruit injury by approximately 100 DD, therefore allowing first cover sprays to be delayed by this same period.
6. New formulations of malathion did not extend the longevity of residues against CM.
7. Delegate did not show ovicidal activity against CM eggs but when it was used at the petal fall timing it delayed the onset of CM injury much like residual ovicides.

Methods:

Methods used in this project were outlined in last year's new project proposal and have not changed significantly enough to warrant their repetition here. If there are specific questions with regard to methods, consult the 2007 new proposal or contact the PI for more information.

Results and Discussion:

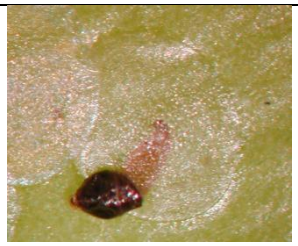
Baseline Bioassays: Laboratory bioassays help to characterize the inherent toxicity of insecticides against pests and, therefore, establish baseline data on susceptibility for future reference when questions of resistance arise. Table 1 summarizes results of laboratory bioassays for several registered and experimental products evaluated against CM and *OBLR* in 2008-09.

Table 1. Summary of baseline bioassays conducted in 2008-09.

Chemical	Year	Source	n	Slope (SE)	LC_{50}-ppm (95% CL)
CM larval screening (fruit injury)					
Malathion	2009	LAB	400	1.6 (0.4)	24.3 (7.3-45.6)
CM ovicidal screening – Egg dip test (topical application)					
Delegate	2009	LAB	2557	0.7 (0.04)	10.9 (6.5-1635)
Cyazypyr	2008	LAB	1541	0.4 (0.8)	955.7 (n/a)
CM ovicidal screening – Apple dip test (residual application)					
Delegate	2009	LAB	3689	1.4 (0.7)	141.8 (110.6-181.1)
Cyazypyr	2008	LAB	1431	2.1 (0.2)	27.7 (19.8-36.8)
CM larval screening – Diet incorporation (larval mortality)					
Delegate	2008	LAB	210	2.2 (0.6)	0.04 (0.02-0.07)
Success	2008	LAB	210	2.9 (0.8)	0.26 (0.12-0.39)
Altacor	2008	LAB	210	2.0 (0.5)	0.07 (0.01-0.13)
Altacor	2008	LAB	245	2.1 (0.4)	0.05 (0.03-0.08)
Cyazypyr	2008	LAB	210	3.5 (0.8)	0.07 (0.03-0.11)
Cyazypyr	2008	LAB	280	3.0 (0.6)	0.06 (0.04-0.08)
CM adult screening – Laboratory reared adults (adult mortality)					
Delegate	2008	LAB	366	0.6 (0.09)	471.9 (239-966)
Guthion	2008	LAB	125	2.5 (0.4)	232.0 (144-4000)
Success	2008	LAB	226	1.0 (0.2)	770.6 (485-1871)
Lorsban	2008	LAB	102	1.8 (0.4)	206.1 (108-684)
OBLR adult screening – Laboratory reared adults (adult mortality)					
Delegate	2008	TF LAB	198	1.5 (0.2)	12.9 (n/a)
Guthion	2008	TF LAB	71	3.0 (0.7)	148.2 (100-228)
Success	2008	TF LAB	194	1.4 (0.9)	45.0 (n/a)
Lorsban	2008	TF LAB	95	3.4 (1.0)	116.1 (53.8-165.2)

The combined ovicidal, ovi-larvicidal and true larvicidal activity (Fig. 1) of different products helps explain their potency against this CM. In previous studies Altacor (rynaxypry) was shown to be highly toxic to CM eggs as a residue (LC_{50} - 6.1 ppm) but less toxic when applied topically (LC_{50} - 55.2 ppm). Another experimental insecicide, cyazypyr, in the same class showed activity similar to rynaxypry, that is it was more toxic to CM eggs as a residue than when applied topically (Table 1). Another insecticide, Belt, showed poor ovicidal activity against CM eggs, which at least partially explains why it does not provide robust control in the field. Delegate showed more toxicity when applied topically to CM eggs compared to when eggs were exposed to residues, LC_{50} values or 10.9 ppm and 141.8 ppm, respectively. At the field rate it was estimated that 63% of eggs would die when treated topically (sprayed) versus 18% if eggs were deposited on a residue. Bioassays were also conducted against different formulations of malathion designed to extend the life of this insecticide. Results showed no improvement of longevity compared to a standard malathion formulation.

Figure 1. Examples of the effects of insecticides on the egg, ovicidal, or the larval stage, ovi-larvicidal or larvicidal, of codling moth.

		
True ovicidal activity. The larva died within the egg.	Ovi-larvicidal activity. The larva died in the process of exiting the chorion.	True larvicidal activity. The larva exited the egg, fed briefly, and died in close proximity to the egg.

Selecting for Resistance: One way to determine the risk of resistance development is to select populations in the laboratory over successive generations and determine if and at what rate tolerance to a chemical develops. We selected 2,000 OBLR leafroller neonates with an LC_{70} concentration each generation. The concentration (LC_{70}) of insecticides increased as the tolerance of the selected populations increased. Selection with Altacor resulted in a significant increase in the LC_{50}, resistance ratio of more than 2, while after five generations the LC_{50} value had increased almost seven fold relative to the unselected laboratory colony (Table 2). After four generations of selection with Delegate the LC_{50} value had increased only about 3.5 times (Table 2) but this represented a significant resistance ratio.

Table 2. Results of probit analyses for diet incorporation bioassays with *C. rosaceana* neonate larvae from Altacor and Delgate selected populations.

Selected Generation	Chemical	n	Slope (± SE)	χ^2	LC_{50} (ppm) (95% FL)[1]	LC_{90} (ppm) (95% FL)[1]	LCR-LC_{50}[2] (95% CL)[3]
1	Altacor	450	1.02 (0.39)	20.74	0.16 (0.07-0.32)	2.94 (1.41-8.37)	2.2 (1.02-4.65)*
3	Altacor	350	1.72 (0.17)	17.10	0.26 (0.20-0.34)	1.46 (1.00-2.43)	3.1 (2.12-4.43)*
5	Altacor	210	1.19 (0.17)	10.31	0.77 (0.31-1.48)	9.26 (4.40-33.02)	6.6 (3.27-13.24)*
6	Altacor	180	1.88 (0.36)	7.71	1.03 (0.50-1.66)	4.93 (2.88-14.19)	6.6 (3.68-11.79)*
1	Delegate	450	2.56 (0.37)	4.18	0.10 (0.07-0.12)	0.31 (0.23-0.48)	1.26 (0.86-1.85)
2	Delegate	350	2.53 (0.33)	3.96	0.12 (0.09-0.15)	0.39 (0.29-0.59)	2.3 (1.59-3.26)*
4	Delegate	350	3.63 (0.58)	2.98	0.17 (0.14-0.20)	0.38 (0.30-0.56)	3.5 (2.37-5.09)*
6	Delegate	210	3.01 (0.48)	2.52	0.22 (0.17-0.29)	0.59 (0.43-1.02)	3.64 (2.42-5.46)*

The heritability (h^2) declined in the Altacor selected population after only five generations indicating that much of the heterogeneity in the population has been selected against (Fig. 2). The heritability (h^2) had not declined in the Delegate selected population by the fourth generation indicating that there was more heterogeneity in the population yet to be selected against, but by the sixth generation heritability had declined to levels similar to that of the Altacor selected population.

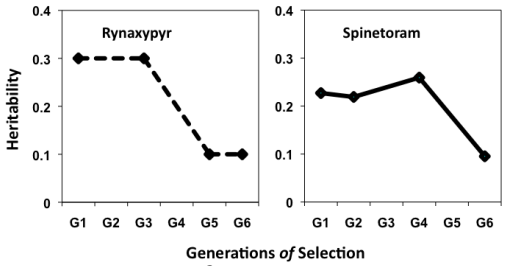


Fig. 2. Heritability (h^2) of Altacor (left) and Delegate (right) resistance in a laboratory population of *OBLR* selected for resistance.

The mean values of the response quotient (*Q)* for resistance against Altacor and Delegate were 0.11 and 0.07, respectively (Fig. 3). These results indicate that resistance evolution would be slower against Delegate than that against Altacor, and thus Delegate would be more durable than Altacor against this particular population of *OBLR*.

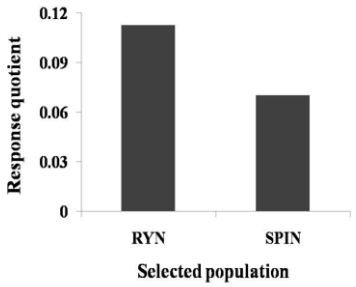


Fig. 3. Response quotients of the Altacor and Delegate selected populations of *OBLR*.

These data demonstrate the risk of these two new insecticides to resistance development and underscores the need to follow sound resistance management strategies to at least slow the development of resistance in the field.

Reversion of resistance: A cohort of *OBLR* populations selected for resistance were removed from selection pressure and evaluated each generation to determine if susceptibility would return. Selected populations were susceptible to Altacor (rynaxypyr) after five generations and to Delegate (spinetoram) after six generations (Fig. 4). It is encouraging that reversion to susceptibility occurred with both insecticides as it suggests that resistance can be managed through rotation with each other, or possibly other products with different modes of action.

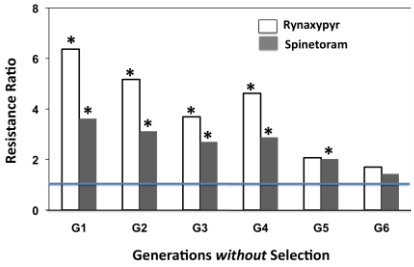


Fig. 4. Reversion of resistance in *OBLR* removed from selection.

Surveys of Field Populations: A survey of CM populations from across the state revealed no concerns with tolerance to Delegate or Altacor. Delegate data were from topically treated adults using technical spinetoram dissolved in acetone. The demonstration that this method can provide reliable and repeatable dose-response lines will allow us to use moths captured in pheromone traps to assess more codling moth populations than is possible if bioassays are restricted to larvae.

We have participated in an international project looking at susceptibility of CM to Altacor. This has been a very good collaborative experience and data thus far shows no major difference in tolerance between field and laboratory (susceptible) populations (Fig. 5).

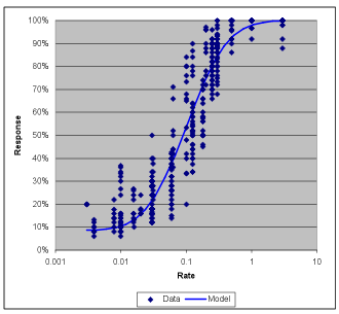


Fig. 5.

Field populations of *OBLR* were collected from six different orchards. Populations were reared in the laboratory and tested using a diet incorporation bioassay to determine their susceptibility to Delegate and Altacor. Results of these bioassays are shown in Fig. 6. Every population of *OBLR* collected in the field showed significant, though low, levels of resistance to Altacor (rynaxypyr) relative to the laboratory colony. Populations showed varying levels of resistance to Delegate (spinetoram) with some populations being resistant while other were more susceptible than the laboratory colony. The response of *OBLR* populations to Delegate mirrored that of Success (spinosad), indicating cross-resistance between these products. These data demonstrate that resistance to Success will be conferred on Delegate as was expected. These data did not suggest any correlated cross-resistance between Altacor or Delegate and the organophosphate insecticide Guthion (azinphosmethyl). Low levels of resistance to Proclaim (emamectin benzoate) was documented for the first time since its registration in 2005. Previous data had not shown any sign of resistance in field-collected populations of leafrollers.

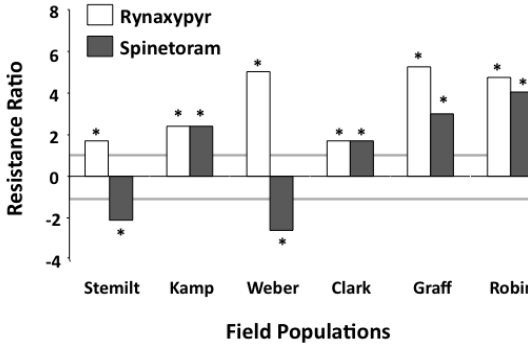


Fig. 6.

A new chemistry in the same class as Altacor was evaluated for toxicity to five field populations of CM. Results showed a high level of toxicity to all populations and LC_{50} values similar to those from a susceptible laboratory population.

Field-collected populations will continue to be reared in the laboratory and used in further experiments to determine if the mechanism of resistance noted from the selected laboratory population (see below) are also expressed in the field populations. It is possible that two different mechanisms are functioning in these populations.

Mechanisms of Resistance: We used colorimetric microplate assays to assess the activity of detoxification enzymes in resistant (selected) as well as susceptible (unselected) populations of *OBLR* in order to determine the mechanisms of resistance. We used a total of 30 third instar larvae from each of the selected and unselected populations to determine total proteins using Bio-Rad protein assay, and the activity of non-specific esterases, mixed-function oxidases, and glutathione-*S*-transferases using α-naphthyl acetate (α-NA), *3,3′,5,5′*-tetramethylbenzidine (TMBZ), and *1*-chloro-*2,4*-dinitrobenzene (CDNB) as substrates, respectively. The results of detoxification enzyme assays indicate that the activity of esterases was significantly increased in Altacor (Fig. 7 - left) selected population *(p = 0.004)* whereas the level of oxidases was significantly increased in the Delegate (spinetoram) selected population *(p = 0.039)* (Fig. 7 - right). There was no increase in glutathione-*S*-transferases activity for Altacor selected populations but Delegate selected populations showed an increase in activity though not significantly different from unselected populations *(p = 0.054)*. These results indicate that the laboratory selected populations that showed resistance to Altacor and Delegate do not share resistance mechanism. It further suggests that these two reduced-

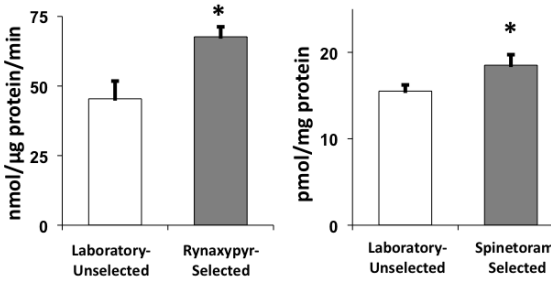


Fig. 7.

risk insecticides can be used in resistance management program involving the use of these products in rotation. Further studies will examine field-collected populations to determine if they show the same levels of enzyme activity as the laboratory selected populations. It is always possible that field populations will have a different pattern of biochemical resistance than the selected populations.

Development of Molecular Markers: No progress has been made in developing molecular markers for resistance in *OBLR* to Altacor or Delegate. It was likely too ambitious of a goal to set to be able to identify molecular markers within the scope of this project. However, now that we have resistant populations it should be possible with additional time and funding to move forward with this objective. We will not be asking the commission to fund this work.

Efficacy Evaluations: Twenty field trials to evaluate new insecticides for efficacy, timing and rates against CM and *OBLR* were conducted in the 2008-09 period. Most of these efforts were supported through gift grants from private chemical companies. Data from these trials form the basis for recommendations in WSU Extension Bulletin EB-0419 "Crop Protection Guide for Tree Fruits in Washington".

A key finding showed that Altacor had ovicidal activity, which provides flexibility in its use pattern in apple and the opportunity to coincidentally control CM eggs and leafroller larvae early in the season. Cyazypyr, a new chemical made by the same company that developed Altacor, showed similar activity against CM (eggs and larvae) and *OBLR* in field trials. However, a closely related product, Belt (flubendiamide), was shown not to have ovicidal activity against CM and most likely accounts for its lower level of efficacy against this pest.

Table 3 is a summary of numerous field trials over the last 5 years comparing control of CM based on insect growth regulators (IGRs) with programs based on neonicotinyls (Assail or Calpyso) and azinphos-methyl (AZM - Guthion). IGRs worked well when directed against the first CM generation but were never as good as the neonicotinyls or Guthion, even though they were applied three versus two times. The level of control with the IGRs declined in the second CM generation, especially for

Table 3. A summary of insect growth regulator and oil programs for control of CM compared to those using neonicotinyl or azinphos-emthyl programs.

		Timing (DD[a], + Retreatment Interval)		Avg Reduction in Codling Moth Injury Relative to UTC (SEM)		
Insecticide	Rate (gm AI/A)	1st Generation	2nd Generation	1st Generation	2nd Generation	n
Rimon	95	100, +14d, +14d	1000, +14d, +14d	85.9 (4.7)	81.9 (3.4)	7
Intrepid	113	100, +14d, +14d	1000, +14d, +14d	88.0 (2.9)	77.1 (2.3)	4
Esteem	50	100, +14d, +14d	1000, +14d, +14d	73.7 (9.2)	55.5 (4.8)	3
Mineral Oil	1% v:v	200, +14d, +14d	1200, +14d, +14d	74.9 (6.7)	55.2 (10.8)	4
Neonic.[b]	Various	250, +21d	1250, +21d	96.5 (6.4)	84.5 (1.3)	6
Guthion	454	250, +21d	1250, +21d	96.9 (1.1)	94.8 (1.4)	9

[a], Timing reported as accumulated codling moth degree-days (Celsius) from biofix unless followed by 'd' indicating the calendar day interval between applications.

[b], Compilation of all trials that relied on season-long applications of Assail or Calypso.

n, Number of trials.

Esteem. Oil alone provided reasonable control of CM by killing eggs in the first generation but the level of control fell in the second generation. It is likely that control with IGRs and oil in the second CM generation would have been high if one more application had been applied to cover the longer

oviposition period. These data show that IGRs and oil can control CM but do not represent the best stand-alone programs for this pest. It is better to incorporate the benefits of IGRs or oil into programs that incorporate lavicides in order to optimize CM control.

Table 4 gives a summary of how characteristics of IGRs and oil can be incorporated into programs with larvicides to control CM. Two different strategies are outlined in this table. In the first strategy an IGR or oil is applied at the beginning of a CM generation to act as an ovicide. In the first generation the IGR coincidentally controls leafroller larvae. The early ovicide treatment is followed by a tank-mix of an IGR and larvicide, which acts to kill larvae hatching from eggs and to kill eggs deposited after the treatment. The value of this approach is to reduce trips through the orchard and it has been a very powerful program against very high CM populations, especially if followed by an additional larvicide 14-17 days after the tank-mix application. The second strategy uses an IGR or oil early in each generation as in the first strategy, but follows it with a delayed (100 DD) larvicide, which is repeated 17 days following the first. This strategy is effective because it puts the most active residues of the larvicides on the target when most of the CM egg hatch is occurring.

Table 4: Summary of field trials that incorporated ovicides and larvicides for control of CM.

Ovicide Class[a] (Timing[b])	Timing (tank-mix[d] or larvicide)	Retreatment (17 days later)	Avg reduction in CM injury relative to UTC (SEM) 1st Gen	2nd Gen	n
Tank mix strategy with delayed first cover					
IGR	IGR + Larvicide[c]		88.8 (3.5)	89.9 (1.3)	8
HMO	IGR + Larvicide		91.6 (3.5)	83.6 (3.6)	9
Ovicide early with delayed first cover larvicide treatments					
IGR	Larvicide[c]	Larvicide	97.7 (1.4)	92.6 (1.4)	3
HMO	Larvicide	Larvicide	75.1 (8.7)	77.1 (5.3)	5

[a], Ovicide class, IGR is either Rimon, Intrepid, or Esteem. HMO is horticultural mineral oil.

[b], Timing for IGR at 100 or 1000 CM degree-days (DD) from biofix. Timing for oil is 200 or 2000 DD.

[c], Larvicide is either Assil, Calypso, Delegate, or Altacor.

[d], Tank-mix timing is delay cover; 350 DD in first generation or 1350 DD in second generation.

n, number of trials.

In 2008 two large plot field trials (un-replicated) Altacor and Delegate provide excellent control of *OBLR*. In 2009 we evaluated five insecticides in large un-replicated field trials against *OBLR* that were applied by a grower. All the insecticides provided very good control in this test (Table 5). We have also conducted several replicated small plot trials in 2008 and 2009 against overwintered and summer *OBLR* that showed very good results for these products.

Table 5: *OBLR* control following a single petal fall application, 2009.

Trt	Insecticide	Rate (form/acre)	Post-treatment Evaluation (20 DAT)- OBLR/100 shoots Feeding sites	Live larvae	Pupae	Dead Larvae
1	Proclaim 5SG	4 oz	20.4	0.0	0.0	4.5
2	Delegate 25WG	6 oz	15.8	0.2	0.0	2.6
3	Altacor 35WG	4 oz	14.1	0.5	0.0	1.8
4	Belt 480SC	5 fl oz	20.8	0.1	0.0	4.8
5	Tourismo*	15 fl oz	15.7	0.1	0.0	2.0

* - Tourismo is a pre-mix of flubendiamide and a buprofezine.

We showed that *OBLR* larvae were controlled by a blossom application of limesulfur. While these data were from a hand-gun applied treatment, and are thus preliminary information that need to be validated using standard airblast equipment, they do show that if a grower is applying limesulfur as a blossom thinner they likely would not need a specific leafroller control at petal fall.

Reduce use of Lorsban (chlorpyrifos) is in response to grower sensitivity to using organophosphate insecticides due to farm worker concerns and because there are many effective alternatives for leafroller control that can be used later in the season, e.g. at petal fall. Some questions have arisen about the impacts of eliminating Lorsban from the pre-bloom control and we conducted a test in 2009 to address some of these questions. The test was a replicated small plot design. The treatments are shown in Table 6. Lorsban and different oils were the primary insecticide treatments. While different tools were used to assess the impact of different treatments the focus of this discussion is on their effects on aphids and their natural enemies. Where Lorsban was included as a treatment there

Table 6: Aphid shoot samples associated with Lorsban and oil treatments, 2009.

			Average number of aphid infested shoots/2 minute sample						
		Rate (form. per	28-May			15-Jun		30-Jun	7-Aug
Trt	Treat.	acre)	AGA	GAA	RAA	GAA	RAA	RAA	WAA
1	Citrus Oil Lorsban	2 qrt 2 qrt	1.0a	0.7a	0.0b	3.7a	0.7b	1.0b	1.3b
2	Supreme Oil Lorsban	5 gal 2 qrt	0.0a	0.0a	0.0b	6.0a	0.3b	2.7b	0.0b
3	Citrus oil Supreme Oil Lorsban	2 qrt 2 gal 2 qrt	1.3a	0.0a	0.0b	4.0a	1.3b	0.3b	0.7b
4	MSO Lorsban	2 qrt 2 qrt	2.0a	0.0a	0.0b	6.7a	0.3b	0.0b	0.3b
5	MSO Supreme Oil Lorsban	2 qrt 2 gal 2 qrt	0.7a	0.0a	0.3b	5.0a	0.3b	0.0b	0.0b
6	EXP Oil Lorsban	2 qrt 2 qrt	1.7a	0.3a	0.7b	4.0a	0.3b	3.3b	0.0b
7	Supreme Oil	5 gal	1.0a	0.0a	0.0b	4.3a	0.3b	3.7b	12.0a
8	Citrus oil	3 qrt	0.3a	0.7a	1.0b	5.3a	1.3b	2.0b	12.7a
9	Supreme Oil Assail	5 gal 1.7 oz	0.0a	0.7a	0.0b	9.0a	0.0b	0.3b	25.0a
10	UTC		2.0a	0.0a	5.0a	3.3a	9.7a	13.0a	8.3a

Means in the same column followed by the same letter are not significantly different (P=0.05, Student's t test).

were lower levels of rosy apple aphid (RAA) but not of apple grain aphid (AGA) or green apple aphid (GAA). There were also lower levels of woolly apple aphid (WAA) in the August sample. It appears that Lorsban used in the delayed-dormant had some impact on WAA densities. It is also interesting to note that we could not identify any negative impact on natural enemies, such as, the WAA parasite from Lorsban applications based on limb taps, yellow sticky cards or shoot samples. We did sample fruit injury in this test and found that where Lorsban was included in delayed-dormant treatments San Jose scale infestation on fruit was significantly less than where only oil was applied.

Executive Summary

Baseline toxicities have been established for CM and *OBLR* for all the newly registered insecticides. These data provide an understanding of the inherent toxicity of these products as well as the basis for evaluating suspected resistance development in the field. Additional information on the residual activity of new insecticides has also been developed through field-aged bioassays. These data helped to define the effective residue life of different chemicals and also provides another tool for assessing suspected resistance. Field populations of CM and *OBLR* have been evaluated for their susceptibility to several newly registered insecticides. While no resistance was detected in CM populations most populations of *OBLR* evaluated were found to have low to moderate levels of resistance, in some cases before those populations were exposed to the products. A susceptible *OBLR* population was selected in the laboratory with Delegate and Altacor. After one and four generations *OBLR* showed significant levels of resistance to Altacor and Delegate, respectively. The biochemical basis for resistance in *OBLR* to Altacor and Delegate was due to increased levels of esterases and oxidases, respectively. These findings support the concern that new chemistries will be susceptible to resistance development, especially in *OBLR*, and points to the need for sound resistance management programs. There did not appear to be a strong correlation between resistance to new insecticides and OP insecticides in *OBLR* populations, however, there was strong cross-resistance between Delegate and Success. Numerous field trials have been conducted to evaluate new insecticides for control of CM and *OBLR*, both as individual product comparisons and in programs that mix different products. The results of these trials are represented in WSU recommendations found in EB-0419 and in educational materials associated with the Pest Management Transition Project.

FINAL PROJECT REPORT
WTFRC Project Number: CP-07-708

Project Title: Interaction of dispersal and management of CM and OBLR

PI:	Vince Jones	**Co-PI(2):**	Jay Brunner
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Total project funding request: **Year 1:** $60,112 **Year 2:** $62,452 **Year 3:** $65,213

Other funding sources: None

Budget 1:

Organization: WSU-TFREC		**Contract Administrator:** ML. Bricker; Kevin Larson	
Telephone: 509-335-7667; 509-663-8181 x221		**Email:** mdesros@wsu.edu; Kevin_Larson@wsu.edu	
Item	Year 1: 2007	Year 2: 2008	Year 3: 2009
Salaries[1]	$28,332	$28,332	$29,151
Benefits	$10,200	$11,049	$11,660
Wages	$12,000	$12,480	$12,979
Benefits	$1,380	$1,959	$2,336
Equipment	$0	$0	$0
Supplies[2]	$6,000	$6,300	$6,615
Travel[3]	$2,200	$2,332	$2,472
Total	$60,112	$62,452	$65,213

Footnotes:
[1] Tawnee Melton 0.75 FTE
[2] Supplies include general lab supplies and ELISA-specific supplies, field supplies including traps, lures, markers, cell phone charges
[3]Travel costs are within-state travel to field sites and vehicle costs

Objectives:

3. Evaluate methods to age-grade CM caught in traps throughout the season. This will be used to evaluate times of peak reproductive potential during which control measures should be optimized.
4. Determine the effect of flight of specified distances on the reproductive ability of CM and OBLR males and females using laboratory assays.
5. Evaluate the effects of different cover sprays on dispersal of CM using protein markers and investigate the effects of border sprays of kaolin on movement patterns.

Significant Findings:

We developed a method to age-grade CM males and females collected in traps that is based primarily on the condition and appearance of the reproductive system.

OBLR age-grading was not possible because there was no significant variation in appearance of any character.

Our CM age-grading showed in the overwintering generation that the middle 50% of young moths occurred in a relatively short interval, while middle-aged and older moths were captured over a much longer period. These differences were largely not present during the first summer generation.

CM males and females were completely unaffected reproductively by flights of ≈ 6,200'.

OBLR reproduction was heavily affected if neither sex was flown, reducing reproduction ≈2.5 fold compared to when either one or both sexes were flown before mating.

Our field tests of cover sprays on dispersal of CM were highly variable and showed no statistically significant differences between the Assail and Guthion treatments. Flight mill studies were shown to be cheaper and a more sensitive method to evaluate effects of cover sprays.

Flight mill studies showed that sublethal (LD_{10}) doses of Assail significantly reduced average flight distance, number of flights, and flight duration of CM females and males.

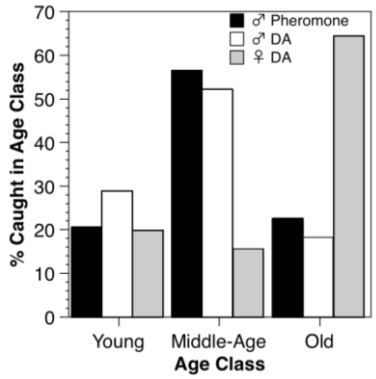
OBLR females exposed to sublethal doses of Assail and Guthion showed significant reductions in flight distance, number of flights and flight duration. Males were unaffected.

Our kaolin studies showed that a three tree border can reduce CM migration significantly, even in the face of high population pressure.

Results and Discusssion:

Objective 1. We used a combination of statistical procedures to classify all the males and females caught in our 2007-2008 seasons as young, middle aged, or old. For each moth, we evaluated mating status, size of fat body cells, amount and color of the fatbody, size of the abdomen and color of the reproductive tract. Our initial clustering analysis indicated that female classification required the size of fatbody cells, the amount of fat body, color of the fat body, and color of the reproductive tract for accurate classification. Male classification required the same variables, but also used the size of the abdomen. Using these factors, we age-classified each moth and then used discriminant analysis to evaluate the error rate. Our data showed that the error for females was only 3%, male error rate was 1.5% when moths were collected using the Trécé DA Combo lure and 0.3% when males were collected using pheromone traps. There were only minor differences in the percentage male age classes caught between the pheromone and DA Combo lures, with >50% the males being in the middle age group (Fig. 1). However, for females captured in Combo DA lures baited traps, only 35% of the were in the young or middle group, with 65% being old. These

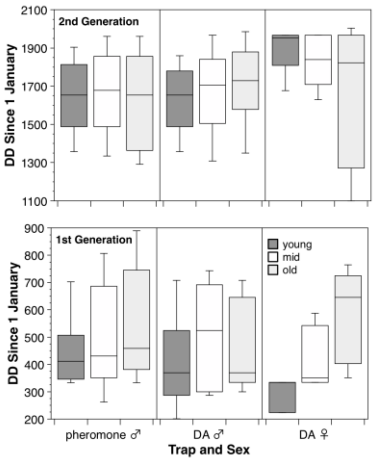
Fig. 1. Age classes of moths captured in 2007-2008 by trap type.



data show that the DA Combo trap has a bias towards sampling primarily older females.

With the classification system applied, we were able to determine the DD at which moths of different ages were caught throughout the season (Fig. 2). We found that in the first generation, the middle 50% of the distribution of young adult males was caught between 350 and 500 DD for pheromone baited traps and 290-525 DD for traps beited with DA combo lures (Fig. 2). The same values for moths classed as middle aged or old are greatly expanded particularly towards the end of the generation. Interestingly, if we target the center 50% of the collection of young adults, that would be near the timing of the delayed first cover if an ovicide or oil is used early in the season (525 DD since 1 January or 350 DD since first moth). Females tended to show a much greater separation in capture of the different age classes.

Fig. 2. DD at which the different age classes were caught in 2007-2008 by trap type, sex and generation. The boxes show the point at which 25 and 50% of moths were caught. The line in the middle shows 50% catch and the whiskers show 10 and 90% emergence.



Trends in emergence of the different age classes during the second generation do not show the large differences in spread of emergence seen in the first generation, but the tendency of slightly increasing median and broader spread is seen in the male emergence patterns (Fig. 2). The emergence of females in the second generation as measured by the DA Combo lures is considerably distorted by low number of females caught that were in good enough condition to classify, especially in the young and middle-aged groups (4 and10 moths, respectively). All the other groupings except the old males in the DA Combo lures (70 moths) had between 100 & 1000 moths and likely accurately reflect the emergence patterns in the field.

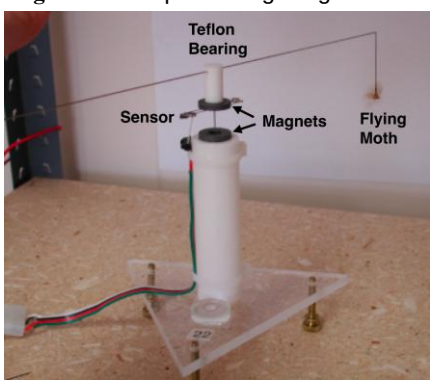
Objective 2. We have built 24 digital flight mills that can be used to evaluate dispersal capabilities of CM and OBLR and the effect of dispersal on reproduction. We are able to run 24 insects simultaneously and the data are recorded by a computer. This data allows us to determine how far and fast they fly, whether they stop and rest, the number of flights, and the duration of flights.

The flight mills use magnetic levitation and Teflon bearings to reduce friction to a minimum (Fig. 3). A one-foot long hollow tube is attached to the bearing and moths are attached to the arm by gluing a small insect pin to their back on the thorax (to prevent interference with the wing motion). The pin is then inserted into the hollow tube. As the moths fly in circles, a sensor detects a small magnet attached near the bearing on every revolution. After flight, the moths are easily separated from the insect pins so that they can be used in our reproductive experiments.

Effect of flight on reproductive output of CM and OBLR

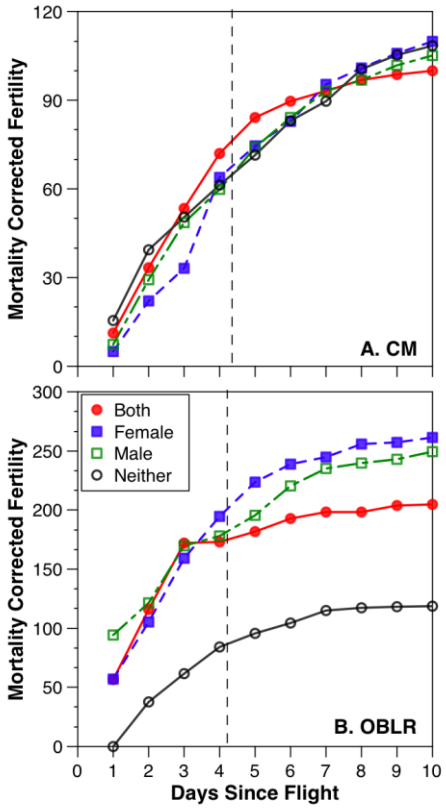
To test the effect of flight on reproductive output, we reared them until the adult stage and the day after emergence, we flew them for roughly 6,200 feet, then paired them in one of four ways: (1) both members of the pair were flown, (2) females were flown and paired with males that were tethered, but not flown, (3) males that were flown and paired with females that were tethered, but not flown, or (4) both pairs were tethered, but neither flown (control group). We then measured their daily mortality, and egg production, and the egg hatch. We used life tables to analyze the data and present the mortality corrected fertility (l_xM_x) as the effect of flight on reproductive rate.

Fig. 3. Close-up of a single flight mill.



Because moths can only take in a limited amount of energy by feeding on honeydew in the orchard, we had hoped to see that moths that flew would have had reduced survivorship, reduced sperm packet sizes (males) or egg production (females). However, what we found was that there were no significant differences in reproductive rate or mortality between CM pairs that flew and those that did not (Fig 4A). This is one of the worst case scenarios from the standpoint of population biology. Basically, if moths fly 6,200 feet, their reproduction and longevity is completely unaffected, meaning that moths migrating into the orchard can easily affect population dynamics in the new location. The 6,200 foot figure indicates that moths may move and infest >2,772 acres without an reproductive disadvantage.

Fig. 4. Effect of flying ♂ and/or ♀ CM and OBLR on reproductive output. A. CM . B. OBLR. Dotted line is average longevity in the field in the summer.



OBLR had a different response to flight than CM, with the individuals that were not flown having the lowest reproductive rate, followed by those pairs where both members of the pair were flown (Fig. 4B). There were no differences between the pairs where ony one sex was flown. This sort of result is nearly as bad as the response from CM - basically, migrating individuals have unimpared reproduction (as with CM). The only bright side is that if moths within the field do not fly much, they will have a lower reproductive rate. Unfortunately, that means the impact of the migrating moths is proportionally greater (≈ 2-2.5 fold higher) on population growth when they arrive at a new location compared to moths that do not fly before mating. Thus migrating indivduals carrying a gene for pesticide resistance should be able to easily pass that on in the area where they settle.

Objective 3*. Cover Spray Effects on CM Fligth Distances: Methods (field studies)*

2007. We concentrated on evaluating the effects of Assail and Guthion on CM movement using the protein markers. Two plots were set up in a large Brewster, WA orchard. In the first plot, trees were planted on a 12 x 18' spacing, and were 24 x 18' spacing in the second. The trapped area of both plots was roughly 12.6 acres, but the first plot was longer and narrower (1476' x 360') than the second (1115' x 492'). We placed 60 traps in the first plot and 44 in the second; in both plots, we used the Combo DA lures in standard LPD traps. Both plots were under mating disruption using a full rate of Isomate C+ dispensers.

Traps were placed uniformly throughout both blocks and checked 3 times per week during a three-week period of the first and second flights. All moths captured were dissected to determine mating status and age of the moth using the classification scheme reported in objective 1.

We applied 150 gallons of a 10% egg whites solution to the center 1.5 acres in both plots on the same dates. Egg whites were applied once per generation at roughly 25% adult emergence. In the first generation, Assail was applied to plot 1 and Guthion to plot 2. During the second generation, the treatments were reversed, so that Assail was applied to plot 2 and Guthion to plot 1.

During the first generation, we had a small amount of rain one week after treatment, and then an additional 0.3" fell (according to the Brewster Flat PAWS station) during the third week, that would have affected the data. In the second generation, 0.2" fell two days after our treatments were applied, again potentially affecting marking throughout the experiment.

2008. We set up two different plots to evaluate the effect of Assail and Guthion cover sprays on the dispersal of codling moth. The first plot was set up in Manson during the first generation, but only 14 moths were caught in 120 traps. The second plot was set up during the second generation in Quincy, and we captured 1,651 moths, of which roughly 10% (171) were positively marked.

The second plot consisted of two blocks: one was 19.5 acres (≈ 1487' long x 560' wide) and treated with Guthion and the second was 12.9 acres (≈794' long x 687' wide) and treated with Assail. Treated areas in each block were 350' long by 112' wide and situated on one end of the block, thus the furthest distance outside the marked area that could be recorded in each block was 1375' in the Guthion block and 575' in the Assail block - after those distances, there was a wide road (85'-105') between the blocks and adjacent orchards. We set up four transects in each plot away from the marker treated areas with equal distances for the full length of the Assail block; in the longer Guthion block, we added a lower density of traps out to the end of the block. For comparison of dispersal in the two plots, we initially only looked at the moth captures in the transects out to the 575' in the Guthion block, then compared the dispersal in the full range of traps with the understanding that any trap capture beyond 608' in the Guthion block would *a priori* cause higher mean dispersal distances.

We used the Trécé DA Combo lures so that we would obtain at least a small number of female moths and to have consistency in trap catch as both blocks were under mating disruption. Each moth captured was sexed and then dissected to determine moth age and mating status.

Results - 2007 Cover Spray Effects: We caught 333 moths in the two plots over both generations; 144 in plot 1 and 189 in plot 2. In plot one, 137 (95%) were caught in the first generation. In plot 2, 61% were caught in the first generation. The overall sex ratio was 89.2% males, which is similar to other studies we've performed with the Combo DA lures.

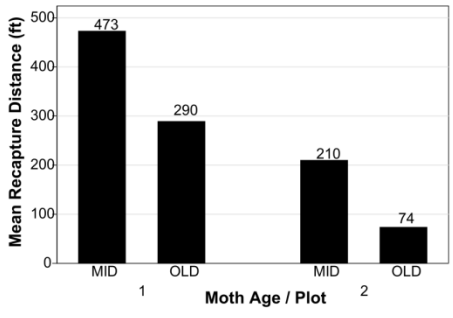
We caught 29 marked moths, 13 in plot 1 and 16 in plot 2. The low percentage marking is likely a result of the rain and restricted the complexity of the analysis that we could perform. We found that there were no significant differences in dispersal distances related to the pesticide. This likely related to the low power of the test caused by the low marking rate.

Using the age classification system, old moths dispersed an average of 160' and middle-aged moths averaged 364'. In each plot, the average dispersal distance was significantly lower for older individuals compared to middle aged ones (fig. 5). Only 3 marked females dispersed out of the area treated with egg whites, and they showed similar results to the males, with the two mid-aged females dispersing an average of 567' versus 154' for the single old female.

The evaluation of mating status also suggested that mated moth fly further than unmated ones. The results were especially pronounced in the first plot (higher tree density plot) with mated moths flying twice as far.

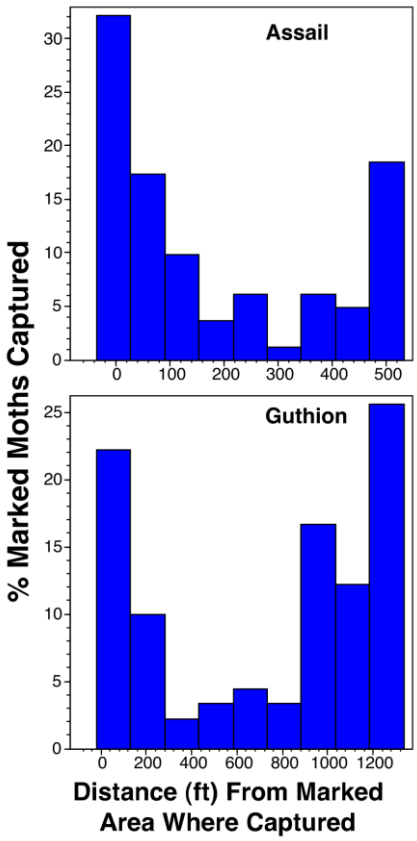
In terms of plot differences, the average dispersal distance in the first plot (395') was significantly higher than the second plot (142') (Fig. 5). This was consistent between generations and age classes, regardless of which treatment was applied. The reasons for these differences are unclear, but may be related to tree density. The first plot was planted at roughly 2x the density of the second plot and it was not on flat ground (it sloped downward from east to west roughly 82'). Our previous studies have shown that wind velocity in higher density orchards tends to be lower than in low

Fig. 5. Average recapture distance for middle-aged and old CM in Brewster 2007.



density orchards, suggesting that moths may be able to fly a greater percentage of the time (our studies in another project showed flight at wind speeds > 3.3 mph is rare and that moths are unable to locate lures at those wind speeds). The potential difference in the amount of time moths are flying would tend to allow them to move further in the high-density orchard where wind velocity would be < 3.3 mph a greater percentage of the time.

Fig. 6. Percentage of marked moths captured at different distances from the marked area. In both plots, moth captures rose sharply at the end of the plot where a wide drive row was present.



Cover Spray Effects 2008

As usual, there was a strong bias towards males (91% males) in the DA Combo lures with no significant difference in sex ratio between the two treatments. Because of the low percentage of females captured, unless mentioned, all analyses are restricted to male moths. Analysis of the age distributions of marked moths captured in each block also showed no significant differences between the blocks and averaged 11% young, 54% middle aged, and 35% old. This ratio is similar to that of unmarked moths caught in the plots.

In terms of the average distance moved, there were no significant differences resulting from either pesticide ($p = 0.3$) or age ($p = 0.08$) when the Guthion plot data were restricted to the same trapping grid size as the found in the Assail plot.

Probably the most interesting factor in the data was the differences in trap capture within the plot (Fig. 6). When evaluating the data for moth capture in both treatments, individuals dispersing from the edge of the plot tended to be found at high levels near the marking area and at the furthest edge of the block. Moth behavior is apparently strongly affected by the wider drive rows found at the end of the block. A possible explanation might be the trap density (and hence competition between traps) is effectively lower at the ends where traps are not found outside the plot, but our larval distribution studies performed a few years ago for improving the Taiwan sampling program showed the same trends of heaviest populations being found on the borders in roughly 80% of the orchards sampled.

Cover Spray Effects Using Flight Mills:

The variability obtained in the field experiments means that it is difficult to show statistically significant differences related to pesticide applications, but does not mean that they don't exist. In the field, we cannot control moth age, weather patterns, trap catch, marking rates, and tree density, size and other factors except within broad limits. The costs of doing the large experiments also preclude doing large numbers of them at any one time. To address these issues, the flight mill technology we developed for objective two seemed like an obvious method to try. In the lab studies, we can control all the different variables, and relatively quickly (and cheaply) determine how sublethal doses affect flight. Clearly, testing moths on the flight mills is extremely artificial and is not indicative of normal behavior in the field. However, flight mill data gives us good information on the physiological capabilities of the moths under a given set of conditions, in our case when exposed to sublethal doses of Assail and Guthion. Being able to evaluate moths in the laboratory means that we have a much less expensive way to test pesticides than using field experiments.

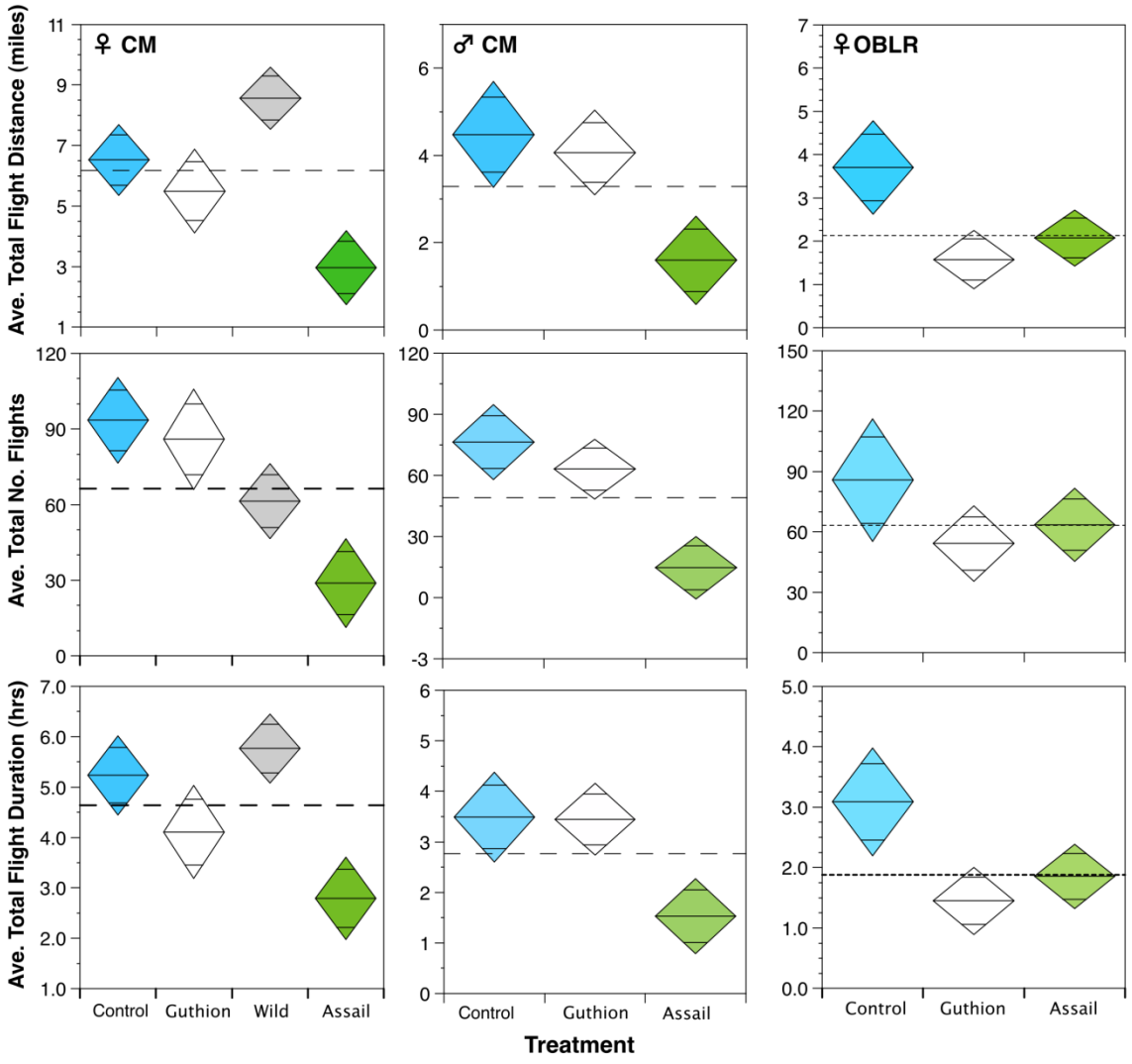
In this study, we applied a sub-lethal dose of guthion or assail to the container the moths were held in for 24 hours before flight. Moths were then attached to the flight mill and flight distances, number of

flights, and total flight duration were recorded. In the case of codling moth, we used moths from the USDA-Wapato colony, but also flew female moths that were emerging from bands that had been collected the previous fall. These wild moths can serve as a second control that provides information on how well the Wapato colony reflects wild-type flight characteristics. We flew males and females separately. OBLR tested came from the TFREC colony.

Results: The wild CM female moths flew significantly further than the Wapato female control moths; there were fewer flights, but they were longer duration on average (Fig. 7). The guthion-treated female moths showed a non-significant reduction in the total flight distance, number of flights and average flight duration compared to the control moths. However, the assail treatment significantly reduced all three parameters 54, 69 and 47% for flight distance, number of flights, and flight duration, respectively. Clearly, Assail even at low doses strongly affected moth flight. Effects on male CM were similar in terms of the effects and significance of them (Fig. 7).

The OBLR females treated with either guthion or assail had significantly lower flight distances, duration and number of flights compared to the control moths (Fig. 7). There were no significant differences between female moth flight parameters between the two pesticide treatments. In complete contrast, the OBLR males showed no significant differences between control, guthion or assail treatments in any flight parameter.

Fig. 7. Mean diamond plots for CM and OBLR flight distance, number of flights, and flight duration. Dotted lines on plots indicate the overall mean for a comparison, the line in the middle of the diamond is the mean, the upper and lower lines indicate where if ther is no overlap that treatments are signficantly different at $p = 0.05$.



These studies clearly show that migratory ability and movement in the field can be affected by the pesticide used, even when applied at very low levels. Evaluating a range of differnet pesticides could be used to help manage codling moth and OBLR, particularly when movement into the plot from an outside source is a key factor in a particular management situation. In situations where high levels of movement are suspected, Assail would be a much better choice than guthion for codling moth, either material would reduce flight of female OBLR.

Cover Spray Effects: Kaolin

2008. We set up two different trials to evaluate the ability of a three tree row barrier to reduce CM movement from high population to low population areas. In the first trial, a large abandoned orchard was removed adjacent to a commercial block. Although the grower applied a Surround treatment to the border, it was only applied once at a low rate and washed off completely within a small amount of time. We sampled 795 trees at the orchard, but in all plots, damage was less than 0.05%, so no analysis was possible.

The second trial was run in a small planting at WSU-TFREC with extraordinary levels of codling moth pressure, coming from surrounding infested blocks as well as present within the block. Early in the first generation, we started treating the bottom half of the plot with insecticides and left the top part of the plot untreated. A three-row barrier of surround was applied twice to the center of the plot and to one edge (Fig. 8). We sampled every tree in the block by evaluating 60 half-fruit per tree for CM damage at the end of the first and second generations. There were no sprays applied during the second generation. We compared the damage found on either side of the surround barrier, looking at both overall damage in each subplot, and looking at the differences within a plot in the three tree rows adjacent to the surround barrier and then the three rows furthest from the surround barrier.

2009. We set up two studies, one at WSU-TFREC that had high pest pressure and one in east Wenatchee where two plots occurred side-by-side with one of the growers complaining that high pressure was coming from his neighbor for the past several years.

The plot at WSU-TFREC was treated with Kaolin at 50 lbs/acre a total of three times so that we had a clearly demarked area from what we felt was the direction of movement. We sampled the plots three times (1st, 2nd generation, harvest), evaluating ≈ 53,000 fruit for damage over the three periods. Unfortunately, we made the mistake of not treating the entire plot for first generation CM, because we knew it had been treated the year before (but obviously not well!). This resulted in very high damage that occurred early on that masked any movement restriction by kaolin treatments.

The test in east Wenatchee consisted of three apple blocks of 14.5, 18, and 17.5 acres (Fig. 9). The experiment was set up so that we had areas where kaolin was applied between the different plots so that we could see if migration from one plot was limited and egg laying occurred. We used 28 pheromone traps to evaluate flight and sampled >189,000 fruit over the three sampling periods (1st, 2nd generation, harvest). Kaolin was applied three times in the first generation and three times in the second to portions of the presumed moth source block (grey boxes in figure 9). The idea was to see if we could stop movement and damage in the areas across from the kaolin treated two rows. We treated two rows within the source block and in the lower right quadrant, the two rows across from the source block; the idea being to test if we could hold populations within the source block versus keep them in the outer two rows in the bottom right block.

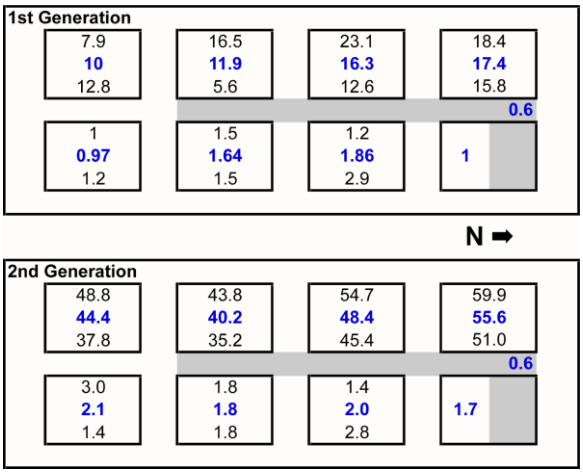
2008 Results: The heavy insecticide treatments of the lower four plots in the first generation resulted in very low damage levels in those plots compared to the top four plots that were untreated (Fig. 8). Overall, damage was highest on the north (right) border of the block and decreased in each successive plot to the south (left). There were no significant differences between the upper and lower three rows, except in the second plot from the left in the top row.

In the second generation, the average damage in the top plots increased significantly (3-4 fold) over the levels in the first generation, but the lower block damage levels did not increase significantly from

the first generation. The greatest increase was in the lower left plot which was not protected by the surround barrier, but that still remained very low (Fig. 8).

The data show that the surround barrier is very good at reducing migration between plots. The three plots on the bottom right were unaffected by the high populations on the top side of the Surround border. The bottom left plot that was not protected by the barrier is the only question. It appears that the TFREC blocks to the north of our test block were the source of pressure and the kaolin border stopped migration from those blocks and then funneled the moths down the tree rows in the upper plots. However, it is still a question why moths that laid eggs on the top left block did not move down into the unprotected block below in greater numbers.

Fig. 8. Layout of kaolin experiment at WSU-TFREC. Each square represents a plot; gray areas were treated twice with Surround in the first generation. The three numbers represent the average % damage for the plot (center number) and the three rows furthest and closest to the Surround border. The one number in the lower right plot is the average for the plot; not enough trees were present to tabulate.



2009 Results: The moth catch was heaviest in the presumed source block with relatively low levels occuring in the other two apple blocks. Damage did not correspond precisely to the trap catch, where the highest catches occurring near the kaolin treated areas in the presumed source block (Figs. 9 and 10). Overall, the damage was extraordinarily low (no damage to the apple block to the south west, and 0.7 and 0.4% damage in the source and north east block, respectively).

In regards to the kaolin treatment in this experiement, we did not see a reduction in damage that could be directly attributable to the kaolin treatments, other than in the kaolin treated rows, which had no damage present. The orchard block to the south west had no damage occur within it, despite some high moth counts and high damage in the adjacent presumed source block. This shows that CM treatments in conjunction with mating disruption can reduce or prevent damage when used correctly.

The lack of results in this situation is perplexing compared to the work the pervious year where we found the kaolin treatments allowed us to strongly channel moth movement away from parts of the plot, even on a relatively small scale and in the face of heavy pressure. Hindsight suggests that it might have been better to have modified the spray so that we would have prevented moths moving north -south from cutting behind the treated rows by placing additional treated strips (dotted boxes in Fig. 10) - this would not be needed in a normal commercial application where treated rows would not be broken up as our experimental protocol dictated.

Overall, the use of kaolin sprays as border treatments to reduce codling moth movement from a high level area to a low level area still needs more work, but the results from our studies last year and in 2005 strongly suggest that it can stronly reduce movement and damage with only a few boarder rows needing treatment. The most difficult aspect of this project is finding plots where damage is sufficient to show differences and where other aspects of orchard design (e.g., row spacing, different tree sizes, row direction differences) do not potentially complicate the results or mask the effects when applied on an experimental scale.

Fig. 9. Kaolin plot in East Wenatchee; numbers indicate the number of CM adults captured season long in the 28 traps spaced between the different plots. Gray boxes show where kaolin was applied.

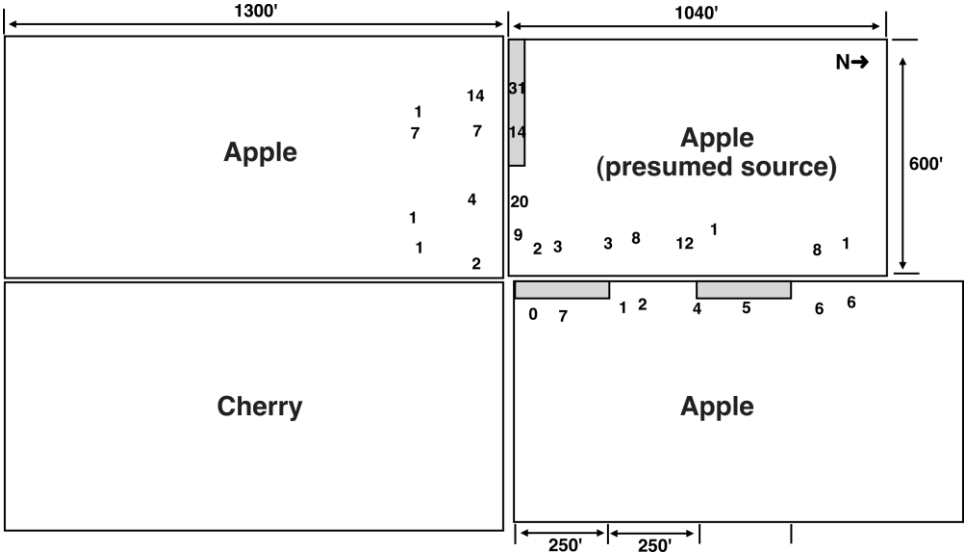
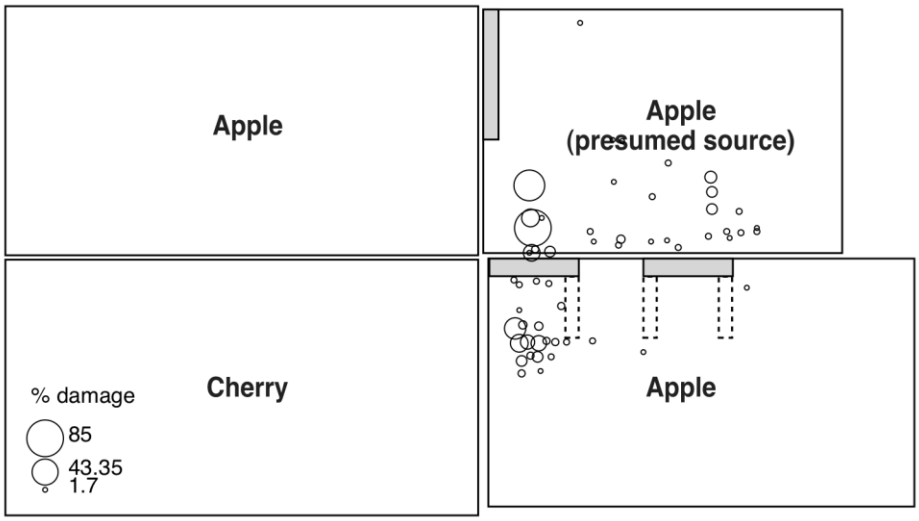


Fig. 10. Kaolin plot in East Wenatchee; circle size is proportional to damage (scale shown in lower right corner), samples with no damage are not shown for clarity. Gray boxes show where kaolin was applied; dotted boxes are where we should have added to the barrier to prevent moths moving north-south from slipping behind the kaolin barrier.



Executive Summary – Significance of This Project to the Industry.

Objective 1. The classification system provides us with information that is useful in understanding the overall population dynamics of codling moth. For example, it suggests an additional reason why the delayed first cover strategy is effective. Our data suggests that targeting the period when young moths ocurr in the first generation might be a useful tactic, but that tactic probably is considerably less useful in the second generation, in part because of the rapid aging that occurs during the summer because of high daily DD accumulations. The large number of old moths caught late in each generation suggests that control measures applied at that time are relatively less useful because the reproductive potential of older moths is so low compared to younger or middle age moths.

Objective 2. The 6,200 foot flight was chosen as one that would be a reasonable distance for moths to fly based on our studies from the pesticide effects study (objective 3). Our data in the reproductive effects sections show that for both CM and OBLR moths within a fairly large area can be considered to be a single interacting population, although clearly the probabilility of mating between moths decreases as the distance between them increases. For CM, flying the 6,200 feet did not affect population growth. For OBLR, non-flying pairs had a lower reproductive rate than those were either sex or both sexes of the pair were flown. This may mean that migrating individuals that carry a gene for resistance will be able to easily pass it on to the population in the area where they finally establish; their growth rate is roughly 2.5 fold higher than the non-migrating pairs.

Objective 3. Our field studies were highly variable and did not provide statistically significant differences in the flight distances of CM between plots treated with guthion or assail. This is likely caused by variability between blocks, small elevational differences, low capture rates, and a range of other factors inherent in field studies. However, use of our flight mills showed that it was easy to detect effects of sub-lethal doses on flight duration, distance, and number of flights. CM males and females were both strongly affected by Assail, but not Guthion. OBLR females were strongly affected by Assail and Guthion, while OBLR males were unaffected. Choice of Assail for CM cover sprays would greatly reduce movement potential for either CM or OBLR.

Kaolin barriers were shown in 2008 to be highly effective in channelling migrating moths around areas and reducing damage significantly. In 2009, we did not see this, primarily because of poor choices in the layout of our experimental plots. The 2008 data along with data from a previous project results strongly suggest that 2-3 rows around the border adjacent to a source of migrating moths will significantly reduce damage from migrating individuals.

Future Studies

Objective 1. The classification system is complete and will likely play a role in helping us understand how to focus our control programs for better efficacy or to evaluate current programs more effectively. Further focused studies are not needed for this objective.

Objective 2. The reproductive effects studies are complete and strongly suggest that both CM and OBLR flying ≈6,200 feet are not sacrificing either longevity or reproductive output to fly that distance. These data mean that control of these migrating individuals is necessary. Further studies on this area are not needed.

Objective 3. Our flight mill studies showed that pesticide choice can strongly effect the flight behavior of both CM and OBLR. Further studies in this area would be very productive and reasonably priced, if only to evaluate the current suite of insecticides as to their effect on migration.

Kaolin studies would also be worthwhile if performed on a very large scale (e.g., muliple plots per year for multiple years). These studies are expensive, and difficult to set up and highly dependent upon the likelihood of true migration coming from a particular direction versus a person's tendency to "blame the neighbor" for damage within a particular orchard.

FINAL PROJECT REPORT **WTFRC Project Number:** CP-07-701

Project Title: Augmenting fungal control in apples with natural compounds

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Other funding sources: **None**

Total Project Funding: $100,706

Budget History:

Item	Year 1: 2007	Year 2: 2008	Year 3: 2009
Salaries	$20,276	$21,880	$23,924
Benefits			
Wages			
Benefits			
Equipment	$7,146	$4,491	$4,449
Supplies	$3,857	$5,987	$6,357
Travel	$1,142	$1,197	
Miscellaneous			
Total	$32,421	$33,555	$34,730

RECAP ORIGINAL OBJECTIVES

The goal of this research is to find new, safe natural compounds that effectively improve activity of conventional fungicides for pre/ post-harvest treatment of apple. We are trying to identify molecular targets of these compounds using genomic tools and determine effective methods for delivery of discovered compounds. This research will greatly improve the ability to suppress decay of apples, a priority identified by the WTFRC. Our specific objectives are:

1. Determine an effective method for delivery of newly discovered natural compounds, leading to a target-specific strategy for a safe and economic approach to fungal pathogen control in the field or during processing and storage.
2. Identify the most efficient molecular targets [*e.g.*, mitochondrial superoxide dismutase (Mn-SOD), cellular antioxidation system, *etc*.] for newly discovered compounds using functional genomics approaches.
3. Augment antifungal activity of natural compounds, through synthesis of structural derivatives, based on structure-activity relationships of analogs of identified antifungal natural compounds.

SIGNIFICANT FINDINGS (Last 3 years):

[A] Identify the most efficient molecular targets:

- Fungal response (tolerance) to 2,3-dihydroxybenzaldehyde or 2,3-dihydroxybenzoic acid was found to rely upon mitochondrial superoxide dismutase (*SOD2*) or glutathione reductase (*GLR1*), genes regulated by the *HOG1* signaling pathway, respectively. Thus, certain benzo analogs can be effective at targeting cellular oxidative stress response systems.
- The *SLT2* and *BCK1* genes, encoding mitogen-activated protein kinase (MAPK) and MAPK kinase kinase (MAPKKK) in cell wall integrity pathway, respectively, were essential for fungal tolerance to thymol, *o*-coumaric acid, 2,3-dihydroxybenzaldehyde and berberine hemisulfate.

[B] Determine an effective method for delivery of newly discovered natural compounds:

- Activity of conventional antifungal agents, fludioxonil, strobilurin and antimycinA, which target the oxidative and osmotic stress response systems, was elevated by co-application of certain benzo analogs (aldehydes and acids).
- The activity of conventional antifungal compounds interfering with cell wall integrity, *i.e.*, Congo red or calcofluor white, was elevated by coapplication with thymol or 2,3-dihydroxybenzaldehyde, demonstrating the chemosensitizing capacity of the identified compounds on fungal growth.

[C] Overcome the tolerance of *Penicillium expansum* to fludioxonil and strobilurin through chemosensitization by using natural compounds:

- We investigated the mechanism of two fludioxonil resistant strains of *Penicillium expansum*, *i.e.*, FR2 and FR3, provided by Dr. Xiao. Our study indicates that fludioxonil resistance of the FR2 and FR3 strains is a result of UV-induced mutation of cellular redox homeostasis.
- Increased sensitivity of FR3 to oxidizing agents, compared to its parental strain (W2), indicated the oxidative stress response system of FR3, such as the mitogen-activated protein kinase (MAPK) pathway, had become defective as a result of UV-treatment.
- Alternatively, the other resistant strain, FR2, showed higher tolerance to oxidizing agents compared to its parental strain (W1), suggesting fludioxonil resistance of FR2 was based upon gain-of-function, possibly increased antioxidation activity.
- To overcome the resistance in both strains, redox-active natural phenolics were successfully used as chemosensitizing agents that targeted various elements of the oxidative stress-response

pathway. Co-application of certain natural phenolic compounds with fludioxonil overcame fludioxonil-resistance in two mutant strains, FR2 and FR3, of *P. expansum*.

- Natural phenolics were also found that served as chemosensitizing agents for overcoming resistance to strobilurin.

Methods:

Chemicals. Test chemosensitizing agents: thymol [5-methyl-2-(isopropyl)phenol], 2,3-dihydroxybenzaldehyde, gallic acid and ester analogs (methyl-, ethyl- and octyl- gallates); antifungal agents: fludioxonil, kresoxim-methyl, antimycin A; and oxidizing agents: menadione, hydrogen peroxide (H_2O_2), diamide; other compounds such as alkaloid, cell wall interfering agents (Congo red, calcofluor white) were purchased from Sigma Co. (St. Louis, MO). Each compound was dissolved in dimethylsulfoxide (DMSO; absolute DMSO amount: < 2% in media) except H_2O_2 and diamide, which were dissolved in water, for incorporation into media.

Microorganisms and culture condition. *Penicillium expansum* FR2 and FR3, fludioxonil resistant mutants (Li and Xiao, 2008) and their parental strains, W1 and W2, respectively, were grown at 28 °C (82.4 °F) on potato dextrose agar (PDA). *Aspergillus fumigatus* AF293, wild type, and *A. fumigatus* MAPK deletion mutants *sakA*Δ and *mpkC*Δ (Xue *et al*. 2004; Reyes *et al*. 2006) were grown at 37 °C (98.6 °F) on PDA (5 to 7 days). *P. expansum* NRRL974 and *A. flavus* NRRL3357 (obtained from National Center for Agricultural Utilization and Research, USDA, Peoria, IL) were cultured at 28 °C (82.4 °F) on PDA (5 to 7 days).

Saccharomyces cerevisiae wild type BY4741 (*Mat* a *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) and selected deletion mutants lacking genes in (a) antioxidative stress response/multidrug resistance systems or (b) cell wall construction/integrity system were obtained from Invitrogen (Carlsbad, CA) and Open Biosystems (Huntsville, AL), as follows (See also Kim *et al*., 2005, 2007, 2008b): Gene regulation mutants: *yap1*Δ, *msn2*Δ, *msn4*Δ, *hot1*Δ, *sko1*Δ, *rim101*Δ; Transporter/assembly protein mutants: *flr1*Δ, *yor1*Δ, *pdr5*Δ, *vph2*Δ, *tfp1*Δ/*vma1*Δ; Signal transduction mutants: *sho1*Δ, *sln1*Δ, *ste50*Δ, *ste20*Δ, *ypd1*Δ, *ssk1*Δ, *ptp2*Δ, *ptp3*Δ, *hog1*Δ, *hog4*Δ, *ssk22*Δ, *ssk2*Δ, *ste11*Δ; Antioxidation mutants: *ctt1*Δ, *cta1*Δ, *osr1*Δ, *trr1*Δ, *trr2*Δ, *tsa1*Δ, *grx1*Δ, *grx2*Δ, *trx1*Δ, *trx2*Δ, *glr1*Δ, *gsh1*Δ, *gsh2*Δ, *sod1*Δ, *sod2*Δ, *ahp1*Δ; DNA damage control/energy metabolism mutants: *rad54*Δ, *sgs1*Δ, *acc1*Δ, *gpd1*Δ, *hor2*Δ;Osmoregulation mutant: *hog1*Δ (MAPK), *hog4*Δ (MAPK kinase; MAPKK; Scaffold activity), *ssk22*Δ (MAPKK kinase; MAPKKK), *ssk2*Δ (MAPKKK), **ste11*Δ (MAPKKK); Cell wall construction/integrity mutant: *slt2*Δ (MAPK), *mkk1*Δ (MAPKK), *mkk2*Δ (MAPKK), *bck1*Δ (MAPKKK), *wsc1*Δ (Sensor-transducer), *mid2*Δ (Sensor for cell wall integrity signaling), **kss1*Δ (MAPK), **ste7*Δ (MAPKK), **ste11*Δ (MAPKKK); Morphological switch mutant: **kss1*Δ (MAPK), **ste7*Δ (MAPKK), **ste11*Δ (MAPKKK); Mating response mutant: *fus3*Δ (MAPK), **ste7*Δ (MAPKK), **ste11*Δ (MAPKKK), *ste5*Δ (Scaffold protein), *ste2*Δ (Receptor for "alpha" factor pheromone), *ste3*Δ (Receptor for "a" factor pheromone); Sporulation mutant: *smk1*Δ (MAPK); PKC-signaling pathway mutant: *rom2*Δ (GDP/GTP exchange protein), *fks1*Δ (β-1,3-D-glucan synthase), *fks2*Δ (β-1,3-D-glucan synthase), *rlm1*Δ (Mcm1p-Agamous-Deficiens-Serum Response Factor <MADS>-box transcription factor), *swi4*Δ (Transcription factor), *pkc1*Δ (Protein serine/threonine kinase; dipoid), where the asterisk (*) indicates overlapping biological roles of the marked gene in more than one pathway (Reference for the description of each deletion mutant: www.yeastgenome.org). Yeast strains were grown on YPD (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose) or SG (0.67% Yeast nitrogen base w/o amino acids, 2% glucose with appropriate supplements: 180 µM uracil, 200 µM amino acids) medium at 30 °C (86 °F; 5 to 7 days).

Antifungal bioassays. Yeast cell dilution bioassays were performed as described (See Kim *et al*., 2005, 2007). Sensitivities of filamentous fungi to the compounds were based on percent radial growth of treated (T) compared to control (C), receiving only DMSO, colonies and/or based on the Vincent equation [% inhibition of growth = 100 (C-T)/C, C: diameter of fungi on control plate; T: diameter of fungi on the test plate] (Vincent 1947), if necessary. Fungi (5 x 10^3 spores) were diluted in phosphate

buffered saline and spotted on the center of PDA plates (triplicates) with or without antifungal compounds. Growth was observed for 3 to 7 days. Effectiveness of chemosensitization by thymol (0.2 to 0.6 mM), octylgallate (0.05 to 0.2 mM) or 2,3-dihydroxybenzaldehyde (2,3-D; 0.05 to 0.3 mM) was assessed by incorporating each compound into growth media with fludioxonil or strobilurin (kresoxim-methyl; 0.02, 0.04, 0.06 mM). Radial growth was recorded as described above. Oxidizing agents, menadione (0.001 to 0.512 mM), hydrogen peroxide (0.5 to 5 mM) or diamide (0.5 to 5 mM) were incorporated into media at respective levels and fungal sensitivities were measured by fungal radial growth, as described above. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of a compound where no fungal growth was observed.

RESULTS & DISCUSSION:

Fungal tolerance to benzo analogs depends on cellular Mn-SOD. *Based on yeast cell dilution bioassays, 2,3-dihydroxybenzaldehyde had the highest antifungal activity,* i.e.*, no visible growth of wild type* S. cerevisiae *at ≥ 80 μM, among eight benzo analogs tested. Highest to lowest antimicrobial activity was, as follows: 2,3-dihydroxybenzaldehyde > 2,5-dihydroxybenzaldehyde > 2,4-dihydroxybenzaldehyde > 3-hydroxybenzaldehyde > vanillin, 4-hydroxybenzaldehyde, veratraldehyde > benzaldehyde. An almost identical relationship in the relative antifungal activities of the analogs was observed among the various fungi,* i.e.*,* Penicillium *and aspergilli tested.*

Among forty-five mutants of *S. cerevisiae* examined, where genes in oxidative stress response/multidrug resistance systems were individually deleted, the *sod2*Δ [mitochondrial superoxide dismutase (Mn-SOD) deletion] mutant showed hypersensitivity to 2,3-dihydroxybenzaldehyde (at 10 μM) compared to the wild type strain. This greater sensitivity strongly indicated Mn-SOD activity is crucial for fungal response/tolerance against toxicity of benzaldehyde derivatives. It appears this gene is a promising candidate as a potential target for fungal control.

The *sakA*Δ mutant of *A. fumigatus* is hypersensitive to benzaldehyde derivtives. *Aspergillus* can be used as a model filamentous fungal pathogen to validate the target or mode of action of natural compounds identified through yeast screening in view that the entire genome of several species of *Aspergillus* (*i.e., A. nidulans, A. fumigatus, A. flavus*) have been sequenced and annotated to a great extent, and manipulation of its genome has been well established, allowing the studies of functional genomic responses to treatments. Responses of *sakA*Δ and *mpkC*Δ mutants derived from *A. fumigatus* AF293, to the benzo analogs was also examined. The MAPKs SakA and MpkC are orthologous proteins to Hog1p of *S. cerevisiae* (Xue *et al*., 2004; Reyes *et al*., 2006). Growth of AF293 and *mpkC*Δ was inhibited by 32 to 72% with 0.2 to 0.25 mM 2,3-dihydroxybenzaldehyde, respectively. The *sakA*Δ strain was more sensitive to these treatments, showing 56 to 100% reduction, respectively, in radial growth. Like Hog1p in *S. cerevisiae*, SakA may play a role in regulating Mn-SOD activity and, thus, tolerance to 2,3-dihydroxybenzaldehyde. The same trends were observed with 2,5-dihydroxybenzaldehyde, but higher concentrations (0.8 to 1 mM) were needed to achieve similar levels of growth inhibition.

2,3-Dihydroxybenzoic acid inhibits fungal growth by disrupting cellular glutathione homeostasis. The acid derivative of 2,3-dihydroxybenzaldehyde, 2,3-dihydroxybenzoic acid, was also examined in order to investigate structure-activity relationships with regard to acid or aldehyde moieties. The 2,3-dihydroxybenzoic acid inhibited growth of *S. cerevisiae* (MIC in wild type ≥ 7 mM). Also, growth of a number of *S. cerevisiae* deletion mutants was inhibited by 2,3-dihydroxybenzoic acid at 4 mM, including *glr1*Δ, *gsh1*Δ, *gsh2*Δ, *vph2*Δ (vacuolar ATPase assembly protein deletion), *vma1*Δ (vacuolar ATPase deletion). Also, like in treatments with 2,5-dihydroxybenzoic acid in our prior study (Kim *et al*., 2007), exogenously supplemented GSH resulted in a strong recovery of growth of these *S. cerevisiae* strains. These findings suggest the mechanism of antifungal activity of 2,3-dihydroxybenzoic acid is, as with the 2,5- analog, disruption of cellular GSH homeostasis. Thus, the GSH reductase gene (*GLR1*), another gene relatively downstream within

the *HOG1* signaling pathway, may play an important role for fungal tolerance to this, or related, compounds. The sensitivities of the *vph2*Δ and *vma1*Δ mutants may result from disruption of the normal ability for transportation, sequestration and detoxification of toxic compounds in vacuoles.

The concordance of these results demonstrates there is a structure-activity relationship between the acid and aldehyde moieties in that they affect different target genes in the *HOG1*-signaling pathway. The 2,3- and 2,5- dihydroxybenzaldehydes targeted *SOD2*. Whereas, 2,3- and 2,5-dihydroxybenzoic acids targeted *GLR1*, disrupting glutathione homeostasis.

Chemosensitization to conventional fungicides by 2,3-dihydroxy benzaldehyde and benzoic acid derivatives: targeting cellular signal transduction/oxidative stress response systems.

Some fungi having mutations in certain MAPK genes, involved in signal transduction of oxidative stress responses, can escape toxicity of phenylpyrrole fungicides, such as fludioxonil (Kojima *et al*., 2004). In this regard, we found MAPK mutants, *sakA*Δ and *mpkC*Δ, of *A. fumigatus* were tolerant to fludioxonil toxicity. However, co-application of 2,3-dihydroxybenzaldehyde (at 0.2 mM) or 2,3-dihydroxybenzoic acid (at 11 mM) with fludioxonil effectively prevented these mutants from developing this tolerance to fludioxonil. This prevention of tolerance by co-application of either of these compounds may result from the disruption of genes downstream in this MAPK pathway. In particular, based on the results with the deletion mutants of *S. cerevisiae* it is likely that these aldehyde and acid analogs target the antioxidative gene *sod2* and the glutathione homeostasis genes.

The potential chemosensitizing effect of 2,3-dihydroxybenzaldehyde was also tested on the activity of kresoxim-methyl, a strobilurin fungicide, and antimycin A. Co-application of 2,3-dihydroxybenzaldehyde enhanced the antifungal activity of both fungicides against the filamentous fungi examined, *A. fumigatus*, *A. flavus* and *P. expansum*. Co-application of 100 or 200 µM 2,3-dihydroxybenzaldehyde to kresoxim-methyl (25 µM; **Figure 1a**) or antimycin A (5 µg mL^{-1}; **Figure 1b**) resulted in complete (100%) inhibition of fungal growth, except *A. flavus* (70% inhibition). Whereas, if any of these compounds are applied alone at these rates fungal growth is only slightly inhibited.

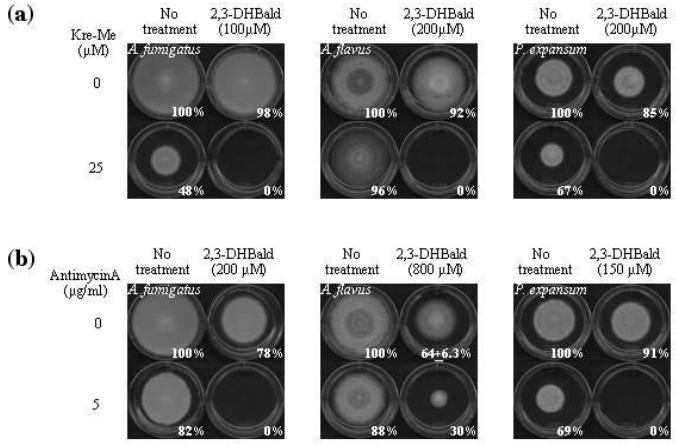

Figure 1. Targeting the mitochondrial antioxidative stress system with 2,3-dihydroxybenzaldehyde in combination with (**a**) strobilurin (kresoxim-methyl) or (**b**) antimycin A had an enhanced antifungal effect against the filamentous fungi, *A. fumigatus* AF293, *A. flavus* NRRL 3357 or *P. expansum* NRRL 974. Standard deviation: <5%, except where noted.

Natural compounds to which *slt2*Δ/*bck1*Δ mutants of *S. cerevisiae* showed sensitivity: chemosensitization of cell wall-interfering agents. We then tested the antifungal effects of natural

compounds by using *slt2*Δ, *bck1*Δ, *wsc1*Δ and *swi4*Δ mutants (See Methods) of *S. cerevisiae*. We found that *slt2*Δ and *bck1*Δ mutants showed the highest sensitivity (10^3 to 10^4 times) to thymol, 2,3-dihydroxybenzaldehyde, *o*-coumaric acid and berberine comparing to the wild type or *wsc1*Δ/*swi4*Δ strains.

In the bioassay using *S. cerevisiae*, thymol showed chemosensitizing effect to Congo red, where co-application of thymol (0.5 mM) and Congo red (100 and 500 μg/ml) resulted in ~10 to 10^4 times higher sensitivity of yeast cells (wild type, *slt2*Δ and *bck1*Δ mutants) comparing to the independent treatment of each compound. Results also showed that co-application of Congo red (0.4 to 1.0 mg/ml) with thymol (1.0 mM) or 2,3-dihydroxybenzaldehyde (0.2 mM) enhanced its antifungal activity against *A. fumigatus* (*i.e.*, ~95% to 100% growth inhibition; **Figure 2A**), while no chemo-sensitizing effect was observed with *o*-coumaric acid or berberine (data not shown), indicating thymol or 2,3-dihydroxybenzaldehyde may affect common cellular target of cell wall-interfering agents. We found that 100% of growth inhibition can be achieved in *A. fumigatus* AF293, *A. flavus* 3357 and *P. expansum* 974 (**Figure 2B**) by co-application of thymol and 2,3-dihydroxybenzaldehyde, strongly indicating these two compounds affect common cellular target.

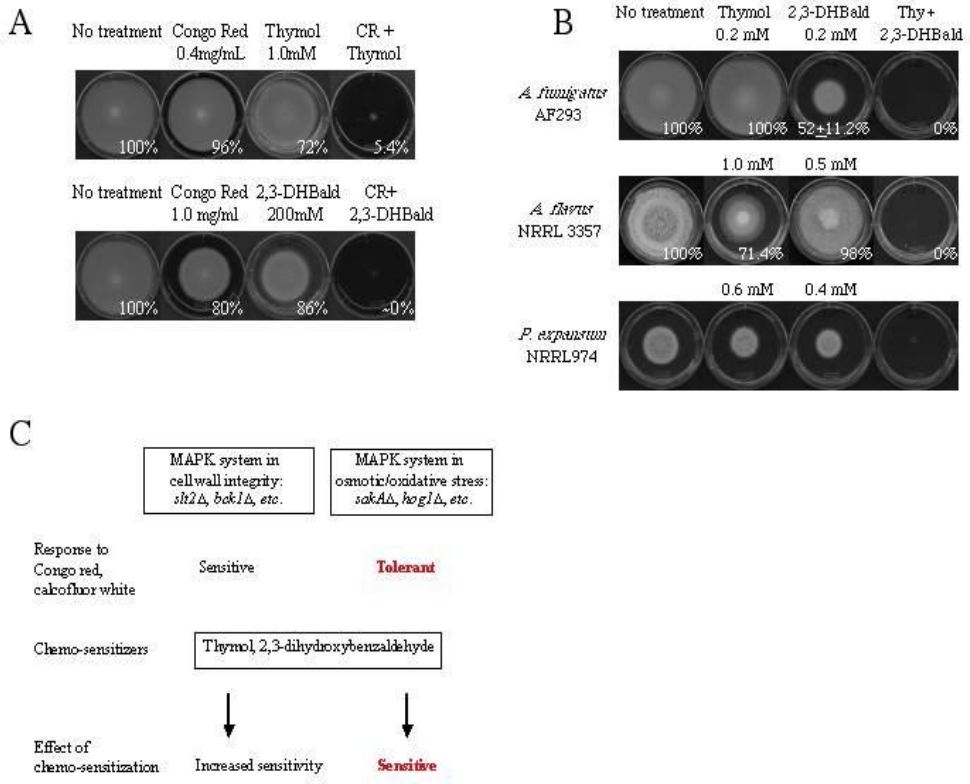

Figure 2. (A) Co-application of Congo red (0.4 to 1.0 mg/ml) with thymol (1.0 mM) or 2,3-dihydroxybenzaldehyde (0.2 mM) enhanced its antifungal activity against *A. fumigatus* (*i.e.*, ~95% to 100% growth inhibition), (B) Co-application of thymol and 2,3-dihydroxybenzaldehyde achieved 100% growth inhibition in *A. fumigatus* AF293, *A. flavus* 3357 and *Penicillium expansum* 974, indicating these two compounds affect common cellular target. (C) Diagram summarizing the chemosensitizing effect of thymol and 2,3-dihydroxybenzaldehyde on fungi.

Overcoming fungal tolerance to antifungal agents by using natural compounds: *characteristics of* P. expansum *fludioxonil resistant mutants.* UV-induced generation of *P. expansum* FR2 and FR3, fludioxonil-resistant mutants, was previously reported elsewhere (Li and Xiao, 2008). Further characterization of these mutants performed in this study is summarized as follows:

(A) Reduced growth rate of FR2 and FR3 on normal medium: FR2 and FR3 mutants showed reduced radial growth on PDA (w/o fludioxonil) compared to their respective parental strains, *i.e*., W1 and W2, respectively (FR2: 11-17% reduction; FR3: 25-30% reduction). Results indicated that FR2 and FR3 possessed inherently lower hyphal-growth activity.

(B) Fludioxonil resistance of FR3: When grown on fludioxonil-containing medium, FR3 showed higher resistance to fludioxonil than W2. FR3 showed only a 3-14% growth reduction as compared to a 17-100% growth reduction of W2 on media containing 0.02-1.0 mM fludioxonil compared to W2 controls. Interestingly, the growth rate of FR3 exposed to fludioxonil always exceeded that of FR3 controls [*i.e.*, 19-35% higher radial growth with fludioxonil (0.02-1 mM) than without fludioxonil].

(C) Fludioxonil resistance of FR2: FR2 also showed higher fludioxonil resistance than W1, with FR2 showing a 21-38% growth reduction compared to a 66-100% reduction of W1 under fludioxonil treatments (0.02-1 mM) compared to growth of W1 controls. However, unlike FR3, the growth rate of FR2 exposed to fludioxonil was always lower than that of untreated FR2 [4-25% lower growth exposed to fludioxonil (0.02-1 mM) compared to control FR2]. Sporulation of fludioxonil treated FR2 was very poor, whereas that of fludioxonil treated FR3 was normal. Hence, though both strains exhibited resistance to fludioxonil, different mechanisms were probably involved between the strains.

Differential responses of* P. expansum *mutants to oxidizing agents. We hypothesized that the mechanism(s) of fludioxonil resistance in *P. expansum* FR2/FR3 mutants resulted from a mutation in the HK-MAPK signaling system. Some fungi having mutations in certain MAPK genes can escape toxicity of fludioxonil. We observed in FR2/FR3 the characteristics of fungi having a mutation in the HK signaling system, showing reduced hyphal growth while resistant to fludioxonil (Hagiwara *et al*. 2007). Since the HK-MAPK pathway is the key signaling system for fungal defense against environmental stresses, such as oxidative stress, we reasoned that the FR2/FR3 mutants should be hypersensitive to exogenously applied oxidizing agents.

Menadione, a redox cycling quinone, is a source of toxic superoxide radicals (Castro *et al*. 2007; Fernandes *et al*. 2007). FR3 was approximately twice as sensitive to menadione than the parental W2 strain (MICs for menadione: 0.256 mM < W2 < 0.512 mM *vs.* 0.128 mM < FR3 < 0.256 mM; **Fig. 3**). FR3 was also more sensitive to other oxidizing agents, H_2O_2 (3 mM) or diamide (3.5 mM; thiol-oxidizing agent) than W2 (**Fig. 3**).

Interestingly, the response of the FR2 mutant to the oxidizing agents was directly opposite to that of the FR3 mutant. Unlike FR3, FR2 was almost twice as tolerant to menadione than its parental strain, W1 (MICs for menadione: 0.128 mM < W1 < 0.256 mM *vs.* 0.256 mM < FR2 < 0.512 mM; **Fig. 3**) and was also relatively tolerant to H_2O_2 (at 4 mM), where W1 showed no growth (**Fig. 3**), or diamide (at 2.5 mM) (FR2: 29% less growth than FR2 w/o diamide; W1: 42% less growth than W1 w/o diamide; **Fig. 3**).

The hypersensitivity of the FR3 mutant to oxidizing agents, in addition to characteristic reduced hyphal-growth, indicates the HK-MAPK oxidative stress response pathway was defective in this strain. Since fungicidal activity of fludioxonil is exerted through a normal/functional MAPK system, mutation of the HK-MAPK pathway may explain why the FR3 strain was able to escape the mode of action of fludioxonil (Kojima *et al*. 2004).

On the other hand, FR2 appeared to represent a gain-of-function phenotype. This strain showed increased tolerance to the oxidizing agents. This increased tolerance suggests an increased antioxidative capacity that possibly could ameliorate cellular redox fluctuations under fludioxonil treatment. However, the energy expenditure for heightened antioxidative activity, under conditions without stress, may explain the lower hyphal growth of FR2. We previously observed that overexpression of the antioxidation gene *sodA*, encoding mitochondrial superoxide dismutase (Mn-

SOD) of *A. flavus*, in the *S. cerevisiae* wild type strain resulted in reduced growth on normal growth medium (*i.e*., w/o oxidative stress) (Kim *et al*. 2006). Collectively, our results indicate that disruption of normal cellular redox homeostasis, either through up- or down-regulation of antioxidation activity, can be at least one mechanism of fludioxonil resistance in *P. expansum*.

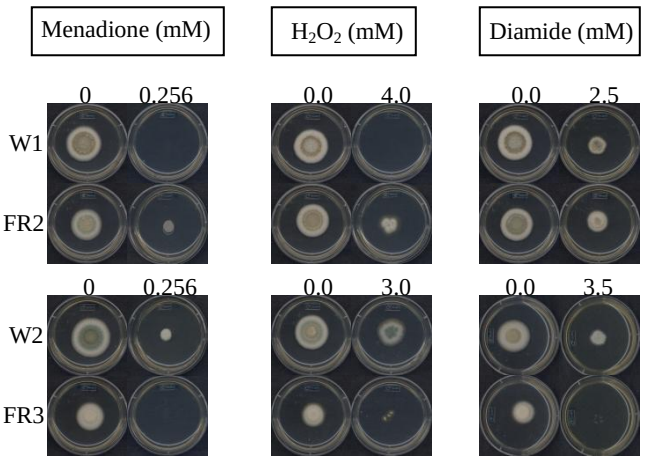

Figure 3. Differential responses of *Penicillium expansum* parental strains, W1 and W2, and respective fludioxonil-resistant mutants, FR2 and FR3, to oxidizing agents, menadione, hydrogen peroxide (H_2O_2), or diamide.

Chemosensitization of fludioxonil resistant strains using redox-active natural compounds. Chemosensitization using redox-active natural compounds, such as thymol or 2,3-dihydroxybenzaldehyde (2,3-D), was found to be effective in overcoming fungal resistance to conventional antifungal drugs (Kim *et al*. 2008a,b). We examined the chemosensitizing activity of octylgallate, thymol and 2,3-D in co-applications with fludioxonil. Growth of FR2, FR3 and their parental strains was almost completely inhibited when fludioxonil (0.02 or 0.06 mM) was co-applied with 0.15 mM of octylgallate (**Fig. 4**). Thymol or 2,3-D also exhibited some chemosensitizing activity to fludioxonil in the *P. expansum* strains. However, this activity for 2,3-D was negligible in the mutant strains (**Table 1;** See below).

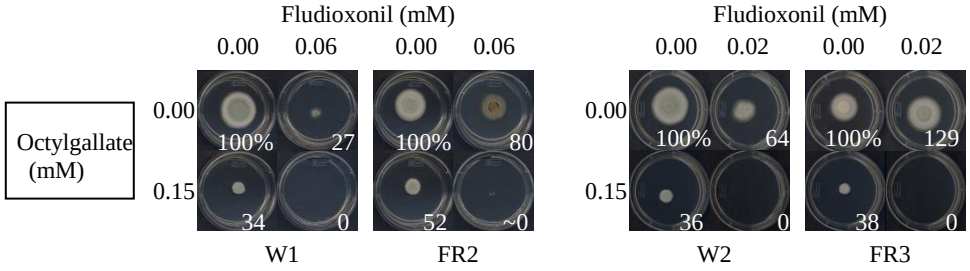

Figure 4. Representative bioassay showing growth of FR2, FR3 (fludioxonil-resistant mutants of *P. expansum*) and their respective parental strains, W1 and W2, in co-applications of fludioxonil (0.02 or 0.06 mM) and octylgallate (0.15 mM). (See also Table 1). Note: Radial growth rates of each strain w/o any treatment was considered as 100% growth (control), and the relative growth rate in each treatment was determined accordingly (SD < 5%).

Chemosensitization of* P. expansum *to a mitochondrial respiration inhibitor. The responses of FR2 and FR3 to the oxidizing agents (See **Fig. 3**) showed the redox homeostasis systems of these mutants were abnormal. We decided to further investigate the responses of these mutants to another fungicide, strobilurin (kresoxim-methyl). The responses of these mutants to strobilurin treatments were not substantially different from those of the respective parental strains (**Table 1**). Hence, there was no indication the mutant strains were resistant to this fungicide. The mode of action of strobilurin is different from that of fludioxonil. Strobilurin inhibits complex III of the mitochondrial respiratory chain, resulting in a disruption of energy production. Coinciding with this disruption is an abnormal

release of electrons that additionally damages cellular components by oxidative stress. Mn-SOD plays an important role in protecting cells from such oxidative damage. We were interested to see if co-applying redox reactive chemosensitizing agents, targeting Mn-SOD, could augment the fungicidal effects of strobilurin. As shown in **Table 1**, octylgallate (as low as 0.05 mM) in combination with strobilurin (0.02 mM), resulted in complete growth inhibition of both parental and mutant strains of *P. expansum*. This chemosensitization also occurred with co-application of 2,3-D or thymol (**Table 1**).

Table 1. Percent growth of strains of *Penicillium expansum*, fludioxonil-resistant mutants (FR2 and FR3) and respective parental strains (W1 and W2), to fungicides fludioxonil (Flud) and strobilurin (kresoxim-methyl) (Kre-Me) and redox-active natural phenolics, alone and in combination (co-applied). The phenolics include octylgallate (OcGal), 2,3-dihydroxybenzaldehyde (2,3-D) and thymol (Thy). Numbers in parentheses are concentrations (mM) of each compound used, and percent numbers (%) are relative growth rate (radial growth) of fungi compared to "no treatment" controls of each strain (=100%) (SD < 5%). *P. expansum* strains were grown at 28°C (82.4°F) on potato dextrose agar. Growth was observed for 3 to 7 days.

	Fungicide alone	Chemosensi-tizer alone	Co-applied		Fungicide alone	Chemosensi-tizer alone	Co-applied
W1	Flud (0.06) 27%	OcGal (0.15) 34%	0%	**W2**	Flud (0.02) 64%	OcGal (0.15) 36%	0%
	Flud (0.06) 14%	2,3-D (0.20) 71%	~0% (few colonies)		Flud (0.04) 78%	2,3-D (0.30) 75%	0%
	Flud (0.04) 44%	Thy (0.60) 50%	0%		Flud (0.04) 26%	Thy (0.20) 58%	0%
	Kre-Me (0.02) 54%	OcGal (0.05) 69%	0%		Kre-Me (0.02) 52%	OcGal (0.05) 73%	0%
	Kre-Me (0.02) 50%	2,3-D (0.15) 81%	0%		Kre-Me (0.02) 54%	2,3-D (0.20) 79%	0%
	Kre-Me (0.02) 44%	Thy (0.60) 40%	0%		Kre-Me (0.02) 47%	Thy (0.40) 50%	~0% (few colonies)
FR2	Flud (0.06) 80%	OcGal (0.15) 52%	~0% (few colonies)	**FR3**	Flud (0.02) 129%	OcGal (0.15) 38%	0%
	Flud (0.06) 78%	2,3-D (0.20) 70%	48%		Flud (0.04) 119%	2,3-D (0.30) 85%	96%
	Flud (0.06) 86%	Thy (0.60) 36%	~0% (few colonies)		Flud (0.04) 135%	Thy (0.20) 83%	~0% (few colonies)
	Kre-Me (0.02) 59%	OcGal (0.05) 77%	0%		Kre-Me (0.02) 67%	OcGal (0.05) 79%	0%
	Kre-Me (0.02) 59%	2,3-D (0.15) 77%	0%		Kre-Me (0.02) 65%	2,3-D (0.20) 82%	0%

Kre-Me (0.06) 41%	Thy (0.40) 41%	~0% (few colonies)	Kre-Me (0.02) 61%	Thy (0.20) 83%	~0% (few colonies)

Chemosensitization of* P. expansum *with newly identified cinnamaldehyde. We recently identified cinnamaldehyde as a potent antifungal agent. In combination with thymol, additive antifungal effect was identified in *P. expansum* NRRL974.

Table 2 . Ranges of Minimum Inhibitory Concentrations (MICs) of thymol and cinnamaldehyde (mM), tested alone or in combination against *Penicillium expansum*. Compound interactions were determined as Fractional Inhibitory Concentrations (FIC)[a].

Compounds	MIC: alone	MIC: combined	FIC
	P. expansum NRRL974		
Cinnamaldehyde	$0.4 < n_1 < 0.8$ $0.4 < n_2 < 0.8$	$0.2 < n_1 < 0.4$ $0.0 < n_2 < 0.1$	0.625 additive

[a]Compound interactions were determined as described by Isenberg as follows: FIC (Fractional Inhibitory Concentration) = (MIC of compound A in combination with compound B / MIC of compound A alone) + (MIC of compound B in combination with compound A / MIC of compound B alone). Compound interactions: synergistic (FIC index ≤ 0.5), additive ($0.5 <$ FIC index ≤ 1), neutral ($1 <$ FIC index ≤ 2) or antagonistic ($2 <$ FIC index). n_1, MIC of cinnamaldehyde alone. n_2, MIC of thymol alone. n_3, MIC of cinnamaldehyde in combination with thymol. n_4, MIC of thymol in combination with cinnamaldehyde.

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EXECUTIVE SUMMARY:

During last three years, we identified a potentially effective approach to fungal control using newly discovered natural compounds that have a target-specific basis of activity, as follows:

(1) Identify the most efficient molecular targets: Antioxidative stress response and cell wall integrity systems of fungi can be an efficient molecular target of phenolics for pathogen control.

(2) Determine an effective method for delivery of newly discovered natural compounds: Results also show certain natural compounds are effective synergists to commercial fungicides and can be used for improving control of fungal pathogens. We proved positive interaction between phenolics and conventional fungicides significantly augment the fungicidal effects of commercial fungicides by reducing the costs of application or contamination of the environment.

(3) Overcome the tolerance of *Penicillium expansum* to fludioxonil and strobilurin through chemosensitization by using natural compounds: Certain safe natural phenolic compounds have the potential to serve as potent chemosensitizing agents to enhance activity of conventional antifungal drugs or commercial fungicides. We demonstrated how a number of phenolic compounds greatly improved effectiveness of fludioxonil and activated a process for overcoming fludioxonil-resistance (**Fig. 5**). These compounds also greatly enhanced the activity of strobilurin. Our results indicate this enhanced activity is from disruption of cellular redox homeostasis by targeting the antioxidative stress response systems (*e.g*., Mn-SOD) with redox-active natural compounds. Chemosensitization by safe, natural compounds can lower effective dosages of conventional antifungal drugs and fungicides.

In conclusion, our data proved the effectiveness of targeting cellular stress response system such as oxidative stress response or cell wall construction/integrity pathway for control of fungi. Results also show certain natural compounds are effective synergists to commercial fungicides and can be used for improving control of food-contaminating pathogens. Use of such compounds for fungal control reduces environmental and health risks associated with commercial fungicides, and lowers cost for control and the probability for development of resistance to these fungicides.

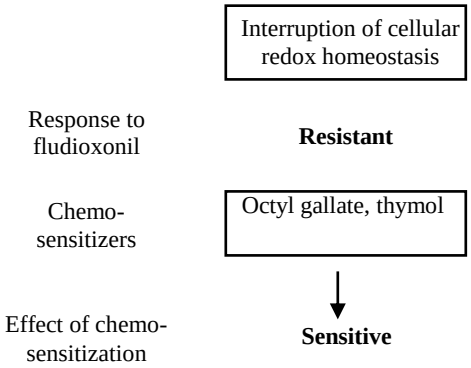

Figure 5. Diagram showing chemosensitizing effects of safe, natural compounds, which enhance antifungal activities of and/or overcome fungal resistance to conventional fungicides such as fludioxonil.