FINAL PROJECT REPORT

Project Title Pesticide resistance in pear psylla

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Cooperators: Elizabeth Beers

Total Project Funding: $48,700

Budget History

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

Item	2014	NA	NA
Salaries			
Benefits			
Wages	$13800		
Benefits	$ 1200		
Equipment			
Supplies[1]	$ 1000		
Travel			
Miscellaneous			
Plot Fees[2]	$ 1000		
Total	$17,000		

Footnotes:[1] Insecticides, collection materials, computer program for DNA analysis. [2]Moxee farm pears-fertilizer

Budget 2 (Shearer and Hilton)
Organization Name: OSU MCAREC **Contract Administrator:** L.J. Koong
Telephone: 541-737-4066 **Email address:** l.j.koong@oregonstate.edu

Item	2014	NA	NA
Salaries[1]	$5,215		
Benefits[1]	$3,454		
Wages[2]	$5,787		
Benefits[2]	$1,739		
Equipment	$0		
Supplies[3]	$346		
Travel[4]	$459		
Plot Fees	--		
Miscellaneous	--		
Total	$17,000		

Footnotes: Footnotes: [1]Salary and Benefits: Faculty Research Assistant 0.75 mo. Bioscience Research Technician 0.75 mo. [2]Wages and Benefits: Summer Technician(s), 10 weeks [3]Supplies: Lab supplies for assay and rearing [4]Travel to field. 0.556/mi.

Budget 3 (Chiu)
Organization Name: University of California Davis **Contract Administrator:** Guyla Yoak
Telephone: (530) 752-3794 **Email address:** gfyoak@ucdavis.edu

Item	2014	NA	NA
Salaries[1]	$5,896		
Benefits[1]	$2,252		
Wages	--		
Benefits	--		
Equipment	--		
Supplies[2]	$3,552		
Travel	--		
Miscellaneous[3]	$3,000		
Plot Fees	--		
Total	$14,700		

Footnotes: [1]Salary and Benefits: Technician (2 months of full time); [2]Supplies: Lab supplies for generating transcriptome sequencing libraries and library quality control including NEB Next Ultra RNA library Prep Kit for Illumina, NEB Next Multiplex Oligos for Illumina, NEB Next Poly(A) mRNA Magnetic Isolation Module, Biorad Experion Nucleic Acid Analysis Kit, and consumables such as pipet tips and microcentrifuge tubes
[3]Miscellaneous: Transcriptome sequencing costs at the UC Davis Genome Sequencing Center

Objectives

1. **Conduct a resistance survey of winterform pear psylla in WA and OR**

2. **Produce and analyze transcriptomes from populations of pear psylla to identify genetic variations that confer insecticide resistance**

Significant Findings

- Admire, AgriMek, Delegate, Nexter, Pounce, and Warrior were screened for activity against winterform pear psylla adults from 9 sites in OR and 11 sites in WA.
- Mortality caused by Delegate and Nexter was the highest of the insecticides tested, but still averaged 56 and 49% for the populations tested.
- Mortality caused by Admire and AgriMek was 25 and 18%, respectively, indicating a high probability of resistance.
- Mortality caused by Pounce (13%) and Warrior (10%) was low overall.
- A single population with very low mortality in both Delegate and Nexter bioassays suggests the possibility of cross-resistance between the two products.
- Development of selectivity ratios (harm to natural enemies balanced against pesticidal efficacy) is important to making sustainable pest management decisions.
- Transcriptomes for 24 populations of pear psylla in Oregon or Washington were sequenced. These are the first transcriptomes produced for pear psylla and will be submitted to NCBI Genbank to facilitate psylla research.
- Mutations in genes involved in neurotransmission were identified in pear psylla populations that exhibited resistance to AgriMek and Pounce. Genetic markers can be developed to identify resistant populations and monitor the spread of resistance.

Results & Discussion

Obj. 1. Methods. We examined the relative efficacy of six insecticides commonly used for control of pear psylla. The materials included Admire Pro, AgriMek, Delegate, Nexter, Pounce, and Warrior. Active ingredients and maximum label rates are given in Table 1.

Table 1. Pesticides screened for efficacy against pear psylla

Product	AI	% AI or lb AI/gal	Maximum label rate (label units)	Maximum label rate (ppm AI)	MOA
Admire Pro	imidacloprid	4.6 lb AI/gal	7 fl oz	302	group 4A
Agri-Mek SC	abamectin	0.7 lb AI/gal	4.25 fl oz	28	group 6
Delegate 25WG	spinetoram	25%	7 oz	131	group 5
Nexter 75WP	pyridaben	75%	16 oz	899	group 21
Pounce 25WP	permethrin	25%	25.6 oz	479	group 3
Warrior II	lambda-cyhalothrin	2.08 lb AI/gal	2.56 fl oz	50	group 3

Using the insecticides listed above, we evaluated mortality of winterform pear psylla from 20 pear orchards in WA (11 orchards) and OR (9 orchards). Psylla were collected in Sept-November of 2014 and 2015. Field collections were performed with either an beating tray and aspirator or a large plastic funnel with a jar attached to the bottom (Fig. 1), speeding collection of large numbers of insects. The funnel was held beneath a branch which was struck sharply with a padded stick. This process was repeated until sufficient adults were collected. Adults were kept cool (40°F) and under short photoperiod (10L:14D) and provided with a moisture source until used in a bioassay.

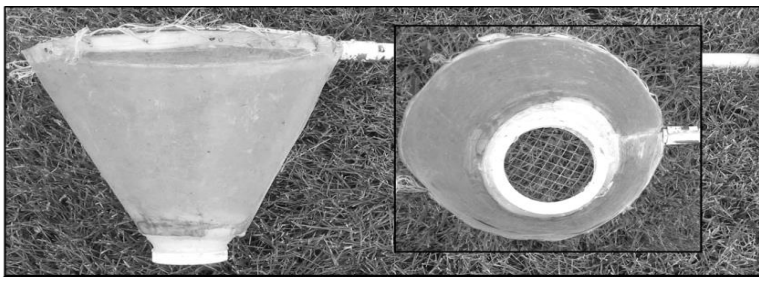

Fig. 1. Plastic funnel used to collect adult psylla

The bioassay format chosen was the slide dip so that data would be comparable to previous work. A group of 25-35 adults (unsexed) were anesthetized with CO_2 and affixed to the slide using double-sided sticky tape. After all adults for a bioassay were placed on slides, they were re-scanned and any dead adults removed. Each dosage was tested with three slides, or 50 to 150 individuals/ concentration. Depending on the numbers of adults available, 2-7 concentrations were tested. The larger number of concentrations is useful for probit analysis, while the reduced number is most appropriate for a diagnostic dose approach. All bioassays included a water check. The slide with adults was dipped in the pesticide solution (or water) for 5 seconds then held at room temperature for 48 hours. After this time, the adults were evaluated for mortality.

Fig. 2. Adult psylla on slide with double-sided sticky tape.

Obj. 1. Results & Discussion. A total of 77 bioassays were performed with psylla from various orchard and insecticide combinations. Twenty-four of those bioassays had a reduced number of doses, and are most appropriate for a diagnostic dose evaluations. The maximum label rate (MLR) was chosen as a means of comparing the various orchard populations. An additional 53 bioassays contained a wider range of concentrations, from which a probit lines were calculated (not shown). A single summary statistic was chosen that represented both bioassay types. If the MLR was included in the bioassay, the percentage mortality from this concentration was used; otherwise, the concentration *nearest* the MLR was chosen by using the minimum of the absolute value of the differences between the actual rate and 1. Two of the bioassays did not contain a concentration sufficiently close to represent the MLR, and were excluded from the summaries. Thus, the figures represent a 'best case' scenario of the mortality that would occur if the material were applied at the MLR (Figs. 3a-f). The results are arranged and color coded by the state from which the population originated.

Mortality caused by Admire was variable but generally low at the MLR (average=25% for all populations, n=12) (Fig. 3a). Average mortality for AgriMek was similarly low (12%, n=12) (Fig. 3b). Mortality caused by Delegate was considerably higher overall (45%, n=12), with only a single Washington population (OK) showing resistance to this material (Fig. 3c). Results from Nexter were

similar (50%, n=10) to those of Delegate; the same Washington (OK) populations that was highly tolerant of Delegate was also highly tolerant of Nexter (Fig. 3d). Most of the populations from Washington and Oregon were resistant to Pounce (Fig. 3e), with an overall average was 13% (n=19). Results from the Warrior bioassays were similar, with an average of 10% (n=10) mortality (Fig. 3f).

The results of these bioassays must be interpreted with a great deal of caution. Only two the materials, the pyrethroids Pounce and Warrior, are typically used against winterform adults, and thus were tested with the most appropriate target stage. Resistance to pyrethroids in pear psylla has been known from the 1970s, and was well documented for fenvalerate in the 1990s. However, neither pyrethroid in the current study was tested with piperonyl butoxide (PBO), an adjuvant commonly used to help overcome resistance mechanisms. Mortality would most likely have been higher overall for these two products with the addition of PBO.

The other four insecticides (Admire, AgriMek, Nexter, Delegate) are typically used after the dormant/delayed dormant period, when egg, nymphs, and (in later generations) summerform adults are present. Nymphs, especially the earlier instars, are likely the most vulnerable to pesticides, and therefore the primary target of these materials. Without bridging information on activity difference between winterforms and nymphs, historical levels of activity, or contemporaneous bioassays of a susceptible population, few conclusions may be drawn other than the variability among the populations tested.

Lastly, the low mortality in one population (OK) for both Delegate and Nexter suggests the possibility of cross-resistance between the two products. However, more populations would need to tested to establish this experimentally.

Selectivity. Most of the insecticides tested would be considered non-selective to natural enemies, and this presents an additional item for consideration in the choice of materials. The 'worst case scenario' is where the insecticide is no longer very effective against the target pest, but retains its toxicity to one or more important natural enemies. For instance, AgriMek is acutely toxic to a psylla parasitoid (*Trechnites* sp) and the predators *Anthocoris* and *Deraeocoris*, even at 25% of the field rate. It is also toxic to the western predatory mite *Galendromus occidentalis*, so disruption of both biological control systems can be expected. Developing a selectivity ratio, which indicates the relative harm (to natural enemies) to relative good (pesticidal efficacy) could help guide grower choices for more sustainable pest management programs.

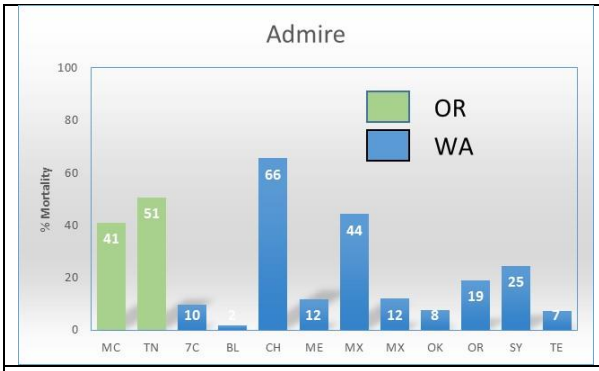

Fig. 3a. Percentage mortality of winterform psylla by Admire at or near the field rate (WA, OR).

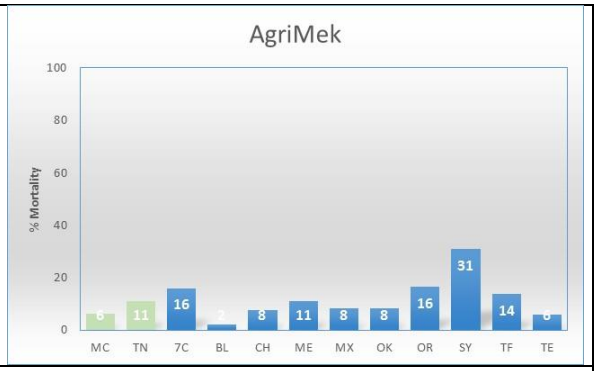

Fig. 3b. Percentage mortality of winterform psylla by AgriMek at or near the field rate (WA, OR).

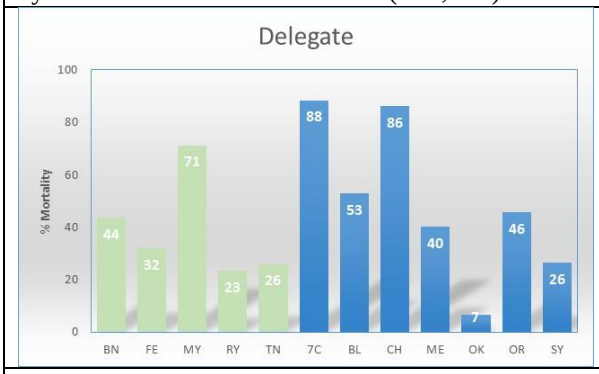

Fig. 3c. Percentage mortality of winterform psylla by Delegate at or near the field rate (WA, OR).

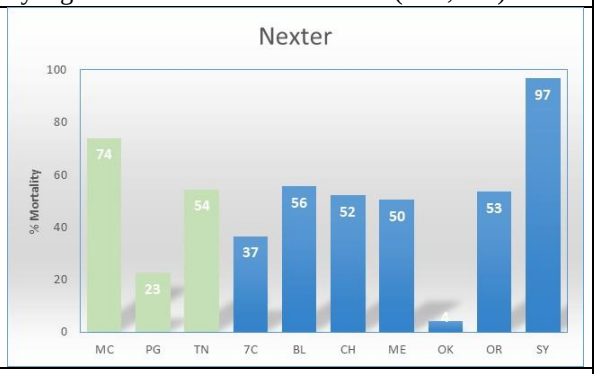

Fig. 3d. Percentage mortality of winterform psylla by Nexter at or near the field rate (WA, OR).

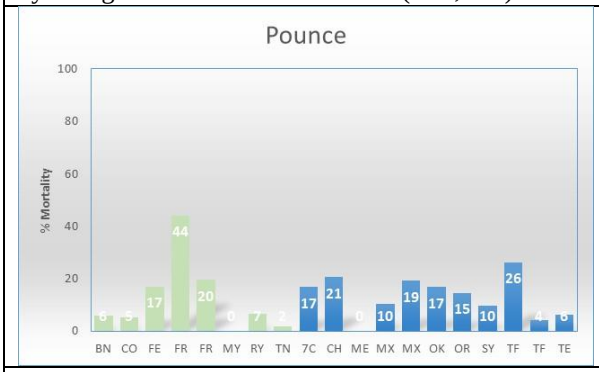

Fig. 3e. Percentage mortality of winterform psylla by Pounce at or near the field rate (WA, OR).

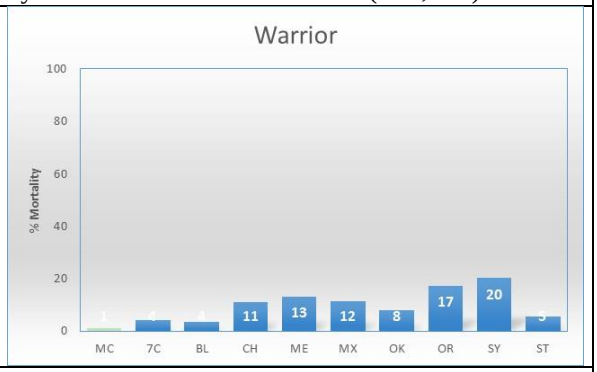

Fig. 3f. Percentage mortality of winterform psylla by Warrior at or near the field rate (WA, OR).

Obj. 2. Methods

The goal of this objective was to identify genetic mutations that could underlie resistance to specific insecticides tested in Objective 1 using RNA sequencing. Specifically, we focused our genetic analysis on AgriMek and Pounce as pear psylla populations that are either susceptible or resistant were available for RNA analysis.

RNA extraction, library preparation, and high-throughput sequencing
Total RNA was extracted from 25 individuals from each collection site using Tri-reagent (Sigma). Following polyA mRNA enrichment, which enriched for RNA from expressed genes, using the Next PolyA magnetic isolation module (New England Biolabs), paired-end sequencing libraries with an approximate average insert length of around 150bp (standard for transcriptome analysis) were created using the Next Ultra RNA library Prep Kit (New England Biolabs). Transcriptome libraries representing 24 populations (2 replicates per population) were sequenced using 100bp paired-end Illumina HiSeq at the UC Davis Genome Center Sequencing facility.

Bioinformatic analysis to identify genetic mutations underlying insecticide resistance
Since the genome sequence of pear psylla is not available, we performed *de novo* transcriptome assembly using "Trinity" (release 2013-02-25). Our experimental and bioinformatic pipeline yielded individual transcriptomes for the different psylla populations. To extract genetic information from our transcriptomes and annotate the genes, we performed comparative sequence analysis against insect genomes in the public database. Finally, we used the program "Freebayes" to identify genetic differences (single nucleotide polymorphism, SNP) between susceptible and resistant populations for (1) AgriMek and (2) Pounce. In particular, our focus was on genes that are known to be associated with insecticide target site, e.g. ion channels and neuro-receptors, or metabolic resistance, e.g. detoxification enzymes.

Obj. 2. Results and Discussion

Sequencing and annotation of pear psylla transcriptome
In addition to the value of our survey for genetic variations that may confer insecticide resistance, the psylla transcriptome resulting from this project will be submitted to NCBI Genbank and shared with other scientists to facilitate basic and applied research on pear psylla. Besides the genetic markers we can now develop to monitor insecticide resistance, especially if these mutations were confirmed in more populations, the transcriptome data can also be used to develop other molecular markers to monitor population dispersal as well as trait variations.

Identification of genetic differences that underlie the response of pear psylla to AgriMek and Pounce
Samples were available to analyze potential genetic differences between populations that were resistant and susceptible to (1) AgriMek and (2) Pounce. In the case of other insecticides, there were not enough susceptible populations to provide the statistical power necessary to identify gene mutations.

Genetic basis of Pounce resistance
Genetic differences were identified between Washington populations (ME, OK, OR, SY, TE) resistant to Pounce as compared to the Oregon TN population based on bioassays performed in Objective 1. Mutations in genes involved in neuronal function and metabolic detoxification were identified in resistant populations. Two of these mutations are non-synonymous mutations, i.e. mutations that are expected to change the sequence of the mutated proteins, and hence may either enhance or disrupt their functions. Confirmation of these mutations in causing Pounce resistance will

require biochemical analysis. We did not find KDR mutations that have been known to cause resistance to pyrethroids, indicating that the mechanisms underlying Pounce resistance in these psylla populations may be through other mechanisms.

Table 2: Select neuronal and detoxification genes that show genetic mutations in psylla populations resistant to Pounce as compared to susceptible populations

Predicted Pear Psylla Gene	E Value	Non-Synonymous?	Function
cGMP-specific 3',5'-cyclic phosphodiesterase	1.81E-86	Yes	Neuronal
cytochrome P450 4c3	7.13E-75	No	Detox
UDP-glucuronosyltransferase 2B10	4.11E-149	Yes	Detox
Kv channel-interacting protein 4	6.11E-128	No	Neuronal
sodium/hydrogen exchanger 8	0	No	Neuronal
voltage-dependent anion-selective channel	0	No	Neuronal
sodium-independent sulfate anion transporter	7.27E-152	No	Neuronal
cation-transporting ATPase 13A3	0	No	Neuronal
calcium-independent phospholipase A2-gamma	4.03E-79	No	Neuronal
piezo-type mechanosensitive ion channel component	6.37E-159	No	Neuronal

Genetic basis of AgriMek resistance

Genetic differences were identified between populations that are resistant (BL, CH, MC, ME, OK, OR, TF, TE) to AgriMek as compared to susceptible (TN) population based on bioassays performed in Objective 1. Although mutations in genes involved in neuronal function and metabolic detoxification were identified, they are synonymous mutations that are not expected to change the sequence of the mutated proteins. However, it is possible that expression level of these proteins could be influenced, even by non-synonymous mutations. This can be verified using quantitative PCR. It is expected that if more susceptible samples were available, then the identification of the causal mutations would be more likely.

Table 3: Select neuronal and detoxification genes that show genetic mutations in psylla populations resistant to AgriMek as compared to susceptible populations

Predicted Pear Psylla Gene	E Value	Non-Synonymous?	Function
cytochrome P450 4c3	4.71E-75	No	Detox
cytochrome P450 4g15	0	No	Detox
ecdysone receptor	4.16E-06	No	Neuronal
ADP/ATP translocase 2	5.42E-11	No	Neuronal
sodium/hydrogen exchanger 8	0	No	Neuronal
serine carboxypeptidase	7.48E-155	No	Detox
proton-coupled amino acid transporter 2	1.19E-44	No	Neuronal

Additional bioinformatic analysis to examine the biochemical basis of the gene mutations identified here can help to validate the causal mutations for AgriMek and Pounce resistance. Finally, more populations with varying degree of susceptibility to the other insecticides will have to be sequenced to order to identify genetic mutations underlying resistance to Admire, Delegate, Warrior, and Nexter. Our results presented here will now enable the development of genetic markers to identify and monitor the spread of pear psylla resistance populations.

Executive Summary

All of the insecticides tested produced low or moderate mortality on the average in winterform adults. Overall, the highest levels of mortality were produced with Delegate and Nexter, the two newest materials. Generally poor mortality was produced by AgriMek and Admire and, which have been used since the late 1980s and mid-1990s, respectively, in pear production. Activity of the pyrethroids Pounce and Warrior was consistently low in both Washington and Oregon populations, although they were tested without PBO.

Transcriptomes for 24 populations of pear psylla in Oregon or Washington were sequenced. These are the first transcriptomes produced for pear psylla and will be released to NCBI Genbank to facilitate psylla research. Mutations in genes involved in neurotransmission were identified in pear psylla populations that exhibited resistance to AgriMek and Pounce. Additional bioinformatic and biochemical analysis can be performed to further confirm the causal mutation that underlie resistance. Genetic markers can be developed to identify and monitor the spread of resistance populations.

The development of resistance in psylla populations despite the availability of multiple modes of action is an indication of failure of insecticide rotation as a substitute for IPM. Even with 5-6 MOAs available to pear growers, our production systems are on the brink of field failure despite the use of all possible MOAs. Without the ecosystem services of natural enemies to clean up resistant individuals, or the availability of novel MOAs, our current system is vulnerable to failure.