2023 Apple Crop Protection Research Review

ESA Biocontrol Tour in 2022. Searching for natural enemies on the bank of a pond in a Zirkle block near Othello. It was planted with plant species designed to attract and host beneficial insects to promote biocontrol in the surrounding orchard.

Photo Source: Tory Schmidt, WTFRC

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Funding Duration:
Amount:
Agency Name:
Notes:

Item			2022
Salaries			$8,000.00
Benefits			
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies			
Travel			
Plot Fees			
Miscellaneous			
Total	$0.00	$0.00	$8,000.00

Footnotes: Salary covers sub-contractor fees for stats analysis

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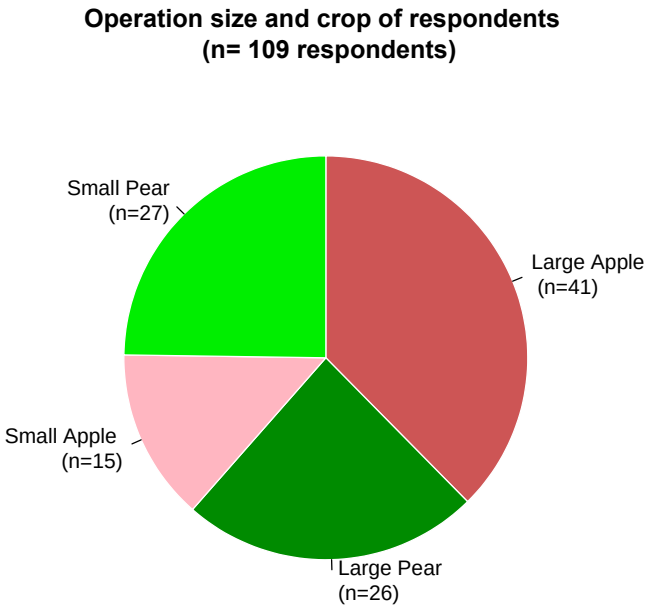
Introduction:
In response to industry concerns regarding a perceived loss of control of Codling Moth (*Cydia pomonella*, hereafter "CM") in apple and pear crops in the Pacific Northwest, the Washington Tree Fruit Research Commission formed the Codling Moth task force in September of 2020. The task force was comprised of growers, industry leaders, and university researchers. The goal of the task force was to 1) better understand what orchard management and CM control tactics are currently being used, and 2) to use a survey data to better understand if there are common practices working/not working. To do this, the committee created and deployed an industry-wide survey. The survey was sent out to apple and pear orchard owner/managers and pest management consultants in Washington. In total, 127 respondents completed enough of the survey to analyze. For each portion of this report, the number of respondents who answered that specific question (which differs from the overall number of respondents of the survey) will be included (notated throughout this report as "n=…").

Structure of this report: Chris Adams, head of the Codling Moth Task Force, narrowed down the survey questions to be analyzed to a series of "Key Questions" from the full survey and requested that for each Key Question a separate report section be given for "apple" and "pear" growers. Furthermore, Chris requested that for both apples and pears, differences between "Small" growers (which we defined as <100 acres) and "Large" Growers (defined as >100 acres) as well as the impact of organic management practices. For each Key Question, we used the most relevant and useful data available to glean some insights into how management differs based on:
-apple and pear growers
-large and small operations
-organic management

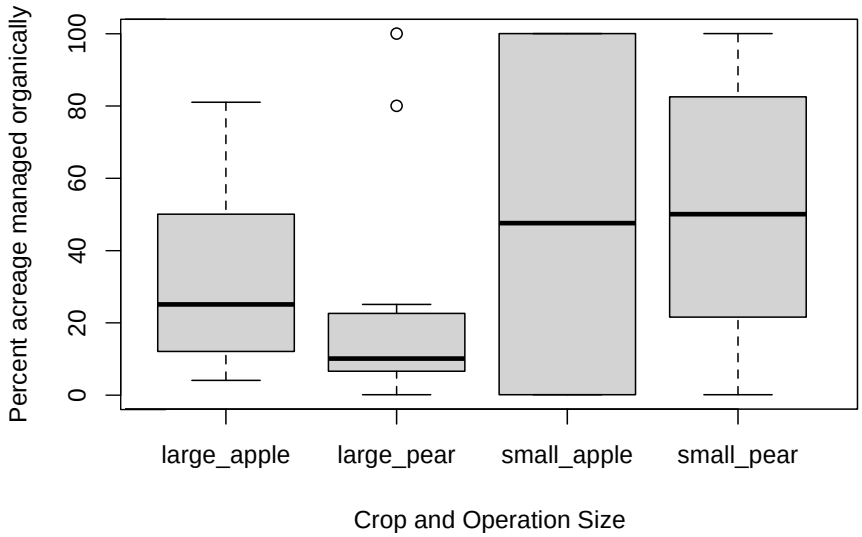
It should be noted, that by parsing out this relatively small dataset in so many ways, this often resulted in comparisons of very low sample sizes. In the chi-square analyses, we were able to avoid the normal approximation, and run the analyses on very low sample sizes, but this should be taken into account when drawing conclusions. Significant differences (or not) are noted with associated p-values (indicated p= 0.0…). For other analyses, specifically regarding comparisons of conventional vs organic management practices, there were simply far too few (sometime only 1-3 responses), which were unable to be analyzed in the ways anticipated. For these examples, graphs were created to visually inspect the differences between treatments. Sample size is noted in the figure title throughout the report. Throughout this report, for each question, the specific test used, or the reason for not using a test are noted.

Who took the Survey?
Of the 182 respondents who logged in to take the survey, 135 completed enough of the survey to use (>8% of the survey, i.e., more than just their name). Of the 112 respondents who reported their role, 74 (66.1%) identified as "manager/owner", and 38 (33.9%) identified as "consultant." Respondents were asked to take the survey for a specific crop, either apples OR pears, not both. Of those 113 who reported their cropping system, 60 (53.1%) responded for "apples", and 53 (46.9%) responded for "pears." Because of the variability in the size (acres) of operations and the potential for management to differ between larger and smaller operations, The committee head requested we group the data into 2 groups: large (>100 acres) and small (<100 acres). For those respondents who reported both cropping system and acreage, here is the breakdown:
Figure 1.



Additionally, the survey asked respondents "What type of orchard management do you perform?" with the ability to fill in acreage percentages managed "conventionally", "organically", or in "transition". Growers were not able to self-identify as "conventional" or "organic" (many manage in both ways) but many did report what percentages of the acreage they farmed for each management type. For some questions, we created and used a continuous "% acreage managed organically" variable from the data available. To answer other questions, we binned growers based on their predominant management acreage (e.g., "mostly conventional" = >75% acreage managed conventionally, "mostly organic" = >75% acreage managed organically). Lastly, for a few other questions, there was survey data specific to "conventional", "organic", or in "transition". For each question, we chose the most relevant approach to tease out any differences that might be important based on farm management preference. This breakdown of how respondents' reported % acreage managed organically is shown in figure 2.

Figure 2.*



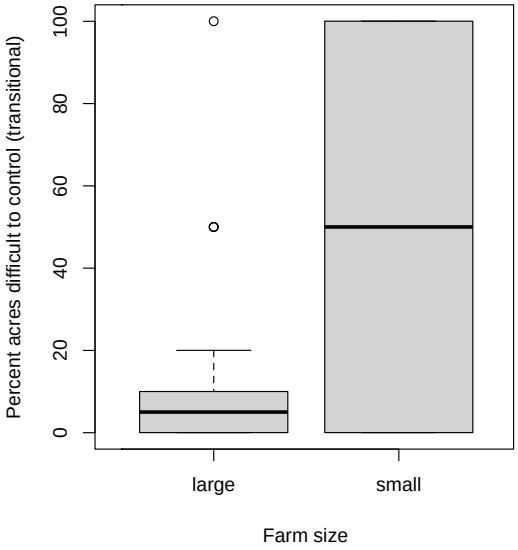
*For those unfamiliar with this type of graph, it's called a "box plot" or "box and whisker plot." The thick black bars indicate the middle (median) of the dataset, the grey boxes indicate the middle quartiles of the data (where 50% of the data are), and the 'whiskers' indicate the upper and lower quartiles (where the upper 25%, and lower 25%, respectively) of the data lie. The rouge datapoints indicated are considered outliers.

KEY QUESTIONS:

Question 5, part 1: **"What percentage of acreage is codling moth difficult to control (for each type of management)?"** To address this question, we used a chi-square test to look at differences between small and large growers on land on organic, transitional, and conventional acreage.

<u>Apple Growers' response</u>
Small vs. Large Operations: On both organic and conventional orchards, there was no difference in the percentage of acreage on which CM was difficult to control between small and large orchards (p= 0.4898 and p= 0.08296, respectively). On transitional orchards, small orchards reported a significantly higher % of acreage on which CM was difficult to control (p= 0.04198) shown in figure 3.

Figure 3.



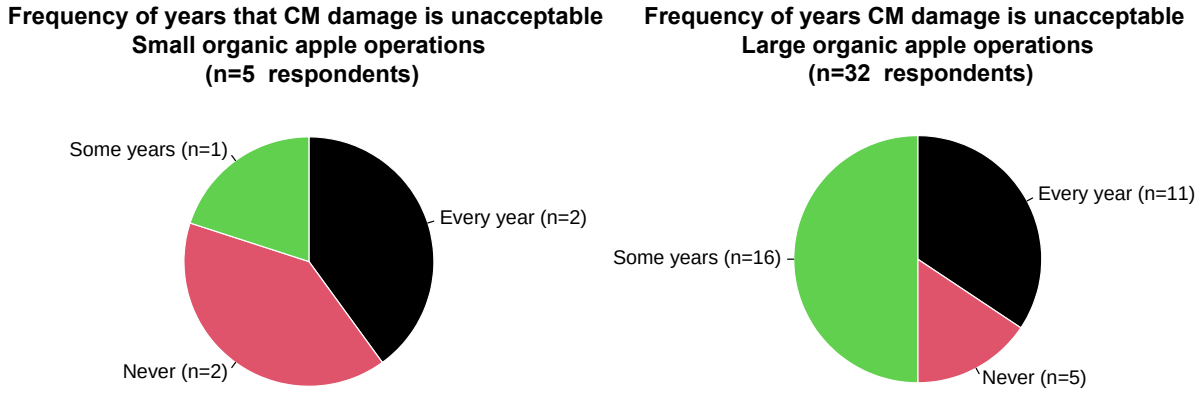
Pear Growers' response:
Small vs. Large Operations: On organic, transitional, and conventional orchards, there was no difference in the percentage of acreage on which CM was difficult to control between small and large orchards (p= 0.1724, p= 0.6037, and p= 0.8836, respectively)

Question 5, Part 1 Summary: Generally, for both apples and pears there were no major differences in the percentage of acreage on which CM was difficult to control. The exception to this was on transitioning apple acreage, where small farms reported a higher percentage of acreage that was difficult to control.

Question 5 - part 2: "What Frequency of years is CM damage unacceptable?" To address this question, we used a chi-square test to look at differences between small and large growers on land under organic, transitional, and conventional orchards.

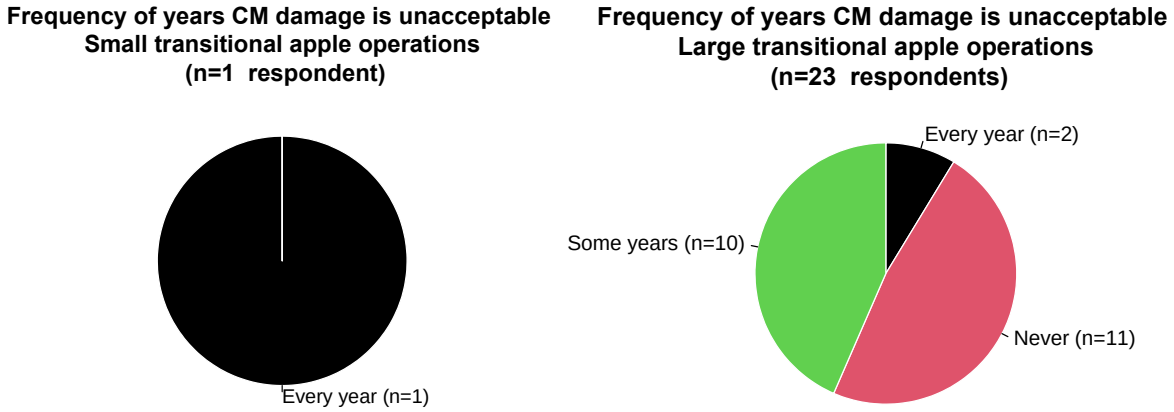
Apple Growers' response:
Small vs. Large Operations: On organic acreage, there was a significant difference in the frequency of years CM damage was found to be unacceptable between small and large orchards (p= 0.0035 *but note small sample size, particularly of small orchards). Larger farms reported a higher percentage of damage in "some years" where smaller farms reported a higher percentage of "never." These differences are shown in Figure 4.

Figure 4.



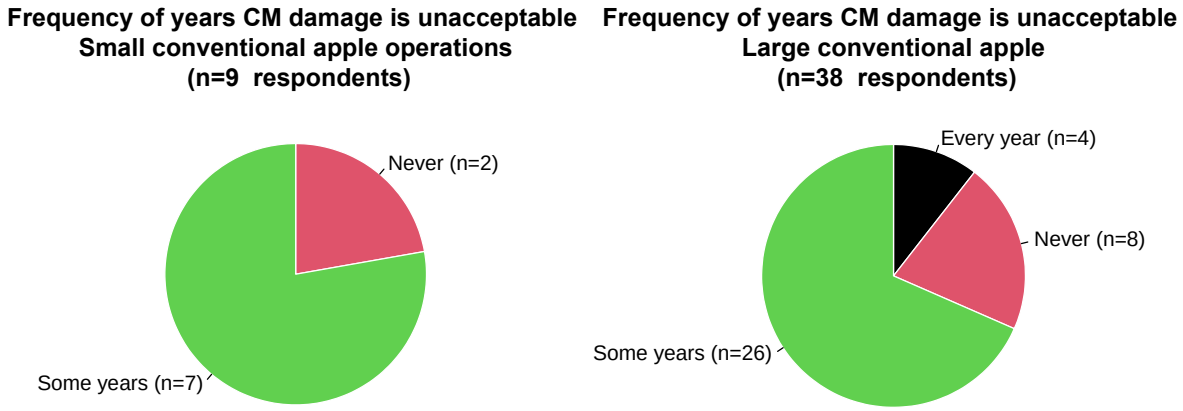
On transitional acreage, there was a significant difference in the frequency of years CM was found to be unacceptable between small and large orchards (p= 0.02949, *but note VERY small sample size, particularly of small orchards). These differences are shown in figure 5.

Figure 5.



On Conventional acreage, there was a significant difference in the frequency of years CM was found to be unacceptable between small and large orchards (p=0.0005). Some percentage of large orchards reported unacceptable damage every year. These differences are seen in figure 6.

Figure 6.



Pear Growers' response*:*

Small vs. Large Operations: On organic, transitional, and conventional acreage, there was no difference found in the frequency of years CM was found to be unacceptable between small and large orchards (p= 0.07346, p= 0.2359, and p= 0.1639, respectively).

Question 5, Part 2 Summary:

Although the response rate for small orchards in all three systems is very low for this question, and there is variability across the 3 systems, it is interesting to note that there is consistently unacceptable damage some years. No interesting differences emerged from the pear growers.

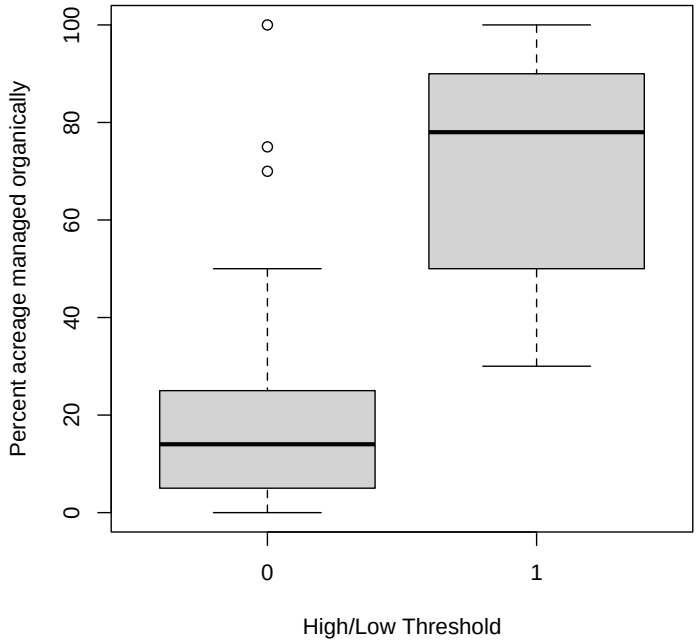
Survey Question 6: "What is your threshold for codling moth damage?" We analyzed data for those who reported a % damage, which excluded some responses. Many folks did not answer this question, many did not provide a percentage, but instead stated a number of stings/trap. Others stated differences between Taiwan market and other markets. I was directed to this approach by the head of the task force. To compare large and small farms we used a chi-square analysis. To compare differences in thresholds based on organic management we took a different approach. In communicating with Tobin Northfield regarding this question, we decided, based on the distribution of the data, to divide the data into 2 groups (<5% damage and >=5% damage) and run a logistic regression.

Apple Growers' response:

Small vs. Large Operations: There was no significant difference in CM thresholds between small and large farms (p= 0.8576).

Organic Management: There was, however, a difference in CM thresholds based on the % acreage managed organically (p= 0.01695); growers with a higher percentage of land managed organically had a higher threshold for damage than those with less land managed organically. This difference is indicated in figure 7.

Figure 7.



Pear Growers' response:
Small vs. Large Operations: There was no significant difference in CM thresholds between small and large farms (p= 0.8266).

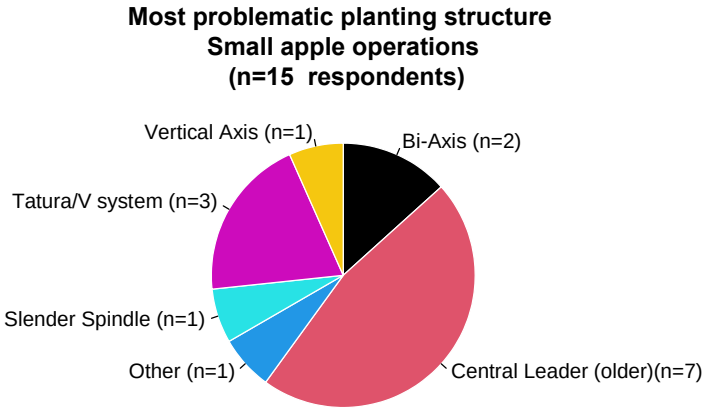
Organic Management: There was no difference in the CM damage threshold based on % organically managed acreage (p= 0.913).

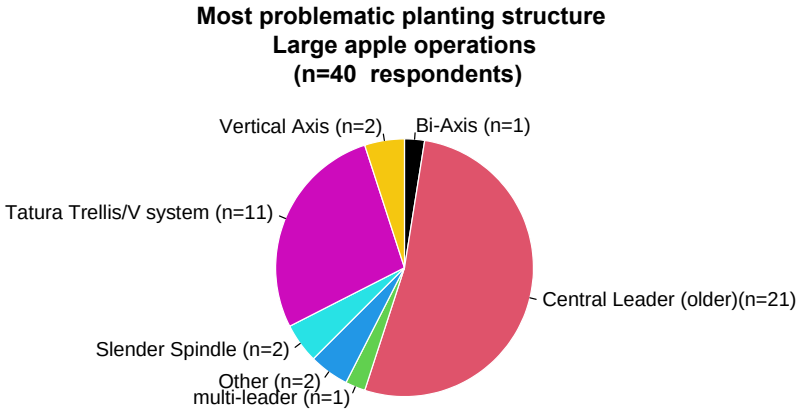
Question 6 Summary:
In both apples and pears, the size of the operation did not seem to matter with regards to a CM damage threshold. However, with apples, the more organic acreage a respondent reported, the high CM damage threshold they had.

Question 7: "In your highest-pressure block, what is your tree planting?" It should be noted that many people answered "other", then wrote in "multi-leader". In fact, multi-leader was the only "other" option described. "Other" was replaced with "multileader" where applicable. The small vs. large farm comparison data were analyzed with a chi-square test. For the organic comparison, it doesn't really make sense to ask how the type of tree that is most problematic, changes as a continuous increase in % acreage managed organically, however, we did look at how this changed with subsets of the data that were "mostly organic" (>75% organic) or "mostly conventional" (>75 conventional).

Apple Growers' response:
Small vs. Large Operations: There was a significant difference in the most problematic tree type found between small and large growers (p=0.0001) with small operations reporting a higher amount of bi-axis and larger operations reporting a small percentage of "multi-leader" as the most problematic tree structure. These differences are shown in figure 8.

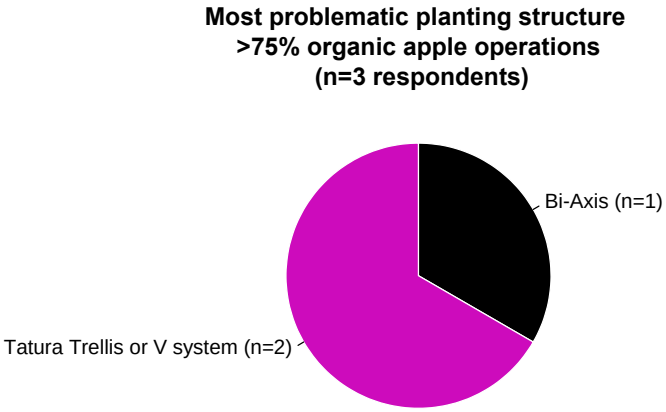
Figure 8.

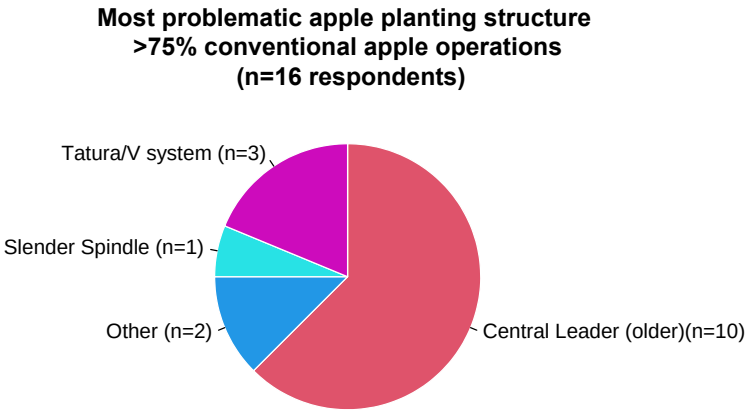




Organic Management: Visual exploration of how mostly organic and mostly conventional operations report problematic tree structure (*note small sample sizes). These comparisons are found in figure 9.

Figure 9.

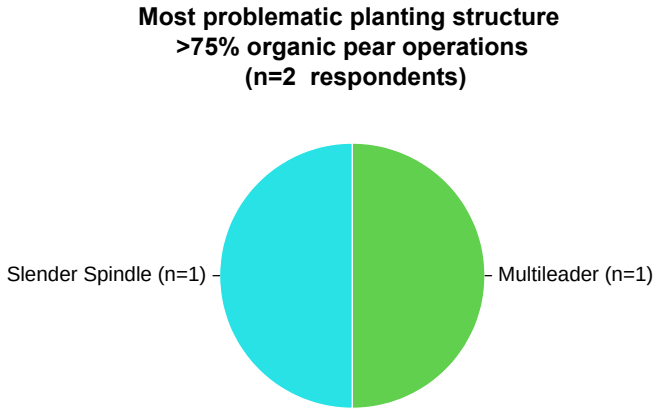


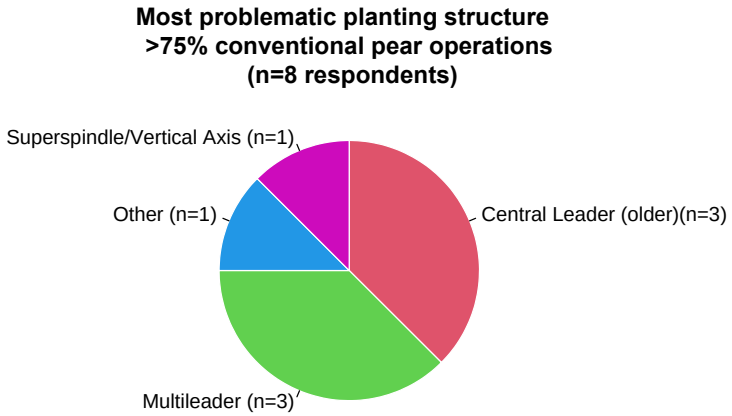


<u>Pear Growers' response:</u>
Small vs. Large Operations: There was no difference in the most problematic tree structure between small and large orchards (p= 0.5897, note, central leader dominant in both).

Organic Management: Visual exploration of how "mostly organic" (>75% organic acreage) or "mostly conventional" (>75% conventional acreage) operations report problematic tree structure (*note small sample sizes). These comparisons are shown in figure 10.

Figure 10.





Question 7 Summary

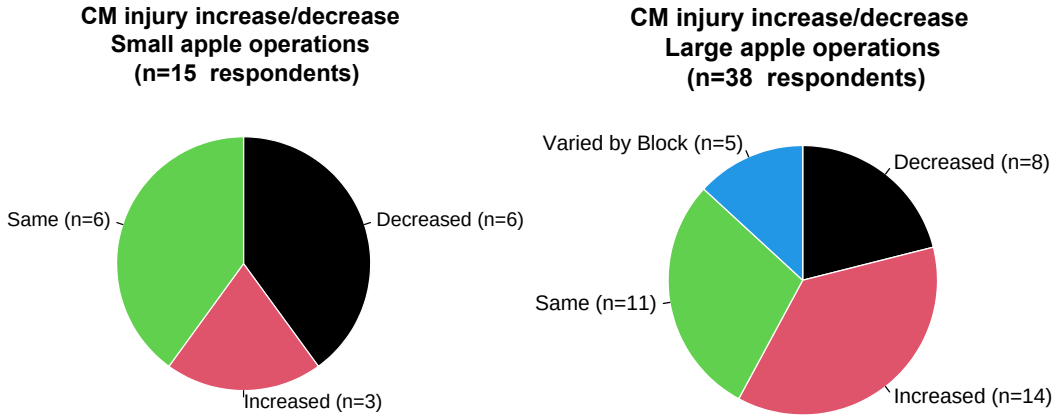
While there is some variability in reporting, both in terms of response rate, as well as tree structures reported, older central leader trees dominated as the most problematic tree structure.

Survey Question 8 "Over the past three years, codling moth injury in the orchard(s) you own or manage has" (select choice – Increased/decreased/remained the same). The small vs. large farm comparison data were analyzed with a chi-square test. For the organic management comparison, we visually explore the differences in changing CM injury for subsets of the data that were "mostly organic" (>75% organic) or "mostly conventional" (>75 conventional).

Apple Growers' response:

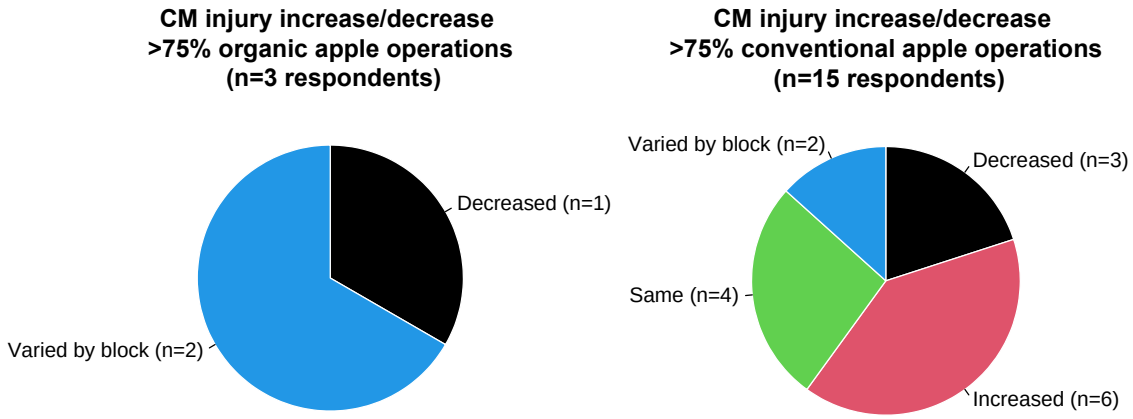
Small vs. Large Operations: There was a difference in whether CM injury increased, decreased, or remained the same on small vs large operations (p=0.0005), with small operations seeing larger percentages of injury staying the same or decreasing, and larger growers seeing injury increasing and some reporting injury to be variable by block. These differences are shown in figure 11.

Figure 11.



Organic Management: Visual exploration of how mostly organic and mostly conventional operations regarding how injury increased/decreased if a grower is "mostly organic" (>75% organic acreage) or "mostly conventional" (>75% conventional acreage). These comparisons are shown in figure 12.

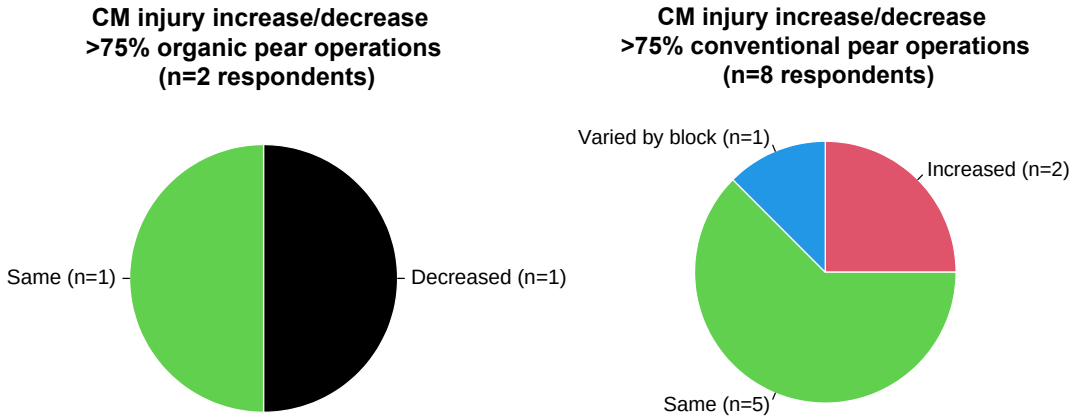
Figure 12.



<u>Pear Growers' response:</u>
Small vs. Large Operations: There were no differences in whether CM injury increased, decreased, or remained the same on small vs large operations (p= 0.5727).

Organic Management: Visual exploration of how mostly organic and mostly conventional operations regarding how injury increased/decreased if a grower is "mostly organic" or "mostly conventional" (*note small sample sizes). These comparisons are shown in figure 13.

Figure 13.



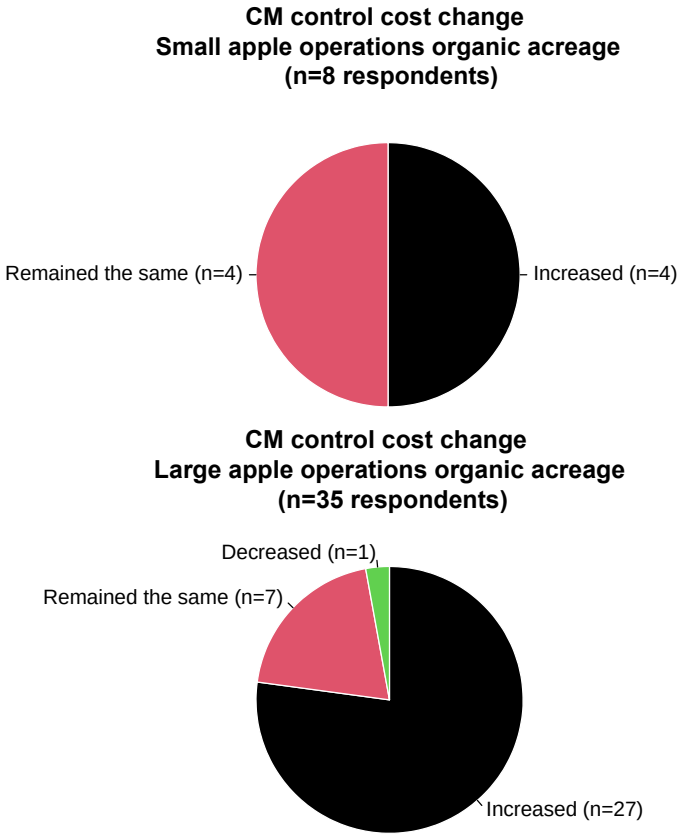
Question 8 Summary:
While apple growers reported quite variable and different results in CM injury changes between small and large operations, pear growers found no differences between large and small operations. In terms of difference in organic and conventional, only conventional growers of both apples and pears reported increases in CM damage, but again, note small sample sizes for mostly organic operations.

Survey Question 9 "Over the past 3 years, how did CM control costs change in your orchard?"
Answers were given for 3 different managements (conventional, organic, transitional). By breaking out the data in this way, the sample sizes are very small (especially for the number of small operations). Small and large operation comparisons were made within each management using chi-square analysis.

Apple Growers' response:

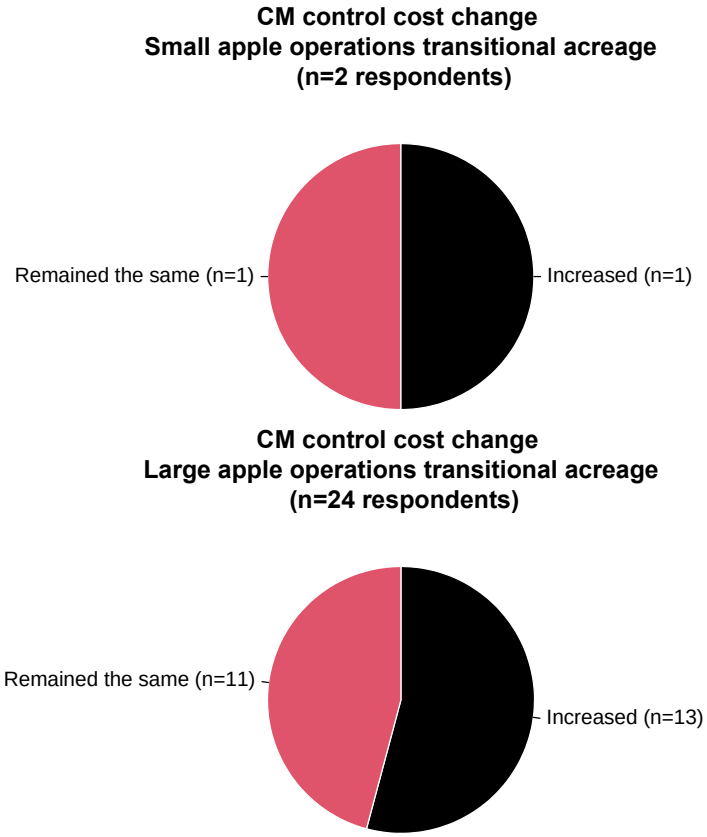
Small vs. Large Operations on organic acres: There was a difference between CM control cost changes on organic acreage, with a higher percentage of large operations reporting increased costs (p= 0.001). These differences are shown in figure 14.

Figure 14.



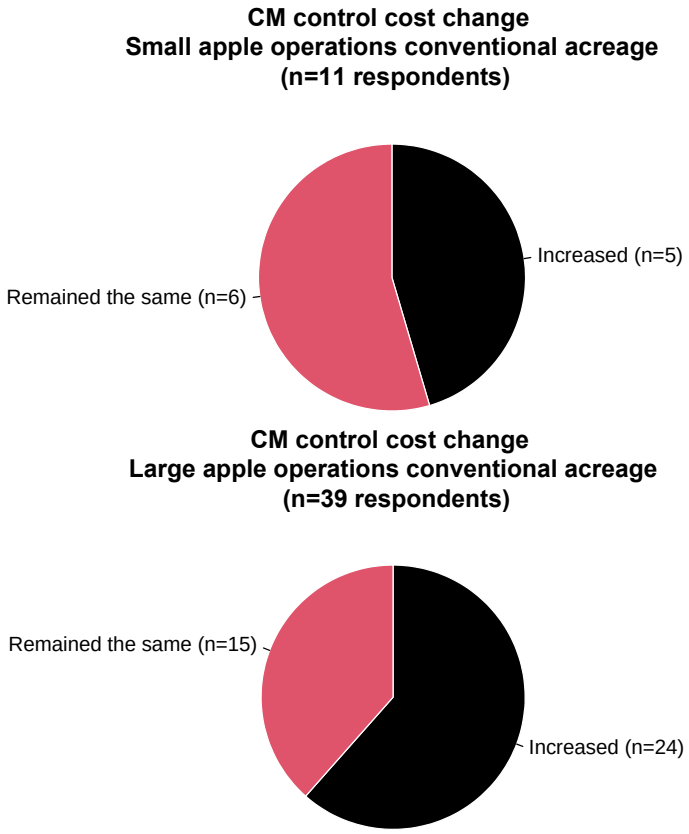
Small vs. Large Operations on transitional acres: There was a difference between CM control cost changes on transitional acreage, with a higher percentage of large operations reporting increased costs (p= 0.0075) (*note low sample size). These comparisons are shown in figure 15.

Figure 15.



Small vs. Large Operations on conventional acres: There was a difference between CM control cost changes on conventional acreage, with a higher percentage of large operations reporting increased costs (p= 0.0005). These differences are shown in figure 16.

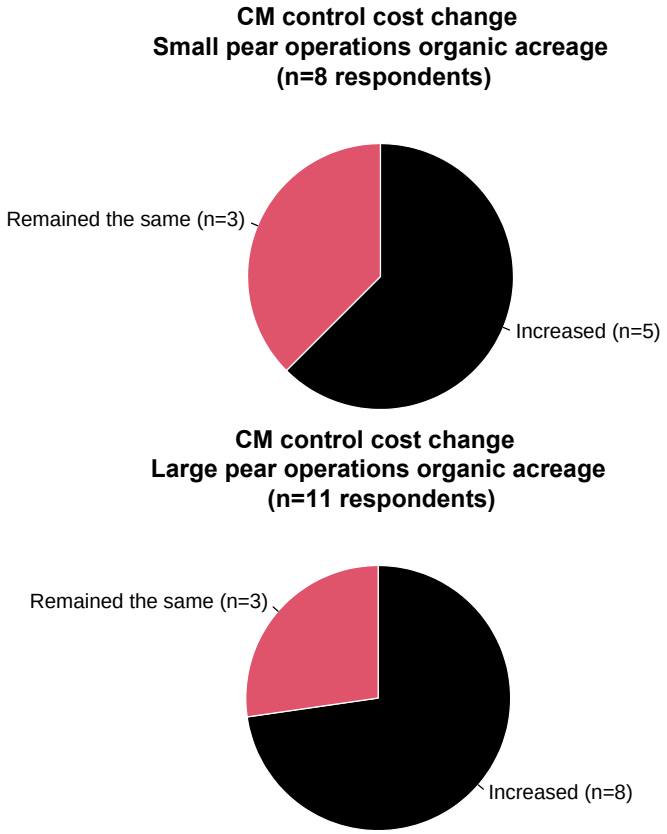
Figure 16.



<u>Pear Growers' response:</u>

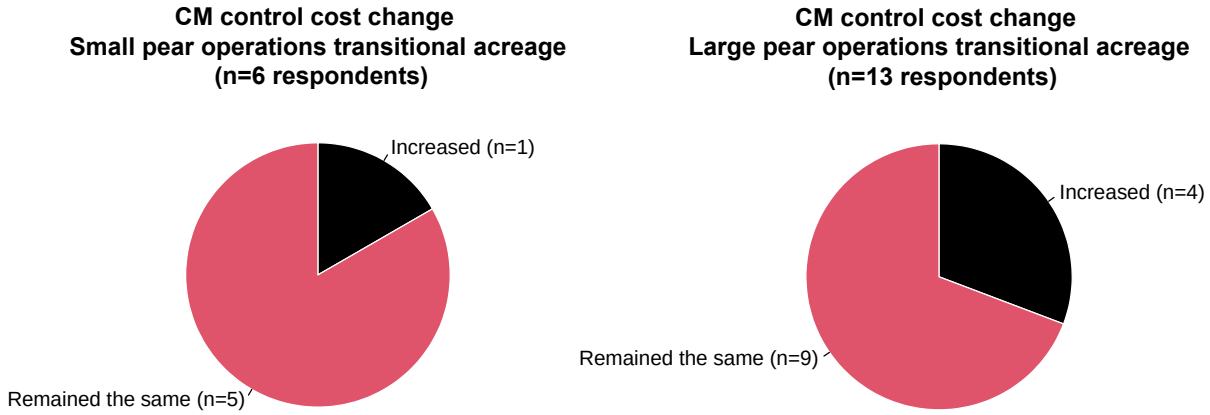
Small vs. Large Operations on organic acres: There was no difference between CM control cost changes on organic acreage (p= 0.6197). These comparisons are shown in figure 17.

Figure 17.



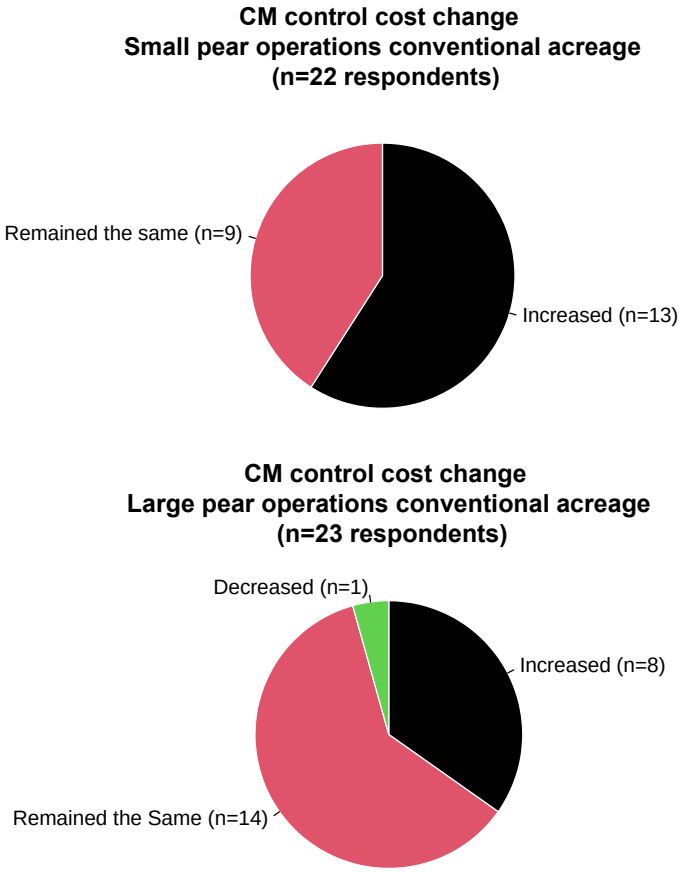
Small vs. Large Operations on transitional acres: There was no difference between CM control cost changes on transitional acreage (p= 0.09495). These comparisons are shown in figure 18.

Figure 18.



Small vs. Large Operations on conventional acres: There was no difference between CM control cost changes on conventional acreage (p= 0.2849). This comparison in shown in figure 19.

Figure 19.

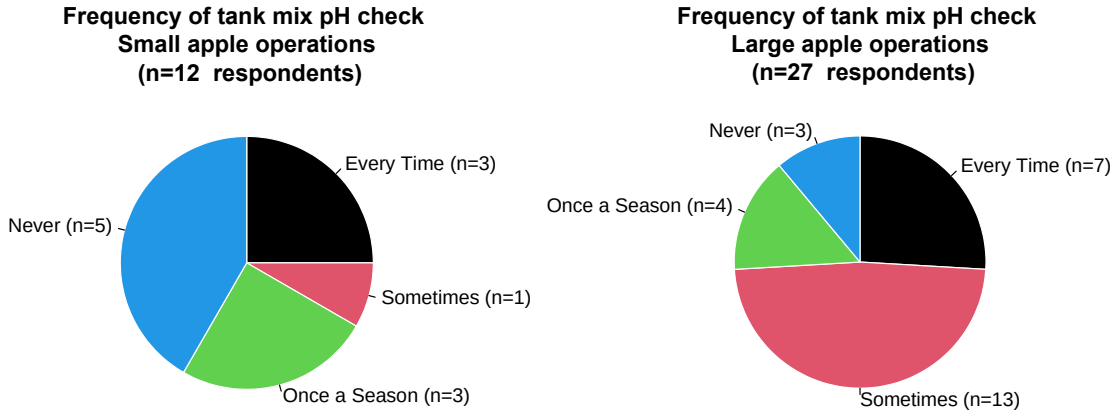


Question 9 Summary:
Interestingly, across all managements, large apple operations reported significantly increased costs associated with CM control. Pear operations did not experience this same difference.

Survey Question 35: "Most sprays, especially biologicals, are affected by the pH of the water used in the tank. How frequently do you check the pH level of the tank mix?" For the small vs large operations comparison, we used a chi-square test, and for the organic management, much like in Q7 and Q8, sample sizes were very small and differences are only visualized.

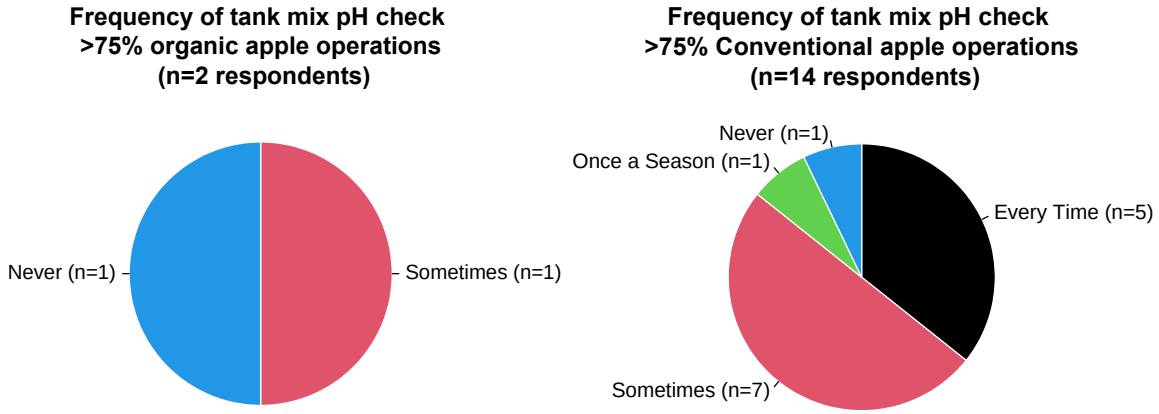
Apple Growers' response:
Small vs. Large Operations: There was a difference (p= 0.0025) in the frequency of tank pH checks between large and small growers. A majority of small operations checked the pH "Never", and the majority of large operations checking the tank pH "Sometimes". These differences are shown in figure 20.

Figure 20.



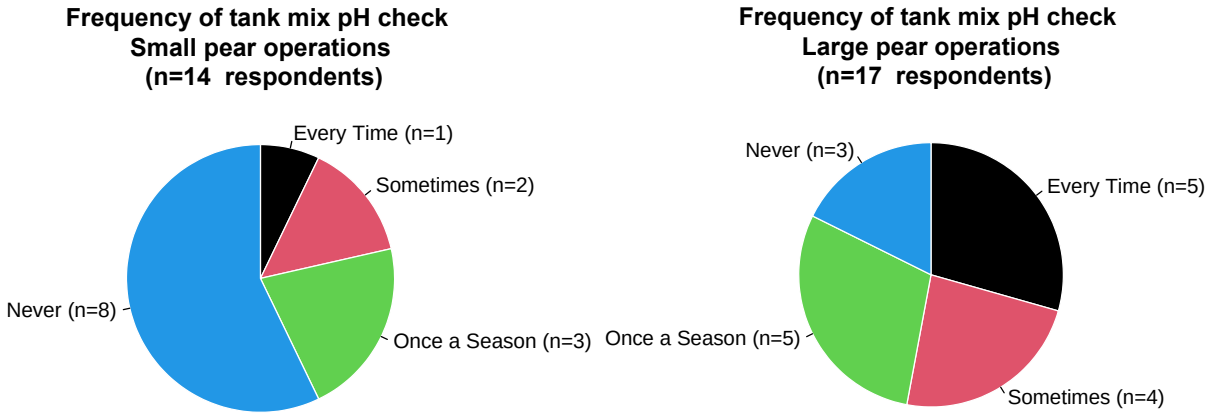
Organic Management: Visual exploration of how frequently "mostly organic" and "mostly conventional "operations check the tank pH. (*note small sample size). These comparisons are shown in figure 21.

Figure 21.



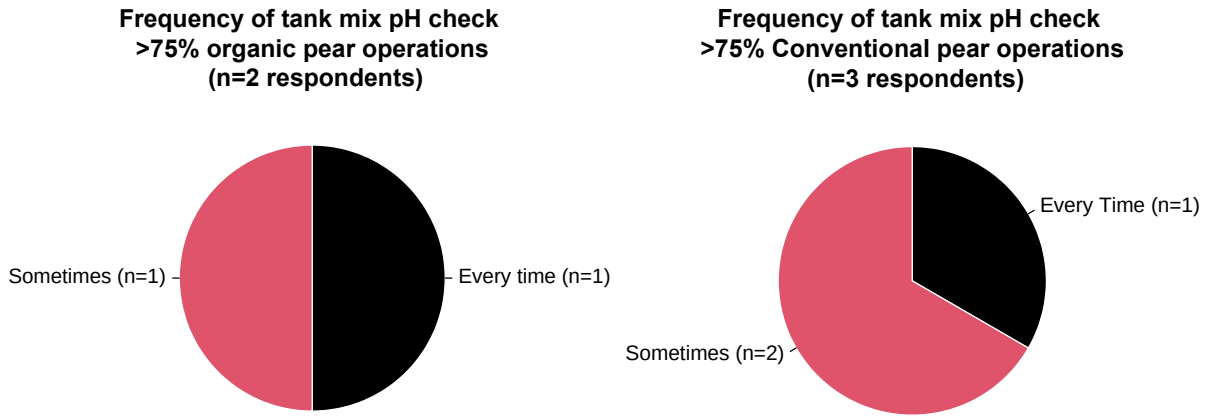
<u>Pear Growers' response</u>:
Large vs. Small operations: There was no difference found between small and large operations regarding the frequency of tank pH checks (p= 0.1609). This comparison is shown in figure 22.

Figure 22.



Organic Management: Visual exploration of how frequently "mostly organic" and "mostly conventional" operations check the tank pH. (*note small sample sizes). This comparison is shown in figure 23.

Figure 23.



Q35 Summary- It appears that across apples and pears of all sizes, about 3/4 of respondents could be checking the pH more regularly

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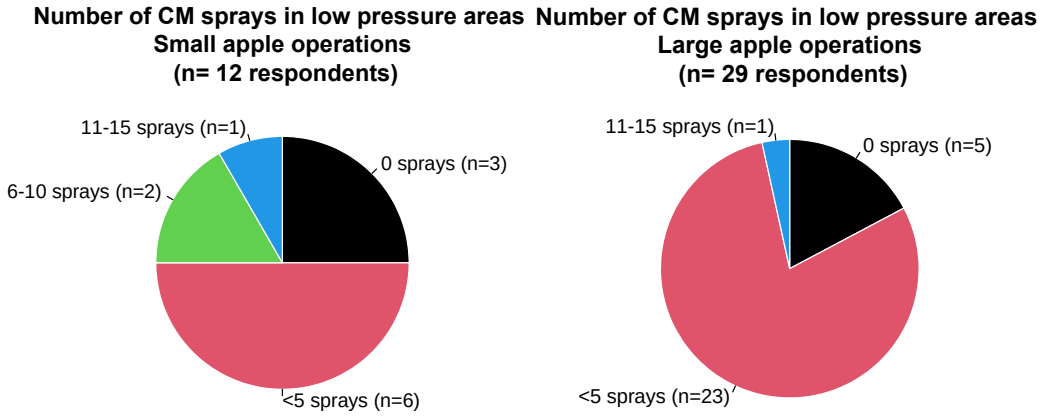
Survey Question 36 "How many codling moth sprays did you apply in low pressure areas in 2020?"
We analyzed the small vs large operations with a chi-square test, and then visually explored the organic management differences.

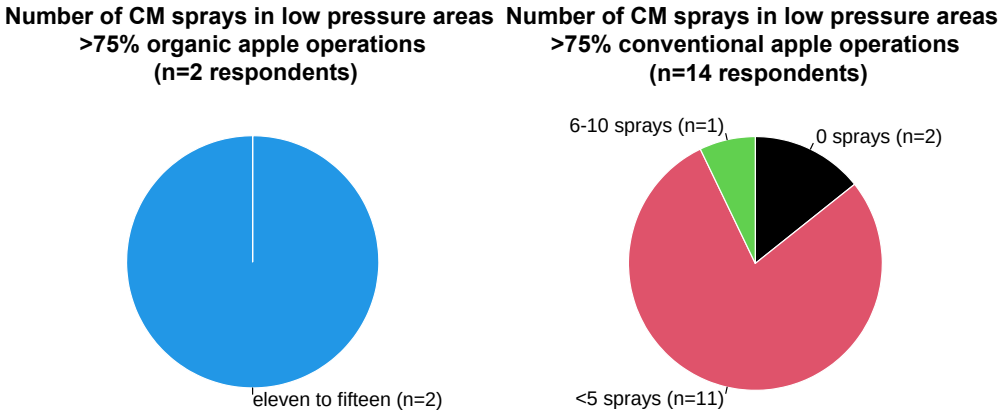
Apple Growers' response:
Small vs. Large Operations: There was a difference in the number of CM sprays in low pressure areas between small and large operations (p= 0.01649), with a higher percentage of large operations sprayed for CM <5 times, and a larger percentage of small operations spraying 6-10 and 11-15 times. These differences are shown in figure 24.

Figure 24.



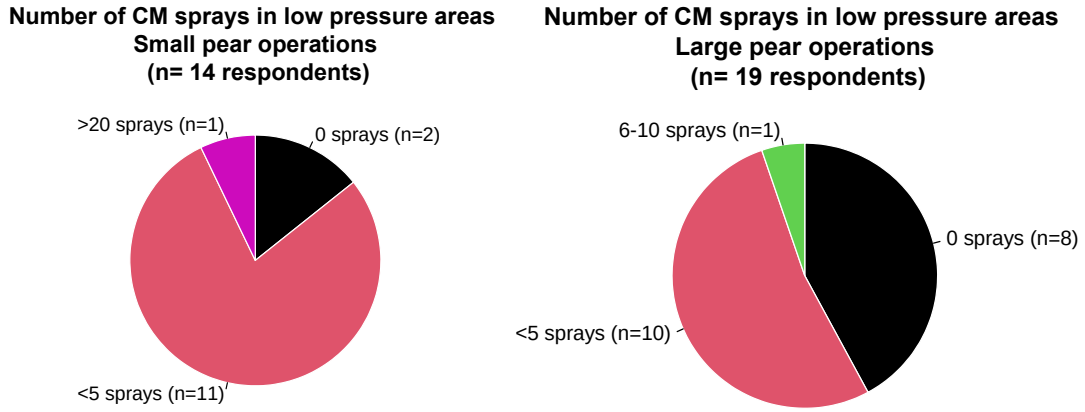
Organic Management: Visual exploration of the number of CM sprays that "mostly organic" and "mostly conventional" operations used (note small sample size). This comparison is shown in figure 25.

Figure 25.



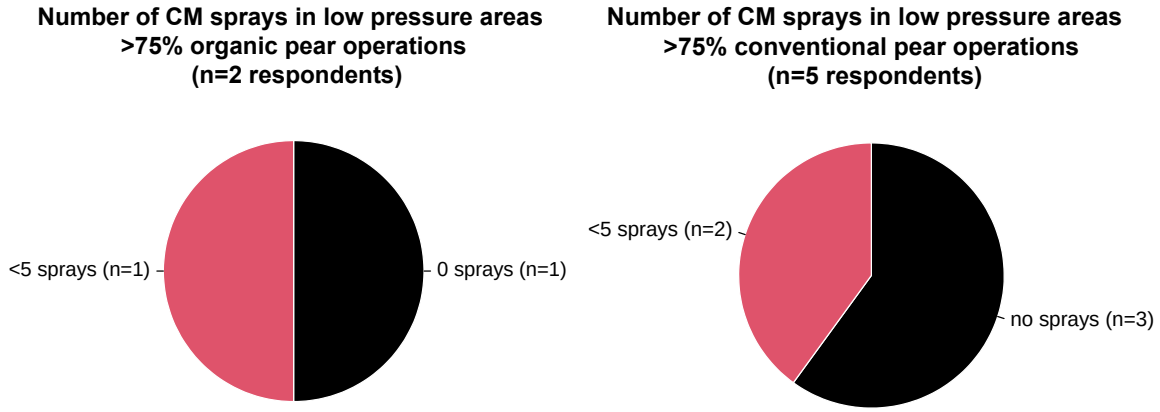
<u>Pear Growers' response</u>:
Small vs. Large Operations: There was not a significant difference in the number of CM sprays in low pressure areas between small and large operations (p= 0.08246). This comparison is shown in figure 26.

Figure 26.



Organic Management: Visual exploration of the number of CM sprays that "mostly organic" and "mostly conventional" operations used (again, note small sample sizes). This comparison is shown in figure 27.

Figure 27.

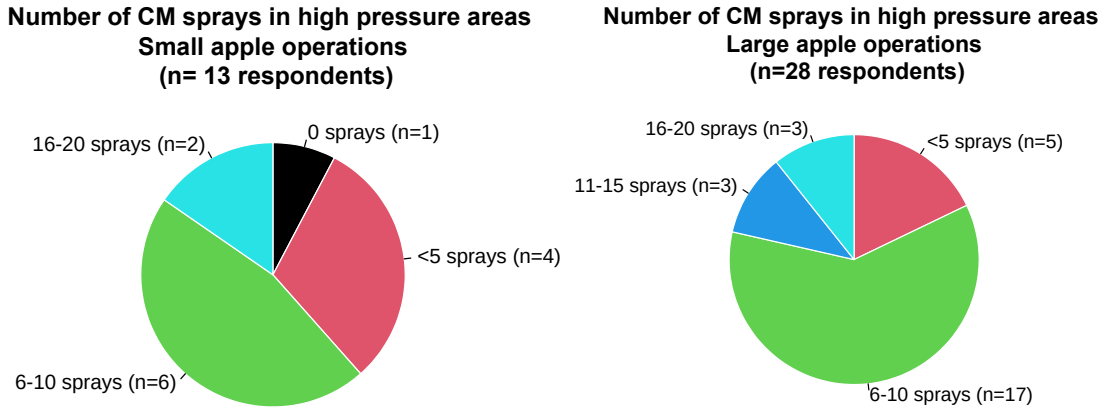


Q36 Summary: while there is some variability in terms of the number of CM sprays in low pressure areas, the majority of apple and pear operations are spraying <5 times, with small percentages spraying more.

Survey Question 37: "How many codling moth sprays did you apply in high pressure areas in 2020?" We analyzed the small vs large operations with a chi-square test, and the visually explored the organic management differences, due to very small sample sizes.

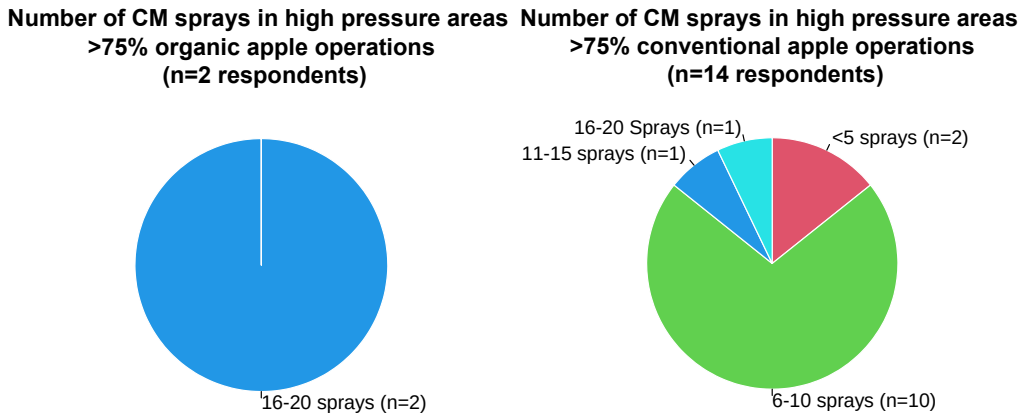
Apple Growers' response:
Small vs. Large Operations: There was a significant difference in the number of CM sprays in high pressure areas between small and large operations (p= 0.02699), with large farms spraying more. This difference is shown in figure 28.

Figure 28.



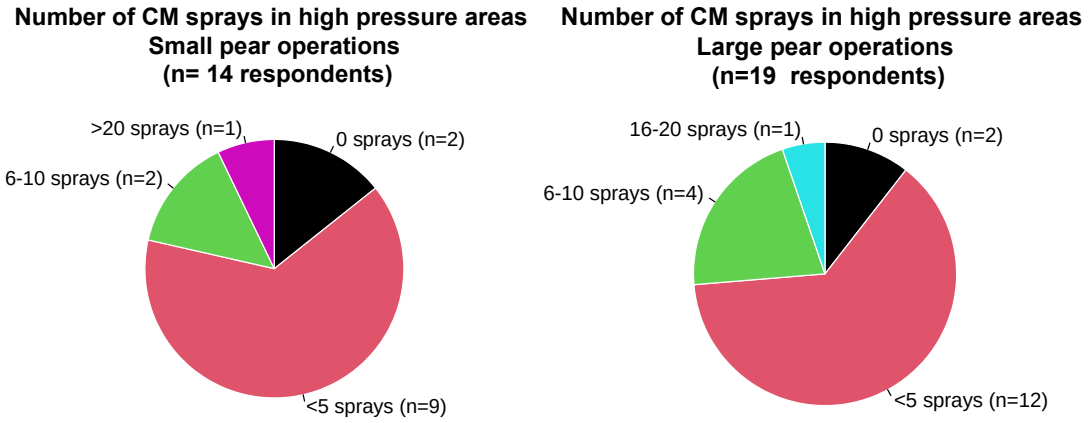
Organic Management: Visual exploration of the number of CM sprays that "mostly organic" and "mostly conventional" operations used (again, note small sample sizes). This comparison is shown in figure 29.

Figure 29.



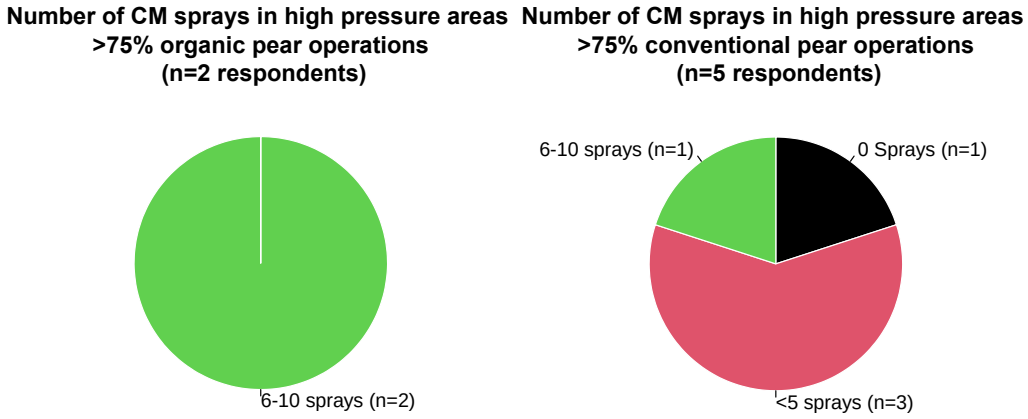
<u>Pear Growers' response</u>:
Small vs. Large Operations: There was no difference in the number of CM sprays in high pressure areas between small and large operations (p= 0.4603). This comparison is shown in figure 30.

Figure 30.



Organic Management: Visual exploration of the number of CM sprays that "mostly organic" and "mostly conventional" pear operations used (again, note small sample sizes). This comparison is shown in figure 31.

Figure 31.



<u>Q37 Summary:</u> In general, larger apple operations appear to be spraying their high pressure areas more times. Additionally, higher percentages of "mostly organic" operations (both apples and pears report spraying more times (although again… note small sample sizes).

Overall Report Summary:

Despite these survey data being relatively messy and the samples sizes being quite small, there are a few very interesting takeaways:

- Apple operations are experiencing consistent, unacceptable codling moth damage in a way that pear operations did not report.
- As apple operations grow a higher percentage of their acreage in organic, their threshold for CM damage goes up
- Central Leader (older trees) dominate as the most problematic tree structure across apples and pears
- Across organic, transitional, and conventional acreage, large apple operations reported significantly increased costs associated with CM control, relative to smaller operations.
- Most folks should be checking their tank pH more regularly
- Larger apple operations look to be spraying their high pressure areas more often than small operations.

The survey was created and distributed by the Codling Moth Task Force Committee appointed and supported by the Washington Tree Fruit Research Commission in 2020. The committee was comprised of growers, university researchers, and industry leaders. Data were compiled by Chris Adams. Analysis was done by Matt Jones (Cascade Agroecology, LLC) and Tobin Northfield (WSU-TFREC).

Project Title: Can we get codling moth females to stop laying eggs on apple?

Report Type: Final Project Report

Primary PI: William B. Walker III
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Cooperators: Dr. Paul Bergeron, Nature's Life, Yakima, WA (Scientific Collaborator); Dr. Jason Pitts, Baylor University, Waco, TX (Scientific Collaborator)

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 56,110
Total Project Request for Year 2 Funding: $ 58,817
Total Project Request for Year 3 Funding: $ 61,610

Other related/associated funding sources: Awarded
Funding Duration: 2018
Amount: $35,000
Agency Name: USDA-ARS, Pacific West Area Office
Notes: Area Office awarded money to previous project manager, Dr. Stephen Garczynski, to purchase a flight tunnel and Track3D system. The Track3D system is comprised of cameras and software to monitor insect behavioral responses in a flight tunnel. No other funds have been sought for this project.

WTFRC Collaborative Costs: None

Budget 1
Primary PI: William B. Walker III
Organization Name: USDA-ARS
Contract Administrator: Mara Guttman
Telephone: 510-559-5619
Contract administrator email address: mara.guttman@usda.gov
Station Manager/Supervisor: Rodney Cooper
Station manager/supervisor email address: rodney.cooper@usda.gov

Item	(Type year of project start date here)	(Type year start date of year 2 here if relevant)	(Type year start date of year 3 here if relevant)
Salaries	$37,306.00	$39,282.00	$41,322.00
Benefits	$13,804.00	$14,535.00	$15,288.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$5,000.00	$5,000.00	$5,000.00
Travel			
Plot Fees			
Miscellaneous			
Total	$56,110.00	$58,817.00	$61,610.00

Footnotes: Salaries and benefits are requested for a full-time GS-6 Lab Technician. Supplies are for cloning ORs, oligonucleotide primer synthesis, Cas9 mRNA and kits for making CRISPR guide RNAs.

AMENDMENT

This section serves notification that there was a change in PI for this project. After completion of the first year of the project, the lead PI, Dr. Stephen F. Garczynski passed away in February 2019. Dr. Garczynski had drafted the first continuation report for this proposal. There was an agreement between USDA-ARS and WTFRC that this project could be paused while Dr. Garczynski's Research Geneticist position was vacant. New PI William B. Walker was indicated on the first continuation report as a "Cooperator" and was hired in October 2020 to fill the vacant Research Geneticist position. Dr. Walker has been working to complete the project as planned and was advised to submit the continuation report as drafted by Dr. Garczynski. During the time this project was paused, no further project work was performed. The equipment items mentioned in "other funding sources" were obtained by Dr. Garczynski and are now available to facilitate completion of the project.

OBJECTIVES

1) Identify and clone Odorant Receptors expressed in the abdomen tip of codling moth females.
Over thirty odorant receptors (OR) were identified in a transcriptome generated from abdomen tips of codling moth females. A majority of ORs identified in the transcriptome have been confirmed to be expressed by molecular cloning and DNA sequencing. Gene expression assays have also been conducted to compare OR expression in mated versus non-mated codling moth females. In the coming years, several of the cloned ORs will be used in expression assays (not funded by this proposal) to determine activating plant compounds; this will facilitate future odorant-based lure research and development.

2) Knock-out OR genes using CRISPR/Cas9 genome editing.
CRISPR/Cas9 genome editing is fully developed for codling moth, and is being used to knock-out genes expressed in the female abdomen tip. CRISPR/Cas9 genome editing of ORs facilitates basic research in the laboratory on the function of the codling moth's olfactory system. Information derived from this line of research may be utilized by integrated pest management strategies including odorant lure development and push-pull applications. A single OR gene that is expressed in female antennae and abdomen tip has been genome edited with the CRISPR/Cas9 approach to induce inactive, functionally deleted OR protein. Laboratory populations of codling moth with the inactive OR gene are being generated for use in oviposition assays.

3) Determine which genes are essential for oviposition site selection.
The hypothesis tested here is that inactive OR genes for specific plant volatiles will alter female codling moth oviposition site selection. Oviposition assays for codling moth are under development and will be used to assess the effects of OR gene knock-out populations. Identification of ORs that directly mediate oviposition behavior will facilitate behavioral testing odorants that are detected by the OR.

SIGNIFICANT FINDINGS

- In addition to the odorant receptors (ORs) identified in the female abdomen transcriptome, several other odorant receptors of interest, including candidate pheromone receptors, were PCR amplified, cloned, and confirmed to be expressed in female abdomen tips
- Three ORs showed expression differences in abdomen tip of mated versus unmated codling moth females
- CRISPR-mediated editing has been achieved for a female specific OR that is expressed in female antennae and abdomen tip, but not in male codling moth.
- Functional assays of this OR resulted in identification of activating fruit odorants not previously known to be detected by codling moth

RESULTS AND DISCUSSION

Identification and cloning odorant receptors expressed in female codling moth
We have prepared a transcriptome (a compilation of all genes expressed in a particular tissue) from abdomen tips dissected from pre-adult (pupal) and unmated and mated codling moth female adults. Transcripts encoding 38 different ORs were discovered. In year two of this project, during the past year, attempts were made to clone and sequence full length transcripts of all of these ORs. Of the 38 ORs identified in the transcriptome, we were able to confirm full-length transcript expression of 24 from female codling moth abdomen tips of either unmated, mated or both (24/38 = 63%). These transcripts were cloned to verify expression and DNA sequence for the design of guide RNAs for use in future CRISPR genome editing experiments to determine functional roles for these receptors.

We were not able to detect or confirm expression in adult female abdomen tips for 14 of the 38 ORs (36%) identified in the abdomen tip transcriptome. It is possible that some of these ORs are not expressed in adult female abdomen tip, but rather only in the abdomen tip of pupal stage, since pupal

abdomen tips were also used to generate the source transcriptome. Given the overall objectives of this project, we did not conduct any PCR screening of ORs in the pupal stage. Alternatively, failure to detect OR expression by PCR in the female adult abdomen tips may be due to technical reasons, for example faulty oligonucleotide primers, or expression below detection thresholds. To rule these out, for ORs that we were unable to detect during initial PCR assays, we designed and tested additional oligonucleotide primers, and moreover, each primer set was subjected to serial rounds of PCR amplification in order to detect expression of transcripts that were otherwise below threshold detection levels after the first round of detection.

It was previously reported that a candidate pheromone receptor, OR1, was detected in female codling moth abdomen tips (Garczynski et al., 2017). Interestingly, we did not observe OR1 transcripts in our female abdomen tip transcriptome. It may be the case that OR1 displays restricted expression in codling moth abdomen tip neurons below thresholds levels for detection in our transcriptome. Because of this, we decided to conduct additional PCR screening of all candidate pheromone receptors, regardless of whether they were identified in our female abdomen tip transcriptome. In our previously published codling moth antennal transcriptome (Walker et al., 2016), a majority of these candidate pheromone receptors displayed either male-specific or female-specific antennal expression and are thus hypothesized to influence sex-specific behaviors such as egg-laying in case of the female specific ORs. Furthermore, one candidate pheromone receptor, OR3 was previously reported to be activated by host-plant volatiles, not pheromones (Bengtsson et al., 2014), opening up the possibility that other candidate pheromone receptors may also respond to host-plant odorants, and mediate behaviors such as egg-laying.

A total of 15 candidate pheromone receptors were screened, regardless of their identification-status in the female abdomen tip transcriptome. Of these, 13 were confirmed to be expressed in abdomen tip of unmated or mated female codling moth, including OR1 which was not identified in the female abdomen tip transcriptome, and OR3, which was. In total, we confirmed 34 ORs expressed in female codling moth abdomen tip tissue, regardless of mating status. While a sizable percentage of ORs identified in the transcriptome were not confirmed by this approach, we nonetheless confirmed and sequenced a substantially large repertoire of ORs in adult female abdomen-tip to provide ample substrate to pursue the main objective of this proposal.

Further analysis of the abdomen tip transcriptome has revealed expression of at least 23 gustatory receptors (GRs), which function in the sense of taste, and 17 ionotropic receptors (IRs), which are known to function in either the sense of smell or the sense of taste. Due to the importance of the sensory systems of both smell and taste in influencing insect oviposition behaviors, future research will examine in greater detail the expression and function of GRs and IRs in codling moth abdomen tip physiology and oviposition behaviors.

Expression analysis of odorant receptors in abdomen tip of unmated and mated female codling moth

For all odorant receptor transcripts that were successfully PCR amplified and cloned, PCR amplification attempts were made on biological samples containing abdomen tips of both unmated and mated codling moths. For some of the odorant receptors, we were initially only able to successfully generate PCR products from either unmated or mated samples but not both. These observations led us to hypothesize that for some of the ORs, expression might be regulated by mating, and furthermore, ORs for which expression is induced or increased by mating may have special relevance to detection of odorants that influence egg-laying behavior. We thus sought to utilize quantitative real-time PCR (qRT-PCR) assays on a subset of ORs that displayed preliminary indications of expression differences in abdomen tip samples of unmated versus mating codling moth. This methodology allowed us to more precisely assess whether mating could induce or restrict expression of specific ORs in female abdomen tip.

Oligonucleotide primers were designed for qRT-PCR assay to assess relative expression levels of 19 ORs in abdomen tip of unmated versus mated codling moth females. Initial testing using multiple

sets of primers for each OR revealed that for 10 of these 19 ORs (53%), expression was not consistently detectable with the qRT-PCR assay. For these ORs, it is likely that their expression levels in adult female abdomen tip are below the threshold of detection by the qRT-PCR assay. We thus pursued fully replicated qRT-PCR assays of the remaining 9 ORs (47%) from this subset to determine if mating affects their expression. It was determined that for one of the ORs, expression was significantly increased in abdomen tip of mated versus non-mated female codling moth, and for two other ORs, expression was significantly decreased in abdomen tip of mated versus non-mated female codling moth (Figure 1).

These ORs will be subjected to further research on their role in codling moth egg-laying behavior through assays on OR protein function as well as CRISPR knock-out experiments. None of the other seven ORs that we assayed showed statistically significant differences in expression in abdomen tip of mated versus unmated codling moth females.

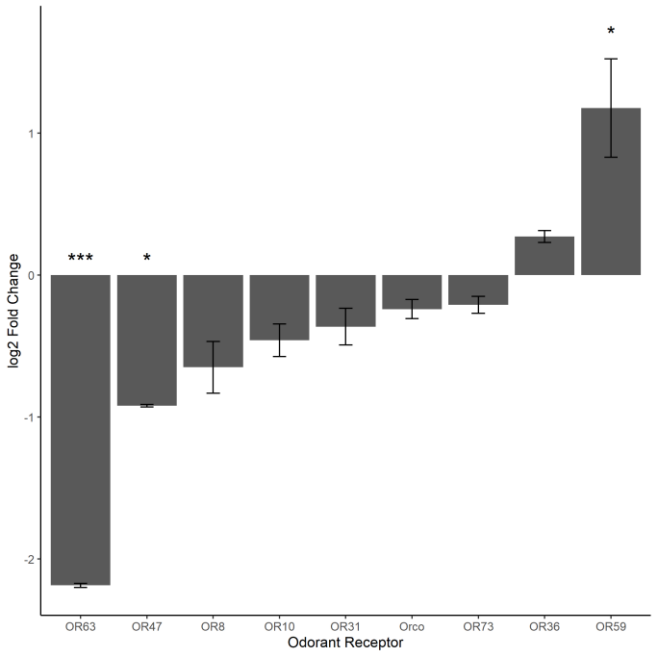

Figure 1. Relative expression of odorant receptors (ORs) in female abdomen tips of mated codling moths versus unmated codling moths. For each OR, three replicate biological samples each from mated and unmated codling moth females were used in quantitative real-time PCR assays. OR gene expression was normalized to gene expression of two control genes and binary log differences in expression values are shown in mated relative to unmated samples. Asterisks indicate statistically significant differences indicated with p value < 0.05 (*) or $p < 0.005$ (***). Error bars indicate standard error values.

Due to the fact that a majority of ORs initially screed by qRT-PCR assay were not detected by this methodological approach, this study is currently being expanded to test expression for all ORs identified in the abdomen tip transcriptome or otherwise detected through molecular cloning assay. Experiments are underway, and results are thus not yet available. Furthermore, GRs and IRs also identified in the abdomen tip transcriptome will also be screened. These additional experiments will provide an expanded view of the effects of mating on smell and taste function in the codling moth female abdomen tip.

Functional characterization of receptors expressed in female abdomen tip

Prior to initiation of this project, research was being conducted on functional characterization of ORs that displayed female-specific expression in antennae of codling moth. One of these female-specific antennal ORs, was determined to be activated by aldehyde odorants present in apple volatile collections, including nonanal (Walker et al., unpublished data), which has been shown to stimulate egg-laying behavior in codling moth (Witzgall et al., 2005). Further research on the functional capacity of this OR, in collaboration with the laboratory of Dr. Jason Pitts at Baylor University, has revealed that it is also strongly activated by specific lactone compounds, in addition to activation by aldehydes present in apple volatile collections. Lactones are known to provide distinct odor characteristics present in other fruit that codling moth can infest, such as peaches and plums, however detection of these types of compounds by codling moth has not been previously reported.

Furthermore, our research on abdomen tip expression of ORs in female codling moth revealed that the aldehyde/lactone sensitive OR is also expressed in female abdomen tip, in addition to the antennal female-specific expression profile previously reported (Walker et al., 2016). Therefore, it is hypothesized that this OR may have a dual role in detection of odorants that mediate both host-seeking behavior at as distance as well as close-range oviposition behavior. Current research efforts are focused on CRISPR editing of this female-specific OR to disrupt its functionality and evaluate specific roles it plays in mediating olfactory behaviors in codling moth females.

Preliminary CRISPR experiments on female-specific odorant receptors

Five different guide RNAs (sgRNAs) were designed to target editing of different genomic regions of the codling moth female-specific OR. Approximately 200 freshly laid codling moth embryos were injected with each sgRNA combined with the CAS9 mRNA. 40-60% of all injected embryos survived injections through at least neonate larval hatching. Larvae were provided with unrestricted access to standard artificial diet. In order to assess relative efficacy of CRISPR editing for each sgRNA, two different biological samples containing approximately 10 pooled third-instar larvae were taken from each sgRNA cohort. Genomic DNA was extracted from each sample, the appropriate genomic region was PCR amplified and subjected to high-throughput DNA sequencing in order to evaluate degree of editing induced by each sgRNA.

CRISPResso analysis revealed that substantial CRISPR editing was induced by three of the five sgRNAs in one or both of the biological samples selected for each sgRNA. These three sgRNAs have been selected for further experimentations. One of the sgRNAs is present in the exon 2 region of the OR gene, while the other is present in the exon 3 region (Figure 2). For initial CRISPR editing experiments, the exon-2 sgRNA was co-injected with either of the two exon-3 sgRNAs. The objective was thus to induce deletion of the genomic region across exon 2 and exon 3, resulting in a non-functional OR protein. PCR analysis of genomic DNA across exon 2 and exon 3 in CRISPR-injected individuals revealed the presence of several genotypic variants that contained genomic deletions across the targeted region (Figure 2). Furthermore, analysis of the sequence of mRNA for the target OR in the offspring of CRISPR-injected individuals revealed partial or whole deletions of entire exons including exon 2 and exon 3. These results provide further support that CRISPR-based genome editing of odorant receptors is robust in codling moth, and will serve as a viable approach to study gene function directly within codling moths.

Current efforts are focused on crossing CRISPR-edited codling moth insects that contain deletions of the target OR gene in order to generate codling moth colonies that are homozygous, or fully edited, with non-functional copies of the OR gene. Once these colonies have been generated and shown to be stable, CRISPR-edited insects will be subjected to oviposition behavioral assays that are currently under development.

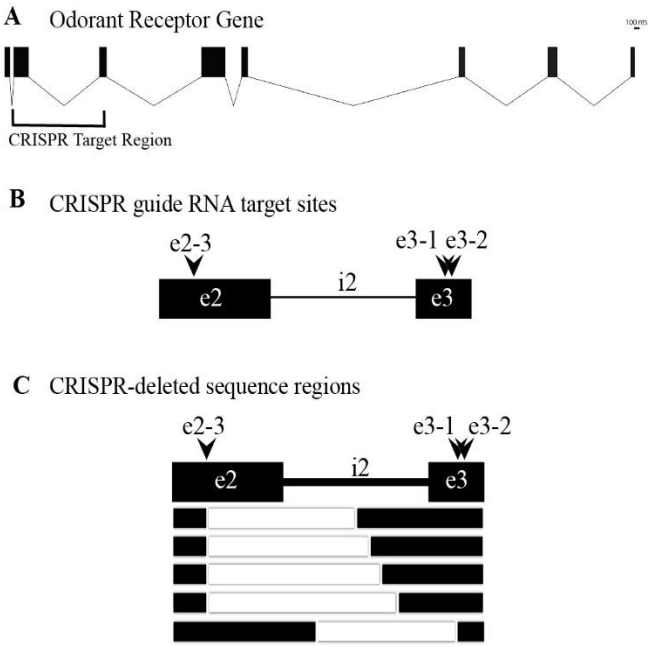

Figure 2. CRISPR editing of female odorant receptor (OR). **A)** Gene structure of target OR, with exons shown as black bars and introns shown as stick lines. Target region for CRISPR-editing encompasses exon 2 and exon 3. **B)** target site for selected guide RNAs (sgRNA) in exon 2 (e2) or exon 3 (e3). The aim is to delete intervening sequence between e2 and e3, including the second intron (i2). One sgRNA from each exon are both injected simultaneously. **C)** Five examples of CRISPR editing genotypes from injected individuals. The black bars (below exon/intron structure) indicate genomic regions not deleted, while intervening white bars indicate deleted regions.

Preliminary female codling moth oviposition behavioral assays

Initial efforts at developing a codling moth oviposition assay have not been successful. As the central aim of this project is to divert codling moth egg laying behavior from apple, we attempted oviposition assays using a plastic container with an apple wedge in it. Codling moth females that had been mated with males were placed into the contain and allowed to lay eggs freely within the container during an 8-hour period largely during their preferred hours for egg-laying, including the 3-4 hours prior to darkness. A majority of moths tested laid their eggs, however, not on the apple, but rather on the plastic container. This behavior may be attributed to the fact that moths used were from our codling moth colony, which has been maintained in our laboratory for more than a decade; behaviors of colony-kept insects is well known to deviate from those of wild insects due to a variety of factors including lack of natural conditions and inbreeding effects from restricted population size.

An attempt was made to assay wild-caught codling moths with our behavioral oviposition assay, and also introgress them into our colony. Codling moth larvae were harvested from a source of apples from a Yakima neighborhood tree. The intent was to rear the larvae to adulthood, then use a subset in behavioral assays and introduce the remainder into the colony. However, this population was heavily infested with parasitic wasps and very few adult moths emerged. Greater efforts will be made during the next growing season to obtain varied sources of wild codling moth. Additionally, optimization of the behavioral assay is underway, using colony insects, for example, with different types/sizes of containers.

Conclusions

Analysis of odorant receptor (OR) expression in the female codling moth abdomen-tip transcriptome has been completed. A repertoire of over thirty ORs expressed in the abdomen tip has been confirmed through molecular cloning and sequencing analysis. Rigorous quantitative expression analysis has resulted in identification of three ORs that display expression patterns in the abdomen tip that are modulated by mating. Stemming from this project, other chemosensory gene families that function in the sense of smell and taste are also being investigated. Odorant and tastant receptors that display increased or reduced expression after mating are strong candidates to have a prominent role in pre- or post-mating behaviors, such as food- or mate-seeking and egg-laying.

A single OR that is expressed in both female antennae and abdomen tip, and is not expressed in males, is observed to respond to fruit-derived odorants, including some that have not been previously reported to be detected by codling moth. Ongoing and future research is aimed at evaluating how these odorants affect codling moth behavior in both the laboratory and apple orchards. Current efforts in the laboratory are focused on CRISPR editing of this female-specific OR gene and subsequent consequences on olfactory behaviors when this gene is disrupted. Behavioral oviposition assays are under development in order to facilitate this aim. Future research is planned for CRISPR-editing disruption of other OR genes that display mating-affected expression patterns in the abdomen tip. Collaborative research efforts will be aimed at identification of odorant ligands that activate these receptors, as well as testing the effects of CRISPR-based disruption of them on codling moth oviposition behaviors.

Executive Summary

Project Title: Can we get codling moth females to stop laying eggs on apple?

Keywords: codling moth, olfaction, oviposition, CRISPR, lures

Abstract: Despite its prominence in the USA for over 200 years, codling moth remains a primary pest in apple cultivation to this day. In recent decades, control of codling moth in orchard production systems has been greatly aided by targeting the codling moth's olfactory system, exploiting the insect's keen sense of smell. Until now, this has largely been focused on disrupting male moth attraction to female through use of pheromone dispensers to induce mating disruption. However, it is the female moth that lays the eggs to ultimately propagate the next generation. Therefore, further attention to the female olfactory system is necessitated, ultimately aimed at disrupting female codling moth olfactory-based behaviors and thus complementing strategies that disrupt male codling moth behaviors. Research on insect olfactory systems has largely investigated the function of the insect's nose, the antennae. However, recent discoveries in other insects have cultivated an appreciation for an olfactory role for the female ovipositor, located on the abdomen tip, in guiding behaviors such as egg-laying, known as oviposition. In recent decades, much attention has been given to insect odorant receptors (ORs) due to their central role in the detection of odorants from the insect's environment.

For this project, analysis of OR expression in the female codling moth abdomen-tip has been completed. A repertoire of over thirty ORs expressed in the abdomen tip has been confirmed through molecular cloning and sequencing analysis. Rigorous quantitative expression analysis has resulted in identification of three ORs that display either increased or reduced expression in the abdomen tip subsequent to mating. These odorant receptors are strong candidates to have a prominent role in pre- or post-mating behaviors, such as food- or mate-seeking and egg-laying.

A single OR that is expressed in both female antennae and abdomen tip, and is not expressed in males, is observed to respond to fruit-derived odorants, including some that have not been previously reported to be detected by codling moth. Ongoing and future research is aimed at evaluating how these odorants affect codling moth behavior in both the laboratory and apple orchards. CRISPR-based editing of this female-specific OR gene has been achieved, and future efforts will be focused on the consequences of disruption of this gene on olfactory behaviors of female codling moths. Behavioral oviposition assays are under development in order to facilitate this aim. Future research is planned for CRISPR-editing disruption of other OR genes that display mating-affected expression patterns in the abdomen tip. Collaborative research efforts will also be aimed at identification of odorant ligands that activate these receptors, as well as testing the effects of CRISPR-based disruption of them on codling moth oviposition behaviors.

Proposal Title: Assessing effects of orchard management on codling moth ecology

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Cooperators: Tobin Northfield, WSU Entomology/TFREC

Project Duration: 3-Year

Total Project Request for Year 1 Funding: $82,000
Total Project Request for Year 2 Funding: $85,000
Total Project Request for Year 3 Funding: $88,000

Other related/associated funding sources: None

WTFRC Collaborative Costs: None

Budget 1:

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Organization Name: Washington State University
Contract Administrator: Michael Ababurko
Telephone: 509-335-5521
Contract administrator email address: Michael.ababurko@wsu.edu

Item	2022	2023	2024
Salaries[1]	$58,000	$60,320	$62,733
Benefits[2]	$20,671	$21,498	$22,358
Wages			
Benefits			
Equipment			
Supplies[3]	$1,329	$1,182	$909
Travel[4]	$2,000	$2,000	$2,000
Miscellaneous			
Plot Fees			
Total	$82,000	$85,000	$88,000

1 – Salary for a postdoctoral scholar (100% FTE) who will oversee the project
2 – Benefits for the postdoctoral scholar include health and life insurance, retirement benefits, etc.
3 – Funds to purchase trapping materials for collection of codling moth data
4 – Funds will be used to support rental of a motor pool vehicle to support regular travel to field sites

Justification: Effective codling moth management relies on assessing population dynamics and phenology in orchards. For example, growers and consultants use phenology models to estimate the timing of codling moth life stages in the field so insecticide sprays are timed to when eggs and new larvae are present. However, the validity of codling moth models has been questioned recently because codling moth trap catch data from commercial orchards often fails to mirror predictions from models; ***growers and consultants often note in particular that trap catch of first-generation adults lags what is predicted by phenology models***. In this project we are assessing factors that affect codling moth ecology and the potential fit (or lack thereof) between trap catch and predictions of phenology models. ***Our project will produce more flexible models that growers can use to assess codling moth ecology and make management decisions.***

Objectives: The impacts of modern management practices on codling moth ecology will be investigated with two research objectives, with data leveraged into a third extension objective. Our three complementary objectives are:

(1) Assess dynamics of codling moth populations across orchards with variation in intensity of mating disruption and early-season insecticide use

(2) Improve predictive capacity of codling moth phenology models by incorporating factors that may affect population dynamics, such as mating disruption and insecticide use

(3) Conduct outreach to show how codling moth ecology is affected by management practices

Progress on Objectives (2022)

(i) Objective 1: Assess dynamics of codling moths populations across orchards with variation in intensity of mating disruption and insecticide use.

At the beginning of the project team we brought on Robert Curtiss, who was a postdoctoral scholar in co-PI Nottingham's program, to lead the sampling effort on the project. Our original goal was to sample codling moth populations across rchards reflecting variability in production conditions and management practices that are typical of Washington (i.e., sites will be selected across gradients from North to South and East to West, with variable elevations, precipitation, etc.). We were able to achieve our goals with site selection, except we were unable to identify any orchard blocks that had no mating disruption or early-season insecticide sprays (see the paragraph to follow for more details on site selection); while our study thus lacked a true "untreated control", by designing our study in commercial orchards we feel confident our study reflects practices used in Washington orchards. Across the blocks sampled there was variability in the use of mating disruption and sterile insect releases, which mimicked the disruptions we expected might cause deviation between phenology models and trap capture.

Plots: In spring 2022 WSU postdoc RT Curtiss identified seven commercial apple orchards that were appropriate for this study. They were selected based on both their geographic location and the type of mating disruption employed. The orchards were divided into three geographic blocks corresponding to latitudes and longitudes 46-47°N and 119-121°W, 47-48°N and 119-121°W, and 48-49°N and 119-121°W. The Northern- and Southern-most blocks each had one passive

and one active mating disruption plot, while the central block had one passive, one active, and one no mating disruption plot. We plan to continue to monitor these orchards in 2023 and select additional orchards that may meet particular conditions. In particular, we will continue to seek out any orchards that do not use mating disruption or sterile insect releases, as we expect codling moth populations to mirror phenology models most closely at unmanaged sites.

Sampling: Throughout 2022, led by postdoc Curtis, our team conducted weekly sampling of codling moth populations in each orchard. Each orchard had a total of ten traps that were placed both along block edges and towards the center of the block (Figure 1). Captures of male and female codling moths were quantified using Orange Pherocon VI delta traps (Trécé Inc.) baited with a PHEROCON® CM-DA COMBO™ Lure + AA Lure (Trécé, Inc.) designed to attract both male and female moths. The 2-part lure was held above the replaceable sticky liner with a pin through the top of the trap. To maximize catch, traps were placed within the top 1/3 of pre-marked trees and lures were changed every eight weeks. Traps were monitored every 7 days. Trap sticky liners were removed and replaced if moths were present when traps were checked, and they were examined in the lab to determine sex of captured moths. Due to the proximity of sterile moth releases, all captured moths were inspected for the presence of internal red dye to discern sterile from wild moths.

From our first year of data, codling moth was rare across our 7 orchard blocks in 2022. Codling moth was fairly rare, with only 360 total moths collected across all sites; only 20% of the moths collected were wild type moths and 80% were indicated as sterile moths. These data are being used to construct phenology curves for each site for use in modelling

Figure 1. Example of sampling design in an orchard. Traps were placed throughout the block and were checked weekly to assess moth population dynamics.

Additional Data Collection and 2023 plans: To supplement our own field work, we are working with the OK-SIR program to gather additional codling moth trap data from fields managed with variability in sterile insect releases. These data, which go back over a decade, provide a more broad set with which to test our modeling hypotheses. We hope to identify other commercial partners who are willing to share trap catch data in 2023 as part of the project. We will continue our sampling work in 2023 to gather more data from commercial fields in Washington.

(ii) Objective 2: Improve predictive capacity of codling moth phenology models by incorporating factors that may affect population dynamics, such as mating disruption and insecticide use

In the fall of 2022 we brought on Gengping Zhu, who is a postdoctoral scholar in the Crowder lab, to work part-time (40% FTE on the project; this complements the 60% FTE used to support postdoctoral scientist Curtiss). As an initial step in modeling, we have gathered temperature data and produced predictions of the codling moth phenology model for each of our seven sites. Output of the model shows predicted % emergence of different codling moth life stages; our trap data will be used to test how these predictions matches up with adult trap catch. Zhu is also working to gather the weather data from the hundred of sites provided by the OK-SIR program.

Once we have all datasets prepared we anticipate the modeling phase will accelerate in Fall of 2023 once we have an additional year of field data.

(iii) Objective 3: Conduct outreach to show how codling moth ecology is affected by management practices

Crowder gave several presentations on codling moth ecology and phenology, and how growers and consultants should consider data on both as part of an integrated management program. The first talk was in February 2022 at the WSU Weather School and reached over 200 subscribers. Crowder also presented work on codling moth at the 2022 Hort Show, the largest industry conference of the year. Additionally, Robert Curtis provided extension talks at four meetings in 2022 in relation to the impact of management practices on codling moth ecology. Finally, Liesl Oeller, who is the coordinator of the WSU DAS, gave a talk on codling moth and the decision aid system at the 2022 Entomological Society of America Annual Conference.

In 2023 we will continue to produce outreach talks and we hope to work with the codling moth task force to provide additional material in a variety of formats. We will also work to integrate any new modeling information into the WSU Decision Aid System platform.

Executive Summary

Project Title: Assessing effects of orchard management on codling moth ecology

Keywords: Codling moth, mating disruption, phenology, population dynamics, pest management

Abstract: This project is designed to provide more comprehensive information about how growers and consultants can manage codling moth by linking predictions of phenology models with trap catch data. While much of the Washington tree fruit industry uses phenology models in their management, it is often unclear how growers should integrate trap catch data with models to make informed spray decisions. Our program will provide baseline data on the variability observed in codling moth populations across realistic Washington growing conditions, and show how trap catch data may not always mirror predictions of phenology models. We hope to show that effective early season management using mating disruption, insecticides, or sterile insect releases may actually cause observed trap catch to lag significantly from what is predicted from models. We hope to be able to integrate this information to provide better models that allow growers to conduct more responsive management that links real-time trap data with models.

Our wok in 2022 established a sampling program across 7 commercial orchards, and our team began a process of collecting data from a larger set of orchards for modelling. Our project is supporting two part-time postdoctoral scholars (both work on other projects as well) that are working collaboratively to complete the field objectives and have the data available for modelling. In 2023 we will expand our sampling work and begin to make progress on modelling efforts while continuing to conduct outreach.

Project Title: Genetic engineering of moth viruses for enhanced insecticidal efficacy

Report Type: Continuing Project Report

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Project Duration: 3 Year

Total Project Request for Year 1 Funding: $58,196
Total Project Request for Year 2 Funding: $60,000
Total Project Request for Year 3 Funding: $61,804

Other related/associated funding sources: None

WTFRC Collaborative Costs: None

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Item	2021	2022	2023
Salaries	$40,089.00	$41,425.00	$42,762.00
Benefits	$14,031.00	$14,499.00	$14,967.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$4,076.00	$4,076.00	$4,075.00
Travel			
Plot Fees			
Miscellaneous			
Total	$58,196.00	$60,000.00	$61,804.00

Footnotes: Salaries and benefits are requested for a full-time GS-6 Lab Technician. Supplies are for molecular cloning, viral genotyping and DNA sequencing, cell culture and viral culture/purification.

OBJECTIVES

1) Develop genetic hybrids of CpGV that display increased efficacy in codling moth larvae.

It was initially proposed that admixtures of different strains of CpGV will be used to co-infect codling moth cell culture lines. However, in conversations with Prof. Johannes Jehle, from which the CpGV cell line (Cp14) was obtained, it was determined that this approach is not feasible. The efficiency of viral replication and speed of infection are very low in the CpGV cell line; this results in failure to product large amounts of virus from the cell line. Because of this, the proposed co-infection experiments will be carried out in codling moth larvae instead. Larvae will thus be exposed to admixtures of the different strains, and efficacy trials will be conducted to screen for faster or more potent killing compared to baseline rates. Viral extracts will be made from larvae exposed to mixtures that display enhanced effectiveness, and will be genetically characterized to identify any genetic hybrids that may contain properties of the different virus strains combined in novel ways. Isolates of these hybrids will be cultivated, exposed to codling moth larvae and further screened for efficacy. with eventual applicability in both conventional and organic orchards. For authorized use in organic orchards intended products would be submitted to appropriate Material Review Organizations for official registration.

2) Genetically engineer CpGV to include the spider toxin, Hvt.

Standard molecular cloning and genetic engineering methods will be used to splice the spider toxin gene into the genome of a CpGV strain currently used for codling moth control. Genetically transformed viruses will be cultivated in cell culture and then exposed to codling moth larvae and screened for efficacy. It is hypothesized that the presence of the spider toxin in CpGV will enrich the effectiveness of commercial formulations. Moreover, the presence of an additional virulence factor with a unique mode of action may serve as a safeguard against eventual development of resistance in codling moth populations. Eventual applicability would be designated for conventional orchards. Use of this spider toxin has previously been patented, however the patent has expired, and the toxin may be used freely.

3) Co-infect codling moth larvae with CpGV and CrpeNPV.

The identification of a novel virus, CrpeNPV, that can infect codling moth provides new opportunities to explore enhanced formulations of viral control of codling moth utilizing both CpGV and CrpeNPV concurrently. Fundamental research on coinfection of codling moth with CpGV and CrpeNPV is required, as is further research on the amenability of cultivation of CrpeNPV in standard codling moth cell culture systems. Cultivars of CpGV and CrpeNPV would be combined and exposed to codling moth larvae and then screened for efficacy. Parameters for stable mass production of CrpeNPV in insect cell culture would also be investigated, with eventual applicability in both conventional and organic orchards. In addition to registration for organic use as described above in objective number one, appropriate measures will be taken as necessary for registration of use of CrpeNPV in codling moth control.

SIGNIFICANT FINDINGS

Administrative delays on multiple fronts have delayed the initiation of this project until recently. Thus, there have been no significant findings until now. A detailed status update of the project has been provided in the “Results and Discussion” section below.

METHODS

1) CpGV Hybridization Studies

Procedures: Five CpGV strains, CpGV-M (A), CpGV-E2 (B), CpGV- I07 (C), CpGV-I12 (D), and CpGV-S (E), representative of the five different known CpGV genomic subtypes, A through E (Eberle et al., 2009, Gebhardt et al., 2014), will be obtained and used for genetic hybridization experiments. To establish baseline mortality metrics to compare with genetic hybrids, infection assays will be done with each of the five strains independently, using newly hatched, neonate larvae, as it is this stage in which larvae externally feed on leaves before entering the apple; non-infected neonate larvae will also be

assayed as a further control. Viral titers will be standardized to compare equivalent concentrations of virus across strain types, and will be used to directly inoculate codling moth larval diet (artificial diet or apple leaves). Dose response and time-course studies will be conducted, in which larvae will be permitted to feed on virus-inoculated food and scored for mortality at various time points after initiation of the experiment (3/7/10/14 days post infection (dpi)). Minimum exposure experiments may also be conducted, whereby larvae are exposed to viral treated food for varied lengths of time (10 minutes, 30 minutes, 1 hour, 2 hours, 5 hours), then transferred to non-treated food, and scored for mortality. Larval death rates at various dpi, average post-exposure time to morality and percentage of larvae dead due to granulovirus infection will be measured: precise measurements may be taken with use of video tracking equipment present in our laboratories. These will provide mortality metrics that may inform applicability of treatment in orchard settings (Hinsberger et al., 2020) wherein successive generations of codling moth larvae experience shorter periods of external leaf-feeding before entering the apple (Burgerjon, 1986).

For the genetic hybridization experiments, aliquots of all five strains will be mixed in equal ratios and used to inoculate and infect a codling moth larvae. After a sufficient viral inoculation period (3-5 days), sub-lethal infected larvae will be used as substrate for viral plaque assays (Harrison and Lynn 2008) that have been developed to allow individual viral hybrid genotypes to be purified. Pure isolate strains may then be re-cultured and subsequently used for codling moth infection assays, which will be conducted as described in the preceding paragraph for the parental viral strains. For hybrid strains that are more effective against codling moth larvae than parental strains, genotyping experiments will be performed to determine how different parental strains have hybridized, and which genes have been affected. This would include analysis of restriction fragment profiles of hybrids as compared to parental strains (Winstanley and Crook, 1993) as well as DNA sequencing.

Expected Results: During the first year of the project, initial experiments will be conducted, aimed at generating hybrid strains of CpGV. While hybridization of different strains of CpGV have, to our knowledge, never been studied, it is expected that hybrid strains will be recovered for further testing in larval infection assays. These assays will initially be conducted with larvae from an in-house codling moth colony that has no known resistance to CpGV. During the second and third years of the project, and in consultation with local growers, codling moth specimens from local orchards with suspected/demonstrated CpGV resistance or diminished CpGV efficacy will be collected, reared for one generation, and emergent neonate larvae will be tested in the same way as our laboratory colony. Furthermore, specimens from lab colonies known to be resistant to CpGV (Asser-Kaiser et al., 2007, Sauer et al., 2017a, 2017b) will be obtained and tested in our quarantine laboratory to examine how hybrid viral strains may facilitate resistance-breaking. These experiments would be critical to assess if hybrids that contain genetic material from multiple parental strains are able to successfully infect resistant codling moth populations that their corresponding parental viral strains cannot. Considering that five different CpGV genome group subtypes have been identified and different resistance-breaking patterns have been observed across these different groups, it is expected that hybrid stains cultivated across the subgroups may yield improved efficacy against codling moth larvae.

Potential Problems and Contingencies: It is widely known within the field of research on insect baculoviruses that the insect larvae themselves may be used to cultivate baculovirus stock. In this case, multiple parental strains may be used to co-infect individual codling moth larvae, after which potential hybrids may then be purified from infected larvae and used for further research. It has previously been demonstrated for other GV types that genetically hybrid virus isolates may be purified from infected larvae (Smith and Crook, 1993) without the use of cell culture cultivation and plaque assay purification. In this case larger fifth-instar larvae would be used to culture the virus. This methodology may thus be wholly suitable for laboratory research within the scope of this project.

In order for genetic hybridization of different viral strains to occur during cell culture experiments, it is required that individual cells be infected with two or more viral particles of different strains at the same time. To our knowledge, this has not previously been directly studied with different strains of CpGV, so it is uncertain to what degree it may happen. For NPV baculoviruses it has been

reported that hybridization can occur at very high frequencies (Hajós et al., 2000). Hybridization has also been reported for GVs, with indirect evidence for hybridization of CpGV with another GV viral type (Jehle et al., 2003). It is thus likely that our hybridization experiments will be successful after optimization.

2) Genetic engineering of CpGV and codling moth larval mortality bioassays.

Procedures: The genetic sequence of the *Hvt* toxin of the Blue Mountains funnel-web spider is publicly available through scientific literature and sequence databases. Using standard methodology, DNA sequence of the *Hvt* gene will be synthesized for molecular cloning into the CpGV genome. Genetic engineering of the *Hvt* gene into CpGV genome is facilitated by a baculovirus genomic construct, known as a bacmid. The entire genomic content of the CpGV-M1 strain has been engineered as a bacmid to facilitate insertion of any exogenous genetic material, such as a foreign gene (known as transgene), into a specific intergenic location of the genome where the transgene will be expressed by a viral promoter while not disruptive of any of the native CpGV genes (Hilton et al., 2008). This methodology utilizes a commercially available Bac-to-Bac Baculovirus Expression System (Thermo Fisher Scientific), in which the target transgene is first inserted into a carrier vector of DNA (known as "pFastBac"), which will be mixed with the CpGV genomic bacmid, and through genetic transformation in *E. coli* bacteria, the transgene will be inserted into the target site in the CpGV bacmid. Using this methodology, the *Hvt* toxin gene will be inserted into the CpGV bacmid (henceforth CpGV-*Hvt*), which will then be purified from the *E. coli* cells and used to directly transfect the Cp14 cell culture stocks.

Inoculation of the Cp14 cell line with the CpGV engineered bacmid is sufficient to cause infection of the cell culture with a 12-day incubation period (Hilton et al., 2008). Infected cell culture can then be used to directly inoculate codling moth larval diet (artificial diet or apple leaves) for the larval mortality bioassays. Cp14 cell cultures will be inoculated with CpGV bacmid with or without the *Hvt* gene for direct assessment of the efficacy of CpGV-*Hvt* in killing codling moth larvae. For these experiments, neonate larvae will be used. Larval death rates after three hours post infection (hpi), average post-exposure time to morality and percentage of larvae dead due to granulovirus infection will be measured; precise measurements may be taken with use of video tracking equipment present in our laboratories. Efficacy of CpGV-*Hvt* will be fully assessed with dose response assays, in which experimentally determined dilutions of viral-infected cell culture are applied to the larval food source. Moreover, time-course studies will be conducted, in which larvae will be permitted to feed on viral-inoculated food for varied lengths of time (10 minutes, 30 minutes, 1 hour, 2 hours, 5 hours).

Expected Results: During the first year of the project, CpGV-*Hvt* will be generated, and initial larval infection experiments will be conducted with an in-house codling moth colony that has no known resistance to CpGV. During the second and third years of the project, and in consultation with local growers, codling moth specimens from local orchards with suspected/demonstrated CpGV resistance or diminished CpGV efficacy will be collected, reared for one generation, and emergent neonate larvae will be tested in the same way as our laboratory colony. As a positive control, specimens from lab colonies known to be resistant to CpGV (Asser-Kaiser et al., 2007, Sauer et al., 2017a, 2017b) will be obtained and tested in our quarantine laboratory to examine how *Hvt* toxin may facilitate resistance-breaking by the engineered viral strain.

Both the Lead PI, Walker, and the Co-PI, Neven, have direct experience with utilizing insect cell culture systems for viral expression. Recently Lead-PI Walker served as a visiting researcher during a two-year period (2017-2019) at the Lund University Protein Production Platform, using the Bac-to-Bac Baculovirus Expression system to express engineered insect proteins. Our lab has generated preliminary data on production of transgenic viruses with spider toxin genes in a different insect baculovirus. Given this previous experience of Walker and Neven with viral genetic engineering and insect culture-based viral production, it is expected that there will be no difficulties in generating the CpGV-*Hvt* specimen. Previous spider toxin expression systems using *Hvt* expressed in plants targeting different types of insects including moth worms indicate that this toxin is a strong candidate for controlling insect damage (Khan et al., 2006, Javaid et al., 2016). It is thus expected that *Hvt* toxin will enhance CpGV lethality through introduction of an additional biopesticidal mode of action. Empirical

research in this project will determine to what degree this is realized. Dose response and time-course studies will inform product formulation and application. respectively.

Potential Problems and Contingencies: Based upon previous experience of Walker and Neven with viral genetic engineering and insect culture-based viral production, it is anticipated that the goals of this objective will be achieved. However, potential methodological problems may arise. The Bac-to-Bac baculovirus engineering system is standard to the point of being commercialized, so no problems are anticipated in the genetic engineering phase of the project. However, if problems do arise in the methodology, other methods of viral genetic engineering, such as homologous recombination (Hilton et al., 2008) may be utilized to insert the *Hvt* gene into the CpGV bacmid.

As mentioned in the previous section, potential problems with cell culture methodology may occur. This is not anticipated, though if substantial problems occur, culturing the viruses may be done within the codling moth larvae themselves. Furthermore, it has been demonstrated that engineered CpGV bacmid may be directly injected into codling moth larvae to generate genetically modified virus cultures (Hilton et al., 2008) which may then be purified from infected larvae and used for further research.

Another potential problem is that the CpGV bacmid is derived from a CpGV-M strain, and it has been well documented that some codling moth populations display resistance to this strain (Asser-Kaiser et al., 2007). Moreover, codling moth resistance to this strain is mediated via blocking viral replication (Asser-Kaiser et al., 2011), so introduction of a toxin gene alone may not simply overcome the resistance to the M strain. While our laboratory codling moth colony is not known to be resistant to any type of CpGV formulation, it is important to assess how CpGV with *Hvt* impact CpGV resistant codling moth populations. Thus, specimen from codling moth colonies known to be resistant to CpGV-M strains will be obtained to test if presence of *Hvt* impacts the codling moth resistance. If these codling moth remain resistant to CpGV-*Hvt*, it would be compelling to co-infect CpGV-*Hvt* with another strain that can break CpGV-M type resistance and facilitate replication of both viral strains (Graillot et al., 2016).

3) CpGV and CrpeNPV co-infection studies.

Procedures: It is hypothesized that co-infection of CpGV and CrpeNPV in codling moth larvae will result in enhanced infectivity and mortality, above and beyond that observed with CpGV alone. This principle has been demonstrated in other viral/host infection systems, such as with co-infection of fall armyworm, *Spodoptera frugiperda*, with a GV and NPV (Cuartas-Otálora et al., 2019). For these experiments, the most effective parental CpGV strain identified from objective one will be utilized, and CrpeNPV will be obtained from the source laboratory that initially reported it (Marsberg et al., 2018). To establish baseline mortality metrics to compare with the co-infection studies, infection assays will be done with CpGV and CrpeNPV individually, using neonate larvae. Codling moth larval co-infection studies of CpGV and CrpeNPV will be conducted with an empirically assessed range of mixtures of different ratios of the two virus types, combined.

These mixtures will be used to directly inoculate codling moth larval diet (artificial diet or apple leaves). Dose response and time-course studies will be conducted, in which larvae will be permitted to feed on virus-inoculated food and scored for mortality at various time points after initiation of the experiment (3/7/10/14 days post infection (dpi)). Minimum exposure experiments may also be conducted, whereby larvae are exposed to viral treated food for varied lengths of time (10 minutes, 30 minutes, 1 hour, 2 hours, 5 hours), then transferred to non-treated food, and scored for mortality. Larval death rates at various dpi, average post-exposure time to morality and percentage of larvae dead due to granulovirus infection will be measured; precise measurements may be taken with use of video tracking equipment present in our laboratories.

Expected Results: During the first year of the project, optimization of CpGV and CrpeNPV co-infection parameters will be assessed with experiments conducted in an in-house codling moth colony that has no known resistance to CpGV nor CrpeNPV. Given that co-infection of moth larvae with GV and NPV viruses has been reported to increase viral efficacy (Cuartas-Otálora et al., 2019), it is

expected that we will observe this in codling moth as well. During the second and third years of the project, and in consultation with local growers, codling moth specimens from local orchards with suspected/demonstrated CpGV resistance or diminished CpGV efficacy will be collected, reared for one generation, and emergent neonate larvae will be tested in the same way as our laboratory colony, using optimally determined ratios and dosages as determined during the first year's experiments. As a positive control, specimens from lab colonies known to be resistant to CpGV (Asser-Kaiser et al., 2007, Sauer et al., 2017a, 2017b) will be obtained and tested in our quarantine laboratory to examine how mixtures of CpGV and CrpeNPV may facilitate resistance-breaking by the engineered viral strain.

Potential Problems and Contingencies:

It is unknown whether CrpeNPV and CpGV would be capable of co-infecting the same cells. The principle of superinfection exclusion refers to the ability of an established virus to interfere with a second viral infection. This has been observed for closely related NPV viral species (Beperet et al., 2014), though it is not known whether this principle would apply to GV and NPV viruses, considering that they are of different baculovirus families. This would need to be assessed further here. Even if co-infection were possible, it is unlikely that the GV and NPV would undergo hybridization on account of their differing phylogenetic classifications, though it could not be ruled out, as numerous viral genes are common across all baculovirus genera (Jehle et al., 2006). Assessment of this could be made with restriction fragment pattern analysis of viral DNA isolates after co-infection studies.

RESULTS AND DISCUSSION

Administrative delays in hiring a dedicated technician persisted until the end of summer (2022) resulting in substantial delays in launching this project. The process to initiate hiring on our side was launched during the first year of the project directly after the funding became available, and was finally resolved with the onboarding of the technician in August of 2022. Competency training was provided to the technician during the autumn of 2022. The first round of experimentation is underway, aimed at establishing baselines of viral-mediated killing rates of codling moth larvae with different strains of CpGV (Objective 1) as well as with CrpeNPV (Objective 3). Currently, using CpGV virus provided by BioTepp Inc. (strain E) a preliminary dose response curve has been generated with data from one trial (Figure 1). Data collection is ongoing and will be completed for other strains of CpGV (e.g. strains A-D), as well as CrpeNPV (which was finally obtained from River Bioscience during the summer of 2022). Dose-response curves and mortality time-course data for each virus species type and CpGV strain type will be used as baseline for comparison to data generated in CpGV hybridization studies (Objective 1) and CpGV/CrpeNPV co-infection studies (Objective 3), which will be performed directly after relevant baseline data has been generated.

Specific CpGV genetic materials required for genetic engineering of spider toxin (Objective 2) are only available from a single laboratory in Germany. A Material Transfer Agreement (MTA), which was required to obtain these materials, was finalized in December of 2021, and necessary materials were received in March of 2022. Necessary molecular biology experiments (and troubleshooting) required for genetic engineering of CpGV have commenced in our genetics laboratory, and are currently in progress. However, at present time, there are not yet any results from this to present and discuss.

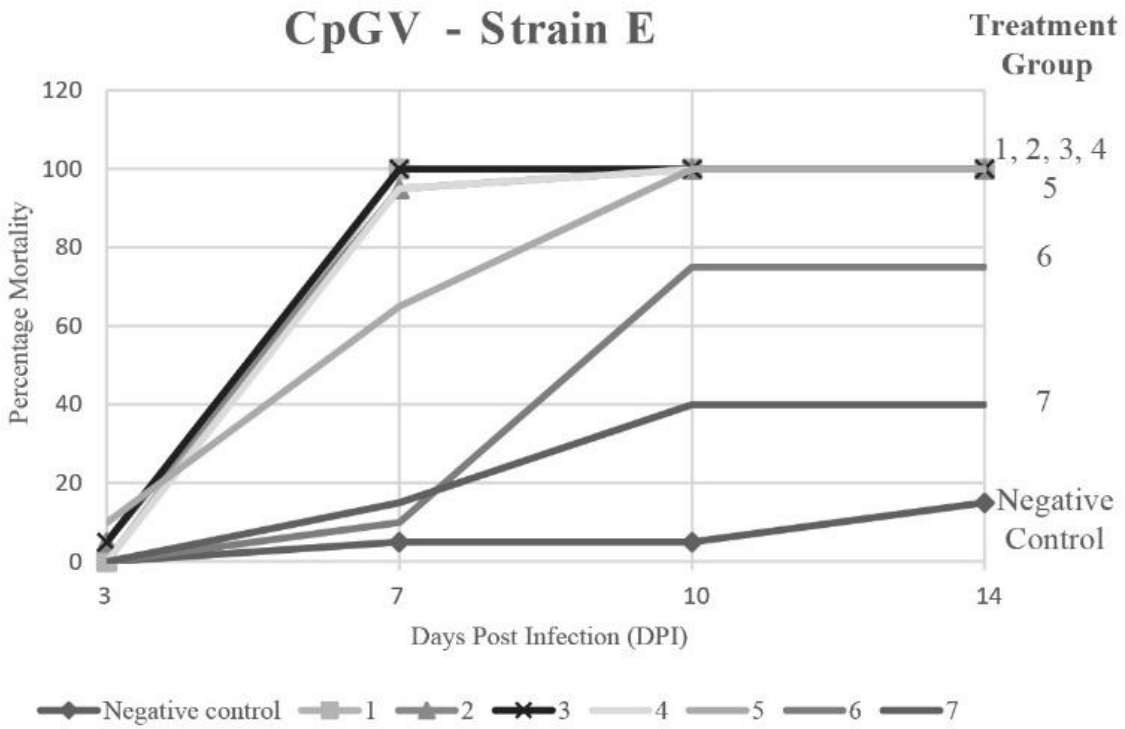

Figure 1. Dose-response mortality assay for codling moth neonate larvae exposed to CpGV - strain E. Twenty larvae tested per treatment (n=20). 10 microliters of virus solution were applied at specified concentrations for the following treatment groups: 1 – 3.7 x 10^5 OB/mL; 2 – 3.7 x 10^2 OB/mL; 3 – 1.85 x 10^2 OB/mL; 4 – 3.7 x 10^1 OB/mL; 5 – 1.85 x 10^1 OB/mL; 6 – 3.7 OB/mL; 7 – 3.7 x 10^{-1} OB/mL. Dilutions were made in autoclaved distilled water, which was also used as the negative control. Treatments were applied evenly to the surface of artificial diet in assay vials and allowed to dry for two hours, after which a single newly hatched neonate larvae was transferred to each assay vial. Larvae were scored for mortality on days 3, 7, 10 and 14 after initiation of experiments.

Project Title: Quantifying codling moth capture, lure plume reach, and trap area

Report Type: Continuing Project Report

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Project Duration: 3 Year

Total Project Request for Year 1 Funding: $207,430
Total Project Request for Year 2 Funding: $188,216
Total Project Request for Year 3 Funding: $195,530

Other related/associated funding sources: Pending
Funding Duration: 2023 - 2025
Amount: **$ 347,287**
Agency Name: Western SARE
Notes: Preproposal accepted and full proposal submitted. Notification in March 2023.
WTFRC Collaborative Costs: none

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Item	2022	2023	2024
Salaries[1]	$96,601.00	$86,901.00	$90,377.00
Benefits[2]	$41,301.00	$36,776.00	$38,247.00
Wages[3]	$12,000.00	$12,480.00	$12,979.00
Benefits[4]	$1,173.00	$1,220.00	$1,269.00
Equipment[5]			
Supplies[6]	$46,855.00	$41,339.00	$43,158.00
Travel[7]	$9,500.00	$9,500.00	$9,500.00
Miscellaneous[8]			
Plot Fees[9]			
Total	$207,430.00	$188,216.00	$195,530.00

Footnotes: [1]Salaries for project technician (1@ 1 FTE), and Postdoc (yr1 1@ 0.9175 FTE, yr2,3 1@ 0.6618 FTE); [2]Benefits for technician @ 41.32%, Postdoc @45.54%; [3]Wages for time slip ($15/hr in yr 1, $15.50/hr in yr2, and $16/hr in yr 3) for 20 weeks/summer; [4]benefits for time slip employees (9.8%); [6]Supplies: computer, printer/software; lab/office supplies, electronics; video camera/accessories, sterile moths (400 dishes/week yr1, 300/wk yr2,3), traps and sticky bottoms, lures. [7]Travel to plots, motor pool rental, fuel, per diem, other related travel.

ORIGINAL PROJECT OBJECTIVES:

1. Research: Compare codling moth lures in commercial apple orchards with mating disruption.
    a) Analyze codling moth capture in traps with 5 commonly used lures under 3 mating disruption regimes (mark-release-recapture study: 15 treatments with 18 replications each).
    b) Determine the number of traps needed per acre when using each lure for accurate monitoring under the three types of mating disruption (from recapture data analysis).
    c) Estimate codling moth population density based on moth capture data in a monitoring trap baited with each (lure) x (mating disruption) type (from recapture data analysis).

2. Extension: Produce practical guidelines for field application of these findings by growers.
    a) Create a decision matrix incorporating economic costs and efficacy (potential returns) of each combination of lure x mating disruption.
    b) Communicate findings to the industry via extension presentations at field days, grower meetings, and updated webpage with project-related factsheets added to the Tree Fruit Extension website.

SIGNIFICANT FINDINGS

Objective 1 – 2022 key findings

- 100 total releases in 2022 resulted in variable capture by lure and MD type
- Early spring capture is poor with all lures
- Passive mating disruption (hand applied reservoir dispensers) suppressed capture for 4 out of 5 lures
- The CMDA+AA lure had the most consistent capture across the three MD schemes
- More replication in years 2-3 is needed to estimate traps/acre and population densities

Objective 2

- Co-PI Curtiss has presented preliminary findings at 4 grower meetings in 2022
- The decision matrix is in development, but will not be completed until after field season 2024
- The project webpage, and three project-related fact sheets are in development as of the writing of this report.

METHODS

<u>OBJECTIVE 1: Compare codling moth lures in commercial apple orchards with mating disruption</u>

This study involves three years of replicated codling moth field releases under 15 treatment combinations. The field component of the study will be completed by the end of the third field season and then through data analysis we will determine mean capture, number of traps needed per acre, and estimated codling moth population density per treatment.

Plots: Experiments will be conducted in commercial apple orchards in geographically diverse locations across Washington State during the summers of 2022, 2023, and 2024. Orchards contained a variety of apple cultivars, rootstocks, irrigation schemes, and tree training systems on 8-10-acre plots. All orchards were treated with codling moth pheromone mating disruption using 1) actively dispensing aerosol emitters (i.e., ISOMATE® CM Mist Plus (Vancouver, WA)) at 0.5-1/acre, 2) passively dispensing reservoir dispensers (i.e., ISOMATE® CM Flex, and Scentry NoMate® CM

Spiral (Billings, MT)) at recommended rates, or 3) no mating disruption (Figure 1). Conventional chemical controls were applied as needed by farmers.

Experimental design and moth releases: The experiment released externally marked sterile codling moths (300 dishes/week for 20 weeks/year @ $6/dish) for on-farm evaluation of codling moth lures. Sterile, mixed-sex codling moth adults were obtained from the Okanagan-Kootenay Sterile Insect Release (OKSIR) facility in Osoyoos, British Columbia, Canada. Upon eclosion, moths at the OKSIR facility were immediately placed in petri dishes at an approximate ratio of 1:1 males:females (ca. 800 moths/petri dish) and treated in a Cobalt-60 irradiator. The dishes of irradiated moths were then packed into battery-powered coolers (2.8 Cu. Ft. Portable Fridge/Freezer: Edgestar co. Austin, Texas) held at approximately 2-5 °C (36-41 °F) and shipped to Washington State. Moths arrived before noon the same day they were packed allowing for immediate release into field plots. Because moths were transported as mixed-sex batches in chill coma directly from the shipper to field sites for immediate release, the sexes could not be separated prior to release.

Immediately upon arrival at field sites, moths were dispensed into 540-ml polystyrene cups (Fabri-Kal Corp. Kalamazoo, MI) in batches corresponding to the number being released at each distance, but never more than 4,000/cup. Moths for each release distance were uniquely colored using ca. 1.25 ml/800 moths with Dayglo florescent pigments (ECO11 Aurora Pink®, ECO15 Blaze Orange™, ECO18 Signal Green™, ECO19 Horizon Blue™) (DayGlo Color, Cleveland, OH), allowed to warm to ambient temperature, and then released at pre-marked locations at distances of 20, 40, 60, and 80 m (66, 131, 197, 262 ft) and from the central pheromone-baited trap location. Moths were gently tossed by hand from the containers of colored moths ca. 1-2 m (3-6 ft) into the canopy of pre-marked trees.

Toriani Kent, Project Technician, releasing pink moths into the orchard canopy (R. Courtney, Good Fruit Grower Magazine)

The experiment employed a cardinal-direction mark-release-recapture design with a single central trap following protocols from Curtiss (2021) (Figure 1). Release locations were marked with flagging tape in the four cardinal directions from the single trap at distances of 20, 40, 60, and 80 m (66, 131, 197, 262 ft) . In each replicate, approximately equal numbers of females and males were released, and the number of moths was increased with increasing distance. Each of the four 20 m (66 ft) release points received ~400 sterile males/~400 sterile females, the four 40 m (131 ft) release points each received ~800 sterile males/~800 sterile females, the four 60 m (197 ft) release sites each received ~1600 sterile males/~1600 sterile females, and each of the four 80 m (262 ft) release sites received ~3200 sterile males/~3200 sterile females.

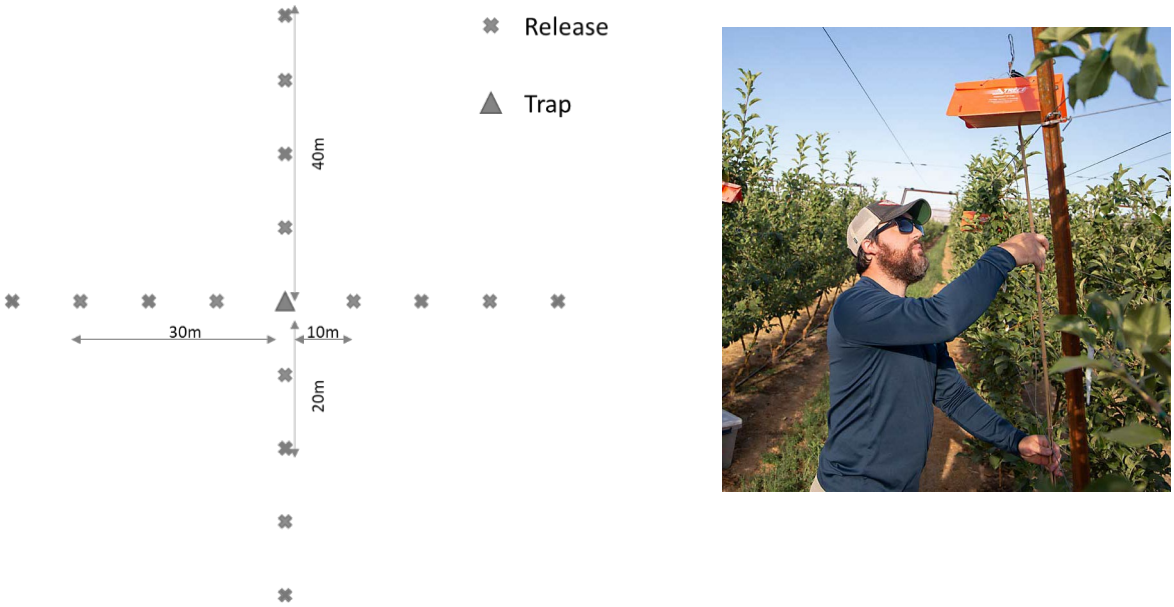

Figure 1. Cardinal-direction mark-release-recapture with a single central trap experimental layout. RT Curtiss is shown hanging a trap in the orchard canopy (R. Courtney, Good Fruit Grower Magazine).

Sampling: The uniquely colored pre-marked moths released at each distance were recaptured at the central trap location. Recaptures of sterile male and female marked moths were quantified using Orange Pherocon VI delta traps (Trécé Inc., Adair, OK) baited with a PHEROCON® CM-DA COMBO™ Lure + AA Lure (Trécé, Inc.) designed to attract both male and female codling moths. The 2-part lure was held above the replaceable sticky liner with a pin through the top of the trap. To maximize catch, traps were placed within the top 1/3 of pre-marked trees. Lures were changed every six weeks. Traps were monitored for 14 days following release. Trap sticky liners were removed and replaced if moths were present when traps were checked weekly and were subsequently be examined in the laboratory using UV illumination (400-405 nm, 12 UV LED bulb flashlight, BioQuip Products, Rancho Domingo, CA) to determine the color and sex of marked moths. Each treatment will be replicated 18 times over the course of the three-year study (6 replications of each treatment/year) due to limitations in weekly availability of moths and test sites. One full replication of all treatments spanned a nine-week period because only 300 dishes of moths were available weekly for this experiment and each individual release requires 60 dishes (Figure 2).

		Lure 1	Lure 2	Lure 3	Lure 4	Lure 5
Block 1	Passive MD	Wk 1,4,7,10,13,16	Wk 3,6,9,12,15,18	Wk 3,6,9,12,15,18	Wk 2,5,8,11,14,17	Wk 1,4,7,10,13,16
Block 2	Active MD	Wk 2,5,8,11,14,17	Wk 1,4,7,10,13,16	Wk 2,5,8,11,14,17	Wk 1,4,7,10,13,16	Wk 2,5,8,11,14,17
Block 3	No MD	Wk 3,6,9,12,15,18	Wk 2,5,8,11,14,17	Wk 1,4,7,10,13,16	Wk 3,6,9,12,15,18	Wk 3,6,9,12,15,18

Figure 2. Example experimental layout and timeline.

Data analysis: Analysis of mark-release-recapture experiments provided estimates of codling moth dispersive distance, plume reach of lures, and trapping area related to males and females independently. To ensure that only reliable and robust data are used for analysis, only replications with at least two recaptured moths from each release distance were used; typically, 10-40% of replications are not acceptable (Curtiss et al., in prep). Males and females were analyzed separately. Data analysis will be plotted following the quantitative methods of Miller et al. (2015) to provide: 1) an untransformed graph of the released moths over distance from trap, 2) plot of 1/proportion of released moths recaptured over distance of release from central trap (MAG plot), and 3) (annulus area)*(proportion of codling moths recaptured)/distance of release from central trap (Miller plot). The untransformed plot confirms that release distances are selected appropriately when a concave line with an asymptotic approach to zero catch is observed. The slope of the MAG plot, linear over close release distances, is used to determine plume reach of monitoring trap lures using the standard curve of Miller et al. (2015), Fig. 4.12. The maximum dispersive distance for 95% of the responding population is estimated by a second-order polynomial fitted to the Miller plot data with the point at which the line crosses the x-axis estimating the maximum distance 95% of the population can disperse (Adams et al., 2017a). The average proportion caught out of all insects in the full trapping area (Tfer) for these experiments will be calculated by dividing the mean of the proportion caught at a specific distance (spTfer) × annulus area by the mean annulus area [mean (spTfer × annulus area)/mean annulus area] (Eq. 5.2, Miller et al., 2015), and will be used to estimate population density per trapping area. Areas of trapping annuli will be calculated as per Miller et al. (2015).

Anticipated results and potential pitfalls: One-third of the total planned replications of each treatment will occur in each year, so major analysis will not occur until the end of the third field season. The most apparent differences will be in attractivity among lure types and efficacy of different types of mating disruption, whereas varied combinations may reveal less impactful on efficacy. Additionally, we anticipate data will suggest the need for higher trapping densities for orchards under the more efficacious lure types and mating disruption.

There are limitations on the number of available moths on any particular day, so if a large number of replications do not have adequate capture for meaningful analysis, some (lure) × (mating disruption) treatments may have less robust data from which to draw conclusions. In 2022, most replications were acceptable.

OBJECTIVE 2: Produce practical guidelines for field application of these findings by growers

Products: The important products of this study are 1) recommendations on the minimum number of traps needed per area to accurately monitor codling moth in apple orchards treated with any of the mating disruption and lure combinations tested, and 2) interpretation of moth capture in those monitoring traps, i.e., what is the density of moths within the trap area if a single moth is captured in a monitoring trap. To deliver useful information to the industry at the end of this project, we will create a decision matrix displaying lure types and mating disruption technologies and corresponding pest density estimates. From these data, IPM thresholds can be clarified to account for estimated pest densities, and management decisions can be more informed and save money and effort.

Dissemination: Our progress on this project will be shared through at least 5 grower events per season, but likely more often based on requests from the industry (i.e., distributor and packing house meetings) and extension events (field days, fruit schools, workshops, etc.). At the end of year 1, a project webpage will be created, housed in the WSU Tree Fruit Extension website, to ensure that growers have free access to our continuing efforts, results, and interpretations. At the conclusion of this project, we will produce full summaries for the website along-side practical strategies for field application of these findings. This will include guidelines on how best to employ the techniques, and a moth capture density decision matrix that accounts for lure type and mating disruption scheme.

RESULTS AND DISCUSSION

OBJECTIVE 1: Compare codling moth lures in commercial apple orchards with mating disruption

Sterile codling moth releases in 2022 were conducted in 45 commercial orchards. Orchards were divided into three geographically distinct blocks corresponding to latitudes and longitudes 46-47°N and 119-121°W, 47-48°N and 119-121°W, and 48-49°N and 119-121°W. Fifteen orchards were in each geographic block, with five blocks for each treatment: no mating disruption, passive mating disruption, and active mating disruption. All releases were performed when scheduled.

There were 100 total releases performed over 20 weeks of the summer 2022. Each orchard (lure × mating disruption combination) received at least two releases resulting in 6-8 replications of each combination across the three geographic blocks. There were no statistical differences in moth capture due to geography or treatment type, likely due to the low overall replication of only one years' release (Figure 3). However, some trends are starting to emerge. Capture in the early spring and late fall is poor across all lures, indicating that growers may not be receiving accurate population data when populations are low and weather conditions are not favorable for flight. Passive mating disruption appears to suppress trap-finding more than active mating disruption, indicating that active mating disruption may be deployed at too low densities to fully suppress mating in our plots. The CMDA+AA lure (4th data set from the left) had the most consistent capture across the three mating disruption schemes and provided the overall highest combined capture (Figure 3).

Preliminary population density estimates based on the 2022 replications also show some trends. The CMDA+AA and Megalure 4k lures both appear to detect codling moth at low population levels (Figure 4, smaller bars indicate detection at low populations, i.e., lure is apparent with mating disruption). The CML2, 10x, and CMDA lures had more variable capture, but do not appear to detect codling moths when mating disruption is present until populations are high (Figure 4, larger bars indicate high populations needed to detect codling moths, i.e., lure is masked by mating disruption).

Although the results presented in this continuing report are only from a single season, and thus the replication is low, there are some important considerations arising for farmers. First, the lure

used in monitoring programs needs to be carefully matched with the mating disruption program. Second, codling moth capture-based decision making on apple farms cannot be accurate until the results of this study are completed and a better understanding of the interactions between the lures and mating disruption types is gained. Last, spray decision-making based on monitoring traps may be inaccurate in the early spring when accuracy is critical because codling moth responses to traps are poor due to variable and unfavorable weather conditions.

Once completed, this project will provide accurate treatment guidance for industry decision makers. Accuracy in spray decisions can lead to cost savings by preventing unnecessary sprays, and/or inducing a spray to prevent crop losses. The cost savings, and/or gains will contribute to the long-term sustainability of farming apples in Washington. The continued investment of the WTFRC-ACP to complete this study over the next two years will be returned many-fold to the industry through more precise codling moth spray decisions.

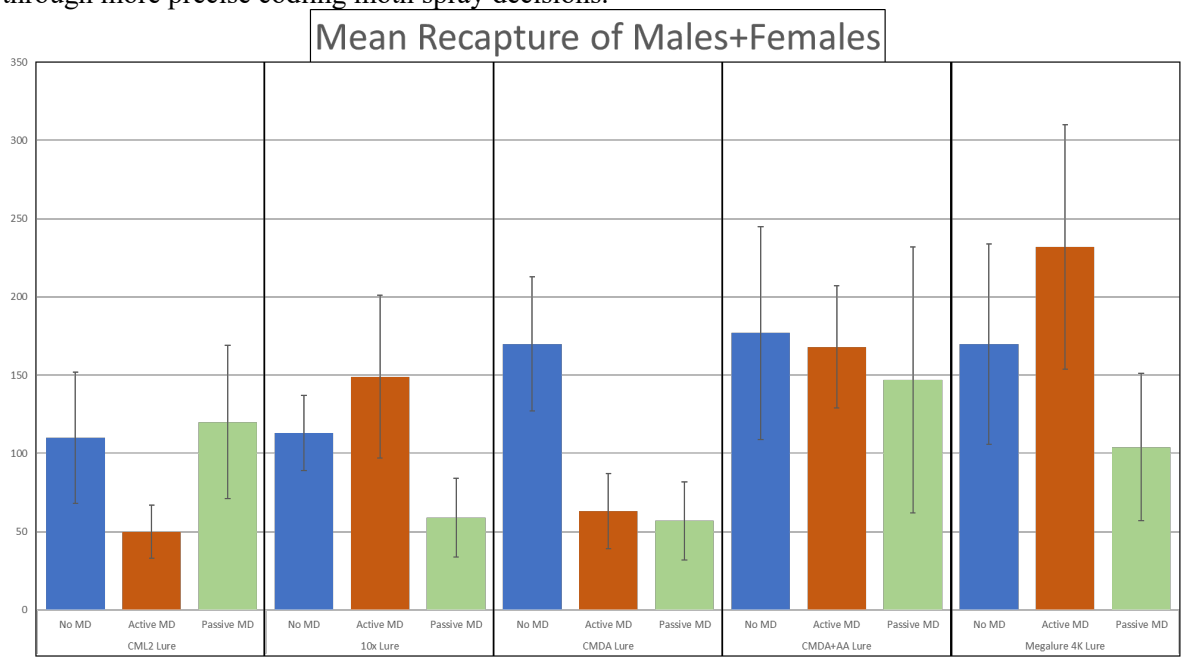

Figure 3. 2022 Preliminary results on mean combined recapture of males and females by lure × mating disruption type. The data sets are (L to R): CML2 Lure, 10xLure, CMDA Lure, CMDA+AA Lure, and Megalure 4K Lure. The data bars within each data set are (L to R): No Mating Disruption (MD), Active MD, and Passive MD.

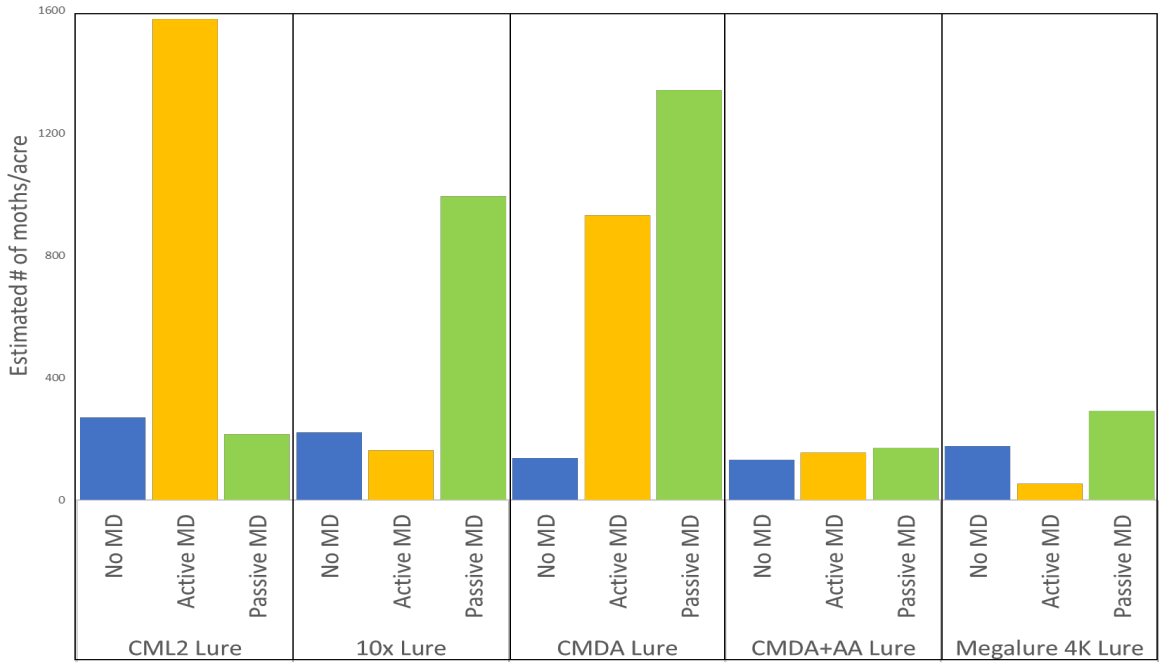

Figure 4. 2022 Preliminary results on codling moth population density estimates when one moth is captured in a trap. There are no Standard Error measures for this estimate, thus there are no error bars on this figure.

<u>OBJECTIVE 2: Produce practical guidelines for field application of these findings by growers</u>

Co-PI RT Curtiss has presented preliminary project findings at four grower meetings in 2022. At least 100 growers and decision-makers were present collectively at these meetings. Although the decision matrix is in development, it will not be completed until after field season 2024 when enough replications are completed to provide meaningful results for analysis. As of the writing of this report, the project webpage and three project-related fact sheets are in development. Project fact sheets will be completed before the summer 2023 field season.

In addition to project-specific activities, we have applied for a Western SARE grant ($347,287) to expand the research aspects of the project in 2023-2024 and add an extension-focused year (2025) to disseminate our findings. Our preproposal was accepted, and we were invited to write a full proposal. Our full proposal was submitted in October 2022, and we await notification in March 2023. The Western SARE proposed project will allow us to expand the scope of this project, cover unanticipated cost increases, and fund additional personnel. Unexpected cost increases include those for sterile moths which have increased considerably since our original quote in summer 2021 (quoted at $24/unit in 2021, cost $30/unit in 2023, increasing to $38/unit in 2023) when this project was in preparation.

Project Title: Novel control of Codling Moth with RNA interference

Report Type: Continuing Project Report

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Cooperators: None

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 69,317
Total Project Request for Year 2 Funding: $ 70,703
Total Project Request for Year 3 Funding: $ 69,680

Other related/associated funding sources: None

WTFRC Collaborative Costs: None

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Item	2022	2023	2024
Salaries	$43,683.00	$44,775.00	$45,894.00
Benefits	$13,979.00	$14,328.00	$14,686.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$11,655.00	$11,600.00	$9,100.00
Travel			
Plot Fees			
Miscellaneous			
Total	$69,317.00	$70,703.00	$69,680.00

Footnotes: Salaries and benefits are requested for a full-time GS-7 Lab Technician. Costs for supplies are for molecular reagents for RNAi, materials for transcriptomic sequencing costs, and also for materials for insect colony rearing and experimental bioassays.

OBJECTIVES

Objective 1. Identify candidate target genes for RNAi through transcriptomic analyses.

Comprehensive knowledge of gene expression in the target organism at the appropriate life stages is a pre-requisite for identification of candidate target genes for RNAi-mediated disruption. In the past decade, whole transcriptomic sequencing has emerged as a robust methodology for examining the sum-total of gene expression in a specific biological sample, representative of different life stages or tissue types. Currently, limited transcriptomic information is available for the codling moth. Therefore, using in-house sequencing equipment and codling moth from our colony, transcriptomes will be generated for different larval stages, pupae, adults and embryos. Analysis of these transcriptomes would lead to identification of candidate genes expressed at each stage that would be targeted for disruption with the predicted outcome of codling moth mortality.

Objective 2. Conduct larval feeding bioassays with RNAi effectors combined with various feeding stimulators to optimize potential deliverables.

Results from objective one will directly be channeled into larval feeding assays. Initially, dsRNA molecules targeting identified candidate genes will be mixed with codling moth artificial diet and provided to codling moth larvae with unrestricted access. Since the RNAi effect is mediated primarily through disruption of expression of specific genes, quantitative real-time PCR (qRT-PCR) assays will be conducted in experimentally treated insects relative to controls to assess efficacy of disruption of gene expression for the targeted genes. At the same time, longevity bioassays will be conducted in experimental codling moth specimen across all stages of development relative to non-treated controls to determine which genes, when targeted for disruption by RNAi, yield the most effective impacts on codling moth mortality and development.

Objective 3. Perform controlled laboratory and field trials on efficacy of RNAi in neonate larvae towards preventing codling moth damage in apples.

Once suitable target genes have been identified through RNAi feeding experiments, controlled experiments will be conducted on apple trees at our experimental orchards in Moxee. Larval behavioral modulators have been developed and used to elicit increased codling moth larval feeding before they entire the apple, thereby increasing exposure to materials that are toxic to them. Experiments will thus be conducted with dsRNA provided in combination with the behavioral modulator and experimental feeding stimulants to assess enhancement of external feeding, and thus uptake of dsRNA. Formulations of dsRNA and the modulators, mixed with water, will be applied through spraying the formulations over apple tree rows during periods where codling moth is active in flight. Codling moth damage to apples will be assessed in treated versus untreated/control areas.

SIGNIFICANT FINDINGS

Administrative delays in hiring a technician to work on this project have delayed the initiation of this project until recently. Thus, there have been no significant findings until now. A detailed status update of the project has been provided in the "Results and Discussion" section below.

METHODS

Objective 1. Identify candidate target genes for RNAi through transcriptomic analyses and injection trials

Procedures: Whole transcriptome datasets will be generated and analyzed for several life stages of codling moth, including early and late embryo, early and late larval instars, pupae and adults. Lead-PI Walker has extensive experience with this approach in entomology research (Walker et al., 2016; Walker et al., 2019). For each life stage an appropriate amount of individual specimen will be collected to ensure that a sufficient quantity of RNA may be extracted to generate high quality transcriptomes. Codling moth specimen will be taken from our in-house codling moth colony. Standard protocols will be used to extract RNA from all sample types and subsequently prepare sequencing libraries that will

serve as substrate for next generation RNA sequencing (RNA-Seq). Sequencing will be conducted in-house with our recently acquired Oxford Nanopore Mk1C sequencer, and the output sequence data will be assessed for quality and arranged into who transcriptome data sets containing consensus transcripts for each gene that is expressed at each life stage. Bioinformatic analyses will be conducted on output sequence data to assess which genes are expressed and relative expression abundances compared to all other genes in each sample. Further analyses will be conducted to compare codling moth expressed genes to transcriptomic data sets of other related insects to characterize unique and conserved genes in the codling moth.

Expected Results: Comprehensive gene expression data sets will be obtained across all life stages of codling moth. Individual transcriptomes will be generated for each life stage for comparison within codling moth and relative to similar data sets already published on record for other species. It is expected that unique life-stage expression profiles will be observed, with a mixture of genes that are expressed across most or all life stages, as well as genes that are expressed in one or few life stages. These datasets will be thoroughly analyzed relative to what is known in relevant scientific literature and body of knowledge to identify suitable gene targets for RNAi-mediated disruption of expression of vital genes across all life stages. Ideally, the most suitable gene targets will be specific to codling moth and few other species.

Potential Problems and Contingencies: State-of-the-art RNA-Seq methodologies and bioinformatic analyses will be utilized on biological samples taken from our internal codling moth colonies. There is thus a very low risk of substantial problems with this stage of the project. The high volume of data generated for each life-stage transcriptome may indeed be challenging to work with and efficiently analyze and parse out the most useful information. However, numerous optimized bioinformatic pipelines have been developed with which the lead scientists are experienced with, and bioinformaticians and computational scientists within our organization will be consulted with to ensure that best practices are followed. Assessment of the genetic diversity potential of targeted codling moth populations is essential to identify the best gene candidates for RNAi. Given that our laboratory may not contain representative genetic diversity of codling moth across Washington and the Pacific North West region due bottlenecking of genetic diversity and inbreeding rearing conditions, annual infusions into our colony have been made with wild codling moth from local orchards; these infusions will continue in the future.

Time-Plan: Transcriptome sequencing and analysis will be performed during the first six months of the project.

Objective 2. Conduct larval feeding bioassays with RNAi effectors combined with various feeding stimulators

Procedures: Candidate genes identified in the whole transcriptome datasets will be targeted for disruption by delivery of complementary dsRNA effector molecules via larval feeding. Genes will be targeted that are expressed in larval but also pupal, adult, and embryonic stages of life. For these candidate genes gene-specific dsRNA will be generated in-vitro, using corresponding gene-specific genomic DNA (gDNA) as a template, with standard molecular biology methods (Walker and Allen, 2010, 2011). dsRNA will also be generated from template gDNA corresponding to a plant gene to serve as a negative control to the experimental conditions. Additionally, dsRNA will be generated from template gDNA corresponding to a universal cellular housekeeping gene, inhibitor of apoptosis (IAP), known to be expressed throughout all life stages, and widely across all insects; RNAi against IAP has been shown to induce rapid mortality in a diversity of insects such as mosquitoes (Pridgeon et al., 2008) and plant bugs (Walker and Allen, 2011). Initial RNAi experiments will be conducted targeting disruption of IAP, as a positive control, in order to optimize protocols and methodology (RNAi against IAP would not be expected to serve as an eventual biopesticide target due to its widespread presence across insects and other domains of life such as fungi).

Initial feeding assays will be conducted via topical application of purified dsRNA solution to standard codling moth artificial diet (Wang et al., 2015). To control for effect of dsRNA feeding on

insect mortality, control experiments will be performed through feeding of dsRNA targeting disruption of a selected plant-specific gene that would not be present in the codling moth genome. Initially high concentrations of dsRNA will be applied to the food. For targeted genes that result in successful RNAi outcomes, lower concentrations of dsRNA will be assayed as well in order to assess minimum and optimal concentrations for eventual tree fruit trials. Individual neonate larvae will be placed in feeding chambers and allowed to feed unrestricted, while being monitored for growth, development, and mortality.

Throughout the course of the experiments, mortality, time of development, and size/growth will be measured during all life stages to evaluate persistence and effectiveness of RNAi beyond the larval stage. Furthermore, for genes which are observed to be disrupted by RNAi in codling moth feeding on dsRNA, new experiments will be performed in which larvae are given access to dsRNA admixtures that target multiple genes. This will be done to evaluate whether there is increased efficacy by targeting multiple genes for disruption simultaneously. For all experiments, sufficiently many insects will be assayed in order to be able to statistically demonstrate that increased mortality or development inhibition is due to the RNAi effect and not other experimental factors. Subsets of injected insects will be sampled for extraction of RNA and molecular assessment of target-gene disruption using standard qRT-PCR assay under experimental conditions of RNAi disruption versus controls.

Expected Results: Screening of the RNAi effect in insects via feeding dsRNA on artificial diet has been identified as an easy, effective and efficient way to assess large numbers of genes with assays resembling field conditions (Whyard et al., 2009). In codling moth it has been shown that feeding larvae with dsRNA can result in RNAi-mediated gene disruption and larval growth deficits (Wang et al., 2015), so it is expected that this approach will be successful. In experiments where RNAi is successful, disruption of target genes will result in increased mortality or developmental inhibition relative to control treatments. It is expected that there will be a correlation between RNAi phenotype (mortality or developmental inhibition) and reduction or elimination of mRNA of the targeted gene. Based upon the results of these experiments, genes that display mortality or developmental phenotypes correlate to disruption of their mRNA will be selected for further experimentation in Objective 3.

In the previous report on RNAi in codling moth, only larval-expressed genes were targeted via larval feeding on dsRNA (Wang et al., 2015). This objective expands upon those findings by examination of persistence of RNAi beyond the larval stage. While this has never before been examined in codling moth larvae, there is confidence that persistence of RNAi will be observed. In a closely related species of the same tortricid family of moths, the light brown apple month, *Epiphyas postvitanna*, it was observed that in larvae that were fed dsRNA effectors, the RNAi gene-disruption effect persisted for more than two weeks as the larvae progressed through the pupal and into the adult stage (Turner et al., 2006). Moreover, in codling moth injected with dsRNA in the pupal stage, RNAi-mediated gene disruption was observed into the adult stage (Wan et al., 2019).

Potential Problems and Contingencies:

While RNAi has been demonstrated to work in codling moth after delivery of dsRNA via larval feeding, these observations were limited to one gene in one published report from one laboratory, and for which no strong RNAi phenotype was observed. Further research is indeed necessary to optimize the methodology related to target gene selection, dsRNA dosage, and duration of exposure, among other factors. If positive results are not immediately forthcoming, it may be necessary to confirm the RNAi effect via microinjection of dsRNA across all life stages, as RNAi via microinjections has also been recently reported for codling moth (Wan et al., 2019). This approach would be taken to confirm the efficacy of dsRNA molecules in inducing RNAi in codling moth in order to rule out insufficiency of supplied materials. The aforementioned IAP gene would be used as a control in this case. Embryonic injections of dsRNA would be performed using same methods as done for CRISPR experiments in codling moth (Garczynski et al., 2017). Larval, pupal and adult injections would be made into the midgut region as described for codling moth (Wan et al., 2019) and other insects (Walker et al., 2010, 2011).

It is well known that when attempting RNAi, not all genes may be disrupted equally, and some genes may not be disrupted at all. Furthermore, some targeted genes may not be disrupted sufficiently to result in a predicted phenotype, such as mortality in this case. Concordantly, for this project, candidate genes will be selected based upon the hypothesis that RNAi-mediated disruption of these genes will result in codling moth mortality or developmental inhibition, based upon what is generally known about the function of these genes. However, it is possible that even if RNAi mediated knockdown is achieved, there will not be increased/sufficient mortality observed. This may be expected due to biological complexities such as genetic redundancies (multiple genes provide similar functions) or species-specific gene functions in codling moth that diverge from hypothesized expectations. In consideration of these potential problems, multiple genes will be targeted for each life stage, and for each gene, multiple regions will be selected to serve as gDNA template to generate a diversity of dsRNA effector molecule types.

The optimal goal is to utilize RNAi to disrupt gene expression and induce mortality or arrested development in codling moth larvae before they enter the apple. This would be mediated through uptake of dsRNA molecules that codling moth larvae have ingested through feeding on leaf and other plant matter before entering the apple, as is the case for uptake of the codling moth granulovirus (Lacey et al., 2008). It has been remarked that while dsRNA sprayed as a biopesticide was as effective as spinosad in controlling damage by the CPB, it was nonetheless slower (Petek et al., 2020). It may be the case that RNAi may not be completely effective in preventing codling moth from entering the apple and causing initial damage to the fruit. It is thus proposed to target genes expressed in all stages of life. In this way, the RNAi effect will manifest itself over time during the generation it is applied to, resulting in increased mortality and reduced populations. In this way, codling moth damage will be reduced from one generation to the next across growing seasons.

Time Plan: Experiments using RNAi against the IAP gene (positive control) and selected plant gene (negative control) will commence immediately at the start of the project in order to optimize the methodology; the IAP gene for codling moth has been identified in the published codling moth genome (Wan et al., 2019). Subsequently, target-gene RNAi experiments would be conducted as soon as ideal candidate genes are identified from the various life-stage transcriptomes. These experiments would be conducted from the middle of the first year of the project and onward until sufficiently effective target genes are identified and optimized for experimental field bioassays in Objective 3.

Objective 3. Perform controlled laboratory trials on efficacy of RNAi in neonate larvae and adults towards preventing codling moth damage in apples.

Procedures: For this objective, we will test RNAi efficacy using the best functioning candidate target genes that have been validated for gene disruption and codling moth mortality or developmental inhibition through the larval feeding assays in objective two. Target gene dsRNA will be synthesized and diluted in water to concentrations that have been observed to work in artificial diet RNAi assays. The codling moth behavioral modulator "Cidetrak – Da Mec" (Trécé Inc., Adair, Oklahoma) has been commercialized to affect codling moth larval and adult behavior through delaying location and entry of fruit. "Da Mec" will be mixed with dsRNA and tested in the lab to ensure that dsRNA is not degraded in the "Da Mec" solution. If the dsRNA remains intact, formulations will be made for spraying that include tank mixtures of the dsRNA together with the "Da Mec" at appropriate concentrations. Additionally, larval feeding stimulants, such as monosodium glutamate (Pszczolkowski et al., 2002), trans-trans-l-anflnocyclobutane-l,3-dicarboxylic acid (Pszczolkowski and Brown, 2004) and L-aspartate (Pszczolkowski and Brown, 2014) will be tested in formulation with dsRNA alone or together with "Da Mec" in field experiments for efficacy in facilitated RNAi-mediated pest control. Initial trials with these materials would first be tested in the laboratory in controlled behavioral assays on apple leaf and fruit materials to measure the extent to which the various formulations elicit increased feeding behavior by codling moth larvae.

Within our experimental orchards, presence of codling moth will first be assessed with sticky traps baited with codlemone pheromone (Knight et al., 2002). Then, at the onset of codling moth

activity, formulation spraying regiments will be implemented with validated mixtures of target-gene dsRNA, "Da Mec" and/or aforementioned feeding stimulants. Initially, dsRNA will be tested at highest dose observed to be effective in artificial diet feeding assays. Randomized block trial replicates will be utilized with respect to different treatment conditions plus no-dsRNA treatment controls. After each flight period, degree of damage to apples will be assessed and compared across each block trial with appropriate statistical measurements employed to assess effectiveness of dsRNA treatments in reducing or preventing codling moth damage to apple fruit.

Expected Results: If this approach is successful, it is expected that there will be reduced codling moth damage to apple fruit in experimental blocks treated with target-gene dsRNA versus controls. At this stage the efficacy of dsRNA in killing codling moth larvae or otherwise disrupting their development will have been validated in laboratory assays. As such, in properly replicated and controlled field block trials, any reductions in codling moth damage to fruit may be attributed to the RNAi effect

Potential Problems and Contingencies: The most considerable potential problem is that things do not always work in the field as they do in the laboratory, for any number of reasons. Environmental exposure of dsRNA is a primary concern. Preliminary experiments will be conducted during the first two years of the experiment, in which dsRNA formulations with and without external feeding elicitors are sprayed on controlled apple leaf and fruit material. In subsequent days and weeks, samples will be taken to assess persistence of presence of dsRNA. It may be necessary to utilize biodegradable nanoparticle encapsulators, such as "BioClay" (Mitter et al., 2017a; Mitter et al., 2017b). Based upon this information, it may be necessary to make one or more sprays of dsRNA formulations during each flight season to ensure maximum efficacy against codling moth larvae. Experimental trials testing sequential spraying regiments of the formulations onto apple leaf and fruit preparations in the laboratory may be utilized to assess optimal conditions for inducing larval mortality or developmental inhibition. Finally, while it is aimed to identify target genes by which RNAi induces complete mortality in the larval stage, RNAi efficiency or time-frame of activity may be reduced under field conditions. As such, larval mortality or developmental inhibition may be delayed beyond entry of larvae into the apple. Under these conditions, initial RNAi efficacy may be observed via observations of reduction in apple damage during the first flight treatment but would instead manifest through reduced codling moth populations across generations and field seasons. As such, it would be necessary to continue experimentation and assessments beyond the three-year scope of this proposal.

Time Plan: Formulations with IAP dsRNA, "Da Mec, and the feeding stimulants will be made and tested in the laboratory during years one and two to assess viability of the approach of combining these compounds with synthetic dsRNA without degradation of dsRNA. Preliminary assessments of dsRNA longevity in field conditions will also be made during the first two years to better inform spraying conditions during the eventual third year experiments. The field trial experiments in Objective 3 will be conducted during the third year during the times where codling moth larvae and adults are behaviorally active.

RESULTS AND DISCUSSION

There was an initial delay in hiring technical support staff funded by this project that is essential to perform research on the molecular genetics aspects for all of the objectives. The process to initiate hiring on our side was launched months ago directly after the funding became available, however, due to administrative delays in processing of hiring documents, the process was only completed within the past month. Initial efforts have been focused on generating molecular materials to test dsRNA of the control gene, IAP (Objective 2). Two initial bioassays have been run with codling moth neonate larvae exposed to IAP dsRNA, however, no excess mortality was observed for codling moth larvae compared to those exposed to negative controls. Current efforts are focused on optimizing these larval bioassays, and protocols are being adapted from CpGV larval mortality assays to ensure that the codling moth larvae are fully exposed to the dsRNA substrate material. Meanwhile, sufficient neonate larval and adult specimen have been collected from our codling moth colony for RNA sequencing experiments (Objective 1); these samples are currently being processed and prepared for the RNA sequencing to

facilitate the goals of the objective. Based upon these, there are thus, at present time, no substantial results to present and discuss.

Project/Proposal Title: Tactics to improve natural enemy releases in tree fruit

Report Type: Continuing Project Report (NCE)

Primary PI: Rebecca Schmidt-Jeffris
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Cooperators: Steve Arthurs (BioBee); Chuck Weaver (G.S. Long & Parabug); Brent Milne (McDougall Fruit); Dave Keller, Sean Gilbert, Rob McGraw, & Tony Mena (Gilbert Fruit), John Haas & Matt Klaus (G.S. Long), Mike Brown (Gebbers Farms) [note: pear grower cooperators will be specified in pear report]

Report Type: Continuing Project Report

Project Duration: 2-Year

Total Project Request for Year 1 Funding: $ $102,558*
Total Project Request for Year 2 Funding: $106,033*
*50% by WTFRC Apple Crop Protection, 50% by FPC/PPC Pear

Other related/associated funding sources: awarded

Funding Duration: 2020-2023
Amount: $36,614
Agency Name: BioBee
Notes: In-kind match of commercial insectary insects, Artemac (brine shrimp cysts on tape), and shipping costs for beneficials to be used in this project. Itemized estimate provided by BioBee.

Funding Duration: 2020-2023
Amount: $720
Agency Name: Parabug, Chuck Weaver private contractor
Notes: In-kind match of drone pilot labor for releasing insects as part of Obj. 2. ~$18/acre × 10 drone-treated acres per trial × 2 trials (apple & pear) × 2 years.

Funding Duration: 2021-2022
Amount: $29,968
Agency Name: Western IPM Center, project initiation grant

Notes: This project expands the efforts in this grant by providing support to conduct grower input sessions and a needs assessment survey. The WIPMC grant will also be used to start a grant team and stakeholder advisory group that will submit a federal grant application to expand this work (likely to USDA OREI). The data collected in this grant will be used as preliminary data in the OREI submission. The results in this report are due to this grant award.

Funding Duration: 2020-2023
Amount: $348,733
Agency Name: Western SARE
Notes: This is a complementary (non-overlapping) project, specifically focusing on earwig releases in apple and pear, on the ground and by drone.

WTFRC Collaborative Costs: none

Budget 1*
Organization Name: USDA-ARS **Contract Administrator:** Mara Guttman
Telephone: 510-559-5619 **Email address:** mara.guttman@usda.gov
Station Manager/Supervisor: Rodney Cooper **Email Address:** rodney.cooper@usda.gov

Item	2021	2022
Salaries[1,4]	$17,458	$17,894
Benefits[1,4]	$5,587	$5,726
Wages	$0	$0
Benefits	$0	$0
Equipment	$0	$0
Supplies[2]	$6,500	$6,500
Travel[3]	$0	$0
Miscellaneous	$0	$0
Plot Fees	$0	$0
Total	**$29,545**	**$30,120**

Footnotes:
[1]GS-5 technician for 6 months per year, 100% FTE at 32% benefits, Year 2 includes 2.5% COLA increase. Technician would assist WSU postdoc (see below) with sampling in all locations. This technician will also assist the postdoc with surface sterilization and PCR for gut content analysis.
[2]Funds to purchase PCR reagents and other PCR supplies for gut content analysis, trapping supplies, and some commercial nutritional supplement products (others provided as in-kind match).
[3]Fuel to field sites will be provided by USDA base funds and is not requested.
*50% by WTFRC Apple Crop Protection, 50% by FPC/PPC Pear
[4]This funding (both years) has been deobligated by USDA-ARS and WTFRC has made it available for WSU, to partially support a graduate student who is assisting with this project

Budget 2*
Organization Name: WSU
Contract Administrator: Stacy Mondy
Contract administrator email address: anastasia.mondy@wsu.edu
Station Manager/Supervisor: Chad Kruger **Email Address:** cekruger@wsu.edu

Item	2021	2022
Salaries[1]	$52,827	$54,940
Benefits[2]	$18,373	$19,108
Wages[3]	$1,200	$1,248
Benefits[3]	$113	$117
Equipment	$0	$0
Supplies	$500	$500
Travel	$0	$0
Miscellaneous	$0	$0
Plot Fees	$0	$0
Total	**$73,013**	**$75,913**

Footnotes:
[1]Nottingham salary ($7,612.50/mo × 12 mo × 2% FTE = $1,827 Year 1, Year 2 reflects 4% COLA increase) + Postdoc salary ($4,250/mo × 12 mo × 100% FTE = $51,000 Year 1, Year 2 reflects 4% COLA increase). Nottingham to supervise data collection efforts in pear in the Wenatchee area and advise on project methods and data summary. WSU Postdoc will be based at the USDA-ARS facility in Wapato, WA and supervised by Schmidt-Jeffris. The postdoc will be responsible for leading data collection and summarizing project results. Due to difficulties in finding a qualified postdoc candidate, we have expanded our search to also include an associate in research, which would have a similar salary, but be hired at the M.S. level. The associate in research (Daniel Hausler) was hired in early 2022.
[2] Benefits rate for Nottingham is 29.9% ($547 Yr 1, $569 Yr 2). Benefits rate for postdoc is 35% ($17,826 Yr1, $18,539 Yr2).
[3]Summer technician at $15/hr×8 hr/wk ×10 wks, 9.4% benefits rate, salary includes 4% COLA increase in Year 2
*50% by WTFRC Apple Crop Protection, 50% by FPC/PPC Pear

OBJECTIVES

1. Improve retention of released natural enemies. A primary complaint from growers is that natural enemies disperse from the orchard immediately after release. Nutritional supplements such as pollen (Nutrimite, Biobest) and brine shrimp cysts (Artemac, BioBee) are commercially available and have been shown to improve retention and survival of natural enemies in greenhouses, but this has not been tested in tree fruit orchards. Using methyl salicylate lures, which attract natural enemies, in combination with nutritional supplements may further improve natural enemy retention with little additional effort on the part of the grower. We will test supplements and lures in combination and individually in plots where commercially available predators, lacewings and minute pirate bugs, have been released. We will collect data on pest control levels, retention of released natural enemies, and recruitment of resident natural enemies. This objective was modified to test *Ephestia* eggs instead of pollen, due to greater ease of application.

2. Determine cost-effectiveness and efficacy of natural enemy release by drone. One method for reducing natural enemy release labor costs is to conduct releases by drone. However, the ability of natural enemies to survive release by drone into orchards and whether this method significantly decreases natural enemy abundance relative to hand-releases is unknown. We will compare released predator abundance, pest control levels, and labor costs for releases by hand and by drone of lacewings and mealybug destroyers in apples. This objective was modified to include comparison of additional treatments, including mealybug destroyer larvae, lacewing cards, multiple species of lacewings, and releasing lacewings as larvae versus eggs.

SIGNIFICANT FINDINGS

- Thanks to a no-cost extension, we were able to delay the main parts of this the project to begin in 2022. The main delay was due to our inability to find a qualified postdoc. Instead, we readvertised the position as an associate in research, open to individuals with M.S. degrees. Daniel Hausler was hired to manage the project in early 2022. Some data was still collected in 2021 because of funding from other sources.

- **Mealybug destroyers, 2020-2022.** In 2020, mealybug destroyers released by hand either early (mid-May) or late (mid-June) at either 2,000 or 5,000 per acre caused ~3× decrease in mealybug populations, but this effect was highly variable between plots. The drone release did not cause a decrease. In 2021, we examined mealybug destroyer releases in one-acre plots, comparing drone versus ground releases of 1,000 mealybug destroyers per acre to a no-release control. We found very few mealybug destroyers 1 day after release and no mealybug destroyers 8 days after release; they likely dispersed due to low pest density in this orchard. In 2022, mealybug destroyers were not recovered after release, despite the presence of mealybugs in the plots, and no differences were observed between treatments. It is possible that a fire blight spray affected this release. In general, mealybug destroyers do not appear to be a reliable control method for mealybugs in apples and cannot currently be recommended due to their high cost.

- **Lacewings, 2021.** We tested releases of two species of lacewings as eggs or larvae: *Chrysoperla rufilabris* and *Chrysoperla carnea*. We found that the *C. carnea* larvae (which came from a different insectary than the eggs) were actually *C. externa*. While lacewings in the *C. carnea* species group are suited to our arid climate, *C. externa* is not. This quality control issue was reported to the insectary. A release of *C. carnea* as eggs (100,000/acre) was the most successful treatment at suppressing woolly apple aphid and green apple aphid in this study. A release of *C. rufilabris* larvae was also effective (20,000/acre). Seasonal counts of aphid colonies were reduced by 57% and 43% in these treatments, respectively. Low numbers of larvae of the released

lacewing species were found throughout the trial (1-5 per treatment, across 8 weeks of sampling). Therefore, when determining efficacy of beneficial releases, scouts should focus on pest numbers, not necessarily natural enemy recovery.

- **Lacewings, 2022.** We compared releases of (1) *C. carnea* eggs by hand, *C. rufilabris* eggs by (2) hand, (3) card, and (4) drone, (5) *C. rufilabris* larvae, and (6) a no-release control. None of the treatments caused a reduction in aphids. Lacewing larvae were recovered from ground-based release treatments (5-14 total per treatment, across 8 weeks), but were not recovered from the control or the drone treatments.

- **Improving retention, 2022.** In a commercial apple orchard, releases of *O. insidiosus* and *C. carnea* decreased green apple aphid populations. However, the food supplements and Predalure caused an increase in aphids compared to the treatments where they were not used. It is likely that complex interactions between released and resident natural enemies are occurring. Possible interactions will be explored via molecular gut content analysis, which is currently in progress. In the commercial and research orchard trials, Predalure showed potential for recruiting resident natural enemies for pest mite control and decreased brown mite abundance.

- **Grower survey and discussion, 2021-2022**. In collaboration with Tianna DuPont and Ashley Thompson, we collected survey data on apple and pear grower perspectives of releasing natural enemies in tree fruit. 132 growers and consultants responded, representing 43,868 apple and pear acres. 37 respondents (28%) are using biocontrol releases occasionally or annually on 7,842 acres costing them $153 per acre on average. The main natural enemies they are releasing are lacewings (29%), lady beetles (28%), and predatory mites (25%). The main barrier to adoption of releases was lack of knowledge/recommendations on how to release successfully (52%). Five stakeholder input sessions were conducted in 2021-2022 in Omak, Wenatchee, Yakima, Hood River, and Medford with a total of 60 participants. The input sessions identified the following as critical research areas: (1) information to make natural enemy releases more effective/useful, (2) evidence of efficacy, (3) what species to release, (4) where to purchase, (5) release timings, (6) release rates, (7) a list of common release mistakes and how to avoid them, (8) on farm success stories, (9) consistent supply, (10) proper placement in the tree/orchard, and (11) pesticide toxicity to natural enemies. Feedback from the survey will be used to determine future research directions and to obtain federal funding to expand the work in this project.

METHODS

The methods below are for the apple portion of the project only. They have been updated to reflect how the work was conducted in 2022.

Improve retention of released natural enemies.

This two-year (2022-2023) study will be conducted in a commercial organic apple orchard in Wapato, WA (Gilbert Fruit) which was selected due to annually high woolly apple aphid (WAA) pressure. The release day will target when aphid populations begin to rise and the typical timing the grower releases lacewings, approximately mid-May. There will be a total of five treatments made of combinations of lure use (Predalure, methyl salicylate), food supplements (Artemac, brine shrimp cysts + *Artemia* eggs), and releases (100,000 lacewing eggs + 2,000 *Orius insidiosus* per acre): (1) Predalure (methyl salicylate) + Foods + Release, (2) Predalure + Release, (3) Food + Release, (4) Release only, and (5) No-release control. Each combination will be replicated in the orchard 5 times in 0.5 acre plots. One week prior to release, we will conduct precounts of aphids by counting the number of WAA, green apple aphid, and rosy apple aphid colonies per 3 shoots each on 9 trees in the center of the plot. At

Fig. 1. Ladybeetle feeding on Artemia tape

this point, one methyl salicylate lure will be added to one tree in the center of each plot to allow the volatiles sufficient time to dissipate prior to releasing the natural enemies. One week after this, we will apply *Ephestia* and Artemac throughout each plot at the insectary recommended rate. Artemac (Fig. 1) will be applied by tying tape with attached cysts to trees and *Ephestia* eggs will be applied by hanging cards. Then, we will release by hand two natural enemy species across the entire trial at insectary recommended release rates: 100,000 *Chrysoperla carnea* eggs per acre (green lacewing, BioBee) and 5,000 *Orius insidiosus* per acre (minute pirate bug, Beneficial Insectary). Post-release sampling will occur at weekly intervals following release for 4-8 weeks. Aphids will be sampled as previously described. Beat tray samples will be collected from the 9 center trees of each plot. All natural enemies from the tap counts will be collected and stored in ethanol. Lacewings and *Orius* collected will be identified to species in the laboratory to determine if they are from the insectary. These specimens will be used for gut content analysis to determine: 1) if released beneficials are consuming pests at high rates and 2) if either released beneficials or resident natural enemies are consuming the nutritional supplements. We will also place two sticky cards on trees within the center of each plot to count all natural enemies to species. DNA analysis will be conducted on any captured *C. carnea* to distinguish resident from released individuals.

We also conducted an additional study using a similar design in a research orchard in Moxee, WA. Because of the size of the orchard, we removed the no-release treatment and also simplified the other treatments, only testing *Orius insidiosus* releases and Artemac (removing the lacewings and *Ephestia* eggs).

Determine cost-effectiveness and efficacy of natural enemy release by drone.

This objective was split into two separate studies in different orchards, one for mealybug destroyers and one for lacewings.

Mealybugs. In 2022, this study was conducted in a commercial organic apple orchard in Desert Aire, WA (Gilbert Fruit). The treatments were (1) ground release of adults, (2) ground release of larvae, (3) drone release of adults, and (4) no-release control. There were five 0.25-acre replicates per treatment (20 plots total). We released 2,000 mealybug destroyers per acre on May 18 (larvae) and May 20 (adults). Mealybugs were counted by two methods: shoot samples and burlap samples. Shoot samples consisted of 15 shoots collected from 15 trees in the center of each plot. Burlap samples consisted of a 15 cm wide piece of burlap tied to the main limb of the tree, on 10 trees per plot (Grasswitz and Burts 1995). They were be evaluated one week after placement. All sample types were collected once weekly for six weeks following releases. Because of poor mealybug destroyer recovery and performance in past studies, this is the final year of efficacy testing using individual plots. We will be seeking any growers who are conducting releases in 2023 to evaluate mealybug counts before and after release and determine rates of mealybug destroyer recovery.

Lacewings. This study will be conducted in a commercial organic apple orchard in Desert Aire, WA (McDougall Fruit) in 2022-2023. Lacewing eggs will be released at a rate of 100,000 per acre and larvae will be released at a rate of 20,000 per acre. The treatments will be (1) *C. carnea* eggs by hand,

(2) *C. rufilabris* eggs by hand, (3) *C. rufilabris* eggs by card, (4) *C. rufilabris* eggs by drone, and (5) *C. rufilabris* larvae by hand. There will be five 0.25-acre replicates per treatment. Aphids will be counted using the methods previously described. All stages of lacewings will be sampled with beat trays; beat samples will be conducted on 12 trees in the center of each plot. Lacewings collected will be stored in alcohol and examined in the lab to confirm that they are the released species. Two sticky cards will also be hung in each plot and any captured green lacewings will be identified to species. DNA analysis will be conducted on any captured *C. carnea* to distinguish resident from released individuals.

In 2022, we also conducted an additional study using single-tree replicates in a research orchard in Moxee, WA. Individual trees with high infestations of rosy apple aphids were identified for releases, but aphid populations crashed the week after release, likely due to a heat wave (early July). This trial will be repeated in 2023 but conducted in late May instead.

RESULTS AND DISCUSSION

For the purposes of comparison between years, we have included a brief overview of results from previous studies (2020-2021), in addition to the results from the current project (2022-2023).

Mealybug destroyer trials, 2020-2022. In 2020, ground releases of mealybug destroyers decreased mealybug counts by three-fold. Recovery of the mealybug destroyers was moderate 1-2 weeks after release early in the season (~3 per plot, then ~1 per plot, respectively). After this period, only 1 mealybug destroyer was found across all plots each week for the rest of the sampling period. In 2021, neither release treatment (ground or drone) lowered mealybug counts compared to the control. This is likely because mealybug populations were very low (<0.04 mealybugs per trap). Low mealybug populations likely caused the poor establishment of mealybug destroyers. This indicates that mealybug destroyers are only effective predators when mealybug populations are higher and therefore may only be useful in orchards where there is a serious, reoccurring issue with this pest. In 2022, we identified an orchard that had a history of incredibly high mealybug pressure. However, the prior year a very high rate of mealybug destroyers was released. It is possible that this release, which we did not monitor, reduced mealybug populations. Early in our trial, there were 0.20 mealybugs per trap and 0.11 mealybugs per shoot. Per trap numbers never exceeded 0.22 mealybugs. We did not detect any differences between treatments during this year of the study (Fig. 2). Additionally, the mealybug destroyers were never recovered after release, even only one week later. A fire blight treatment was made in the orchard immediately after releases, which may have either directly harmed the mealybug destroyers, caused them to be repelled, or the airblast sprayer may have physically removed them from the trees.

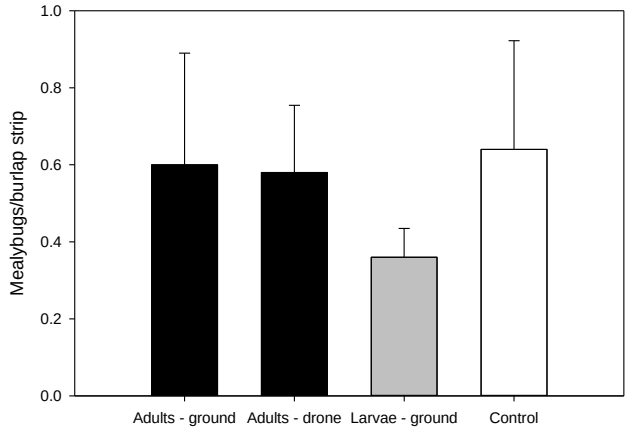

Fig. 2. Seasonal sums (4 weeks post-release) of mealybugs per burlap strip trap in mealybug destroyer release trial, 2022.

We conducted a small trial examining the effect of Serenade via direct contact and fresh resides on mealybug destroyers in the lab. We tested Serenade at maximum field rate, by either directly spraying

the mealybug destroyers with a Potter Tower and moving them to untreated glass Petri dishes, or by spraying the Petri dishes, allowing them to dry, and then adding mealybug destroyers. These treatments were compared to mealybug destroyers sprayed with water and those in water-treated dishes. Each treatment was replicated 10 times (1 Petri dish) with five individuals per dish. A small amount of honey was provided in each dish as a food source. Almost no mortality was observed at 24 h in any treatment. However, at 48 h, mortality was ~50% in all treatments. The mealybug destroyers may have been dehydrated or they may have suffering from other stress related to shipping. Regardless, Serenade did not cause short-term mortality in the insects we tested. We will conduct additional studies in the future to determine if Serenade is harmful to released natural enemies.

Lacewing trials. In 2021, we confirmed the *C. rufilabris* shipments contained the species advertised. The *C. carnea* eggs came from an insectary in Mexico, whereas the larvae came from an insectary in Canada. The *C. carnea* eggs were indeed a species in the *carnea* species group (molecular work is in progress to determine exactly which species). However, the "*C. carnea* larvae" were *C. externa*, which is not a species in the *carnea* species group. This is a known issue with insectaries, as lacewings within the genus *Chrysoperla*, especially in the *C. carnea* species group, are very difficult to identify without the expertise of a specialized taxonomist. In 2022, we did not order *C. carnea* larvae because of this issue. We did confirm that the species we received were correct.

In 2021, the *C. carnea* eggs and *C. rufilabris* larvae resulted in lower aphid populations compared to the control, whereas the other two treatments did not ("*C. carnea*" larvae and *C. rufilabris* eggs) (Fig. 3). Lacewing larvae from the releases were found up to a month after the release occurred. We recovered *C. rufilabris* from the "*C. rufilabris* larvae" treatment the most often, but never found any *C. rufilabris* larvae in the treatment where eggs were released. We found larvae of the correct species in all other plots. We also found several species of native, non-released *Chrysopa* lacewings, which appear to have a healthy population in the orchard. *Chrysopa* larvae were not found until three weeks after our releases and then in lower numbers than our released lacewings. This indicates that our treatments gave this orchard a head start in aphid management compared to the no-release control. All adult lacewings that were found during the course of the trial were *Chrysopa*, therefore we do not yet have evidence that the juvenile lacewings released ever fully developed. However, recovery of lacewings in general was low, so they many have been present and not found.

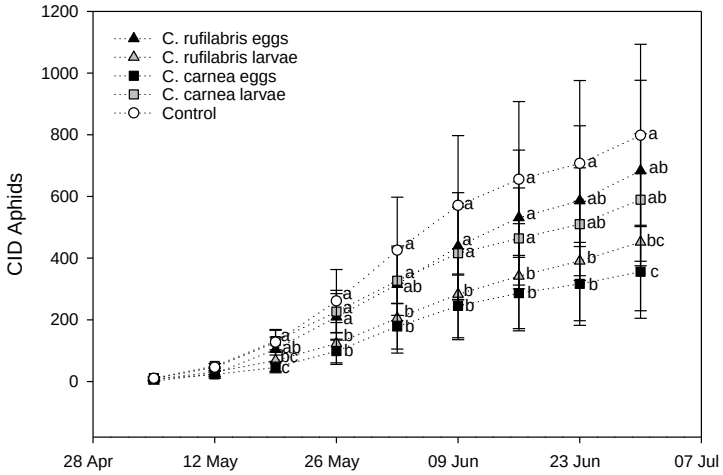

Fig. 3. Cumulative insect days (CID) for aphids in the 2021 lacewing release trial.

There were no differences in aphid colonies per plot in the 2022 lacewing release trial (Fig. 4). Resident natural enemy populations, especially ladybeetles, were high in this orchard. It is possible that the addition of lacewings did not provide much of an addition in terms of biological control. Additionally, unlike the 2021 trial, there were resident populations of *Chrysoperla* species, which also would have contributed to biological control. Recovery of lacewing larvae was actually higher than in 2021. We recovered 12, 11, and 5 larvae throughout the season in the sprinkle eggs, egg cards,

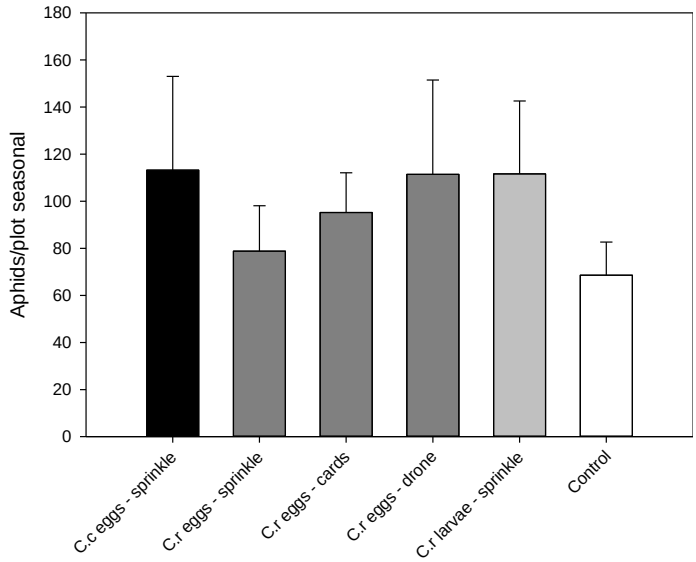

Fig. 4. Seasonal sums of aphid colonies per plot for 8 weeks post-release in the 2022 lacewing release trial.

and sprinkle larvae treatments of *C. rufilabris*, respectively (Fig. 5). We did not recover any *C. rufilabris* larvae in the drone or control treatments. This provides some initial evidence that drones may be a poor delivery mechanism for lacewing eggs, but this should be further evaluated in larger plots. Two adult *C. rufilabris* were found during the trial, indicating that this species can complete development in Washington orchards.

Retention Trials, 2022. In this trial, releases of *O. insidiosus* and *C. carnea* decreased green apple aphids by ~50% compared to the no-release control (Fig. 6). Rosy apple aphids were also present but appeared to be unaffected by our treatments. We speculate that most of the aphid biological control was provided by *C. carnea*. Only 10 lacewing larvae and 1 *O. insidiosus* were recovered during the trial. All individuals will be tested by PCR this winter/spring to determine whether they were consuming pest aphids or the provided food items. Thrips counts were also reduced in the release-only treatment compared to the control by 30%.

Surprisingly, all combinations of the lure and food treatments increased green apple aphid abundance relative to the release-only treatment – bringing aphid levels back to nearly that of the no-release control (Fig. 6). It is possible that these treatments caused changes in the natural enemy community within the plots, potentially resulting in competition or increased intraguild (between natural enemies) predation that may have reduced aphid biological control. We are in the process of performing molecular gut content analysis on a subset of all captured natural enemies to determine if they may have consumed our released predators. Plots with Predalure also had a slight increase (31%) in seasonal thrips populations relative to those that did not. Lures also increased abundance of *Stethorus* by 62%. Pest mite populations were very low in this orchard, so we could not determine if the increased *Stethorus* populations in Predalure plots resulted in improved biological control.

Fig. 5. A released *C. rufilabris* seen in the orchard in post-release sampling

In the retention trial in the Moxee research orchard, treatments with Predalure included had fewer brown mites (Fig. 7). Lures were found to increase abundance of lacewings and spiders (Fig. 8). Only one released *O. insidiosus* was found during the five weeks post-release (lacewings were not released in this trial). It is likely that resident natural enemies, rather than the released *O. insidiosus*, caused the reduction in brown mites. However, it is also possible that *O. insidiosus* contributed to mite biocontrol and we were just unable to recover them during sampling. Predalure use should be further explored for its potential use in recruiting natural enemies for controlling pest mites.

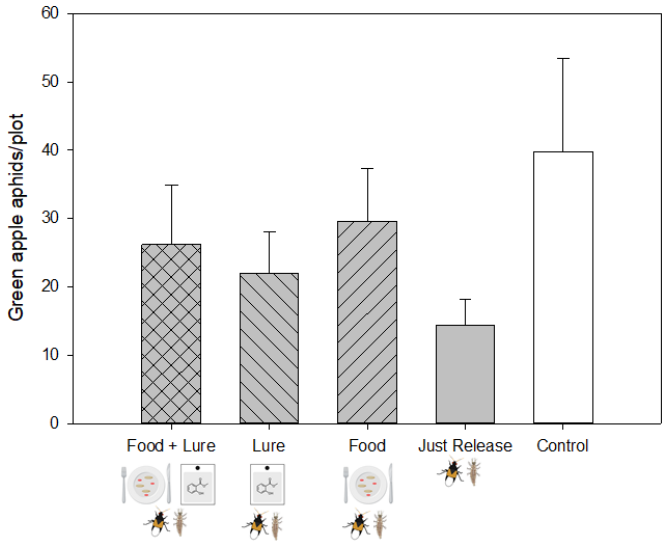

Fig. 6. Seasonal sums of green apple aphid colonies per plot for 5 weeks post-release in the 2022 retention trial in commercial apples.

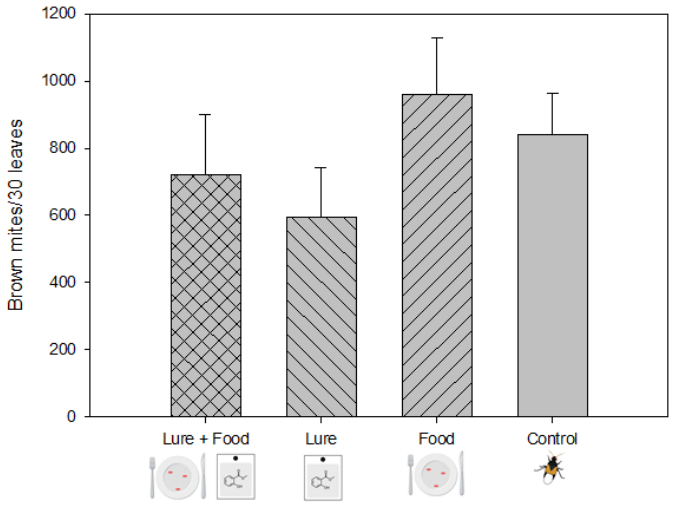

Fig. 7. Seasonal sums of brown mites per plot for 5 weeks post-release in the 2022 retention trial in research apples.

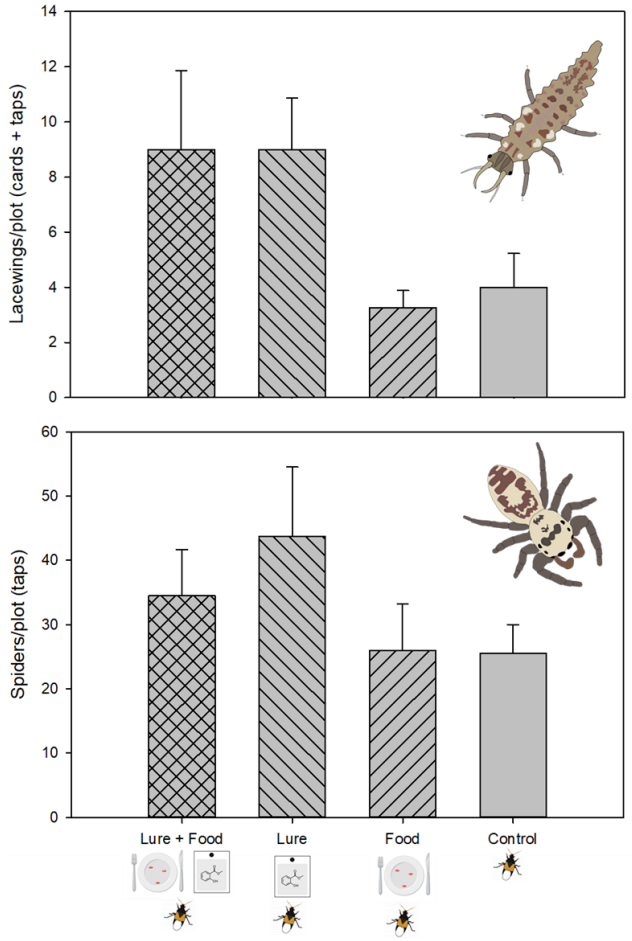

Fig. 8. Seasonal sums of lacewings and spiders per plot for 5 weeks post-release in the 2022 retention trial in research apples.

Project Title: Integrated control of brown marmorated stink bug

Report Type: Final Project Report

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Project Duration: 3 Years

Total Project Request for Year 1 Funding: $96,326
Total Project Request for Year 2 Funding: $99,851
Total Project Request for Year 3 Funding: $103,517

Other related/associated funding sources: Awarded
Funding Duration: 2017-2022
Agency Name: USDA-NIFA-SCRI
Amount: $156,047 for WSU/Beers
Notes: 2017 – 2021, plus a 1 -year NCE due to COVID

Other related/associated funding sources: Awarded
Funding Duration: 2019
Amount: $26,675
Agency Name: Washington State Commission on Pesticide Registration

Other related/associated funding sources: Awarded
Funding Duration: 2020
Amount: $16,505
Agency Name: Washington State Commission on Pesticide Registration

Budget 1
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Item	2019	2020	2021	2022 (NCE)
Salaries[1]	$53,395	$55,531	$57,752	
Benefits[2]	$21,166	$22,012	$22,893	
Wages[3]	$7,800	$8,112	$8,436	
Benefits[4]	$725	$754	$785	
Equipment				
Supplies[5]	$3,000	$3,000	$3,000	
Travel[6]	$5,200	$5,200	$5,200	
Miscellaneous				
Plot Fees[7]	$5,040	$5,242	$5,451	
Total	**$96,326**	**$99,851**	**$103,517**	**$0**

Footnotes: [1]Research Technician (Smytheman), 1.0 FTE, [2]Benefits 39.6%. [3]Time-slip wages 13 weeks@$15/hr., [4]Benefits, 9.3% [5]Laboratory, field and office supplies, electronics. [6]Motor Pool rental, April-October.
[7]Plot fees for Sunrise Orchard apples

Objectives

1. *Investigate the efficacy and non-target effects of insecticide infused netting as a means of monitoring and control of BMSB*. Insecticide-infused netting has several potential applications in monitoring BMSB, and also as a component of behavioral controls. However, the active ingredient in the netting is broad spectrum, thus non-target effects are a possible negative attribute.
2. *Redistribute Trissolcus japonicus (the samurai wasp) where established BMSB populations are identified and monitor its establishment and non-target effects. T. japonicus* was found for the first time in Washington in 2015, allowing us to re-distribute this adventive population; the APHIS permit to release the quarantine strain has not yet been approved. Permit approval is largely based on risk assessment of non-target effects, and the adventive population in Washington provided a rare opportunity to validate laboratory tests in the wild.
3. *Determine development of BMSB on shrub-steppe plants.* BMSB first reached outbreak levels in the US in the mid-Atlantic region, but little is known of its ecology in the semi-arid interior of Washington.
4. *Track the invasion of BMSB in Washington State*. Tracking the spread of an invasive species is key to understanding its invasion ecology. The advent of widely accessible GPS georeferencing, and the combination of targeted sampling and citizen scientist reporting has allowed us to develop a detailed record of the extent and nature of its spread.

Significant Findings

- Captures of BMSB in interior traps in blocks protected by attract and kill (A&K) traps were consistently lower than in blocks not protected by traps. Fruit damage in the protected blocks was 50% lower than the unprotected blocks.
- The use of an insecticide-infused panel trap baited with pheromone provided a significant enhancement to capture over the 'ghost' trap (draped netting). The addition of lights to pheromone traps did not increase trap capture.
- A total of ≈16,000 *T. japonicus* were released in Washington from 2017-2022, with release sites including tree fruit growing areas (mid-Columbia, Yakima, Rock Island, Walla Walla, Prosser), and urban centers (Seattle, Puyallup, Spokane, and Tri-Cities). Yellow sticky cards were deployed to determine if the wasp became established, but only a single specimen was recovered in eastern Washington (Yakima). The original populations in Vancouver, however, are still flourishing.
- Using PCR and morphological methods, we found that *T. japonicus* (samurai wasp) had higher total impact (reproductive and non-reproductive) on the native spined soldier bug (a predator) than on BMSB. Effects on other native stink bug species (all pests) was present, but at lower levels.
- In two years of studies, Washington's native shrub-steppe plants as a diet for BMSB constituted a clear developmental penalty for nymphs reared on native plants in comparison to either a colony diet, or plants prevalent in the mid-Atlantic region. Further, adults experienced substantial reductions in longevity and fecundity on the native diet. This appears to be a clear indication that without crop plants, BMSB will not build to high levels in unmanaged areas of Washington.
- The database tracking the spread of BMSB across Washington has 1,617 records as of December 2022. Thirty of 39 counties have reported BMSB, with the greatest numbers of reports and individual bugs coming from the I-5 corridor centered on Seattle. 2022 was the most active reporting year to date, with 218 reports and ≈9,500 bugs recorded.

Results and Discussion

Obj. 1: Investigate the efficacy and non-target effects of insecticide-enhanced netting as a means of monitoring and control of BMSB

1a. Attract-and-kill for control of BMSB. Much of the initial research on BMSB in the mid-Atlantic area focused on determining the efficacy of various insecticides; this research was critical for enabling growers to prevent crop damage in the short term. Since that time, research efforts have transitioned to exploring longer-term solutions, especially biological control. Although biological control is expected to provide some overall population suppression, it is likely that vulnerable crops will still need a more direct form of protection. Most of the insecticide options will be highly toxic to the samurai wasp and limit its impact in orchards; the primary impact will be in unmanaged habitats. Thus, development of tactics that are compatible with biological control are the highest priority for BMSB.

Behavioral controls have been the most intensively researched alternatives for BMSB control in the past 10 years. The most prevalent of these has been variations on a technique known as attract-and-kill (A&K). The attraction component has been the dual BMSB lure (currently available from Trécé); the means of killing them can be more variable, but often centers on an insecticide component. Initial experiments tested spraying baited trees at frequent intervals, but more recent efforts have focused on the use of long-lasting insecticide nets (LLIN) to cause mortality. This avoids the necessity of weekly sprays; in fact, the toxicity of the netting is projected to last several years.

Methods: We tested a perimeter of A&K traps using 3 pheromone lures and LLIN (Plate 1) to protect an orchard from BMSB fruit damage. Traps were deployed every 50 m (164 ft) on the orchard border next to wooded areas (the latter is presumed to be a major source of BMSB). The traps were deployed in early July and checked every other week until late October. In addition, 3 sticky traps were placed near the center of the orchard to determine penetration of BMSB into the orchard interior. The A&K plots were compared to untreated sections of the same block, separated by a 55 to 756 ft buffer zone (sticky traps only). Adults and nymphs of BMSB retained by the traps were recorded, and a preharvest fruit damage sample (80 fruit/plot) was taken in early August and assessed after ca. 12 weeks of cold storage.

Plate 1. LLIN net in a pear tree

Results and Discussion: Captures in A&K border traps in 2020 were 93 to 99% lower (1 to 3 BMSB/traps/season) than the same blocks in 2019 (44 to 104 BMSB/trap/season), and interior sticky trap catches in 2020 were 61% of those in 2019, indicating lower overall bug pressure in 2020 in this orchard. The A&K traps caught <1 BMSB/trap through most of the season, with no consistent seasonal trend. Surprisingly, the A&K traps caught less than the interior traps throughout the season. The interior sticky traps behind the protective perimeter of A&K traps caught consistently fewer BMSB (Fig. 1), resulting in significantly lower fruit damage at harvest (Fig. 2).

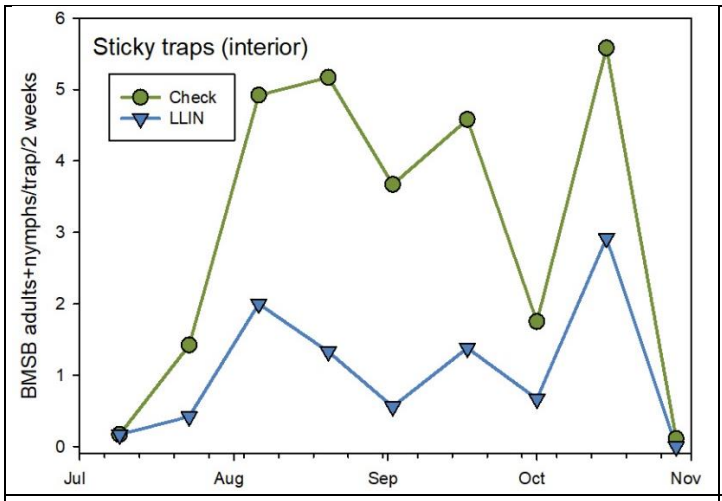

Fig. 1. BMSB captured in the interior of plots protected by A&K traps (LLIN) versus the unprotected (check) plots.

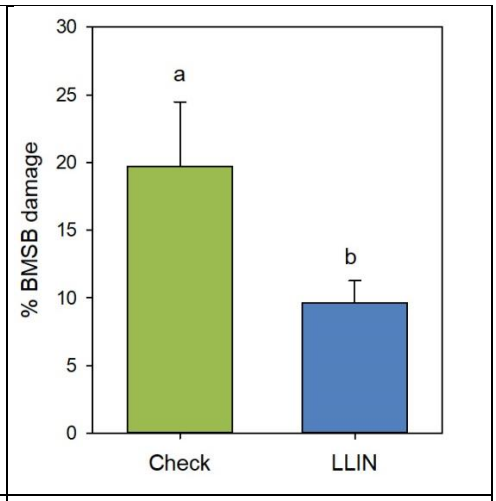

Fig. 2. Fruit damage caused by BMSB in LLIN versus check plots.

1b. Physical exclusion, net barriers. Net barriers were tested to determine if they could intercept stink bugs immigrating into orchards from native habitat. Native stink bugs were used as a proxy for BMSB until populations expanded to a testable level. We built 12 ft high x 150 ft long shade net barriers (white, 20% shade, 2 x 5 mm openings) between apple orchards and unmanaged areas of native habitat (Plate 2a), and studied the movement between the two, and the damage occurring with or without a net. The barriers had a triple row of flaps to retain stink bugs landing on the net and moving upwards. In the second year of the study, the barriers were enhanced with the addition of strips of insecticide infused netting to reduce the possibility of escape of the intercepted bugs (Plate 2b).

Stink bug populations in the orchard were lower in the control plots than in the two barrier treatments, although the differences were not statistically different. Fruit damage was lowest in the deltamethrin net treatment, again without statistical differences. About 6-fold more stink bugs were killed by the deltamethin vs plain net barriers (non-significant), however, higher numbers of some non-target species (Neuroptera, Coccinellids, and Hymenoptera) were also killed by the deltamethrin-augmented nets.

Plate 2a. Net barriers with flaps

Plate 2b. Insecticide-infused netting (black) sewn into flaps of net barrier.

Objective 2: Redistribute *Trissolcus japonicus* (the samurai wasp) where established BMSB populations are identified, and monitor its establishment and non-target effects

2a. Redistribute the samurai wasp in Washington State. *Methods: T. japonicus* was found for the first time in Washington State (Vancouver) in 2015, and we have reared it in colony continuously since that time. Most of the colony's production has been for re-distribution to other parts of Washington. While our initial target was the tree fruit growing areas east of the Cascades, we later expanded that to urban areas which had established BMSB populations. We released *T. japonicus* (mostly as adults, occasionally as parasitized eggs close to hatch) from 2017 to 2022. Our goal for release in urban sites is that when BMSB spread from urban to agricultural areas (the pattern observed in other parts of the nation), their parasitoid will move with them. This may effectively constitute a *pro-active* release for agriculture. In addition, we released *T. japonicus* in two agricultural areas in Klickitat County, and two in Douglas County. As a follow-up to our releases, we deployed yellow sticky traps to determine establishment of *T. japonicus* in several previous release sites.

Plate 3. Release of the samurai wasp in Washington

Results: We released a total of **15,488** adult *T. japonicus* in from 2017-2022, in Prosser (455), Puyallup (700), Rock Island (2,910), Seattle (2,288) Spokane (3,840), Tri-Cities (1,239), Walla Walla (448), White Salmon (3,090), and Yakima (518) (Plate 3, Fig. 3). The numbers released were dependent on the availability of egg masses from the BMSB colony. All of the 2021-22 releases were from the 2020 *T. japonicus* collection from one of the original sites in Vancouver.

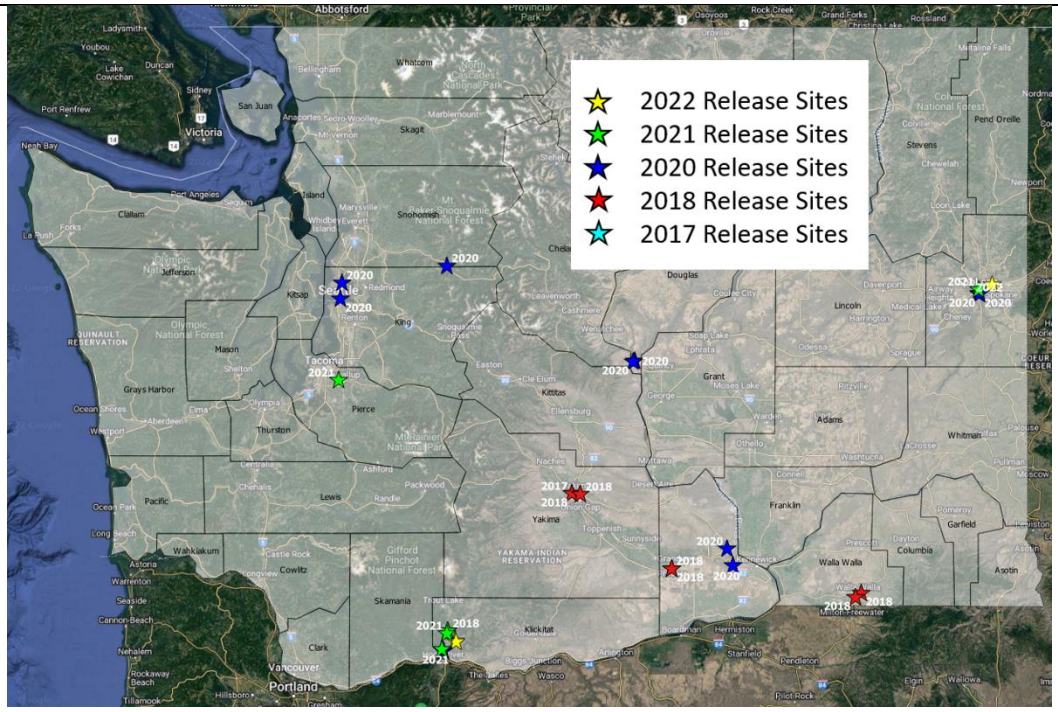

Fig. 3. Release sites of the samurai wasp in Washington, 2017-2022.

From all the years of sampling for recovery of released *T. japonicus*, only a single specimen was recovered from a yellow sticky trap (Yakima, 2022). It is difficult to determine at this point in time if the populations failed to establish, or if they are merely below the threshold of detection. We resampled two of the original sites in Vancouver where *T. japonicus* was first found in 2015-2016. Populations were still present and flourishing; this indicates that at least some of Washington's microclimates are suitable for this parasitoid species. It also validates the use of sticky traps for monitoring recovery of released populations.

2b. Determine permeability of net enclosures to *T. japonicus*. A study was done in 2019 at the Sunrise Research Orchard using released *T. japonicus* from our colony. We used the same 3-tree shade net cages that had been used for BMSB exclusion studies in the past. Sentinel BMSB egg masses (from our colony) were placed in the canopy of an apple tree either inside or outside the net cages. Twenty *T. japonicus* females were released a few feet from the egg mass, with six replicates/treatment. Despite the close proximity of egg masses to the released females, none of the BMSB eggs were parasitized, thus no conclusion can be drawn about net permeability.

2c. Determine the effects of host plant and canopy height by *T. japonicus* (foraging behavior). This objective was explored in two experiments, one in 2019, and one in 2022. The 2019 experiment was performed as described in objective 2b but varying the host plant (apple vs pepper) or the height (1 and 3 meters [3.3 and 9.8 ft] from the ground) in the canopy of an apple tree. Of the 861 sentinel eggs deployed, only 6 were parasitized, all from a single egg mass on pepper, thus no conclusions could be drawn about foraging preferences of *T. japonicus* in relation to egg mass height or host plant (apple vs pepper).

The 2022 experiment was performed at 7 sites in the Vancouver, WA area, which were known or suspected to have an established adventive population of *T. japonicus*. While *T. japonicus* is thought to be arboreal, it can also attack egg masses in vegetable crops (e.g., peppers). Pairs of egg masses were deployed from 3 August to 13 September in adjacent plant canopies either high (6 ft) or low (2 ft) to simulate a tree crop or a vegetable crop. Of the 20 replicate pairs of egg masses deployed, only 4 masses were parasitized (from only 3 sites). Two were from the high treatment, and two from the low treatment. While the attack rate was too low to draw definitive conclusions, *T. japonicus* appears equally capable of finding and parasitizing egg masses regardless of height.

2d. Determine the non-target effects of the samurai wasp on native stink bugs.
Methods: In the summer of 2019 we deployed sentinel stink bug egg masses of three native stink bugs (*Euschistus conspersus Chinavia hilaris*, and *Podisus maculiventris*) and compared them to BMSB to determine attack rate of *T. japonicus*. After allowing completion of egg hatch or development of parasitoids, we characterized the eggs individually using a combination of morphological and PCR methods. The morphological methods used a classification scheme based on appearance where a normally hatched egg and one producing an adult parasitoid were assessed; unhatched eggs were classed based on appearance and subjected to PCR with the new *T. japonicus* primer developed by my lab (Dr. Kacie Athey). The combination of these methods allowed us to evaluate both reproductive (emerged adult parasitoid) and non-reproductive (egg is killed by the parasitoid, but no adult parasitoid is produced) impacts. The latter can be a hidden, but potentially very important non-target effect of a parasitoid, and is an emerging criterium in evaluating natural enemies for classical biological control programs.

Results. *Euschistus conspersus* was not successfully attacked by *T. japonicus* during the course of this study (no adult parasitoids produced); however, it suffered a fairly high rate of non-reproductive effects (22.7%). *Chinavia hilaris*, another pest species, was attacked the least often (7.1% of eggs), with most of the effects being non-reproductive (5.4%). BMSB eggs (*H. halys*) suffered much higher total levels of impact (31%), with most of that successfully producing an adult parasitoid (25.5%). However, the highest level of attack was experienced by the native predator, *Podisus maculiventris* (67.2%), with higher levels of non-reproductive impacts (43.2%) versus reproductive (24.0%) (Fig. 5).

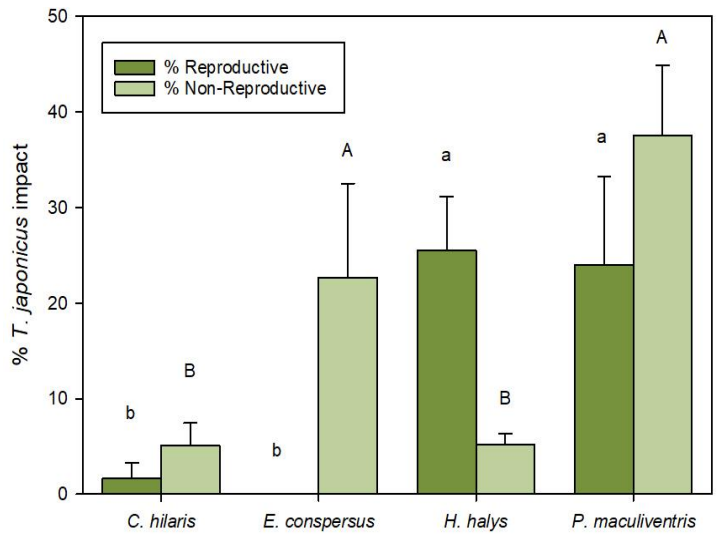

Fig. 5. Total parasitoid impact of *T. japonicus* on BMSB (*H. halys*) and 3 native stink bugs.

Objective 3: Determine development of BMSB on shrub-steppe plants

Methods: In 2019, we compared BMSB raised from the egg to the adult stage on either a typical colony diet (carrots, sunflower seeds, peanuts, bean plants) or plants native to Eastern Washington's sagebrush steppe habitat. Understanding the dietary limitations for development in different regions of the country should help us predict the relative risk of population buildup. The 2019 results indicated clearly that nymphs were slower to develop to the adult stage when fed on native plants, adult weights were lower, and that survivorship was significantly reduced. However, BMSB is unlikely to encounter a typical 'colony' diet in the wild (they do not have access to carrots and peanuts), so we followed up in 2020 with a similar study using plants typical of the mid-Atlantic region compared to our native plants. The mid-Atlantic (or 'Eastern' plants) were cuttings taken from residential areas of Wenatchee, while the native or 'Western' plants were cuttings taken from unmanaged habitats (No. 2 Canyon, Horse Lake Preserve). The assemblage of Eastern plants was fairly consistent throughout the study (maple, tree of heaven, catalpa), while the Western plant assemblage changed as the various species bore fruit. The Western diet was more varied, and included serviceberry, chokecherry (Plate 4), bitterbrush, currant, Oregon grape, elderberry, snowberry, and wild rose at various points during the season. In all cases, both foliage and fruit structures were included in the cuttings; the latter is believed to be essential for the development of BMSB. We followed the nymphal development from 1st instars through adults, noting developmental time, adult weight, and survivorship. Adult weight is believed to associated with reproductive success of the adult; to test this, we took 10 male/female pairs from each of the two diet regimes (continuing with the same diet as the nymphs experienced) and allowed them to mate and lay eggs until the death of the female. This gave us the important measures of fecundity (eggs/female) and longevity.

Plate 4. BMSB nymph on chokecherry.

Results. The survivorship of the Western nymphs to the adult stage was about half the survivorship of those fed the Eastern diet (Fig. 6). The Eastern nymphs reached the adult stage in 42 to 43 days, while the Western nymphs required 49 to 50 days, and their adult weight was 13-15% lower (Fig. 7). The same trends continued for the adults reared on these two diets; the Eastern females laid over twice as many eggs as the Western females (Fig. 8) and lived 59 vs 37 days (Fig. 9). The longevity of the males was 36.1 vs 35.7 days and was not affected by diet.

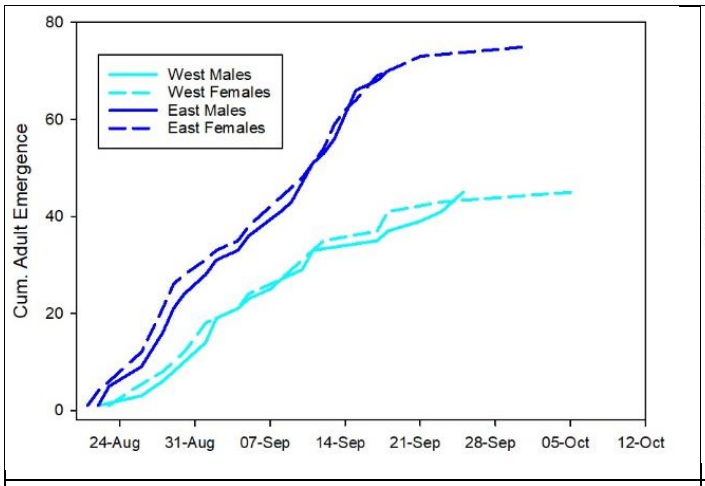

Fig. 6. Adult emergence and survivorship of nymphs fed on host plants from the eastern US vs the arid western US.

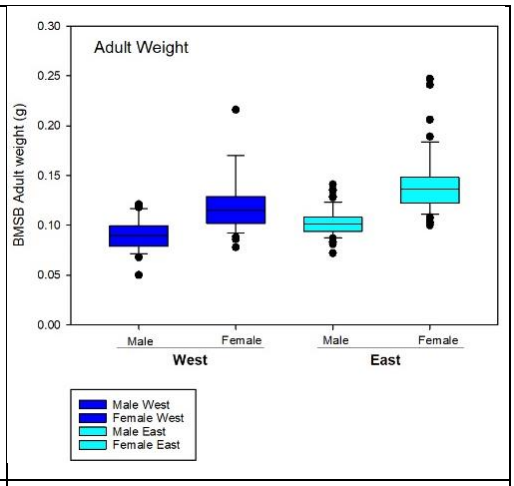

Fig. 7. Weight of surviving adults fed Eastern and Western plant diets (2020).

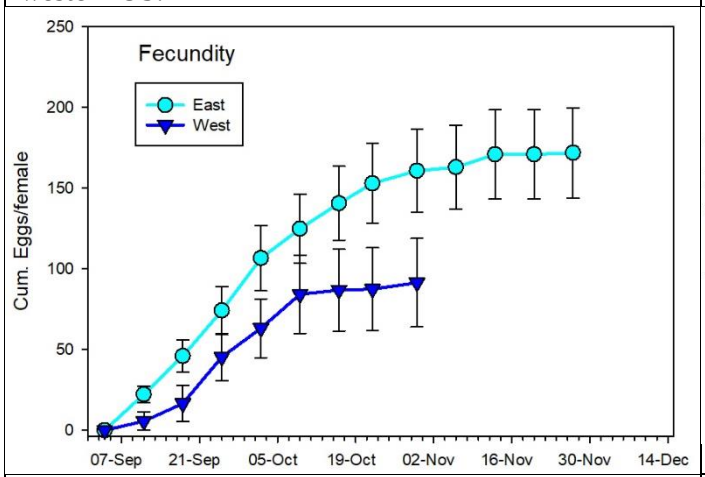

Fig. 8. Fecundity (cumulative eggs/female) from BMSB adults fed on either Eastern or Western plant diet.

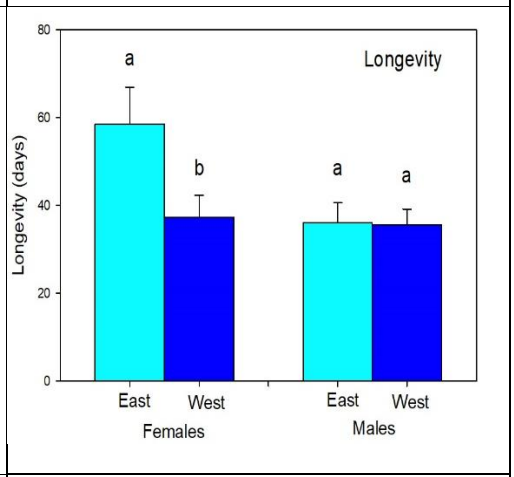

Fig. 9. Adult longevity of BMSB fed on either Eastern or Western plant diet.

Objective 4. Track the invasion of BMSB in Washington State

Methods: We used a combination of targeted sampling with pheromone traps in eastern Washington fruit production areas and logging reports from citizen scientists from around the state to track the spread and relative abundance of BMSB across the state. As of December 2022, the database has 1617 records beginning in 2010 when BMSB was first recorded in Vancouver by K. Sheehan, and in Yakima by Pete Landolt and Dave Horton in 2012. The majority of the records in the database were identified by Peter Smytheman from submitted photographs, and the person submitting the record was asked for a street address; this was used as the georeference point for the maps.

Results: BMSB has been found in 30 (out of 39) counties in Washington state; Island County had its first BMSB report in 2022 (Figure 10).

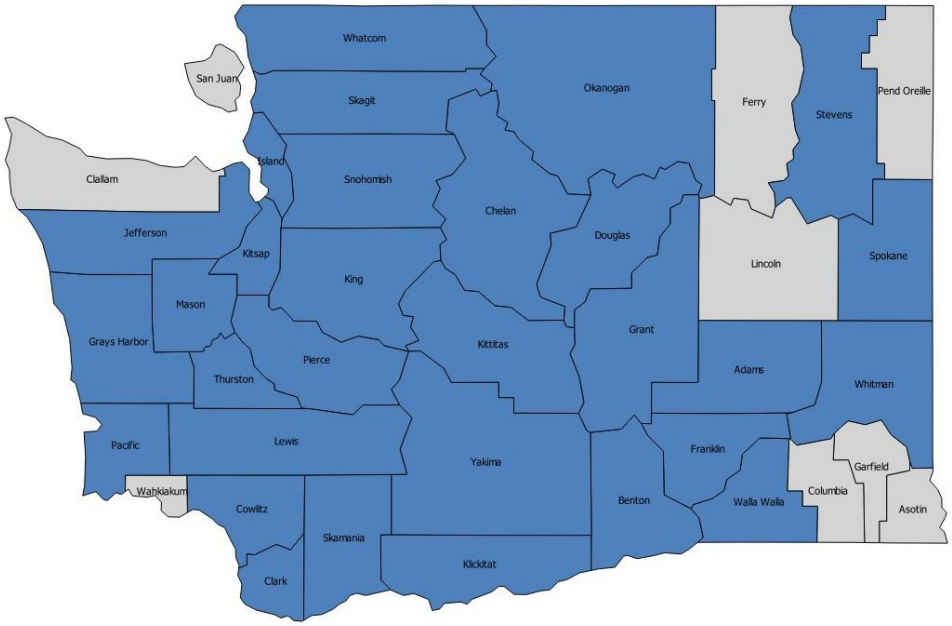

Fig. 10. Counties in blue have recorded one or more BMSB during the reporting period of 2010-2022.

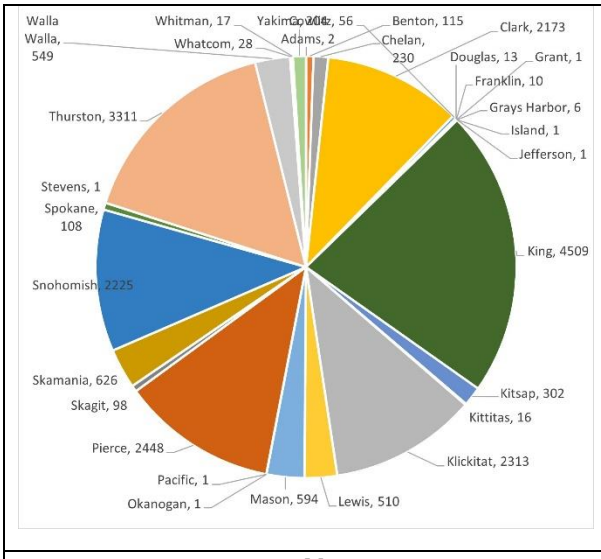

Fig. 11. BMSB reported by county.

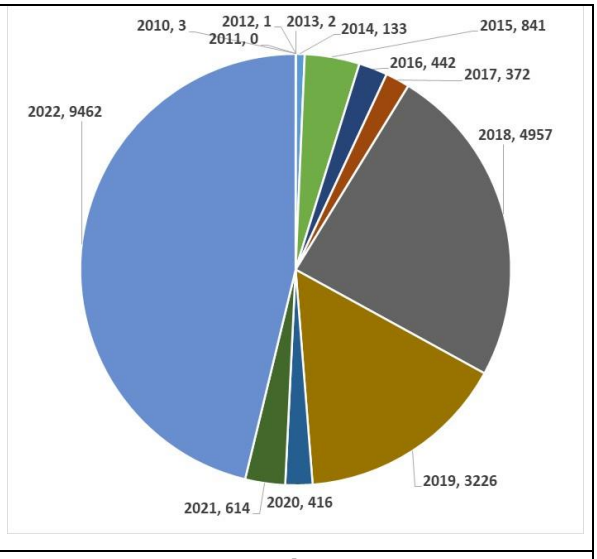

Fig. 12. BMSB reports by year.

Unsurpisingly, the urban counties on the west side of the state had the highest numbers of bugs reported, led by King, Pierce, Thurston, and Snohomish counties. Along the Columbia River, Clark and Klickitat also had numerous BMSB reported (Fig. 11). The number of reports fluctuated greatly from year to year (Fig. 12), with 2018, 2019, and 2022 having the most bugs reported. In general, the

number reported may be a less stable metric than the number of reports, but these two metrics were roughly correlated.

The overwhelming majority (63%) of BMSB reports came from either the interior or exterior of houses (Fig. 13, 14). The next most popular categories were backyards and urban host plants. Only 11% of the reports were from agricultural crops; these numbers are somewhat inflated by the targeted sampling done by my program.

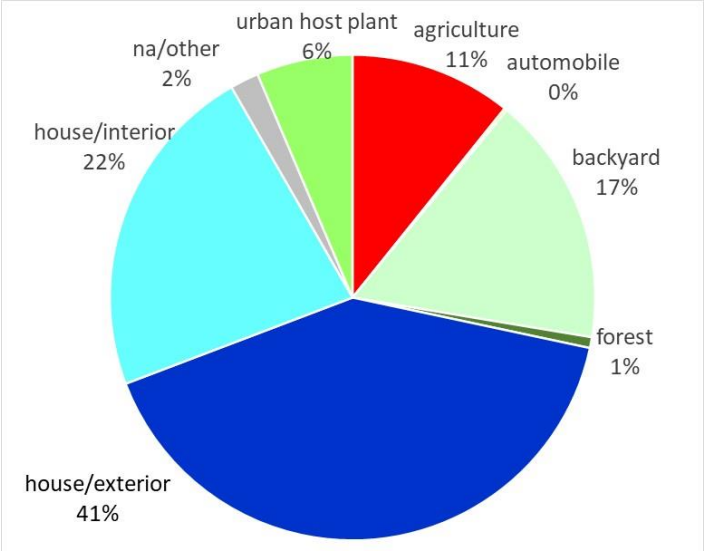

Fig. 13. BMSB reports by location of sighting.

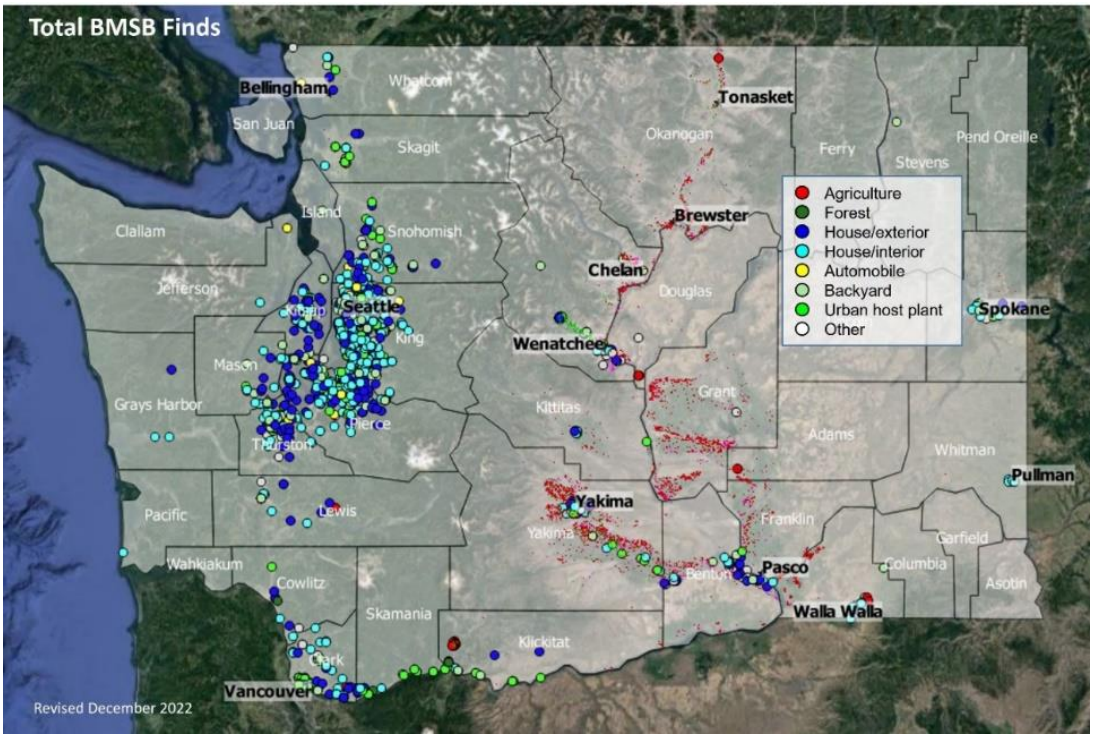

Fig 14. BMSB detection by GPS location. Red, green and magenta pixels represent apple, pear, and sweet cherry acreage, respectively.

The highest concentration of both reports and insects found are from I-5 corridor centered around major urban areas (Seattle, Vancouver). Records east of the Cascades are sparser and more scattered; while it is clear BMSB has invaded the state's interior, populations appear to be localized and relatively small. Most of the finds are in non-native landscaping, where more appropriate host plants are the most plentiful (Fig. 15).

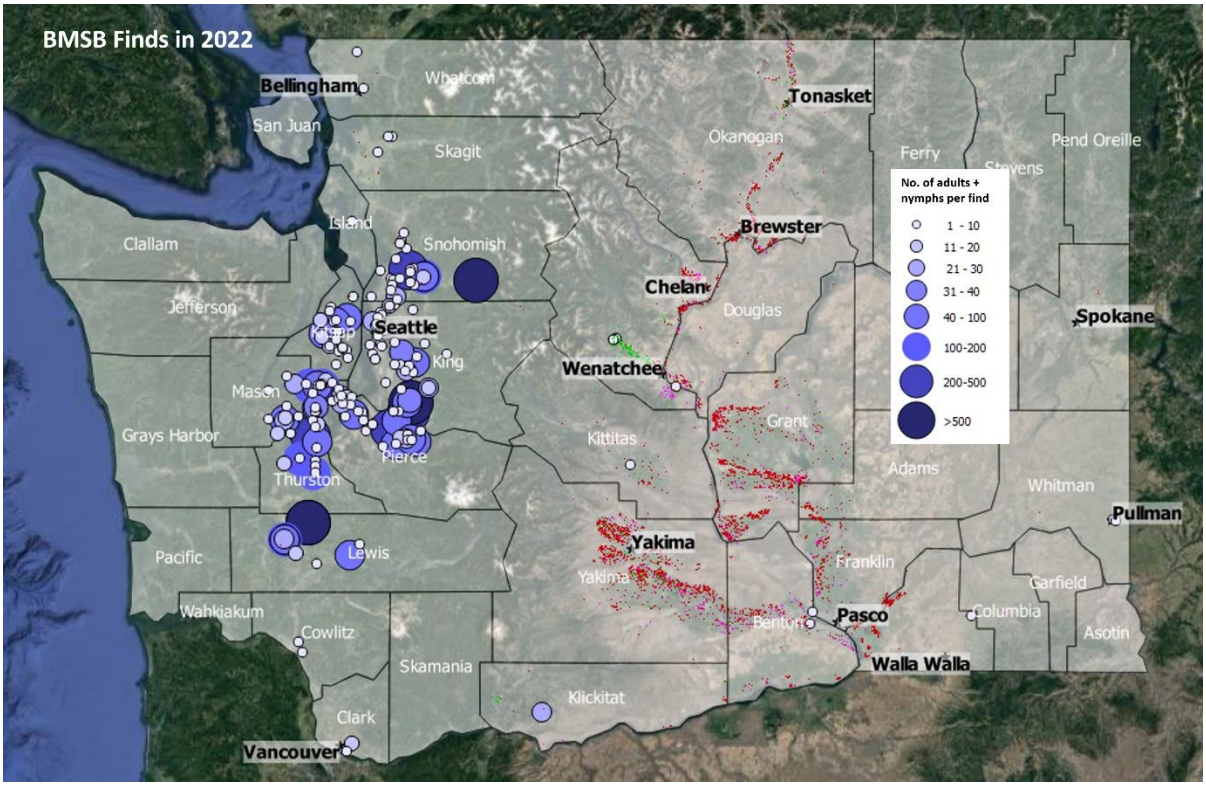

Fig. 15. BMSB reports in 2022; 218 individual reports, totaling 9,500 bugs.

Based on discussions with Justin Bush of the Washington Invasive Species Council (WISC), the BMSB reporting function will be transferred to this organization starting January 1, 2023. This organization has an ongoing mission to track a wide range of invasive species, with greater mapping capability (viz., EDDMapS). Because the WTFRC and SCRI projects are nearing completion, the WISC can provide a long-term archive for this information.

Executive Summary

Project Title: Integrated control of brown marmorated stink bug

Keywords: Invasive species, physical control, biological control, behavioral control, IPM

Abstract: This project, along with the preceding WTFRC grant (CP-16-101), the leveraged projects funded by the USDA-SCRI program and the WSCPR, have established the baselines for a new invasive pest in Washington. We have tracked its spread throughout the state, studied its phenology and nutritional ecology, developed more efficient monitoring tools, explored behavioral and physical controls, and promoted classical biological control where native natural enemies were inadequate. Importantly, we have benefitted by, and contributed to, a national and international research and outreach effort by our colleagues.

Brown marmorated stink bug on apple

The reporting database has established that BMSB is by far more numerous in urban areas west of the Cascades. This may provide a significant advantage for Washington's large agricultural industries which lie primarily east of the Cascades. The most likely explanation is that the semi-arid climate of the interior and its associated flora are inhospitable to this species, and may limit its population growth if not its spread. What remains to be seen is whether the current division we see is permanent, or merely reflecting a delay in invasion and population increase. We can also only speculate whether build-up in unmanaged habitats is a key element in the movement to crop fields and orchards, or if BMSB can achieve pest status without this reservoir.

Moreover, we have clearly identified a possible mechanism why this east-west divide occurs: the poor nutrition provided by our shrub-steppe native plants. Feeding exclusively on native plants imposes a severe developmental and reproductive penalty on BMSB nymphs and adults. When these effects are combined with periods of extreme high temperatures and/or low humidity, mortality at various stages plus poor reproductive success predict greatly slowed population increase in comparison to more favorable climates/regions.

We have successfully used native stink bugs as a model to study control of BMSB, with the added benefit that we now have new potential tools for native stink bugs. Studying control methods before a pest reaches critical levels is difficult, but highly advantageous. With chemical controls already thoroughly explored by eastern colleagues, we focused on behavioral controls using net barriers, insecticide infused netting, and pheromones. The initial results are promising, and may reduce the need for broad-spectrum insecticide applications in areas where BMSB reaches damaging levels.

Lastly, we have made important contributions to the non-target effects of *T. japonicus* (the samurai wasp) because of the well-established adventive population in southwestern Washington. This includes employing new PCR methodology to fully evaluate such effects, and underscore the difference between physiological (lab-derived) host range and ecological host range (that which occurs in nature). This advances the science of introduction of classical biological control (CBC) agents, which may be valuable when future invasive species become candidates for this approach. We have also made significant efforts at re-distributing *T. japonicus*, although to date, without success. Recording our efforts will help future scientists evaluate CBC programs for BMSB, with special emphasis on climate matching of the natural enemy to the invaded region. The same factors that currently limit population growth of BMSB may also limit establishment of *T. japonicus*, either through climatic incompatibility, or simple low host densities.

WTFRC INTERNAL PROJECT – BUDGET SHARED FOR INFORMATIONAL PURPOSES ONLY

CROP YEAR: 2022

CONTINUING REPORT **PROJECT LENGTH (CROP YEARS)**: 2021-2023

Project Title: Pesticide Residues on WA Apples

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Cooperators: Gerardo Garcia, Sandy Stone, Pacific Agricultural Labs, Northwest Hort Council, Cameron Burt

Item	2021 (est.)	2022 (est.)	2023 (est.)
Salaries			
Benefits			
Wages[1]	1450	1500	1550
Benefits[1]	750	775	800
RCA Room Rental			
Shipping			
Supplies/Chemicals	300	300	300
Travel[2]	1000	1100	1200
Plot Fees			
Analytical lab fees	2000	2100	2200
Total gross costs	5,500	5,775	6,050
Anticipated Income (contracts and gift grants)	0	0	0
Total net costs	5,500	5,775	6,050

Footnotes: Schmidt estimates 10% of his time is dedicated to this project on an annual basis
Most pesticides tested are donated by their registrants or an ag chemical supply company
1 Wages & benefits primarily for Garcia (spray applications), crew help for Garcia, and Stone (data entry & review)
2 Travel costs include hauling equipment to & from plots & delivery of samples to Sherwood, OR

2022 WTFRC APPLE PESTICIDE RESIDUE STUDY

Since 2011, the Washington Tree Fruit Research Commission (WTFRC) has conducted annual trials to evaluate pesticide residues on 'Gala' apples. This year, we applied fifteen insecticide/acaricides and six fungicides and one plant growth regulator according to either an "aggressive" protocol intended to generate the highest possible residues while observing label guidelines (maximum label rates at minimum retreatment and pre-harvest intervals) or a "standard" protocol following more typical industry use patterns for rates and timings. Fruit samples were collected at commercial maturity on September 8 and delivered the next day to Pacific Agricultural Labs (Sherwood, OR) for chemical residue analysis.

TRIAL DETAILS

- 15th leaf 'Pacific' Gala / M.9 Nic.29 trained to central leader/spindle on 3' x 10' spacing
- 2 x 25 gal Rears Pak-Blast sprayer calibrated to 100 gal / acre
- All pesticides applied with 8 oz Regulaid / 100 gal water / acre
- A total of 2.35 inches of rain fell on the trial block after the application of Ethephon, but there was no measurable precipitation after the application of all other materials

Measured residues vs. maximum residue levels (MRLs) for STANDARD industry apple pesticide programs in 100 water/acre utilizing typical rates, timings, and retreatment intervals. 'Gala'/M.9 Nic.29, Rock Island, WA. WTFRC 2022.

Chemical name	Trade name	Application rate	Application timing(s)	Measured residue	US MRL[1]	India MRL[1]	Lowest export MRL[1]
		oz per acre	*dbh*	*ppm*	*ppm*	*ppm*	*ppm*
ethephon	Ethephon 2SL	32	126 (May 5)	<0.1	5	0.01*	0.8 (UAE,SAU)
flutianil	Gatten	8	35	<0.01	0.15	0.01*	0.01 (UAE,SAU)
isofetamid	Kenja 400SC	12.5	35	<0.01	0.6	0.01*	0.2 (Kor)
abamectin	AgriMek SC	4.25	35	<0.01	0.02	0.01*	0.01 (UAE,SAU)
benzovindiflupyr	Aprovia	7	35	0.021	0.2	0.01*	0.2 (many)
pydiflumetofen	Miravis	3.4	35	**0.030**	0.2	0.01*	0.01 (UAE,SAU)
tolfenpyrad	Bexar	27	35 & 21	**0.36**	1	0.01*	0.01 (many)
indoxacarb	Avaunt	6	35 & 21	0.090	1	0.01*	0.5 (many)
flupyradifurone	Sivanto prime	14	35 & 21	0.22	0.7	0.01*	0.5 (Tai)
cyflufenamid	Torino	6.8	28	0.014	0.06	0.01*	0.06 (many)
acequinocyl	Kanemite	31	28	<0.025	0.4	0.01*	0.01 (China)
lambda-cyhalothrin	Warrior II	2.56	28	0.024	0.3	0.01*	0.2 (many)
flonicamid	Beleaf 50SG	2.8	28	0.045	0.2	0.01*	0.2 (many)
cyflumetofen	Nealta	13.7	28 & 14	0.11	0.3	0.01*	0.3 (Can,Mex)
sulfoxaflor	Transform	2.75	28 & 14	0.053	0.5	0.01*	0.3 (many)
chlorantraniliprole	Altacor eVo	2.2	28 & 14	0.17	1.2	0.01*	0.4 (many)
afidopyropen	Versys	3.5	28 & 14	<0.05	0.02	0.01*	0.02 (Can,Mex)
buprofezin	Centaur WDG	34.5	21	0.41	3	0.01*	1 (Tai)
phosmet**	Imidan 70-W**	92	14	1.8	10	0.01*	2 (Tai)
mefentrifluconazole	Cevya	5	14	0.078	1.5	0.01*	0.4 (UAE,SAU)
cyclaniliprole	Verdepryn	11	14	0.052	0.3	0.01*	0.2 (UAE,SAU)
cyfluthrin	Baythroid XL	2.8	14	<0.05	0.5	0.01*	0.1 (many)

[1] Top markets for WA apples with established MRLs; 17 Oct 2022. https://nwhort.org/export-manual/, https://bcglobal.bryantchristie.com/

*No tolerance posted; MRL is based on national default value (0.01 ppm in India)

**Imidan 70-W was mixed with a buffering agent to reduce tank pH to 5.5 per standard industry practice

Results of this lone unreplicated trial are shared for informational purposes only and should not be construed as endorsements of any product, reflections of their efficacy against any insect, acarid, or fungal pest, or a guarantee of similar results regarding residues for any user. Apple growers should consult their extension team members, crop advisors, and warehouses to develop responsible pest control programs.

Measured residues vs. maximum residue levels (MRLs) for AGGRESSIVE apple pesticide programs in 100 gal water/acre utilizing maximum labeled rates, and minimum preharvest intervals. 'Gala'/M.9 Nic.29, Rock Island, WA. WTFRC 2022.

Chemical name	Trade name	Application rate	Application timing(s)	Measured residue	US MRL[1]	India MRL[1]	Lowest export MRL[1]
		oz per acre	*dbh*	*ppm*	*ppm*	*ppm*	*ppm*
ethephon	Ethephon 2SL	48	86 (June 15)	<0.1	5	0.01*	0.8 (UAE,SAU))
benzovindiflupyr	Aprovia	7	35	0.018	0.2	0.01*	0.2 (many)
pydiflumetofen	Miravis	3.4	35	**0.034**	0.2	0.01*	0.01 (UAE,SAU)
isofetamid	Kenja 400SC	12.5	35 & 21	<0.01	0.6	0.01*	0.2 (Kor)
acequinocyl	Kanemite	31	35 & 21	<0.025	0.4	0.01*	0.01 (China)
abamectin	AgriMek SC	4.25	28	<0.01	0.02	0.01*	0.01 (UAE,SAU)
lambda-cyhalothrin	Warrior II	2.56	28 & 21	0.043	0.3	0.01*	0.2 (many)
flonicamid	Beleaf 50SG	2.8	28 & 21	0.062	0.2	0.01*	0.2 (many)
tolfenpyrad	Bexar	27	28 & 14	**0.25**	1	0.01*	0.01 (many)
flupyradifurone	Sivanto prime	14	28 & 14	0.13	0.7	0.01*	0.5 (Tai)
indoxacarb	Avaunt	6	21 & 14	0.086	1	0.01*	0.5 (many)
flutianil	Gatten	8	21 & 14	**0.012**	0.15	0.01*	0.01 (UAE,SAU)
chlorantranliprole	Altacor eVo	2.2	21 & 7	0.27	1.2	0.01*	0.4 (many)
cyclaniliprole	Verdepryn	11	21 & 7	0.100	0.3	0.01*	0.2 (UAE,SAU)
cyflumetofen	Nealta	13.7	21 & 7	0.12	0.3	0.01*	0.3 (Can,Mex)
phosmet**	Imidan 70-W**	92	21 & 7	**3.6**	10	0.01*	2 (Tai)
cyflufenamid	Torino	6.8	14	0.018	0.06	0.01*	0.06 (many)
buprofezin	Centaur WDG	34.5	14	0.46	3	0.01*	1 (Tai)
afidopyropen	Versys	3.5	14 & 7	<0.05	0.02	0.01*	0.02 (Can,Mex)
sulfoxaflor	Transform	2.75	14 & 7	0.13	0.5	0.01*	0.3 (many)
cyfluthrin	Baythroid XL	2.8	7	<0.05	0.5	0.01*	0.1 (many)
mefentrifluconazole	Cevya	5	7 & 1	0.19	1.5	0.01*	0.4 (UAE,SAU)

[1] Top markets for WA apples with established MRLs; 17 Oct 2022. https://nwhort.org/export-manual/, https://bcglobal.bryantchristie.com/
*No tolerance posted; MRL is based on national default value (0.01 ppm in India)
**Imidan 70-W was mixed with a buffering agent to reduce tank pH to 5.5 per standard industry practice

CONCLUSIONS

As we have observed in every study since 2011, no spray program produced a residue that exceeded the tolerance level set by the US Environmental Protection Agency; these findings are further evidence that apple growers following directions on product labels should expect their fruit to be in full compliance for domestic sales regarding pesticide residues. Four products we tested, however, did produce **residues which exceed Maximum Residue Levels** (MRLs) set in important export markets for Washington apples: **Miravis, Bexar, Gatten, and Imidan 70-W**. India has yet to post tolerances for most pesticides used by WA apple growers; in the absence of a posted MRL, the default tolerance in India is 0.01 ppm, essentially meaning that any product which produced a detectable residue in our study would potentially violate India's standards.

Results from this year's study found no detectable residues of ethephon, whether it was applied as a chemical thinner (early May) or later in the season (mid-June) to promote return bloom. Not surprisingly, residues tended to be higher in the "aggressive" protocol than in the "standard" protocol. Overall, there were fewer potential violations for residue levels in our samples than we have found in most years, in part because some countries have relaxed some MRLs due in part to effective negotiating efforts from US trade officials and representatives of the Northwest Horticultural Council.

Reports from previous pesticide residue studies on apple and cherry which provide a broader context for these results are available on the WTFRC website at www.treefruitresearch.org. We encourage growers and consultants to stay abreast of current information on international MRLs, which often change in response to trade negotiations and/or political developments. For more information, visit the Northwest Horticultural Council website, www.nwhort.org.

WASHINGTON TREE FRUIT RESEARCH COMMISSION

For more information, contact Tory Schmidt (509) 669-3903 or email tory@treefruitresearch.com

Project Title: Directing plant-microbe relations toward resiliency post-fumigation

Report Type: Final Project Report

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Cooperators: Dr. Mark Mazzola and Dr. David Granatstein

Project Duration: 2-Year
Total Project Request for Year 1 Funding: $ 60, 180
Total Project Request for Year 2 Funding: $ 39, 781

Other funding sources: None
WTFRC Collaborative Costs: None

Budget 1
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Item	2021-2022	2022-2023
Salaries*	NA	NA
Benefits	NA	NA
Wages*	18,359	18,359
Benefits	6,922	6,922
Sequencing Costs	12,800	4000
Lab Supplies	19,125	10,500
Soil Analysis Tests	2,974	NA
Travel	NA	NA
Miscellaneous	NA	NA
Plot Fees	NA	NA
Total	60,180	39,781

*Biological technician with benefits (0.5 FTE as needed, to be hired at beginning of grant)

Brief Overview of Project Goals:

As a replant disease control strategy, pre-plant soil fumigation is the industry standard. Although fumigation significantly reduces pathogen activity and improves tree growth, this benefit is limited to approximately 1 year. Post-fumigation, orchard soil rapidly re-establishes a microbial community indistinguishable from that found in the corresponding non-fumigated replant soil and the proliferation of soilborne pathogens infecting apple, including *Pratylenchus* and *Pythium* spp., is commonly observed (i.e., a chronic disease state). The application of soil amendments following fumigation may be an opportune time to improve the ability of the soil to defend against pathogen reinvasion and improve orchard productivity for an extended period.

The primary objective of this study was to evaluate select soil amendments (listed in Table 1) for the ability to recruit and maintain rhizosphere microbiomes in fumigated soil, that are suppressive to pathogen re-invasion. In addition, this study evaluated the capacity of selected soil amendments to improve other characteristics of soil productivity (nutrient availability, water holding capacity, etc.).

Table 1. Application rates of organic amendments used

Post-fumigation Soil Amendment	Application Rate	Notes
Fumigated alone (block 14b)	NA	Control to "steer" away from
Unfumigated alone (block 12b)	NA	Control to "steer" away from
Unfumigated + 2t Bj/Sa SM	2 tons per acre	Control to "steer" towards
Fum + 1t Bj/Sa SM	1 ton per acre	
Fum + 2t Bj/Sa SM	2 tons per acre	
Fum + CCM (composted chicken manure)	0.7 tons per acre	Rate estimated based on E.C. value
Fum + SMC (shitake mushroom compost)	2% v/v	
Fum + LC (liquid chitin)	2 gal per acre	Rate recommended by manufacturer
Fum + IF (insect frass) *	1.5 cups per ft^{-3} soil	Rate recommended by manufacturer
Fum + 2t *B.napus* *	2 tons per acre	

*Amendment not included in original proposal

Collection of soil: WSU Sunrise Orchard block 14b was fumigated on April 01, 2021 (pers comm Cameron Burt). The old orchard block, previously planted to apple, had been removed in 2017. Telone II (1,3-dichloropropene) was applied at a rate of 122 lbs. per acre; injected at 18 inches depth. Sectagon K-54 (metam potassium) was also applied at a rate of 318 lbs. per acre; injected into the top 6 inches. Fumigated soil was collected on April 26th, 2021 (orchard manager advised a 2-3 week waiting period). Approximately 90 gallons of fumigated soil was removed from the plot by shoveling soil from the top 12" into 5 gallon buckets, which were placed throughout the plot area at regular intervals. Buckets and shovels were disinfected with 10% bleach prior to use to minimize cross-contamination. In addition, approximately 30 gallons of unfumigated replant soil was collected from a nearby block containing apple trees (SRO block 12b). The top stubble within tree rows was removed using a shovel; soil was then *carefully* collected from the top 12", so as not to damage existing tree roots. Lids were placed on all buckets to minimize moisture loss and soil was transported back to the lab. Soil was then stored in 30 gallon bins with lids in a cool, dry place until use.

Soil Amendments: Seed meal formulations (*B.juncea* + *S.alba* and *B.napus*) were ground and passed through a 1 mm^2 sieve, prior to addition to fumigated or unfumigated SR orchard soil according to the application rates listed in Table 1. The *B.juncea* + *S.alba* SM formulation was prepared by blending *B. juncea* and *S. alba* at a ratio of 1:1. Pre-weighed packets of seed meal were added and thoroughly incorporated into the soil by hand. 2.5L of seed meal-amended soil was placed into each pot, moistened with 300 ml autoclaved water and sealed in gas impermeable bags (Bitran) to retain seed meal-generated volatile compounds (e.g. allyl isothiocyanate). The bags were removed after 1 week, and pots were

maintained in the greenhouse for an additional 6-weeks prior to planting to allow for degradation of potentially phytotoxic compounds. All other soil amendments were thoroughly mixed into the fumigated soil 2-weeks prior to planting, according to the application rates listed in Table 1. The amount of material added to each pot was calculated according to an "applied" volume of 6340 cubic feet of soil per acre, based on a high density orchard system with a layout of 12' between tree rows, 3.5 ft wide weed-free strips, and tilling 6" deep. The amendment rate for the composted chicken manure treatment was determined based on a soil EC threshold value of 1.6 mmho/cm (DuPont and Granatstein, 2020). Fruit trees are relatively salt sensitive, suffering decreased growth and yield when EC values in the root zone > 2 mmhos/cm (https://www.bctfpg.ca/horticulture/fruit-tree-nutrition).

Planting/harvest: G.11 rootstocks were used in Experiment 1/Year 1 and Experiment 2/Year 2. Prior to planting, root volume, trunk diameter (16-18 cm above soil line), and total biomass were recorded. For each treatment type (including fumigated, unfumigated, and unfumigated + seed meal controls), there were 7 replicate pots. Pots were set up in a completely randomized block design in the greenhouse and maintained under standard light and temperature regimes (Somera et al., 2021). At the end of each experiment (3 months post planting), the effect of soil treatments on rootstock growth was assessed by measuring increases in trunk diameter, total rootstock weight, root mass, and leader-shoot length. Upon harvest of Experiment 1/Year 1, a variety of chemical and physical properties were measured to assess the influence of the above soil amendments on overall soil health. The measured properties are listed in Table 2.

Significant Findings:

- High nitrate levels in composted chicken manure and liquid chitin led to high salinity as measured by electrical conductivity (EC) when incorporated into fumigated soil. Most notably, composted chicken manure, when used as a post-fumigation soil amendment (even when applied at a relatively low rate, 2 week prior to planting), resulted in the death of all trees.

- Seed meal, shitake mushroom compost (SMC), and insect frass (IF) soil amendments all altered the chemical and physical properties of fumigated replant soil in similar ways including increased water holding capacity, increased pH and increased C:N ratio.

- All amendments, with the exception of insect frass and BjSa SM (1t), were relatively successful in their ability to significantly alter the ***bacterial*** composition of the rhizosphere microbiome and "steer" the community in a positive direction post-fumigation. *B. napus* SM (2t) did not, however, counteract the adverse effects of fumigation on the bacterial rhizobiome as effectively as SMC, LC, or BjSa SM (2t).

- All amendments, with the exception of BjSa SM (1t), successfully altered ***fungal*** community composition and directed the community in a positive manner post-fumigation. In all treatments, a handful of potentially beneficial fungi with activity against specific apple replant disease (ARD) pathogens were significantly enriched relative to the fumigated control. Notably, BjSa SM (2t) increased the potential for fungal-based nematode control in the apple rhizosphere post-fumigation.

- The Fum + LC treatment resulted in ***bacterial*** communities with a high degree of degradative/bioremediation potential. However, liquid chitin was less effective than other treatments at stimulating potentially beneficial ***fungi***.

- Insect frass did *not* effectively shift the ***bacterial*** community away from fumigation-alone or replant control treatments (i.e., chronic disease states). However, with respect to changes in the ***fungal*** community, this treatment moved the community closest to that of the "target" treatment (which is *unfumigated* orchard replant soil amended with BjSa SM at a rate of 2 t per acre).

- Insect frass resulted in a significant increase in trunk diameter relative to the fumigated control.

Results and Discussion:

Experiment 1/Year 1

Table 2. Effect of the different soil amendments on the chemical and physical properties of fumigated replant soil. These metrics are for bulk soil collected 3 months post planting.

	pH	Electrical Conductivity	Cation Exchange Capacity	Na	Ca	Mg	K	Water Holding Capacity	OM	Total N	Total C	C:N	NO_3	NH_4	SO_4^{2-}	P	K	B	Zn	Mn	Cu	Fe
Experimental Treatment		*mmhos/cm*	*meq/100g #*	*percent (%) of CEC*				*in/ft*	*percent (%)*			*ratio*	*mg/kg*									
Fumigated alone control	**6**	**0.3**	**9.1**	**1.3**	**57**	**16**	**11**	**1.28**	**1.4**	**0.08**	**0.65**	**8.7**	**7.9**	**1.1**	**28**	**16**	**393**	**0.2**	**6**	**1.7**	**0.6**	**24**
Fum + 1t Bj/Sa SM	7	0.45	8	2.7	72	22	17	1.4	1.4	0.10	0.89	9.3	35	14	38	40	531	0.2	7.1	2.2	1.1	14
Fum + 2t Bj/Sa SM	6.6	0.74	7.8	4.9	78	26	23	1.95	1.5	0.11	1.12	10.2	70	14	55	40	706	0.3	7	3.7	1.4	17
Fum + CCM (composted chicken manure)	7.8	3.29	9.5	30	84	40	88	1.46	2.6	0.23	1.96	8.7	277	267	271	157	3262	3.4	25	30	15	43
Fum + SMC (shitake mushroom compost)	7.5	0.36	8.5	1.2	72	21	14	1.41	1.9	0.09	0.89	9.7	2.5	4.5	16	23	471	0.2	5.9	1.8	0.5	13
Fum + LC (liquid chitin)	5.2	1.56	9.3	2.7	80	21	13	1.57	1.9	0.12	0.97	8.3	148	8.5	168	44	474	0.2	7.8	22	1	28
Fum + IF (insect frass) *	7.1	0.05	8.7	1.2	66	21	14	1.5	1.6	0.08	0.78	9.3	16	1.5	23	40	489	0.2	6.9	1.2	0.6	13
Fum + 2t *B.napus* *	6.6	0.53	8.2	1.5	71	21	15	1.38	1.3	0.08	0.79	10.1	80	1.4	32	21	477	0.3	6.6	2.6	0.7	27

All analyses were conducted by Soiltest Farm Consultants (Moses Lake, WA).
*Additional treatments not included in original proposal
millequivalents per 100 grams of soil

Effect of amendments on chemical and physical properties of fumigated orchard soil:

Shitake mushroom compost (SMC): The results of the compost-specific analyses indicate that, although neither material is fully composted, "fresh" SMC is more stable and mature than composted chicken manure. Therefore, SMC is likely to benefit soil health by building soil organic matter.

Composted chicken manure (CCM): CCM was moderately alkaline (pH = 8.5), which is typical of manure-based composts. In general, high pH compost should be avoided on soils which are already above the optimum pH for tree fruit (optimum pH = 6-6.5) (Dupont and Granatstein, 2020). The Fum + CCM treatment resulted in an electrical conductivity (EC) value of 3.3 mmho/cm, which exceeds the damage threshold for apple/pear (1.7 mmho/cm). Nitrates (277 mg/kg) made up most of the soluble salts in the EC reading (Table 2). In this experiment, the high EC/nitrates and pH of CCM clearly had a negative impact on plant growth as none of the trees survived. Use of this material as a post-fumigation amendment is likely to negatively affect plant root growth even if the amendment rate is considerably low (as in this experiment).

Test results also point to the potential for carbon and nitrogen loss. Relatively high rates of CO_2 evolution (6.3 CO_2-C/g OM/day) during compost stability testing suggest that a portion of the organic carbon in CCM is being lost as CO_2 gas due to microbial respiration. Moreover, because CCM is moderately alkaline (pH > 7.5), a greater proportion of NH_4 may be exuded in the form of NH_3 gas (i.e., ammonia) leading to a loss of N to the atmosphere (Sullivan et al., 2018). Further, a low C:N ratio (10:1) also indicates the potential for N leaching/loss (although the product is marketed as a "slow nitrogen release plant food").

Liquid Chitin (LC): Similar to Fum + CCM, Fum + LC also had high nitrate/EC values. Although the trees were able to tolerate the extremely high nitrate concentrations (148 mg/kg) resulting from the LC amendment, this treatment would be expected to lead to production of nitrous oxide (N_2O) from microbial denitrification. Nitrous oxide is a greenhouse gas which is approximately 300 times more potent than CO_2. Unlike CCM, LC amendments resulted in a strongly acidic soil (pH = 5.2).

Fum + BjSa (2t): This treatment resulted in the greatest increase in soil C:N ratio and water holding capacity (the amount of water that a given soil can physically hold against the force of gravity), with the lowest cation exchange capacity (CEC) value. That is significant because CEC values refer to the relative ability of a soil to store exchangeable cations (many of which are essential nutrients) and buffer against rapid changes in pH. The most dominant soil cations were Calcium (Ca^{2+}) and Magnesium (Mg^{2+}) in all treatments except Fum + CCM (Table 2).

Amendment-based changes to rhizosphere microbial community composition:

The aim of Experiment 1/Year 1 was to identify materials which could be used to "steer" the apple rhizosphere in favor of a more prophylactic or disease-suppressive state, post-fumigation. **Figure 1** shows how the soil amendments (represented by different colors) altered ***bacterial*** community composition in apple rhizospheres. Assessment of bacterial community sequence data indicated that all those except Insect frass and BjSa SM (1t) were relatively successful in terms of their ability to significantly alter the bacterial composition of the rhizosphere and "steer" the community in a positive direction. **Figure 2** shows how the soil amendments (represented by different colors) altered ***fungal*** community composition in apple rhizospheres. Assessment of fungal community sequence data indicated all those except BjSa SM (1t) successfully altered rhizobiome composition relative to the fumigated control and directed the community in a positive direction post-fumigation.

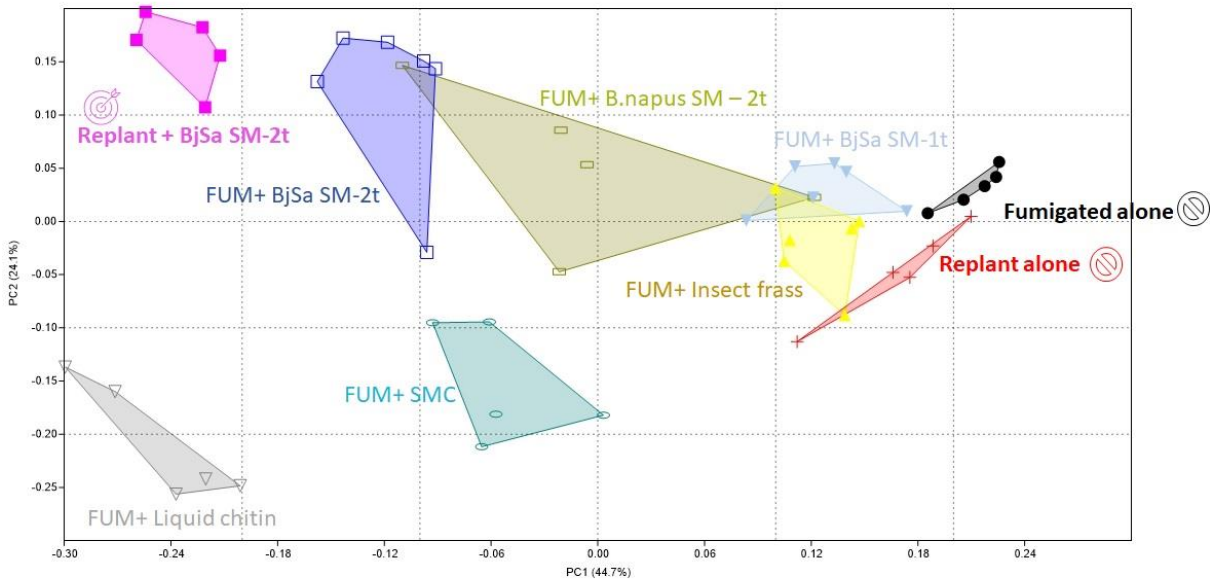


Figure 1. Principal coordinates analysis of bacterial community composition in G.11 apple rhizospheres 4-weeks post-planting at the Order level. Each point represents bacterial community

sequence data generated from an individual soil sample. Filled shapes represent the clustering of replicate samples associated with plants cultivated in the different treatments as labeled.

Bacterial communities associated with plants cultivated in **unfumigated orchard replant soil** (See: Replant alone) and those of plants cultivated in **fumigated orchard replant soil** (See: Fumigated alone) represent community configurations which are conducive to future development of replant disease (Fig. 1). In other words, Replant alone and Fumigated alone represent treatments which are susceptible to infection by root pathogens including those that can incite replant disease and limit productivity of the current orchard. By comparison, the filled shape located in the upper left region of the plot, represents rhizosphere samples from plants cultivated in unfumigated orchard replant soil amended with BjSa SM at a rate of 2t per acre. A large body of research has shown that this particular SM formulation promotes disease-suppressive rhizosphere communities (Mazzola, et al., 2009.; Mazzola, et al., 2015., Wang and Mazzola, 2019.; Somera et AL., 2021). Therefore, this treatment represents a good direction to move towards.

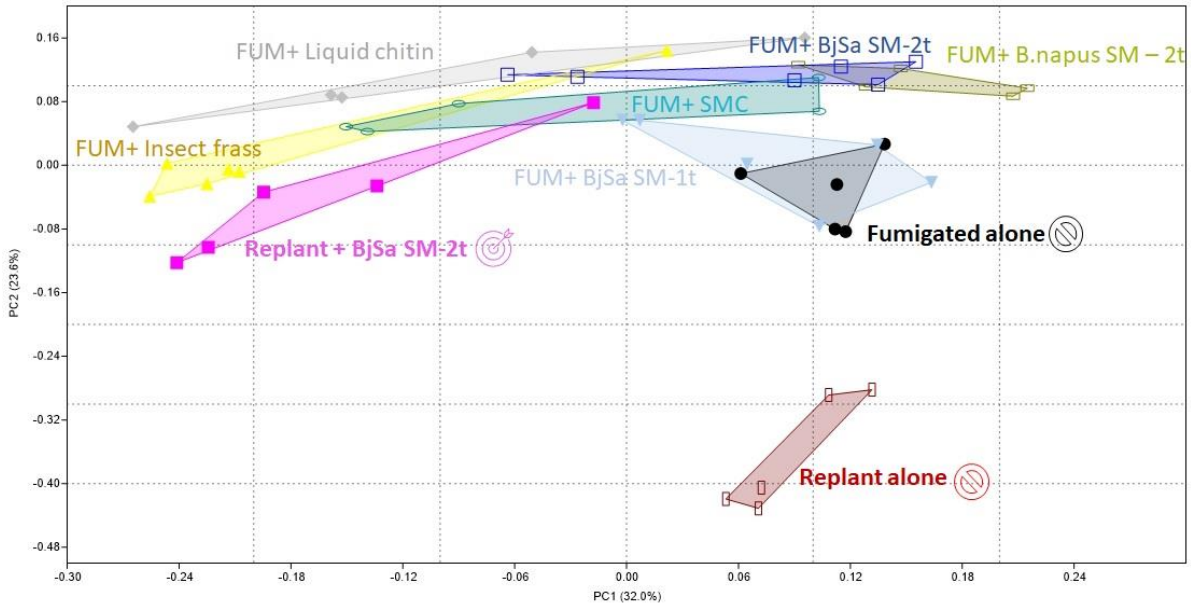


Figure 2. Principal coordinates analysis of fungal community composition in G.11 apple rhizospheres 4-weeks post-planting at the Order level. Each point represents fungal community composition generated from an individual soil sample. Filled shapes represent the clustering of replicate samples associated with plants cultivated in the different treatments as labeled.

Microbial community functional potential varied with amendment-type (Fig. 2-4): Statistical analysis was conducted to identify bacterial and fungal taxa that were significantly enriched in select treatment groups relative to the untreated fumigated control. To explore the functional potential of these systems in more depth, a literature review was conducted on bacterial and fungal species which were identified as being enriched and comparative assessments among treatments were made.

Summary discussion of microbial community steering experiment (in-depth analysis):

Fum + BjSa (2t): This treatment moved the bacterial community closest to that of the "target" treatment (which is *unfumigated* orchard replant soil amended with BjSa SM at a rate of 2 t per acre). A large body

of research has shown that this particular SM formulation promotes disease-***suppressive*** rhizosphere communities. Therefore, the "target" treatment represents one direction to move towards. The target treatment contained the highest levels of metabolic versatility in terms of biocontrol potential, particularly with regard to secondary metabolite/antibiotic production and nematode control (Fig. 3). In Fum + BjSa (2t) the potential for chitinase/fungal control was largely attributed to multiple chitinolytic bacteria belonging to the family Chitinophagaceae and *Paenibacilus polymyxa*. By comparison, in the Replant + BjSa (2t) treatment this functional characteristic was associated with the family Acidobacteriaceae, *P. polymyxa*, *Clostridium* spp. and *Rhixomonas suberifaciens*. Both fumigated and unfumigated BjSa SM amendments appeared to be enriched with a diversity of bacteria associated with heavy metal tolerance and/or uptake. This capability was attributed to Actinobacteria, Bacillales, and Myxococcales in Fum + BjSa (2t) and Acidobacteria, Bacillales, Sphingomonadales in Replant + BjSa (2t). The bacterial community from the unfumigated BjSa SM-treated soil appeared to have the most functional potential in terms of its ability to acquire/compete for iron (e.g. Actinobacteria and Bacialles). Within the fungal community, Fum + BjSa (2t) stimulated the growth of *Fusarium oxysporum* by 30% (relative to the fumigated control). This is noteworthy because *F. oxysporum* has been shown to suppress infection in apple seedlings by the ARD pathogen *Phytophthora cactorum*. The treatment also led to a 5% increase in the relative abundance of *Mortierella* spp., a group of fungi generally well-known for their ability to provide multiple beneficial functions to a variety of plants, including the production of plant growth promoting compounds. In non-fumigated (replant) soil, however, BjSa SM-structured rhizospheres favored *Humicola* sp. (20% relative abundance). Finally, BjSa (2t) was the only post-fumigation amendment with increased potential for fungal-based nematode control.

Fum + BjSa (1t): This treatment was not as successful as other treatments at "steering" the bacterial community away from the degraded states of fumigation-alone or replant control (Fig. 1). This was also the only soil amendment that did not successfully "push" the fungal community out of the post-fumigation state (Fig. 2).

Fum + Shitake mushroom compost (SMC): This treatment worked well at pushing the bacterial community out of the post-fumigation state, but in a different direction than the BjSa SM treatments. The SMC treatment appeared to have a proliferative effect on chitinolytic bacteria, namely those in the family Chitinophagaceae. This result was not unexpected as SMC, which is largely dominated by shiitake mycelium, contains a high level of chitin. Interestingly, this treatment also appeared to have the greatest potential for oomycete control due to the presence of *B. flexus*, *B.subtilus*, and *Rhizobium* spp. This soil amendment tended to support the growth of bacteria with the ability to fix nitrogen from the atmosphere, namely the Hyphomicrobiales (Rhizobiaceae) (Fig. 5). Although the association between this material and the Rhizobiaceae is not entirely clear, the SMC soil amendment was the only treatment which resulted in reduced levels of plant available NO_3 (relative to the fumigated control) in bulk soil (Table 2). Like BjSa (2t), SMC stimulated the growth of *F. oxysporum* by ~ 10% (relative to the fumigated control). Fum + SMC was also one of the only treatments in which *Hypocrea* (i.e., *Trichoderma*) was enriched. This genus contains multiple members known to be antagonistic towards both ARD fungal and oomycete pathogens. However, this treatment also led to an increase in the relative percentage of the aflatoxigenic fungi *Aspergillus parasiticus* in the rhizosphere (0.02% in fumigated soil vs 1 % in FUM + SMC).

FUM + Liquid Chitin (LC): Similar to SMC, this treatment also worked well at pushing the bacterial community out of the post-fumigation state, but in a different direction than the BjSa SM treatments. Like SMC, LC induced proliferation of chitinolytic bacteria, namely those in the Acidobacteriaceae. Members of this group prefer acidic conditions (3.0–6.5 pH), and the low soil pH of this treatment is likely to have contributed to their enrichment. In terms of the ability of the ***bacteria*** living in the rhizosphere to utilize unique, complex compounds for growth (esp. environmental pollutants), this treatment contained the most metabolic versatility (Fig. 5; Degradative). The Fum + LC treatment also promoted the growth of a variety of ***fungi*** with biocontrol potential (including *Hypocrea/Trichoderma* spp.). The majority of fungi

in this treatment (53%), however, were most similar to *Sordaria tomento-alba*, a species of fungi that is not well described in the literature (although not a known pathogen of apple). Similar to SMC, liquid chitin also led to an increase in the relative percentage of *A. parasiticus* in the rhizosphere (0.02% in fumigated soil vs 2.2% in FUM + LC).

Fum + *B.napus* (2t): Compared to all other treatments, the potential for bacterial-based biocontrol (Fig. 3), bacteria known for their ability to cope with excess heavy metals and/or chemical contaminants (Fig. 4), and bacteria associated with nutrient cycling (Fig. 5) was relatively low. Taken together, these results suggest that *B. napus* SM is not as useful at counteracting the adverse effects of fumigation as other amendment options. With regard to fungal community outcomes, however, this treatment led to a 9% increase in the relative abundance of *Mortierella*, a group associated with healthy orchard soils.

Fum + Insect Frass (IF): Comparatively speaking, this treatment did not effectively shift the ***bacterial*** community away from the fumigation alone or replant control states (Fig. 1). However, in terms of changes to the ***fungi***, this treatment moved the community closest to that of the "target" treatment (Fig. 2). Interestingly, this was the only treatment which did not significantly reduce the relative abundance of the soilborne pathogen *Ilyonectria robusta* in the apple rhizosphere. *Chaetomium* sp. became the dominant fungus in this treatment, increasing from 11% (in the fumigated alone control) to 63% in Fum + IF. This genus includes several metabolically gifted members (e.g. *C. globosum*, *C. nigricolor*) possessing the potential to control multiple ARD pathogens including *P.ultimum* and *R.solani*.

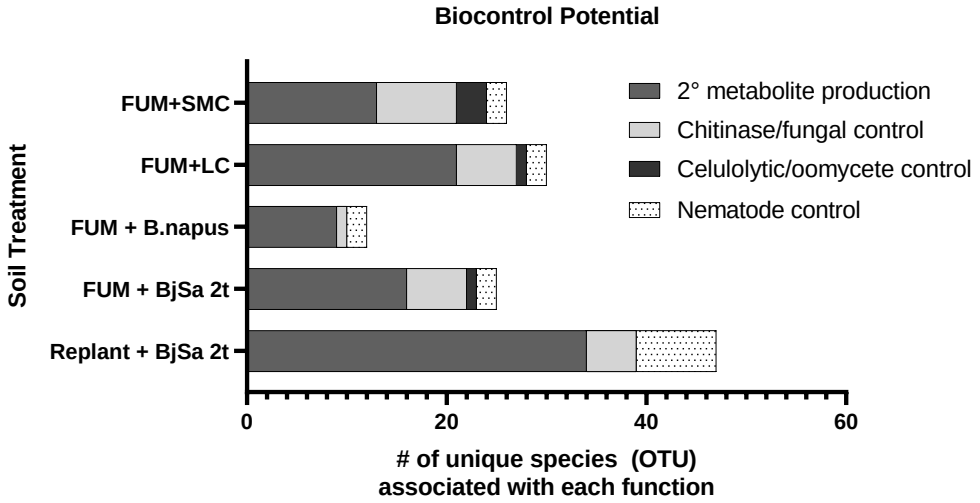


Figure 3. Comparison of the number of unique bacterial species associated with increased biocontrol potential in each treatment (relative to the fumigated control).

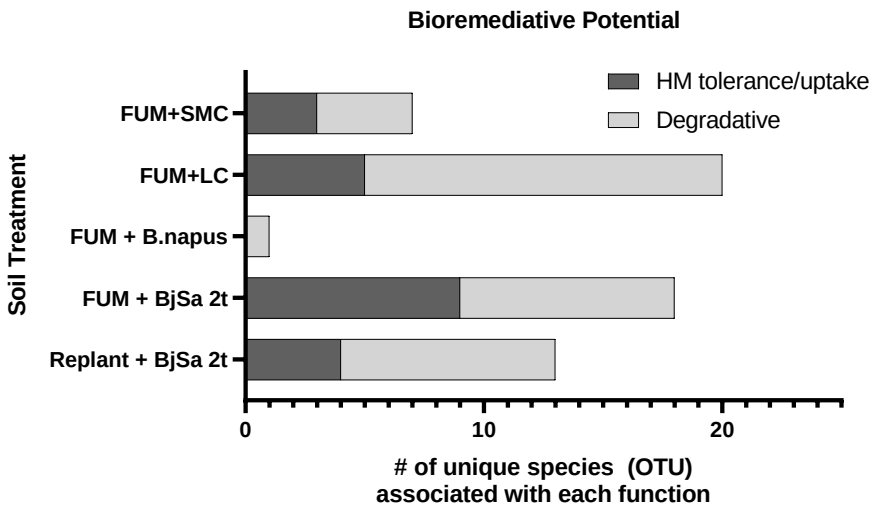


Figure 4. Comparison of the number of unique bacterial species associated with increased heavy metal tolerance/uptake and/or the ability to utilize complex carbon sources in each treatment (relative to the fumigated control).

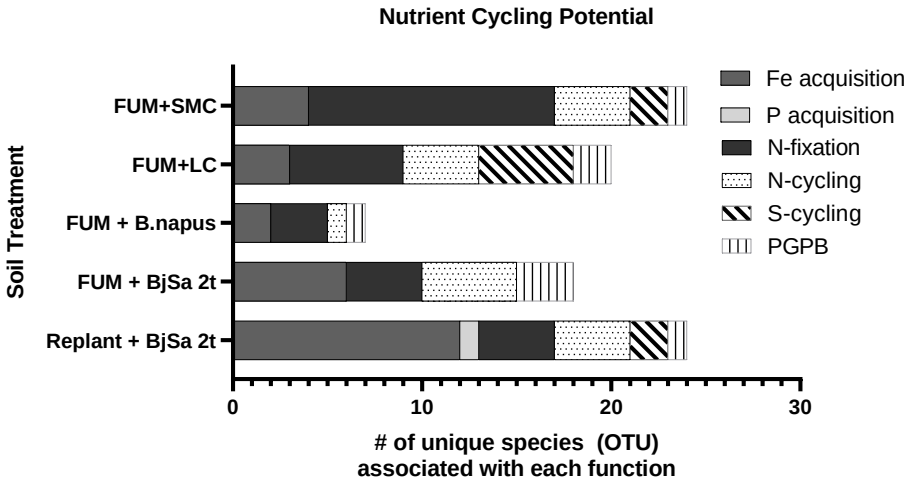
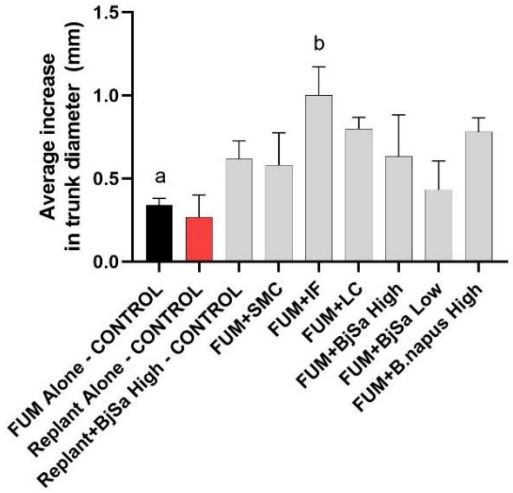


Figure 5. Comparison of the number of unique bacterial species enriched in each treatment relative to the fumigated control associated with a variety of functions related to nutrient cycling and/or characterized as plant growth promoting bacteria (PGPB) in the literature.



The effects of the different soil amendments on rootstock growth were also assessed at the end of Experiment 1. None of the rootstocks planted into the fumigated soil amended with chicken manure compost had any signs of new root or shoot growth. Therefore, CCM is not included in this figure.

Figure 6. Increase in trunk diameter at harvest. Different lowercase letters indicate significantly different means and represent statistical comparisons between the fumigation alone control soil (FUM Alone; black) and all other soil treatments. BjSa High and *B.napus* High = 2 tons seed meal per acre, BjSa Low = 1 ton seed meal per acre, SMC = shitake mushroom compost, LC = liquid chitin, IF = insect frass. Bars represent standard error of the mean.

***Impacts on plant fitness*:**

In general, with the exception of the replant alone control (Fig. 6; red), all amendments resulted in an increase (albeit non-significant) in mean trunk diameter relative to the fumigation alone control soil (Fig. 6; black). Insect frass was the only treatment which resulted in a significant increase in trunk diameter (from planting to harvest) relative to the fumigated control (p=0.027). The amount of wood produced during the growing period (trunk diameter) is an indicator of overall tree health. These results suggest that insect frass may benefit the growth of young trees in fumigated soil.

The second objective of this study was to determine the role of select amendment-modified soil microbial communities in limiting pathogen re-infestation and reducing potential post-harvest pathogens.

Experiment 2/Year 2

Subsequent to the investigations conducted in year 1, the most promising soil amendments (BjSa SM (2t), SMC, LC, and IF) were selected for use in an experiment designed to determine the ability of the altered microbiome to inhibit pathogen re-infestation of the fumigated orchard soil. This experiment utilized soil from the same orchard location as in Experiment 1; a new batch of unfumigated replant soil was collected in the summer of 2022 for use in Experiment 2. This was because the first batch of unfumigated replant soil lacked *P.penetrans*, a result which was likely related to when the soil was collected (April 2021). During early spring, when soil temperatures generally remain below 70°F, nematode populations in soil may be less active due to overwintering. The new batch of replant soil was used in a secondary (i.e., repeat) pre-requisite bioassay to ensure disease control was in fact obtained in fumigated soil. Total plant biomass (Fig. 7), root biomass and shoot biomass (data not shown) were significantly higher in fumigated

soil (a), providing strong evidence of disease control in the fumigated soil. Upon harvest, fine root tissue was also assessed for *Pratylenchus penetrans* abundance. The average number of *P. penetrans* recovered per gram of root tissue was 183 for plants cultivated in replant soil. In comparison, not a single *P. penetrans* was identified in the roots of apple seedlings cultivated in fumigated or pasteurized replant soil.

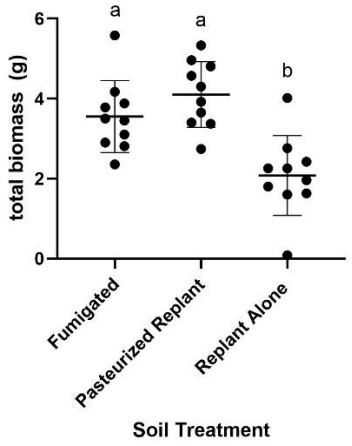


Figure 7. Effect of soil treatment on growth of 4-week old Gala apple seedlings as measured by total plant biomass. Different letters indicate significantly different means and represent comparisons of all three treatments (Kruskal-Wallis followed by Dunn's test; $p<0.05$).

In order to simulate pathogen re-infestation following fumigation and to *directly* test the ability of the apple rhizosphere to limit pathogen re-infestation post-fumigation, a mycelial fragment/spore suspension of the ARD pathogen *Pythium ultimum* was prepared for use as a soil inoculum. In general, *Pythium* spp. populations range from around 60 to 500 propagules in the orchard systems of Central Washington (pers comm. M. Mazzola; Mazzola M., 1998.). The inoculum (300 propagules/g soil) was introduced to pots containing G.11 apple rootstocks cultivated in the select treatments 8 weeks post-planting. Rhizosphere soil samples were collected immediately prior to inoculation (for microbial community sequencing analysis) and bulk soil samples were collected immediately after inoculation (to assess *actual* inoculum density). The experiment was harvested 1 month later (December 2022).

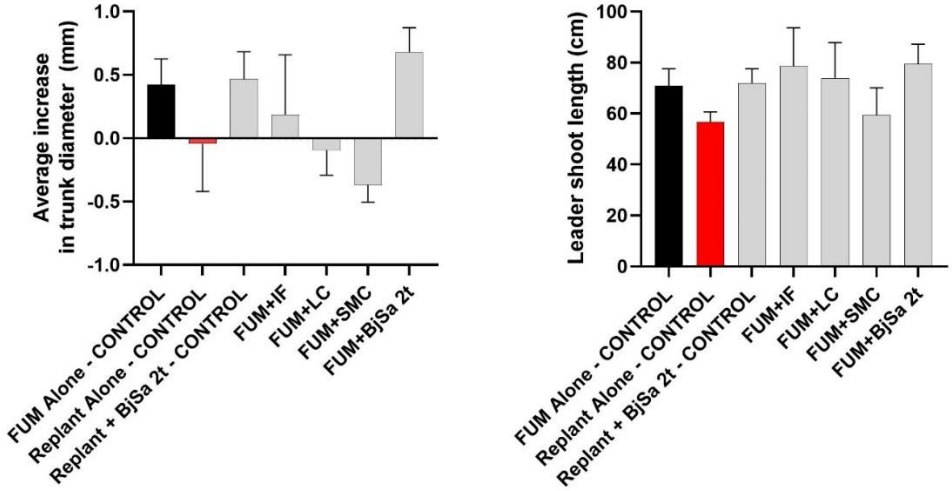


Figure 8. Increase in trunk diameter (left) and leader shoot length (right) at harvest (1 month post inoculation with *P.ultimum*). SMC = shitake mushroom compost, LC = liquid chitin, IF = insect frass. Bars represent standard error of the mean.

In Experiment 2, no treatment resulted in a statistically significant increase in trunk diameter (from planting to harvest) or leader shoot length relative to the fumigated control. However, a positive trend was observed for Fum-BjSa 2t as the treatment resulted in the greatest increase in trunk diameter and leader shoot length. In addition, the SMC amendment resulted in a significant decrease in trunk diameter relative to the fumigated control ($p=0.01$; Kruskal-Wallis followed by Dunn's multiple comparisons test). Three treatments (Replant Alone, FUM + LC and FUM + SMC) resulted in an average trunk diameters that decreased after planting.

Concluding Remarks:

CMC and LC, although relatively inexpensive (~$50 per acre) are likely to be detrimental to plant and soil health when used as a post-fumigation soil amendment. By comparison, the results of these experiments suggest that insect frass and BjSa SM (2t) are both promising treatments for improving multiple aspects of soil health post-fumigation. At this point, however, both of these materials remain costly (~$1,000 -3,000 per acre). The reduced rate (1t per acre) BjSa SM was not enough to effectively "steer" the bacterial community away from that of the fumigation alone or replant control states. The higher amendment rate (2t per acre) is needed to obtain optimal results. When used at this rate, BjSa SM has been shown to consistently provide disease control at levels equivalent to or better than pre-plant soil fumigation. Therefore, use of BjSa SM (2t per acre) as an *alternative* to (rather than in addition to) fumigation is much more cost effective. Future testing of the effects of insect frass on plant/soil health in other soil types and/or at reduced amendment rates is recommended.

REFERENCES

BC Tree Fruit Production Guide. BCFGA; https://www.bctfpg.ca/horticulture/fruit-tree-nutrition/

Dupont T and Granatstein D. 2020. Compost Use for Tree Fruit. WSU Extension Factsheet, FS337E.

Mazzola M. 1998. Elucidation of the microbial complex having a causal role in the development of apple replant disease in Washington. Phytopathology, 88: 930-938.

Mazzola, M., et al. 2009. Interaction of brassicaceous seed meal and apple rootstock on recovery of *Pythium* spp. and *Pratylenchus penetrans* from roots grown in replant soils. *Plant Disease* 93.1: 51-57.

Mazzola M, Hewavitharana SS, Strauss SL. 2015. Brassica seed meal soil amendments transform the rhizosphere microbiome and improve apple production through resistance to pathogen reinfestation. Phytopathology, 105(4):460-9

Somera TS, Freilich S, Mazzola M.2021. Comprehensive analysis of the apple rhizobiome as influenced by different Brassica seed meals and rootstocks in the same soil/plant system. *Appl. Soil Ecol.* 157: 103766.

Sullivan DM, et al. 2018. Interpreting Compost Analysis. OSU Extension Factsheet, EM 9217

Wang, L and Mazzola M. 2019. Field evaluation of reduced rate Brassicaceae seed meal amendment and rootstock genotype on the microbiome and control of apple replant disease. *Phytopathology* 109.8: 1378-1391.

Executive Summary:

Project Title: Directing plant-microbe relations toward resiliency post-fumigation

Key words: Apple, Soil amendment, Soil fumigation, Microbiome, Soil-borne diseases, Replant disease

Abstract:

As a replant disease control strategy, pre-plant soil fumigation is the industry standard. Although fumigation significantly reduces pathogen activity and improves tree growth, this benefit is limited to approximately 1 year. Post-fumigation, orchard soil rapidly re-establishes a microbial community indistinguishable from that found in the corresponding non-fumigated replant soil (i.e., a chronic disease state). The application of soil amendments following fumigation may be an opportune time to improve the ability of the soil to defend against pathogen reinvasion and improve orchard productivity for an extended period. The primary aim of this project was to identify materials which could be used to "steer" the apple rhizosphere in favor of a more prophylactic or disease-suppressive state, post-fumigation. Post-fumigation soil amendments included: *Brassica juncea*/*Sinapis alba* seed meal (2t per acre), BjSa seed meal (1t per acre), *B.napus* seed meal (2t per acre), shitake mushroom compost (SMC, 2% v:v), liquid chitin (LC, 2 gal per acre), composted chicken manure (CCM, 0.7t per acre) and insect frass (IF, 1.5 cups per ft^{-3} soil). In CCM and LC, high nitrate levels led to high salinity as measured by electrical conductivity (EC) and CCM resulted in the death of all trees. Analysis of microbial community sequence data indicated that the remaining amendments, with the exception of BjSa SM (1t), were relatively successful in terms of their ability to significantly alter the microbial community composition of the rhizosphere microbiome and "steer" the community in a positive direction post-fumigation. Insect frass was the only amendment which resulted in a significant increase in trunk diameter relative to the fumigated control. Insect frass and *B. napus* SM (2t), however, did not appear to counteract the adverse effects of fumigation on the bacterial rhizobiome as effectively as SMC, LC, or BjSa SM (2t). By comparison, LC was less effective at stimulating the growth of potentially beneficial fungi. A second experiment designed to *directly* test the ability of the apple rhizosphere to limit pathogen re-infestation post-fumigation was conducted using the ARD pathogen *P. ultimum* as inoculum. At this time, results suggest that insect frass and BjSa SM (2t) are both good candidates for improving soil health post-fumigation; however, *P. ultimum* infection levels in root tissue remain to be determined.

CONTINUING REPORT: YEAR 1

Project/Proposal Title: Assessing Barriers to and Benefits of AMF Colonization in Apple

PI: Dr. Tracey Somera
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Contact Information: Dr. Tracey Somera; 858-344-9750, tracey.somera@usda.gov

Total Project Request for Year 1 Funding: $ 60,046.00
Total Project Request for Year 2 Funding: $ 57,352.00
Total Project Request for Year 3 Funding: $ 54,000.00

WTFRC Collaborative Costs: None

Budget 1
Organization Name: USDA ARS Tree Fruit Research Laboratory
Contract Administrator: Chuck Meyers & Sharon Blanchard
Telephone: 510.559.5769 (CM), 509.664.2280 (SB)
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Station Manager/Supervisor: James Mattheis
Station manager/supervisor email address: james.mattheis@usda.gov

Item	2022	2023	2024
Salaries*	34,002.00	34,337.00	34,337.00
Benefits	14,649.00	14,927.00	14,927.00
Wages	NA	NA	NA
Benefits	NA	NA	NA
Sequencing Costs	4,800.00	NA	NA
Lab Supplies	6,595.00	8,088.00	4,736.00
Travel	NA	NA	NA
Miscellaneous	NA	NA	NA
Plot Fees	NA	NA	NA
Total	60,046.00	57,352.00	54,000.00

Footnotes: *GS 11 post-doc, 0.5 FTE

OBJECTIVES

1. To characterize the capacity of commercially available arbuscular mycorrhizal fungal (AMF) products and pre-existing AMF communities contained in nursery-derived apple roots to compete with native AMF orchard communities.

2. To identify benefits of specific apple rootstock-AMF associations including protection against root pathogenic fungi and tolerance to water stress.

SIGNIFICANT FINDINGS:

Results from preliminary experiments:

- support the idea that plant-AMF relationships are complex and may need to be tailored accordingly

- suggest that matching host genetics with compatible AMF species has the potential to enhance agricultural practices in nursery and orchard systems

- suggest that native AMF and/or pre-established AMF communities strongly influence the AMF communities that assemble within the apple roots after planting and limit effective colonization by mycorrhizal inoculants

Note: Select apple rootstock/AMF associations identified in initial compatibility experiments will be used in subsequent experiments designed to explore functional benefits of AMF colonization including 1) mycorrhizal-mediated resistance to replant disease and 2) *R. irregularis*-mediated tolerance to water stress in the dwarfing rootstock G.11.

METHODS:

Preliminary experiment for Objective 1

An initial experiment designed to assess the ability of commercially available AMF to compete with pre-existing/nursery-derived AMF contained in apple rootstocks was conducted. The experimental set up involved inoculating 2 Malling (M.7 and M.26) and 2 Geneva (G.890 and G.935) rootstocks with a commercially available AMF mix. In order to initially explore the ability of commercial AMF inoculants to displace AMF communities pre-established at the nursery, we wanted a relatively "simple" system (i.e., a *known* community of AMF with a limited number of species). According to the manufacturer, the AMF mixture selected contained a relatively diverse, ecologically relevant consortium of species including: *Glomus clarum* (Order Glomerales), *Glomus monosporum* (Order Glomerales), *Septoglomus deserticola* (Order Glomerales), *Paraglomus brasilianum;* (Order Archaeosporales), and *Gigaspora margarita* (Order Gigasporaceae). As pasteurized potting mix represents an ablated soil microbiome (with no AMF species present), this commercial AMF mixture was hand-mixed into pasteurized potting soil at the recommended field rate (500-600 propagules/tree; 4.5 g/tree) prior to planting. Root tissue was collected for DNA isolation immediately prior to planting (pre-established, nursery-derived AMF community composition) and at 4-weeks post-planting (i.e., new, white roots). DNA was sequenced using the AMF-specific primer set, AML1/2 (18S SSU gene fragment; Pac-Bio amplicon sequencing). DNA from the pasteurized potting mix (PPM) was also sequenced.

Obtaining accurate taxonomic assignments of AMF sequences using current databases (i.e., UNITE, Gen Bank) is challenging (Stefani et al., 2020). We are therefore now in the process of building a phylogenetic tree for Phylum Glomeromycota based on the available 18S SSU gene sequences (Krüger, et al. 2012; Stefani et al., 2020). Tree-based taxonomic assignments will then be used to annotate the sequence data to the species level (i.e., the resolution required in order to make AMF community comparisons).

Preliminary Experiment for Objective 2

A preliminary experiment designed to assess the effect of rootstock genotype and arbuscular mycorrhizal fungal (AMF) species on colonization of apple was completed in July of 2022. In this experiment, we directly tested the ability/efficacy of four different AMF species to colonize four commercially available apple rootstock genotypes. The AMF species used in this experiment belonged to two different families within the order Glomerales: Glomeraceae (*Rhizophagus irregularis* and *Septoglomus deserticola*) and Claroideoglomeraceae (*Claroideoglomus claroideum* and *Claroideoglomus etunicatum*) (Krüger et al., 2012). These AMF were considered to be "ecologically relevant" because they represent species previously documented in apple roots and/or rhizospheres (Ceustermans, et al., 2018; Dalla Costa, et al., 2021; Van Horn, et al., 2021; Hosseini, et al., 2015; Summuna, et al., 2019). Single species inocula were obtained from MycoInTech, Tarragona, Spain. Spores contained in each inoculum were isolated using the sucrose gradient centrifugation technique described on the International Collection of Vesicular Arbuscular Mycorrhizal Fungi: INVAM website (https://invam.ku.edu/spore-extraction). Spore viability and confirmation of species identity were evaluated microscopically under the guidance of Bill Wheeler (International Collection of Vesicular Arbuscular Mycorrhizal Fungi: INVAM; Fig.1). Micro-propagated dwarfing (G.11, G.41) and semi-dwarfing (G.210 and G.890) apple rootstock genotypes were received as plantlets from North American Plants, McMinnville, OR. Tissue-cultured plantlets of uniform size were used because nursery-derived apple rootstocks come with pre-existing AMF communities.

Briefly, micro-propagated plantlets were either inoculated with individual species of AMF or were not inoculated. This was done by placing single species AMF inoculum (commercially prepared by MycoInTech; 830-985 spores/plant) into the root zone prior to planting into pasteurized potting soil (plant roots must be in immediate contact with fungal spores in order for fungal infection to occur). Experimental treatments included all 16 rootstock genotype x AMF species combinations as well as a non-AMF control treatment for each rootstock genotype. In total, there were 20 different treatments (4 x 5 factorial design) with 7 replicate pots each (140 plants). Pots were placed on elevated platforms (to reduce risk of contamination of non-inoculated plants) in a randomized block layout. Each block contained all 20 treatments consisting of the four rootstock genotypes (G.11, G.41, G.210, G.890) crossed with five different AMF treatments (uninoculated control, *R. irregularis*, *S. deserticola*, *C. claroideum*, and *C. etunicatum*).

After 5 weeks (an agriculturally relevant time frame sufficient for colonization to occur), the effects of the interaction between rootstock genotype and AMF species on mycorrhization, plant growth and leaf nutrient concentrations were assessed. Mycorrhizal colonization was assessed via differential staining, in which fungal tissue is stained while background plant cells are not (Giovannetti & Mosse, 1980; Fig. 2). Briefly the presence/absence of stained fungal hyphae, arbuscules, or vesicles was recorded among a field of stained root segments (~2g) using a petri dish (9cm diameter) marked with a 0.5"x 0.5" grid. Percent colonization of AMF was calculated as (# of intersects with AMF present)/(total # of intersects with root tissue) x 100. All 140 samples were examined separately.

RESULTS AND DISCUSSION:

Preliminary Experiment for Objective 1

No Glomeromycota were present in the pasteurized potting mix (PPM), which represented an AMF-free growth medium. The analysis of nursery-derived root tissue indicated that, in most cases, AMF community composition *between* apple rootstock genotypes was significantly different. The only exception was G.935 vs. M.7. After 4 weeks in PPM, samples to which no commercial AMF mixture had been applied had reduced AMF diversity and AMF community composition became more similar between apple rootstock genotypes. By comparison, AMF communities in PPM containing the commercial AMF mixture tended to diverge upon planting into pasteurized potting mix.

Overall, *within* rootstock genotypes, nursery-derived AMF communities demonstrated no significant difference from the no AMF controls or the AMF-amended treatments after 4 weeks. This finding suggests that the nursery-derived AMF remained established/stable in the presence of the commercial AMF inoculum. Nevertheless, some AMF species that were identified in the commercial AMF mixture did appear to effectively colonize Malling rootstocks. The only rootstock in which a statistically significant difference in community composition was identified between pre-planting and AMF-amended treatments was M.26. Therefore, M.26 may be more susceptible than other rootstock genotypes to community shifts by "introduced" AMF species. For example, a species of AMF in the genus *Diversispora* (MaarjAM database, http://maarjam.botany.ut.ee), that was detected in the commercial inoculant, effectively colonized M.26 (10-62% of all Glomeromycota). Another AMF species that was present in both the commercial inoculum and in AMF-amended samples was *Claroideoglomus torrecillas* (identified in M.7 and M.26). One AMF species belonging to the genus *Paraglomus* successfully colonized a wide variety of apple rootstocks, regardless of genotype. This species was not only detected in the commercial mixture, but was also consistently present in the roots of all rootstocks pre-planting (0.7-62% of all Glomeromycota), both after planting into pasteurized potting mix (0.9-100%) and after planting into AMF-amended treatments (0.6%-98%) (Table 1). Sometimes different sequences (representing different AMF species or strains) match the same entry in the database. As previously mentioned, we are now in the process of building a phylogenetic tree for Phylum Glomeromycota. Tree-based taxonomic assignments will be used to definitively distinguish taxa contained in the commercial mix from those colonizing the plant roots.

It is worth noting that none of the DNA sequences detected in the commercial inoculum matched any of the species *purported* to be contained in this mixture (Table 1). From this finding, it is apparent that what the manufacturer advertises is not always what is actually present. For the purposes of our investigation, phylogenetic tree-based taxonomic assignments will be used to resolve/clarify the consortium of species contained in the commercial mixture. Tree-based taxonomic assignments will also be used to further assess shifts in AMF community composition between samples at the species/strain level. The findings from this initial experiment in combination with the creation of a phylogenetic reference tree for AMF will help guide subsequent experiments designed to further assess the ability of commercially available "inoculated" AMF to compete with pre-existing AMF (from the nursery where they are produced) as well as with native AMF/fungi in "live" orchard soil. Following the arrival of a newly hired post-doc, this work is expected to begin in spring 2023.

Preliminary Experiment for Objective 2

The goal of this experiment was not to assess benefits of specific apple rootstock/AMF associations. Instead, this was a preliminary assessment of whether some rootstock genotypes are more susceptible to mycorrhization than others and/or whether AMF species identity influences rootstock compatibility. The findings from this study indicate that the AMF species factor is a significant source of variation affecting percentage of apple root colonization. In other words, optimal mycorrhizal colonization of apple root systems occurs in a rootstock genotype-AMF species specific manner. The highest levels of mycorrhizal colonization were obtained from G.41 x *S. deserticola* and G.890 x *C. claroideum* (Fig. 3). The ability of an AMF to *rapidly* colonize a host is likely to benefit it's competitive ability over time. Therefore, from a practical standpoint, AMF-rootstock combinations that lead to rapid establishment of a relationship (i.e., within 4-6 weeks) show the most promise for successful deployment in natural field settings with existing AMF communities. Percent colonization did not, however, reflect growth outcomes or nutritional status, although significant increases in total Nitrogen were observed in the leaf tissue of G.41, G.210, and G.890 (but not G.11) inoculated with *R. irregularis* relative to uninoculated plants (Fig. 4). Considering that *R. irregularis* appeared to colonize G.11 more effectively than the other AMF species (Fig. 3), this was an unanticipated outcome.

Although many AMF species are generalists and can colonize a range of host plants, functional benefits may be fungus-plant species dependent. This experiment marks an important step towards laying the groundwork for harnessing potential apple rootstock-AMF species preferences in nursery and orchard management systems. Data from this experiment will be used to select compatible apple rootstock/AMF combinations (e.g. G.41 x *S. deserticola* and G.890 x *C. claroideum*) for use in a subsequent experiment. The objective of the ensuing investigation is to provide clear evidence of AMF species directly functioning in beneficial roles with commercially available apple rootstock genotypes. This includes testing the ability of the specific rootstock/AMF associations to enhance plant defense against infection by the fungal replant pathogen *Rhizoctonia solani* AG-5 (lab culture collection, isolate #5-104). In addition, we plan to directly explore the effects of *R. irregularis* on rootstock tolerance to short-term water stress in "live" orchard replant soil using the dwarfing rootstock G.11. Less vigorous apple rootstocks (with relatively small root systems) may become susceptible to water deficits due to the small soil volume exploited by the root system (Tworkoski & Fazio, 2015; Tworkoski et al., 2016, Casagrande-Biasuz & Kalcsits, 2021). As referenced above, preliminary data shows that *R. irregularis* effectively colonizes G.11. In order to assess *R. irregularis* -mediated tolerance to water stress in G.11, a variety of plant physiological characteristics including midday stem water potential and plant stomatal water conduction will be assessed.

Colonization of apple roots by AMF has been shown to result in changes to plant growth physiology that are not necessarily easy to identify or explain. For this reason, we will be collaborating with Dr. Lee Kalcsits at Washington State University who has expertise in the field of tree fruit physiology.

Table 1. AMF species *purported* to be in the commercial mixture vs. those *detected* in the commercial mixture and in root tissue. Taxonomy was determined using the AMF-specific reference sequence database MaarjAM (Opik, et al., 2010).

AMF purported to be in the commercial mixture	Detected in commercial mixture
Glomus clarum (a.k.a. *Rhizoglomus clarum* , *G.clarus* , *Rhizophagus clarus*)	No
Glomus monosporum (a.k.a. *Funneliformis monosporus*)	No
Septoglomus deserticola (a.k.a. *G.deserticola*)	No
Paraglomus brasilianum (a.k.a. *G. brasilianum*)	No
Gigaspora margarita	No
AMF detected in the commercial mixture	**Detected in Apple Root Tissue**
Diversispora sp.	Yes (M.26 only)
Claroideoglomus torrecillas	Yes (M.7 and M.26)
*Paraglomus sp.**	Yes (M.7, M.26, G.890, G.935)
*Rhizophagus irregularis**	Yes (G.890 and G.935)
Claroideoglomus turrini	No
Glomus sp.	No
Glomus sp.	No

* Also identified in root tissue collected pre-planting and in roots from uninoculated controls

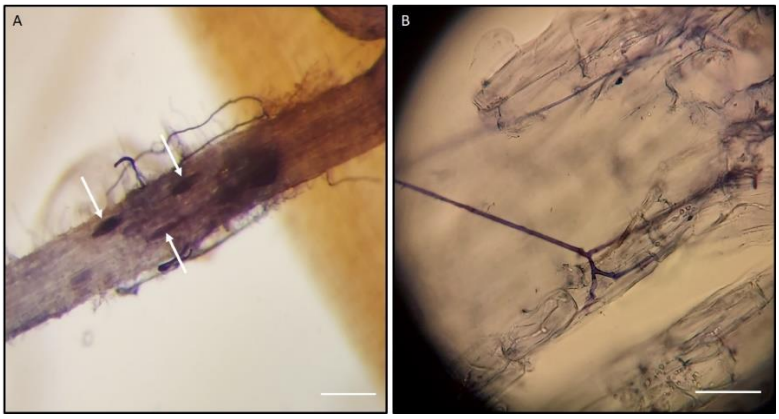

Figure 1. Microscopic visualization of arbuscular mycorrhizal fungi showing vesicles (A; arrows) and hyphal branches extending from apple roots (B). Scale bars: 100 μm (A); 150 μm (B)

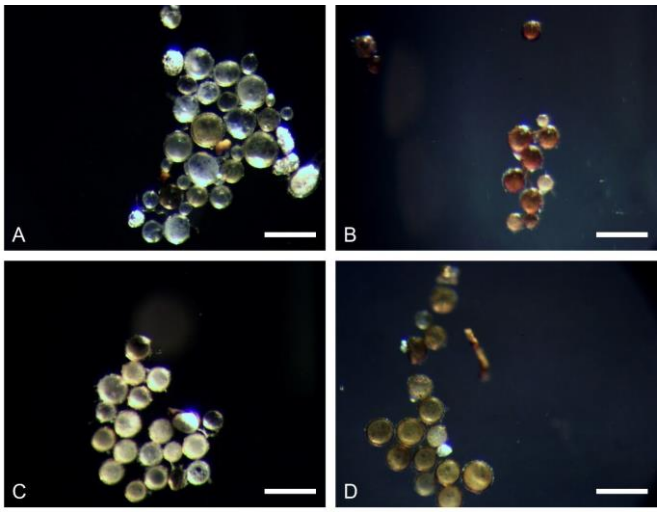

Figure 2. Spores of *Rhizophagus irregularis* (A), *Septoglomus deserticola* (B), *Claroideoglomus claroideum* (C), *Claroideoglomus etunicatum* (D); scale bars = 200 µm

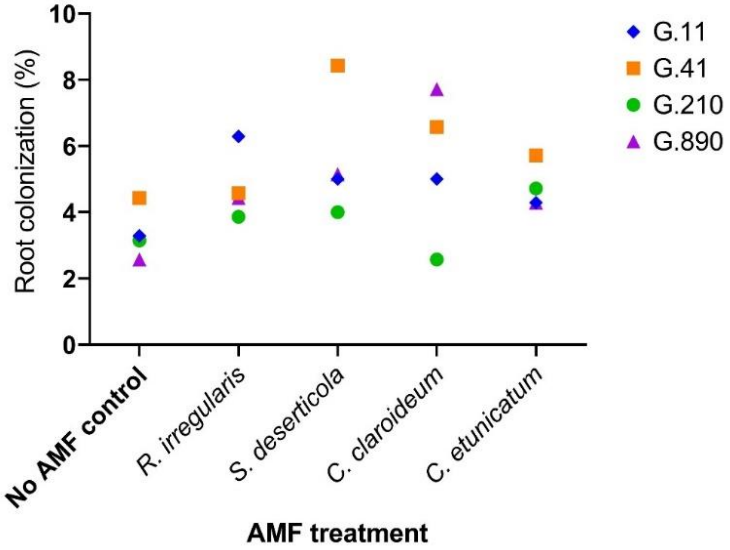

Figure 3. Percentage of root colonization in 4 different apple rootstocks inoculated with 4 different species of AMF at 5 weeks after inoculation. Each data point represents the mean of 7 biological replicates.

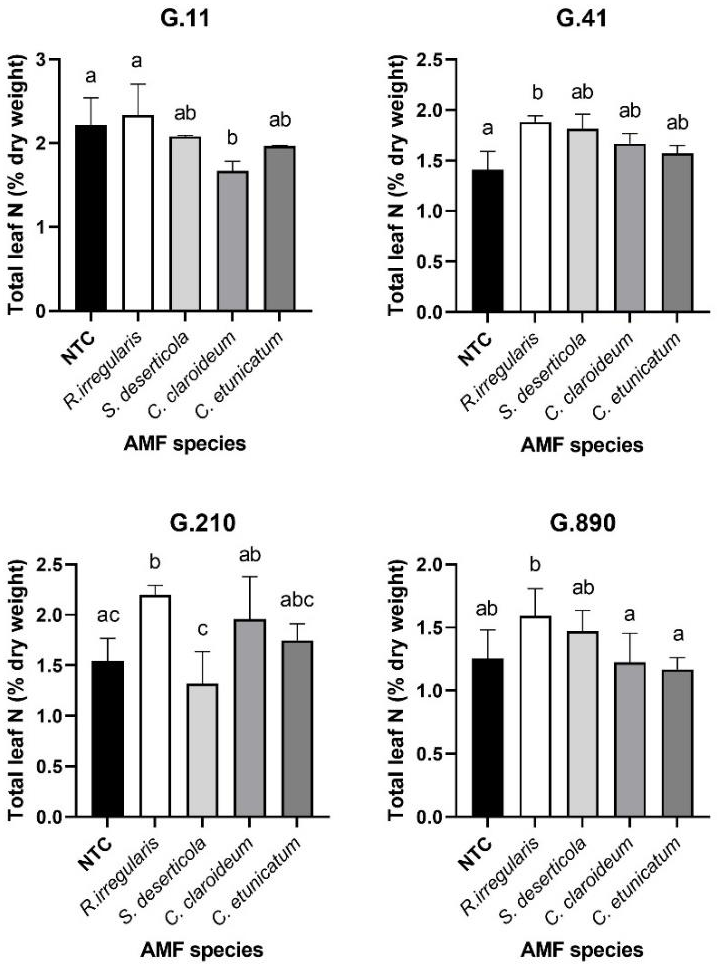

Figure 4. Total leaf N (%) in 4 different apple rootstocks inoculated with different species of AMF, 5 weeks after inoculation. Error bars represent the standard deviation. Different letters above bars indicate statistically significant differences according to Two-way ANOVA followed by Tukey's multiple comparisons test ($p < 0.05$).

REFERENCES

Casagrande-Biasuz E & Kalcsits LA. 2021. Variation in scion water relations mediated by two newly released Geneva series rootstocks. *bioRxiv* .07.20.453132

Ceustermans A, Van Hemelrijck W, Van Campenhout J, Bylemans, D. 2018. Effect of arbuscular mycorrhizal fungi on *Pratylenchus penetrans* infestation in apple seedlings under greenhouse conditions. Pathogens: 7, 76.

Dalla Costa M, et al. 2021. Inoculation with isolates of arbuscular mycorrhizal fungi influences growth, nutrient use efficiency and gas exchange traits in micropropagated apple rootstock 'Marubakaido'. PCTOC, 145, 89-99.

Hosseini A & Gharaghani A. 2015. Effects of arbuscular mycorrhizal fungi on growth and nutrient uptake of apple rootstocks in calcareous soil. Int. J. Hortic. Sci. Technol., 2, 173-185.

Giovannetti M & Mosse, B. 1980. An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytol.*, 489-500.

Krüger M, et al. 2012. Phylogenetic reference data for systematics and phylotaxonomy of arbuscular mycorrhizal fungi from phylum to species level. *New Phytol*., *193*, 970-84.

Opik M, et al. 2010. The online database MaarjAM reveals global and ecosystemic distribution patterns in arbuscular mycorrhizal fungi (Glomeromycota). New Phytol.;188:223–41.

Stefani F, et al. 2020. Taxonomic assignment of arbuscular mycorrhizal fungi in an 18S metagenomic dataset: a case study with saltcedar (*Tamarix aphylla*). Mycorrhiza 30.2: 243-255.

Summuna B. et al. 2019. Diversity and distribution of arbuscular mycorrhizal fungi in pome and stone fruit orchards of Northwestern Himalayan State of Jammu & Kashmir. Indian Phytopathol., 72, 343-350.

Tworkoski T, Fazio G, Glenn DM. 2016. Apple rootstock resistance to drought. *Sci. Hortic.*, 204, 70-78.

Tworkoski T & Fazio G. 2015. Effects of Size-Controlling Apple Rootstocks on Growth, Abscisic Acid, and Hydraulic Conductivity of Scion of Different Vigor, Int. J. Fruit Sci., 15:4, 369-381

Van Horn C, Somera TS, Mazzola M. 2021. Comparative analysis of the rhizosphere and endophytic microbiomes across apple rootstock genotypes in replant orchard soils. Phytobiomes J., 5, 231-243.

Project Title: Comprehensive monitoring and mapping antibiotics resistance in orchards

Report Type: Continuing Project Report

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Project Duration: 2-Year

Total Project Request for Year 1 Funding: $77,898
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Item	2022	2023
Salaries[1]	52,262	54,352
Benefits[2]	10,044	10,446
Wages		
Benefits		
Equipment		
Supplies[3]	10,342	10,187
Travel	5,250	5,250
Miscellaneous		
Plot Fees		
Total	**77,898**	**80,235**

Footnotes: 4% inflation for year 2. [1]Postdoc; one PhD and one MSc student, 1.0 FTE, [2]Benefits Postdoc 32.9%; Graduate students 12.6%. [3] Lab and field supplies, antibiotics, plates, primers, gene sequence services, molecular reagents etc, [4]collect samples, April-October.

Objectives:

1. To collect and screen antibiotics (streptomycin, tetracycline and kasugamycin) resistance in apple orchards throughout the state at the population level;
2. To determine the resistance nature (intrinsic or plasmid-borne) of the pathogen if any;
3. To immediately deliver results to growers and provide guidance on antibiotics use in orchards in the coming years.

Significant Findings:

- 100% *Erwinia amylovora* bacterial populations did not exhibit resistance to streptomycin (cannot grow at 100ppm)
- 100% *Erwinia amylovora* bacterial populations did not exhibit resistance to tetracycline (cannot grow at 25ppm)
- 100% *Erwinia amylovora* bacterial populations did not exhibit resistance to kasugamycin (cannot grow at 200ppm, but 100% can grow at 50ppm)
- 50 samples (each with 3-5 treatments) were collected from Kennewick, Benton City, Chelan, Cashmere, Prosser, Pasco, Wapato, Union Gap, Sunnyside, Othello, Hansen, Wenatchee
- Apple varieties sampled include Gala, Jazz, Fuji, Cripps Pink, Cosmic Crisp, SweetTango, Envy, and WA38 as well as two pear varieties (Concorde and Bosc).
- 85 isolates were stored from shoots (11), suckers (1), cankers (3), leaves (32), petioles (21), fruits(13) and ooze (4).
- Although the model predicted low to modest fire blight occurrence, some orchards in Benton city and Prosser had a bad fire blight year
- 12 non-*Erwinia amylovora* bacterial isolates were collected, which exhibited various degrees of resistance to streptomycin (9), tetracycline (4) and kasugamycin (12)
- Among them, two each showed resistance to all three antibiotics at higher than 1200ppm and higher than 200ppm, respectively
- Among them, two and three showed resistance to streptomycin and kasugamycin at higher than 1000ppm and higher than 200ppm, respectively
- Understanding the molecular mechanism of resistant bacteria from orchards may shield light on whether *Erwinia amylovora* may develop resistance or not.

Methods

Procedures and methodology used:

Objective 1. To collect and screen antibiotics (streptomycin, tetracycline and kasugamycin) resistance in apple orchards throughout the state at the population level.

Sample collection:

Due to lab delay, we missed sampling the blossom blight stage. Since the model predicted a light fire blight year, many orchards did not have many blighted shoot. However, in some organic and conventional orchards near Prosser and Benton city, shoot blight was commonly observed (**Fig. 1**). With the help of growers and crop consultants, we started collecting samples in Mid-June in these areas until early August. Samples were either directly collected by the PI and his associates (postdoc, graduate student, and intern) from orchards or collected by growers, consultants and collaborators and shipped to us. As indicated previously, the latter method proves to be critical for the success of this project (see limitation below about sample collection).

Samples were collected from 12 locations across Washington State, including Kennewick, Benton City, Chelan, Cashmere, Prosser, Pasco, Wapato, Union Gap, Sunnyside, Othello, Hansen, and Wenatchee (**Table 1**). Apple varieties sampled include Gala, Jazz, Fuji, Cripps Pink, Cosmic Crisp, SweetTango, Envy, and WA38 as well as two pear varieties (Concorde and Bosc), which consists of both conventional/organic apples; old/new varieties and young/old orchards. However, when asked whether there were streptomycin-resistant strains or not, the answers were either not sure or did not know. Samples were collected in the orchards and transported in a cooler or packaged in Styrofoam boxes with ice and shipped to the laboratory. Once in the laboratory, samples were immediately processed or stored at 40°F until being processed.

Sample processing:

For each sample, *E. amylovora* was isolated from different plant organs (i.e. 3-5 treatments), including shoots, suckers, cankers, leaves, petioles, and fruit samples showing symptoms of typical fire blight disease with or without ooze. In the first protocol used to isolate bacteria, leaves, cankers, and shoots were surface sterilized in 10% bleach for 30 sec or washed three times with water. Tissues closed to diseased part were cut into small pieces using surgical blade and immersed in 1 ml phosphate-buffered saline (PBS) buffer and the suspension was incubated for 10 min at 80-degree F. Bacteria were then isolated from supernatants of these processed materials by serial 10x dilution plating. Alternatively, using a slightly complicated protocol, tissues from the infection margins close to diseased part were first cut into small pieces. The sample pieces were weighted (0.1-0.5 g) and mixed with ice-cold 10 mM PBS (5-25 mL) in a ratio of 1:50, as recommended by the EPPO standards PM7/20 (EPPO 2013) and the ISPM 27 (ISPM 27, 2016) for the isolation of *E. amylovora*. Samples were then grounded using a sterilized mortar and pestle, and the resulting macerates were serially tenfold diluted in PBS for bacterial isolation. When bacterial ooze was present on the sample, *E. amylovora* was isolated directly from ooze droplets by swabbing the ooze with a sterile cotton swab, suspending in sterile PBS, and streak plating on CCT and LB with antibiotics or by serial 10x dilutions as above.

Determination of resistance at the population level:

For each treatment from each sample, the above serial 10x dilutions were then plated on the semi-selective medium for *E. amylovora* CCT (Ishimaru & Klos, 1954) or on nutrient rich LB plates as a control. To determine antibiotic resistance for each treatment, the above serial 10x dilutions were plated on LB plates amended with streptomycin (100 and 1000 µg/ml), tetracycline (25 and 200 µg/ml), and kasugamycin (50 and 200 µg/ml), respectively. Six dilutions from 10^6 to 10^1 and four 25 µl of each dilution will be added to seven different types of LB plates. Additionally, 50 µl of the 10^{-2} dilution were plated on LB agar containing 25 and 200 µg/mL tetracycline, 200 µg/mL kasugamycin, and 100 and 1000 µg/mL streptomycin. Later we found that 100% *E. amylovora* isolates can grow at 50 µg/mL kasugamycin, so we only kept the higher concentration at 200 µg/ml. Since *E. amylovora* concentrations in macerates from symptomatic samples usually reach > 10^5 CFU/mL on CCT (i.e., 5 x 10^6 CFU/g), we considered that *E. amylovora* isolates were not resistant to antibiotics when no colonies were observed on LB with antibiotics. In addition, some treatments were also analyzed by direct plating plant macerates on CCT and testing antibiotic resistance for 1 to10 *E. amylovora*-like colonies afterward, a protocol like Russo et al. (2008) and Tancos et al. (2016).

Identification of *E. amylovora* isolates:

For each sample/treatment, an aliquot of the plant macerates was mixed in a ratio of 1:1 with 30% glycerol (final glycerol concentration 15 %) and stored at -112°F. When no *E. amylovora*-like colonies were isolated on CCT, we tried to re-isolate the pathogen from the undiluted sample aliquots stored at -112°F and/or from the original samples stored at 40°F. *E. amylovora*-like colonies on CCT and other colonies growing on plates with or without high antibiotic concentrations were purified on King's B (KB) medium and stored at -112°F in 15% glycerol. *E. amylovora* isolates were identified

first based on colony morphology on CCT (purple, domed) and KB (white, mucous, irregular). Final identification of isolates was performed using validated primers for *E. amylovora* using conventional PCR (Taylor et al., 2001). Selected 85 isolates were stored at -112°F for future use (**Table 2**).

Determining the minimum inhibitory concentration (MIC) for non-*Erwinia amylovora* isolates:

In addition, colonies with non-typical *E. amylovora* colony morphology showing resistance to antibiotics were also selected and re-streaked onto LB or LB amended with streptomycin (100 and 1000 μg/ml), tetracycline (25 and 200 μg/ml), and kasugamycin (200 μg/ml). These isolates were also stored at -112°F. To determine minimum inhibitory concentration (MIC), non-typical *E. amylovora* isolates were cultured overnight in LB broth, harvested by centrifugation, and washed twice using PBS. After the final wash, cells were resuspended in fresh LB medium and amended with serial two-fold dilution of antibiotics. MIC was defined as the lowest concentration of a certain antibiotic at which bacterial growth (OD_{600}) was less than 5% of that of no-antibiotic control (**Table 3**).

Objective 2. To determine the resistance nature of the pathogen (in progress)

Since we did not find any apple/orchard/sample/treatment containing any *E. amylovora* isolates that were resistant to the three antibiotics evaluated, we did not perform this task for the pathogen. However, we did discover that selected non-*Erwinia amylovora* isolates showing strong resistance to three antibiotics (**Table 3**). Since the mechanism of resistance in these non-*Erwinia amylovora* isolates were unknown, we attempted to determine the mechanisms for these isolates.

First, universal primers were used to amplify 16S rRNA and sequenced the gene to determine the identity of the isolates. Blast search was conducted to match the closest related species or genus to the unknow isolates. Then known primers for streptomycin resistance (*strA*-*strB*) (McGhee et al. 2011), oxytetracycline resistance (*tetA*, *tetO*, and *tetM* etc.), and kasugamycin resistance (*aac(2')-IIa* gene) were used and PCR were performed to determine whether these strains contain those resistance genes. In addition, if we suspect that resistance to streptomycin and kasugamycin was chromosomal, the *rpsL* and *ksgA* genes could be sequenced and compared to known resistance database. Furthermore, plasmids were isolated from unknown strains to determine whether resistance is plasmid-borne or intrinsic (chromosomal). These experiments are still ongoing.

Objective 3. **To immediately deliver results to growers and provide guidance on antibiotics use in orchards in the coming years**

Normally, after we collected the samples from orchards or received samples from growers, we had preliminary results within a week or two. The main method we used to communicate the results to growers/consultants was email. In the email, we indicated whether we recovered resistant *E. amylovora* isolate or not, what level of resistance was present in the orchard if any, and the percentage of pathogen population exhibited resistance. For the year 2022, we had done this accordingly and promptly informed the results to growers and consultants.

Types and timing of anticipated results:

The anticipated results include, but not limited to, know whether the *E. amylovora* population in survey orchards contain resistance to streptomycin, oxytetracycline, and kasugamycin, determine what is the level of resistance, if any, and understand the mechanism of resistance (plasmid or chromosomal). From these results, we anticipate making science-based recommendations regarding fire blight management decisions for orchards where resistance was detected and helping growers make timely in-season adjustments to their fire blight management programs. These results were already conveyed to growers within the funding year.

The unexpected results were for non-*E. amylovora* isolates, which exhibited high level of resistance to streptomycin, oxytetracycline, and kasugamycin. We expect to know what the identities

of these isolates were, what was the level of resistance, and the mechanism of resistance (plasmid or chromosomal), which should shed light on whether the resistance can be transferred or not and what is the risk for fire blight pathogen to expose to these resistant microorganisms. We expect to obtain these results soon. Upon completion of the project, we would have a clear picture of antibiotic resistance situation in WA and provide us much-needed information in helping growers how to choose and what antibiotics to use or avoid for fire blight management as well as possibility of rotation.

Potential problems or limitations that may be encountered:

- Due to lab renovation and supply chain issues, my lab was basically functional in late June. This led to a shortened season for the project for us.
- Although we put two announcements asking for fire blight samples (in Good Fruit Grower magazine and online newsletter Fruit Matters "Help Needed -- Fire Blight Samples"), the response was not as anticipated. In addition, during the fire blight advisory meeting in February and again in December, we have made and will make further arrangements in hope that this situation will change in next year's growing season.
- Another reason was that 2022 was a light fire blight year in most orchards, which also contributed to our sample collection issue.
- It was mostly unknow whether there was antibiotic resistance in the fields that we collected samples from in 2022. We will try to collect samples in orchards that were previously known to have antibiotic resistance in 2023 or 2024 growing season.

Results and Discussion:

In the current funding year, we started the project later in the season due to lab renovation. However, we managed to collect sufficient samples to conduct the research. Although the model predicted low to modest fire blight occurrence, some orchards in Benton city and Prosser had a bad fire blight year (**Fig. 1**). Samples were mainly collected in 12 locations in Washington State, including Kennewick, Benton City, Chelan, Cashmere, Prosser, Pasco, Wapato, Union Gap, Sunnyside, Othello, Hansen, and Wenatchee; and the samples were mainly from apple, including the following cultivars: Gala, Scifresh (Jazz™), Fuji, Cripps Pink (Pink Lady™), Cosmic Crisp, Minneiska (SweeTango™) and Scilate (Envy™) and WA38 as well as a few samples from 'Concorde' and 'Bosc' pear trees (**Table 1**). The orchards we collected samples from included both conventional and organic productions, and some were old orchards in full production and some were young orchards not yet in production. More than 150 treatments of the 50 samples were processed and a total of 85 isolates were stored from different plant samples, including shoots (11), suckers (1), cankers (3), leaves (32), petioles (21), fruits(13) and ooze (4). These isolates were then properly confirmed to be *E. amylovora* isolates (**Table 2**).

The main results from those processed samples/treatments were that, we found 100% *Erwinia amylovora* bacterial populations did not exhibit resistance to streptomycin (cannot grow at 100ppm), tetracycline (cannot grow at 25ppm), and kasugamycin (cannot grow at 200ppm). However, we found that 100% *Erwinia amylovora* bacterial populations can grow on plates containing 50ppm kasugamycin, which is somewhat troubling. In addition, 12 non-*Erwinia amylovora* bacterial isolates were collected, which exhibited various degrees of resistance to streptomycin (9), tetracycline (4) and kasugamycin (12) or all three of them, which is novel and surprising (**Table 3**). Among them, two each showed resistance to all three antibiotics at higher than 1200 ppm and higher than 200ppm, respectively. In addition, two and three showed resistance to both streptomycin and kasugamycin at higher than 1000ppm and higher than 200ppm, respectively. The rest showed resistance to

kasugamycin alone at 200, 800, and 2000ppm, respectively. We also revealed that some non-*Erwinia amylovora* isolates contain *strAB* and *tetA* genes, suggesting that they may contain streptomycin resistance with two types of mechanisms (**Table 3**). Below we discuss what these results mean to the industry.

Significance to the industry and potential economic benefits:

Antibiotics remain one of the best management tools for managing blossom blight. In WA, more than 95% of growers applied antibiotics for managing fire blight, especially blossom blight. Among the three antibiotics, streptomycin remains the better choice in terms of cost and efficacy in killing pathogens as compared to tetracycline and kasugamycin. However, tetracycline and kasugamycin use is on the rise in WA due to the occurrence of streptomycin resistance of the pathogen in the orchards. Although previously researchers had looked at streptomycin resistance in WA, those studies were mostly conducted from 1970s through 1980s (Loper et al. 1991) and mainly focused on cankers from pear orchards by looking at individual isolates. Since then, however, the extent of resistance in WA has not been comprehensively assessed.

In the current study, we found that 100% *Erwinia amylovora* bacterial populations did not exhibit resistance to streptomycin (cannot grow at 100ppm), tetracycline (cannot grow at 25ppm), and kasugamycin (cannot grow at 200ppm), which are excellent news for the industry. These negative results indicate that at least at surveyed orchards, the fire blight population remains resistant-less; and suggest that the antibiotics management strategy works well by reducing the number of streptomycin application or by using mixture of two antibiotics (streptomycin and tetracycline) in WA. However, we also found that 100% *Erwinia amylovora* bacterial populations can grow on plates containing 50ppm of kasugamycin. These results suggest that it is beyond possible that resistance to kasugamycin may soon be developed in *Erwinia amylovora* population, which is a worrisome phenomenon for the industry and we should pay close attention to.

On the other hand, only about 8% of growers were sure about streptomycin resistance, whereas 75.5% were not sure at all about streptomycin resistance in orchards in WA. During our current year study, this remains true as none of the orchards we visited was sure about whether they had resistance issue or not. Therefore, continuing survey and map antibiotics resistance in orchards in WA remains imperative.

The unexpected novel findings in the current study were that, we recovered 12 non-*Erwinia amylovora* bacterial isolates, which exhibited various degrees of resistance to streptomycin, tetracycline and kasugamycin or two or three of them. This is the first report about environmental microorganisms in orchards that confer multidrug resistance (MDR) to commonly used agricultural antibiotics, which should sound the alarm about the effect of antibiotic use in agriculture. The most troubling fact is that these microorganisms exhibited high level of resistance to two or all three of the antibiotics (>1000ppm), which is unusual. Further understanding this type of resistance will help us prepare if this indeed happens to the fire blight pathogen.

Since 1970s, the widespread multiple spray applications of antibiotics streptomycin, though giving growers one of the most effective means of controlling blossom blight of apples and pears, have led to the development of streptomycin resistant (Sm^R) strains in California, Oregon, Washington, Idaho, Utah, Missouri, Michigan, and New York as well as in Canada, Israel, New Zealand, Egypt, Mexico, and Lebanon. On the other hand, as a promising alternative for fire blight management, kasugamycin (Ksg, $kasumin^R$) was discovered in the 1960s and has been used in agriculture for many years to control rice blast caused by the fungus *Magnaporthe grisea*, rice bacterial grain and seedling rot caused by *Burkholderia glumae*, and rice bacterial brown stripe

caused by *Acidovorax avenae*. Recent study reports the emergence of kasugamycin-resistant *B. glumae* and *A. avenae* isolates, which again highlights the urgent need to monitor kasugamycin-resistant situation in fire blight pathogen population. Similarly, oxytetracycline has been used for fire blight management since 1970s and resistance to tetracycline was recently discovered in pear orchards in California.

In terms of streptomycin resistance mechanisms, the most common mechanism for *E. amylovora* isolates from the western US, including Oregon, Washington, California, Utah, and Idaho, was a point mutation in the *rpsL* gene, which confers high levels of resistance with minimum inhibitory concentrations (MIC) up to 2000 ppm. The most dominant type of streptomycin resistance for *E. amylovora* isolates from the eastern US, including Michigan and New York was the *strA-strB* associated transposon in plasmids pEA34, pEa8.7 and pEA29 or inserted in the chromosome, which confers relatively low levels of resistance with MIC at 500-750 ppm. In our study, we did not detect resistance in any samples of *E. amylovora* infection in 2022.

Like streptomycin, resistance to kasugamycin arises from mutations of its target gene *ksgA*, encoding an adenine demethylase, or present of a kasugamycin acetyltransferase gene aac(2′)-IIa, which acetylates kasugamycin. In contrast, many genes, including *tetABCDE* and *tetOMY*, are involved in tetracycline resistance in bacteria. The question remains is what are the mechanisms that render the 12 non-*Erwinia amylovora* bacterial isolates resistant to all three antibiotics in orchards. Preliminary data suggests the presence of *strAB* and *tetA* genes in some strains, however, the complete picture of the MDR in these non-pathogenic environmental isolates remains to be investigated, which are currently ongoing and the results of these investigation should shed light on the mechanisms of MDR.

In summary, the current results of this project directly benefit the growers of Washington state by providing instant feedback to growers in antibiotics resistance situation in orchards and growers could take immediate actions. Understanding the molecular mechanism of non-pathogenic resistant bacteria from orchards may shield light on whether *Erwinia amylovora* might develop resistance or not, which will help us prepare if this indeed will happen to the fire blight pathogen in the future.

Fig. 1. Examples of ooze drop (left) and severe outbreak of fire blight in a young orchard (right) in 2022.

Table 1. **List of samples, locations, and hosts**

Providers or Orchards	Location	Samples	Host
Gilbert Orchards (Chukar Orchard)	Kennewick, WA	10	Apple 'Gala' (organic); 'Scifresh' (Jazz™); 'Cripps Pink' (Pink Lady™) (Conventiona/organic)
Olsen Brothers Ranches Inc.	Benton city, WA	11	Apple 'Cripps Pink' (Pink Lady™); 'Fuji'
Tianna DuPont (WSU-TFREC)	Wenatchee	1	Unknown host
	Chelan, WA	1	Pear 'Concorde'
	Cashmere, WA	1	Pear 'Bosc'
Zirkle Fruit Company	Prosser, WA	3	Apple 'Gala' (organic)
Graystone Orchards	Pasco, WA	7	Apple 'Gala'; 'Cripps Pink' (Pink Lady™); 'Minneiska' (SweeTango™)
Douglas Fruit	Wapato, WA	1	Apple
GS Long Company	Union Gap, WA	6	Apple
Sportfisher LLC	Sunnyside, WA	6	Apple M9 rootstock; 'Granny Smith';
Teah (Zirkle Fruit Company)	Othello, WA	2	Apple 'Scilate' (Envy™)
	Othello-Hansen, WA	1	Apple 'Cripps Pink' (Pink Lady™)

Table 2. **Number of isolates stored from the field samples**

Plant Organ	# Isolates
Shoots	11
Suckers	1
Cankers*	3
Leaves	32
Petioles	21
Fruit	13
ooze	4
Total	85

Table 3. **Selected non-*Erwinia amylovora* isolates from the field samples exhibited resistance to antibiotics**

Isolate	Streptomycin	Oxytetracycline	Kasugamycin	*strA*	*strB*	*tetA*
Z2176	>2000	1200	>2000	-	-	-
Z2180	>2000	1200	>2000	+	+	+
Z2186	1200	200	300	+	+	-
ABR1	1200	200	200	+ +	+ +	-
Z2173	1600	100	1000	-	-	-
BWC	1400	12.5	1200	-	-	-
ABR5	>2000	12.5	400	+	+	+
ABR10	400	12.5	800	-	-	-
ABR6	400	12.5	200	-	-	-
ABR8	25	12.5	>2000	-	-	-
Z2175	25	12.5	800	-	-	-
EL51	25	25	200	-	-	-

Project Title: Pre- and postharvest disease management in organic apple systems
WTFRC Project Number: CP-19-103A
Report Type: Final Project Report

PI: **Achour Amiri**
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Cooperators: Dr. Wojciech Janisiewicz (USDA-ARS, Kearneysville); Certis, Sym-Agro, Marrone Bio, Wilbur-Ellis.

Project Duration: 3-Years/NCE

Total Project Request for Year 1 Funding: $67,715
Total Project Request for Year 2 Funding: $75,812
Total Project Request for Year 3 Funding: $80,991

Other funding sources: None

WTFRC Budget:

Item	2019	2020	2021
RCA Room Rental	6,300*	6,300	6,300
Total	6,300	6,300	6.300

Footnotes: RCA room(s) will be used to assess the efficacy of regular CA versus DCA for decay reduction.
*RCA rooms have not been used in 2019, so this amount will be used in 2020

Budget 1

Primary PI: Achour Amiri
Organization Name: Washington State University
Contract Administrator: Anastasia (Stacy) Mondy
Telephone: 509-335-2587
Contract Administrator Email Address: arcgrants@wsu.edu

Item	2019	2020	2021	2022
Salaries[1]	38,400	39,936	41,533	0
Benefits[1]	14,008	14,569	15,151	0
Wages	0	0	0	0
Benefits	0	0	0	0
Equipment[2]	10,000	0	0	0
Supplies[3]	2,200	18,200	21,200	0
Travel[4]	1,007	1,007	1,007	0
Miscellaneous	0	0	0	0
Plot Fees[5]	2,100	2,100	2,100	0
Total	**67,715**	**75,812**	**80,991**	**0**

Footnotes:

[1] Salary for a PostDoc at $4,000/ month for 12 months at 0.8 FTE and benefit rate of 36.5%. The PostDoc will work jointly between Amiri' and Zhu's labs as needed.

[2] Funds for Safepod **or** Harvestwatch system for the DCA study in the present project and will be used in planned future research. We expect to obtain additional funds for one of these systems from the pending WSDA-SCBG grant if funded.

[3] Supplies include biological and microbiological reagents for fungi and fungicide tests, manuscript publication fees. In Year 2 and 3, we budget funds for molecular reagents and microbiome analyses work.

[4] Domestic travel to orchards in WA for trials, sampling and outreach activity.

[5] Annual plot fees for an experimental block at Sunrise to be used for the work outlined in the proposal below.

OBJECTIVES

1- Evaluate the adequacy and efficacy of current and novel preharvest management organic strategies.
2- Evaluate the benefits of using dynamic control atmosphere (DCA), GRAS products and biocontrol agents to control rots in storage.
3- Acquire novel knowledge about the impact of different spray regimes and storage conditions on fruit microbiomes pre- and postharvest to enhance management in the future.

SIGNIFICANT FINDINGS:

- The efficacy of 7 organic preharvest materials has been tested in 2019 and four to five of them show very good efficacy. In the 2020-21 season, 15 materials have bee tested preharvest. Efficacy of newer materials is seen, and consistent efficacy of the materials tested in 2019 is confirmed for most organic materials.
- The efficacy of these products was confirmed using artificial inoculations (Activity 1.2).
- Four most effective materials from 2019 trials were selected and tested in 2020 to develop a seasonal spray program (Activity 1.3) to enhance decay management. Overall decay incidence after 9 months of storage was positively proportional to the number of preharvest sprays.
- DCA and static CA (Activity 2.1) showed variability in reducing the incidence and severity of blue mold, gray mold, Mucor rot, Speck rot, and bull's eye rot. Trials conducted in the 2020-21 season confirm the slight advantage of DCA over CA or RA in reducing decays.
- Outreach: Some of the preliminary data have been provided to the stakeholders via meetings in 2020 and 2021.

Results and Discussion

Objective 1:

Activity 1.1. Efficacy of preharvest materials in reducing postharvest decays

Season 2019-20

Nine treatments, including a control (non-treated), a conventional fungicide (Merivon) and seven organic materials sprayed 7 days preharvest on a Fuji block in East Wenatchee in 2019.
After 8 months of storage at 34F in a regular atmosphere, overall decay (all decay types) incidence was 99% in the control and was reduced to below 50% by 5 organic products with Serenade Pro, Double Nickel and Regalia being the most effective (Figure 1). While OSO, provided a good efficacy against gray mold and blue mold when data were analyzed for each pathogen (Data not shown), it reduced overall decay incidence by 30%.

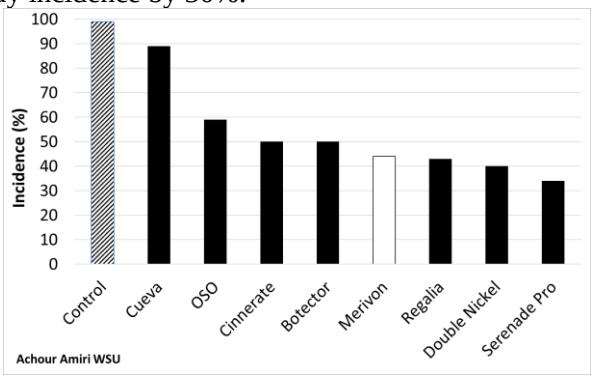

Figure 1. Overall decay (all decays combined) incidence on Fuji treated with the materials 7 days preharvest and stored at 34°F in a regular atmosphere for 8 months. Season 2019-20.

Season 2020-21

In 2020, 12 organic materials were sprayed 7 days preharvest and compared to the conventional Merivon and a non-treated control. The efficacy of most materials tested in 2019 was consistent and the 2020 trial showed that 8 organic materials significantly reduced overall decay incidence after 9 months of RA storage (Figure 2)

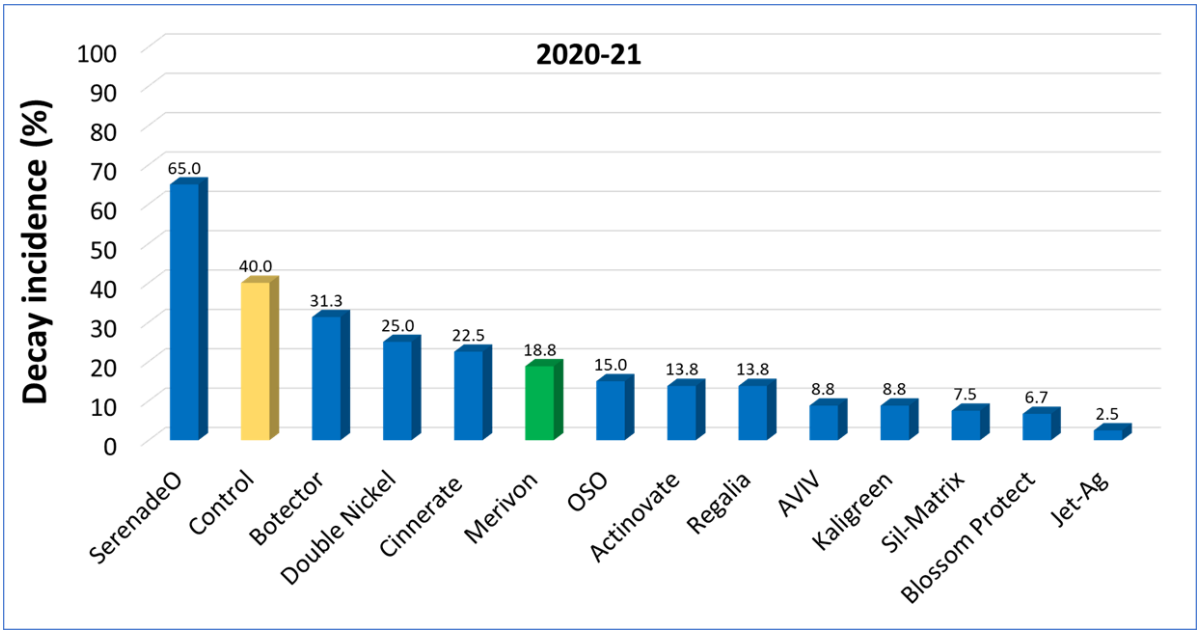

Figure 2. Overall decay (all decays combined) incidence on Fuji treated with the materials 7 days preharvest and stored at 34°F in a regular atmosphere for 8 months. Season 2020-21.

The trial was repeated during the 2021-22 season and data from the 3 or 2 seasons (Kaligreen, Cueva, Sil-Matrix, Jet-Ag, Actinovate) were averaged and shown in Figure 3.

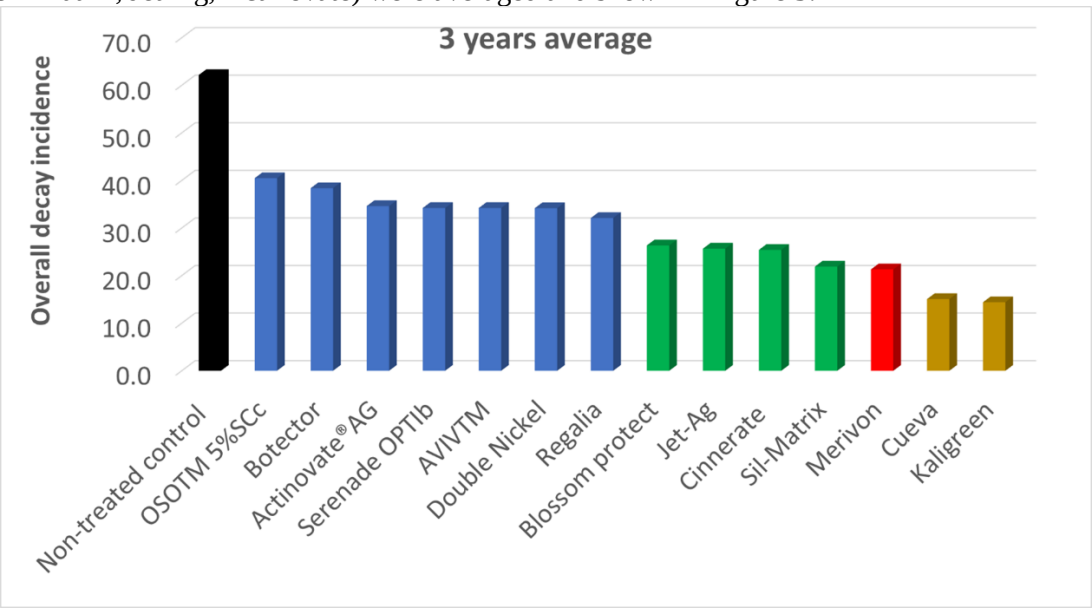

Figure 3. Average decay incidence (2 or 3 seasons) on Fuji treated with the materials 7 days preharvest and stored at 34°F in a regular atmosphere for 8 months.

<u>Activity 1.3.</u> Development of a timely preharvest organic spray program

Four organic materials which showed a good efficacy from Activity 1.1 were selected and sprayed sequentially from petal fall to 7 days preharvest. Results from the 2020-21 and 2021-22 seasons showed that decay incidence after 9 months of RA storage was better optimized with 3 to four sprays after petal fall especially for long term storage (Table 1). Some of the materials which showed a greater efficacy than the ones included in Table 1 have been tested in 2022-23 and data will be shared with stakeholders in 2023. We are also looking at overall spay programs that take into consideration other major preharvest diseases such as mildew and fire blight.

Table 1. Mid- and long term storage efficacies of different organic materials sprayed at different phenological stage.

# of	Spray conducted at				Storage term	
Sprays	Petal Fall	Fruitlet	Green Fruit	7 DPH	Mid-term (6 m)	Long-term (9 m)
3	OSO 5%	Cinnerate		Botector	100	100
3	Cinnerate	OSO 5%		Botector	100	90
4	Cinnerate	OSO 5%	AVIV	Botector	100	77
2	OSO 5%			Botector	91	41
3	OSO 5%		AVIV	Botector	90	64
1				AVIV	90	56
1				OSO 5%	90	27
1				Cinnerate	70	22
3	Cinnerate		AVIV	Botector	61	9
2	OSO 5%			AVIV	27	22
2	Cinnerate			AVIV	24	22
1				Botector	22	12
2	Cinnerate			Botector	-17	-72

Objective 2.

<u>Activity 2.1</u>. Efficacy of ultra-low oxygen (ULO) on decay development

Trials were conducted in 2020 and 2021 on Fuji artificially inoculated with spore suspensions of *P. expansum* (blue mold), B. cinerea (gray mold), *N. perennans* (bull's eye rot), *M. piriformis* (Mucor rot) and *P. washingtonensis* (in 2021, speck rot). Fruit were then stored in 3 different atmospheres, regular (RA), CA (O_2 = 4% and CO_2 = 0.8%), and ULO (O_2 = 1.5% and CO_2 = 0.8%) and stored at 35°F for 6 to 8 months. Results shows that ULO program tested in 2020r had a slight reduction of incidence of blue mold, gray mold, and bull's eye rot but not mucor (Figure 4). In 2021, DCA showed similar efficacy with greater reduction seen for speck rot and bull's eye rot.

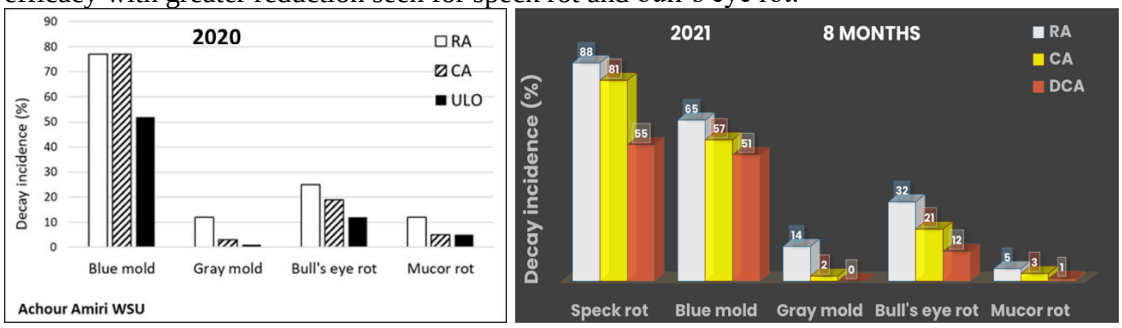

Figure 4. Average decay incidence of 5 major postharvest diseases on artificially inoculated Fuji stored in 3 different atmospheres for 6 months in 2020 and 8 months in 2021.

Objective 3. Acquire novel knowledge about the impact of different spray regimes and storage conditions on fruit microbiomes pre- and postharvest to enhance management in the future.

Apples from the cultivars Gala and Honeycrisp were collected from four orchards, i.e., one conventional and one organic orchard, for each cultivar in 2021 (Gala) and 2022 (Honeycrisp). Twenty Gala apples were harvested at commercial maturity from orchards located near Brewster, WA. Three samples, i.e., peel, calyx-end and stem bowl, were obtained from each fruit. DNA was extracted from the samples and microbiome analyses using 16 S rRDNA (bacteria) and ITS3 x ITS 4 (fungi). Samples from Gala from Washington State were compared to Gala samples from 8 other countries (Figure 5). Gala from Washington state seem to harbor less fungal species compared to Gala from other European countries (Figure 5). Interestingly, organic and conventional Gala apples from WA were not significantly different in term of fungal diversity although the organic apples had a slightly greater diversity.

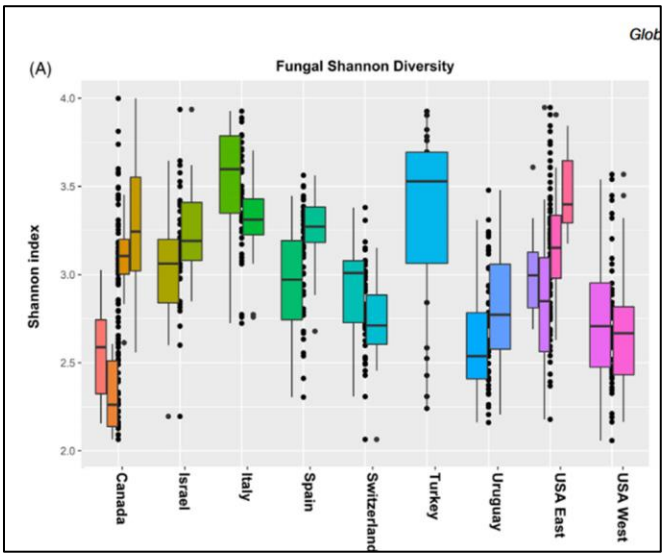

Figure 5. Box plots showing the fungal diversity (Shanon index) of apple samples from WA (right pink bars) in comparison with 7 other countries.

The fungal population of WA Gala apples seems to be the closer from that of Italian Gala than Gala from the US-east coast (Figure 6 b), whereas the bacterial population of WA Gala seem to be distinct from those of East-coast Gala and the 7 other countries surveyed (Figure 6d)

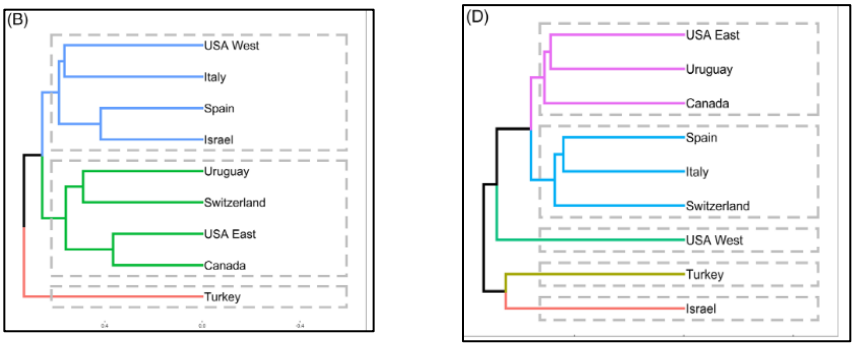

Figure 6. Dendrogram of hierarchal clustering showing the similarity between fungal (B) and bacterial communities of Gala apples from different countries.

Analysis of fungal and bacterial communities from Gala apples form a conventional orchard (B) and an organic orchard in WA, showed strong similarities between tissue types from two different systems with the peel harboring more diverse fungal and bacterial communities and organic apples usually having a slightly more diverse populations but not significantly different or conventional apples (Figure 7).

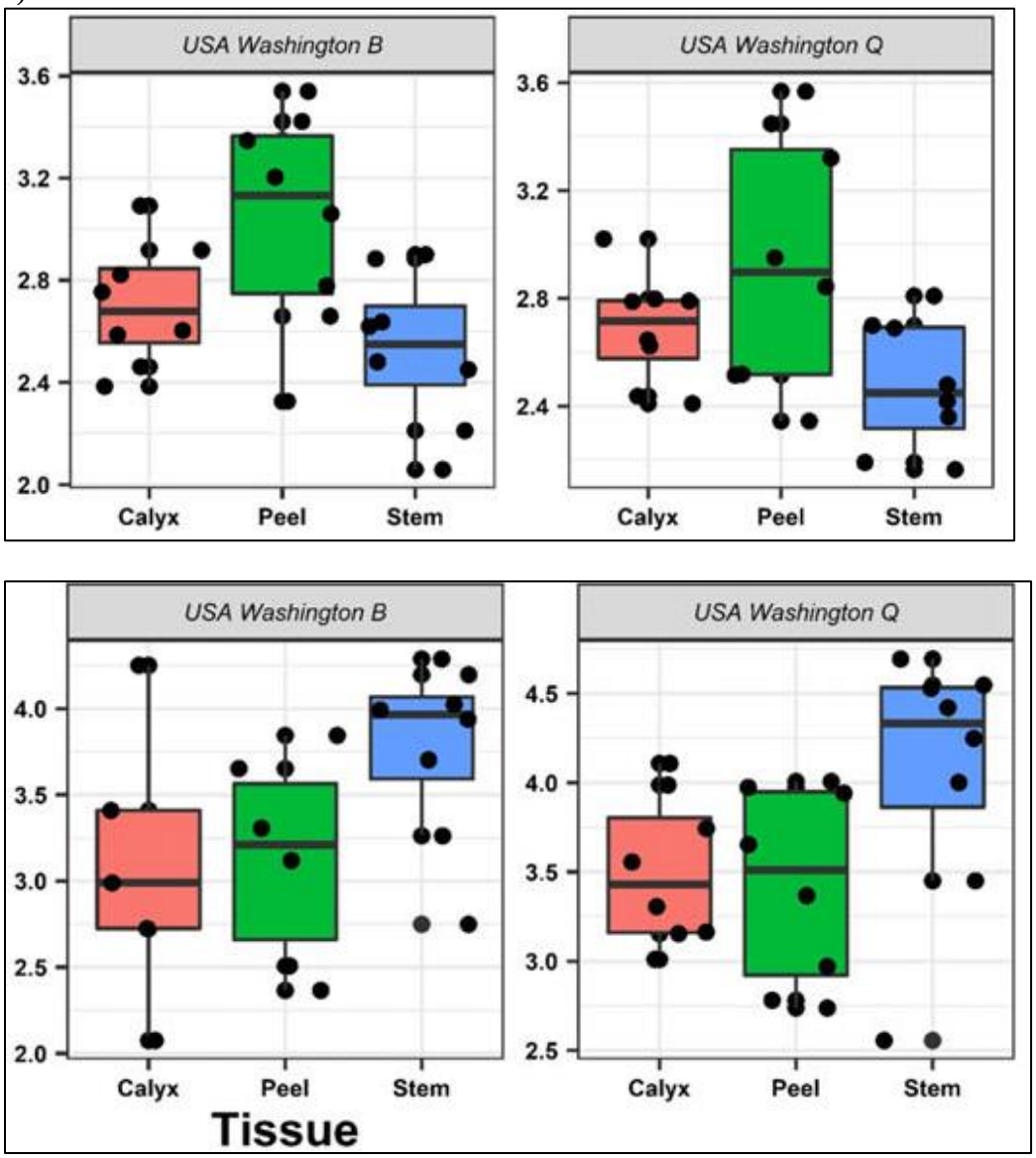

Figure 7. Box plots showing the fungal diversity (top) and bacterial diversity (bottom) among apple tissues, Calyx, peel, stem, of Gala apples collected from a conventional (USA Washington B) and an organic (USA Washington Q) in Washington State in 2020.

Samples (flowers and fruit) from Honeycrisp were obtained from one conventional and one organic orchard in Quincy in 2021. Beside flowers collected at full bloom, fruit were collected at green stage (July), at commercial maturity (harvest), and after 3 months of storage in a regular atmosphere. For fruit, 4 sample types, i.e., cuticle, flesh, stem-end, calyx-end were obtained. DNA was extracted and stored at -80°C (-121°F). Samples are awaiting microbiome analysis which was delayed due to our initial collaborator taking a different position. Results are expected in 2023 and will be shared through extension meeting in 2023 and Workshop in 2024.

Executive Summary

Project title: Pre- and postharvest disease management in organic apple systems

Key words: Decays, preharvest, postharvest, organic management, DCA

Abstract: Herein, we report the efficacy of most current organic material commonly used by organic growers preharvest against major postharvest decays. Fourteen organic materials were screened for 3 consecutive seasons in the field and on detached fruit using artificial inoculations. Materials included biologicals, plant extracts, salts and bio-fungicides. Our results show the ones that are expected to provide a good level of efficacy up to 8 months in storage when applied prior to harvest. The variability in efficacy seen in some cases for the same product between seasons indicate potential inconsistencies known, especially, for biologicals. This also warrants alternation of different materials throughout the season to optimize control in storage. We have also compared different spray programs at different phenological stages to optimize postharvest management. One spray, 7 to 0 day preharvest may be enough for short term storage (up to 4-5 months) but may not protect fruit longer. We found that sprays at petal fall and fruit set, using different materials, in addition to a preharvest spray can significantly reduce decay incidence for up to 8 months. To optimize decay control in organic systems further, we assess the efficacy of a dynamic controlled atmosphere in reducing decays. While the approach still requires some fine-tuning across cultivars, DCA can reduce the development of some latent pathogens such as *B. cinerea* (gray mold), *P. washingtonensis* (Speck rot), and *Neofabraea spp.* (bull's eye rot) better than standard static CA. Based on the results from Gala collected from two orchards in the same location (Brewster), organic and conventional apples from WA do not seem to differ significantly in term of overall bacterial and fungal populations they harbor. While the results need to be discussed in more details in relation to postharvest d pathogens, they could indicate that the last preharvest treatment applied just before harvest may not have significantly impacted the fungal and bacterial populations in the short period of before fruit processing.

Additional Items

Grants

Funds from this grant were leveraged to secure tow extra-mural grants, one from the Organic Research Extension Initiative (OREI) and another from the Western SARE Program to further continue research and extension efforts to support organic growers and packers in Washington.

1. Pre and postharvest disease management of pome fruit to support an expanding organic production in the Pacific Northwest. USDA-Western SARE. $349,887. P.I.: A. Amiri, Co-PIs. K. Gallardo (04/22-08/25).
2. A systems-based approach to enhance quality, safety, and shelf life of organic tree fruit in the Pacific Northwest. USDA-NIFA-OREI. $1,499,887. P.I.: A. Amiri, Co-PI.: C. Torres, F. Critzer, K. Gallardo, B. Sallato (07/21-04/25)

This is equivalent to $8 brought for each $1 invested by the WTFRC in this project.

Abstracts and Talks:

1. Amiri A. 2023. Organic Disease Management: Challenges and Opportunities. OPDMC, Portland, OR. Jan 12th 2023.

2. Amiri A. and Fomba J. 2022. *In vitro* activity of several organic materials against the major postharvest pathogens of Pome fruit. Annual Meeting of the American Society of Phytopathology. Pittsburg, PA. August 2022.
3. Amiri A & Fomba J. 2022. Update on efficacy of organic materials to minimize decay in storage. WA Tree Fruit Association Annual meeting. Dec 6th, 2022.
4. Amiri el. 2022. Webinar on organic decay management: provided 4 talks related to organic decay management. Attendees: 90.
5. Amiri A. 2021. Update on efficacy of organic materials and research needs to minimize decay in Honeycrisp. WA Tree Fruit Association Annual meeting. Dec 7th, 2021.

Project/Proposal Title: Detect Sources of Patulin Contaminations in Processed Apple Products

Report Type: Continuing Project Report Year 2

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Cooperators: Tree Top, multiple packers in Washington state

Project Duration: 3 Years
Total Project Request for Year 1 Funding: $100,638
Total Project Request for Year 2 Funding: $112,312
Total Project Request for Year 3 Funding: $53,372

Other funding sources: Requested
Amount: $50,000/year
Agency Name: Tree Top
Notes: Funds will be split between Amiri and Lee labs based on efforts

WTFRC Budget: ***None***

Budget 1
Primary PI: Achour Amiri
Organization Name: WSU
Contract Administrator: Stacy Mondy
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Contract administrator email address: arcgrants@wsu.edu
Station Manager/Supervisor: Chad Kruger
Station manager/supervisor email address: cekruger@wsu.edu

Item	2021	2022	2023
Salaries[1]	32,760	34,070	35,433
Benefits[2]	13,006	13,526	14,067
Wages	0	0	0
Benefits	0	0	0
Equipment	0	0	0
Supplies[3]	4,900	4,900	3,400
Travel[4]	472	427	472
Plot Fees	0	0	0
Miscellaneous	0	0	0
Total	51,138	52,923	53,372

[1,2] Salaries for a Postdoc scientist (0.65 FTE) at benefits rates of 39.7%. A 4% annual inflation is included for Years 2 and 3.
[3] Supplies include media and regents to grow and maintain fungi to be collected in objective 2. Funds are requested for molecular reagents and sequencing to characterize fungi and strains of *Penicillium* with ability to produce patulin.
[4]Travel to processing facility for sampling and collection and to travel to extension meetings.

Budget 2: Hyun Jung Lee
Organization Name: University of Idaho **Contract Administrator:** Sarah Martonick
Telephone: 208-885-2145 **Email address:** postaward@uidaho.edu
Supervisor or Station Manager name and email address (if applicable):

Item	2021	2022	2023
Salaries[1]	21,934	22,592	0
Benefits[2]	7,705	7,936	0
Wages			0
Benefits			0
Equipment			0
Supplies[3]	18,000	27,000	0
Travel[4]	1,861	1,861	0
Miscellaneous			0
Plot Fees			0
Total	49,500	59,389	0

[1 & 2] Salaries for Co-PI Lee (0.125 FTE) and Co-PI Ryu (0.025 FTE), and benefits rate of 30.7%. Additional salary for a technician (0.25FTE) with benefits rate of 41.8%. A 3% annual inflation is included for Year 2.

[3] Supplies and chemicals for the lab works including instrumental analyses of patulin and screening of isolates for the capacity of patulin production in apples.

[4] Bi-monthly travel to collaborating work sites based on the mileage (440 mi round trip) and per diem ($55/day)..

OBJECTIVES

1. Characterize the different fungal species, occurring on the surface and the core of apples, that may produce patulin in lots/cultivars with history of patulin contamination. Year 1 and Year 2
2. Characterize the ability of the different recovered species and subsamples of each species to produce patulin using biochemical and molecular methods. Year 1 and Year 2.
3. Develop and validate an efficient and quicker strategy for patulin detection in experimental and commercial fruit. Year 2 and Year 3.
4. Update and improve existing SOP to better mitigate patulin and conduct outreach activities for growers, packers, and processors. Year 2-3.

Significant Findings

- Rot-causing fungi were associated with apples recovered from the orchard and at the processing facilities.
- Fungi associated with fruit decay were isolated from both symptomatic and asymptomatic apples from diverse apple cultivars including Gala, Honeycrisp, WA38, Spur Golden, Golden delicious.
- Majority of recovered fungi belonged to *Penicillium* species, however other fungi were recovered and may be important in patulin contamination
- *Penicillium expansum* was the most recovered species, however other *Penicillium* species were isolated from rotten apples
- A one-step cleanup column, MycoSep288 Aflapat column, evaluated for the removal of 5-hydroxy methyl furfural (5-HMF) formed during HPLC was unsuitable due to low recovery of patulin in juice sample.
- A next-generation cleanup column, SupelMIP SPE column, which uses a molecularly imprinted polymer, did not provide sufficient measure of patulin in apples as specified by the manufacturer. However, it provided a viable alternative to traditional solid phase extraction column for quantifying patulin in apple juice

METHODS

OBJECTIVE 1. Characterize the different fungal species, occurring on the surface and in the core of apples. [Amiri, Years 1-2]

Develop a rational sampling protocol to collect fruit from processing facilities. For this study, we will use Honeycrisp and Gala because recent patulin contamination issues have been reported on these two cultivars. Fruit infection by pathogens may vary between seasons and may render pathogen detection challenging if disease pressure is low which may impact the relevancy of our results. To optimize detection, we propose two sampling approaches:

Approach A: Sampling decayed apples prior to fruit processing. This approach will target pathogens that are visible and present at the surface of the fruit. We will coordinate with Tree Top collaborators as they prepare fruit lots for processing. In Year one of this study, 10 lots (growers or packers) will be sampled, and the number can be increased in Year 2 as needed based on data from Year 1. Ideally, the same lots sampled in Year 1 will be included in Year 2. Tree Top staff will randomly collect 50 decayed apples from different bins all from the same lot using a protocol provide by Amiri. Fruit will be placed in clamshells to avoid cross-contaminations and will be transferred to the plant pathology lab in WSU-

TFREC, Wenatchee for further analyses. Fruit will be photographed, and a first diagnostic will be made based on symptoms, lesion consistency, smell, and origin of infection (wound, stem-bowl, calyx-end). A small fruit chunk will be cut at the growing margin of each decayed lesion from each individual apple and will be plated on agar medium amended with antibiotics to eliminate bacterial contaminations (Amiri and Bompeix, 2005). If more than one lesion is observed on a given apple, samples will be taken from as many lesions as present on each fruit. Plates will be incubated for 5 to 10 days (depending on the pathogen) at 68°F and checked for fungal growth. Cultures will be separated into known and unknown pathogens and new fungal cultures will be made from the original plates using a medium without antibiotics for further analyses described in Objective 2.

Approach B: Random sampling of asymptomatic apples prior to fruit processing. A recent study conducted by Tree Top reported high patulin (PAT) levels in apples without visible symptoms nor on the fruit surface neither in the fruit core. To shed light in this issue and to target potential endophyte (inside fruit) infections occurring in the apple cores, we will use a second sampling to complete data from Approach A described above. Because PAT detection, at the processing facility, is conducted on the resulting juices, it is critical to try to link unusual PAT levels with the original fruit lot. Therefore, Tree Top staff will randomly collect 50 asymptomatic (Honeycrisp or/and Gala) prior to starting fruit processing for juice production. In Year one of this study, 10 lots will be sampled, and the number can be increased in Year 2 as needed based on data from Year 1. The juice obtained from these lots will be analyzed by Tree Top Staff for PAT levels, and in case of high patulin levels, the 50 apples sampled for that given "positive lot" will be labeled and transported to WSU-TREC for analyses. Apples will be sliced in two halves to expose the seed pocket for inspection for visible fungal mycelia. In this case, part of the mycelia will be aseptically transferred to agar media, incubated, and used for identification as described above of Approach A. *For both approaches A and B, fruit will be sampled right before fruit are processed to eliminate any interference of additional storage temperature or atmosphere on the PAT levels in the sampled fruit*.

Activity 1.2. Identification of fungal pathogens collected from commercial apples. Fungal colonies obtained from apples sampled in Activity 1.1 will be initially maintained on potato dextrose agar for initial identification using key morphological and microscopic traits used by Amiri Lab. Known species will be immediately frozen in 20% glycerol for long-term storage. Unknown species will be grown and resulting mycelial will be used for DNA extraction and molecular sequencing using ITS markers previously developed (White et al. 2000). All identified species will be numbered and grouped separately to determine the number of strains (from each species) to be used for PAT investigation in Objective 2.

Expected outcomes. The two sampling strategies will provide insights into potential differences between symptomatic and asymptomatic patulin contamination and the fungi that are associated with both type of patulin contamination. Sampling carried out in at least over a two-year period should provide a robust assessment on the species of fungi that are associated with patulin contamination in Washington State processing apple industry. Identification of the fungi using standard procedures (morphological and molecular methods) should confirm the identity of the fungi recovered in the sampling.

Potential pitfalls and limitations. We were very successful in this objective in our first-year sampling and expanded the sampling beyond Gala and Honeycrisp varieties that was earlier reported. We surveyed 11 lots in the first year, comprising both symptomatic and asymptomatic apples and expanded our sampling beyond the proposed Tree Top to Stemilt. While inclusion of other parking houses could improve the breadth of the sampling, TreeTop and Stemilt are two of the leading parking houses in

Washington State and should provide a good insight into drivers of patulin contamination in processing houses. Molecular identification offsets any potential pitfall in morphological identification. However, identification of the few unknowns takes some extra measures in PCR optimization.

OBJECTIVE 2. Characterize the ability of the different recovered species and subsamples of each species to produce patulin using biochemical and molecular methods. [Amiri & Lee, Years 1-3]

Activity 2.1. Selection of fungal strains to assess their ability to produce patulin (PAT). The fungal contaminants that will be collected from sampling and identification outlined in Objective 1, fungi will be grouped into 2 groups: Group I will include species known to produce PAT and Group II will include species not known to produce PAT. Depending on the number of strains in each group and species, sub-samples of strains will be selected, i.e., fruit from different lots, core vs. surface, cultivars, storage duration at the warehouse/processing facility. We expect to collect *Penicillium*, *Alternaria, Aspergillus,* and some *Fusarium* strains, known for PAT production, but we will include a sub-sample from the other species for subsequent analyses. We will initially aim for 10 strains from each species, but the number can be changed in Year 2 to fit the needs of the study.

Activity 2.2. Evaluation of a sub-sample of fungal contaminants for their toxigenic potential on detached apples. To determine the potential of contaminating apples during storage, selected fungal strains identified in Activity 2.1. will be transported to Co-PI's lab to be tested on apples for their capacity to produce PAT.

Apples (cv Honeycrisp, Gala and Golden Delicious) harvested at commercial maturity will be surface disinfected for 5 min in 0.6% sodium hypochlorite solution, rinsed three times with sterile water, and air-dried in the laminar flow hood. Each fruit will be inoculated by two different methods for each testing group: (i) to mimic contamination in the flesh, each apple will be punctured with the point of a 3-mm-diameter finishing nail to a 3mm depth. Approximately 1 hour after puncture, fruit will be inoculated with spore suspension (20 µL of 10^5/mL) or sterile water as a positive control, or (ii) to replicate the fungal contamination in the core, the same amount of spore suspension will be injected into the center of each fruit using a sterile needle and syringe. Inoculated apples will be stored at 1°C for 8 weeks then analyzed for the concentration of PAT formed by HPLC method described below (Objective 3). We will use three cultivars because PAT has been reported to be cultivar dependent (Snini et al. 2016).

Activity 2.3. Molecular investigation of fungal contaminants for presence of know patulin-related genetic markers. While testing a sub-sample of strains on detached fruit is a good way to assess their PAT production ability, we need to screen a large number of strains in order to see the big picture. For example, our recent study from WA warehouses, showed that about 25% of *Penicillium* species causing blue mold decay on pome fruit may not be *P. expansum*. This group has shown different virulence and sensitivity to the postharvest fungicides than the know *P. expansum* species. Their PAT production levels are unknown. For these and the strains to be collected in span of this project, we will screen up to 500 strains for the presence of gene clusters known to be related to PAT production (Tanous et al., 2014; Artigot et al. 2009). DNA will be extracted from the 500 stains using standard lab protocols used in Amiri' lab. Primers developed by Tanous et al. (2014) for *P. expansum* and other to be developed in this project for the other species found in WA will be used to screen for the presence of the PAT-related clusters or part of the clusters.

OBJECTIVE 3. Develop and validate an efficient and quicker strategy for patulin detection in experimental and commercial fruit. [Lee & Ryu, Year 1-2]

*Activity 3.1. Evaluation of analytical methods to detect patulin in apples and apple products***.** To determine practical strategy to test patulin (PAT) levels in the industry, a fast and reliable detection method should be first identified. Among all known analytical methods, immunochemical tests or enzyme linked immunosorbent assays are considered as the only rapid test applicable to commercial

settings. Hence, all commercially available patulin detection kits will be purchased and compared with high-performance liquid chromatography (HPLC) for their accuracy and reproducibility.

HPLC Analysis

Preparation of PAT standard solution – A stock standard solution of PAT will be prepared by dissolving pure crystalline patulin in acetic acid buffer (pH 4.0). The stock solution will be stored at -20°C until use. Working standard solutions will be prepared by appropriate dilution of this solutions with acetic acid buffer (pH 4.0). Acetic acid buffer solution will be prepared by adding 0.45 mL acetic acid glacial to 40 mL of distilled water, then dissolving the 0.245 g acetic acid sodium trihydrate in the above solution, followed by adjusting the pH to 4.0 with acetic acid glacial. The volumes will be adjusted to 50 mL with distilled water after the pH titration procedure. The buffer solution will be stored in an amber bottle.

Matrix solid phase dispersion (MSPD) and extraction procedure – Analysis of PAT on the apple or apple juice concentrate will be carried out by following the method (Wu et al., 2008). Material for MSPD will be C18-bounded silica (mean particle size: 40-75 µm, average pore size: 100 Å) made in Bestown Company in America. The C18 bonded silica material (22 g) will be pre-conditioned with 15 mL hexane, 15 mL dichloromethane, 15 mL methanol respectively before use, then the C18 material will be dried. 0.5 g samples will be blended with 2.0 g of a C18 material using a glass mortar and pestle. After finishing the MSPD blending process, it will be packed into a 10 mL empty cartridge constructed from syringe barrel, containing 0.4 g sodium sulfate anhydrous and a frit that retains the entire sample. The sample will be then compressed to form a cartridge by using a modified syringe plunger. About 0.5 g sodium sulfate anhydrous and a second frit may be placed on top of the material before compression. The principles of performing good chromatography will be always applied: one should avoid channels in the column and not over-compress or compact the material. Then the MSPD column walls will be washed with 3 mL hexane and the column packing dried with a strong stream of air. These elutes will be discarded, and then the receiver replaced by an amber vial with screw cap. The column will be eluted with 3 × 3 mL dichloromethane and the column packing dried with a strong stream of air. The flow of each portion will be stopped for approximately 1 min to allow the solvent sufficient contact time with the column packing. Then the combined solution added to a 1 drop of acetic acid glacial will be evaporated just to dryness in a heating block at 40°C under a gentle stream of nitrogen. The residue will be immediately dissolved in 0.5 mL of acetic acid buffer solution and 20 µL of this solution will be injected into the HPLC system.

Analytical procedure – The final solution will be analyzed under the following conditions by HPLC (Agilent 1260 Infinity HPLC system, USA): the analytical column will be Phenomenex (250 mm × 4.6 mm I.D., 5 µm C18 stationary phase); mobile phase will be acetonitrile-water (1:10, v/v), with flow rate at 1.0 mL/min; UV detector wavelength set at 250 nm; sample injection will be 20 µL.

Calculation of results – the amount of PAT in the final solution will be determined by using a calibration graph of concentration vs. peak area and expressed as mg/kg. The PAT content (C) of the apple juice will be found by using: C (mg/kg) = $C_{sample} \times V \times 1000 / m$; where C_{sample} is the concentration of PAT the final solution (mg/mL), v is the total volume of the final solution (mL) and m is the volume of apple juice taken for extraction (g).

PAT analysis using ELISA

All commercial PAT ELISA kits available in the market, including Patulin ELISA kit (DEIANJ49, Creative-Diagnostics, USA), Patulin (RND99084, Reagen®, USA), and Patulin ELISA test kit (Unibiotest, China), will be used for their performance in detecting PAT from apples and apple products including puree and juice. Samples and standards will be analyzed using the manufacturer's protocol, and the optical density will be read using a microplate reader (Tecan Sunrise, Switzerland).

Performance variables to be considered are: limit of detection (LOD) and limit of quantification (LOQ), range of quantification, inter- and intra-day variability, and total time for analyses.

OBJECTIVE 4. Conduct outreach activities for growers, packers, and processors. [Amiri, Year 2-3]
Talks: Amiri will present data from Year 1 and Year for presentation at local and regional commodity meetings in Years 2 and 3. This will include the Northwest Field Days, Hortshow and Apple Review days.
Publications: Amiri and Co-PIs will publish findings in peer-reviewed journals in Year 3. Amiri will summarize data and publish a newsletter in the WSU Fruit Matter to update the industry on the major findings from this project.

Results and Discussion

Objective 1. Characterize the different fungal species, occurring on the surface and the core of apples, that may produce patulin in lots/cultivars with history of patulin contamination. Year 1 and Year 2

Activity 1.1. Develop a rational sampling protocol to collect fruit from processing facilities. As part of activities to realize the objectives under this activity, we carried out surveys of packinghouses in the 2021-22 season. Sampling was carried out using the two described approaches of sampling decayed and asymptomatic apples. We made extended our survey to other fruit facilities and additional cultivars than the one included in the initial proposal. We obtained samples from 11 lots including five cultivars such as Spur Golden, Golden Delicious, Honeycrisp, Gala and WA38 (Table 1).

Table 1. Number of apples per lot and the associated sources and cultivars

Lot	Source	Cultivar	*n* apples
1	Orchard	Gala	50
2	Orchard	Spur Golden	25
3	TreeTop	Gala	52
4	Stemilt	Gala/Golden Delicious	134
5	Stemilt	WA38	72
6	Stemilt	Honeycrisp	36
7	TreeTop	Gala	52
8	TreeTop	Gala	56
9	TreeTop	Gala	56
10	TreeTop	Honeycrisp	52
11	TreeTop	Golden Delicious	54

We recorded visual observations upon sampling of the lots. Lots 11 (Golden Delicious), 10 (Honeycrisp) and 3 (Gala), which were symptomatic at sampling, showed higher wound frequency (Figure 1a) and decay incidence (Figure 1b). Lots 1 and 2, which were collected directly from the orchard, had no visible wounds but apples from lot 1 developed decay after 21 days incubation at room temperature (Figures 1a,b).

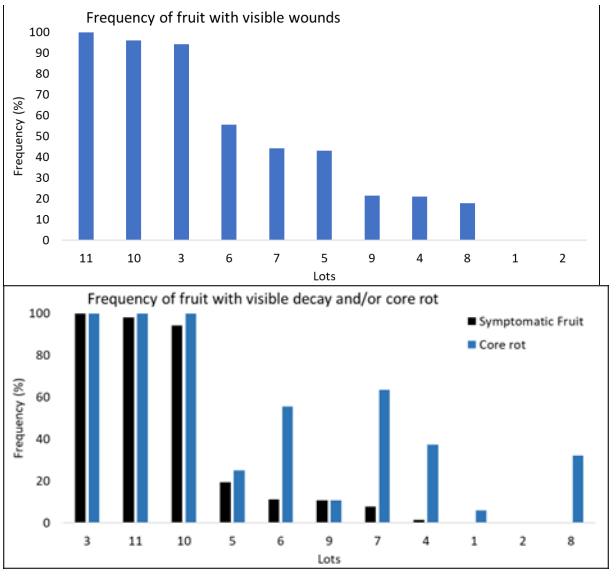

Figure 1. Frequency of fruits with visible wounds at sampling (a) and frequency of fruit with visible decay and core rot (b).

About 544 fungal isolates were recovered from symptomatic and asymptomatic apples from the 11 lots surveyed in 2021-22 (Figure 2a). The number of fungi recovered from the lots were marginally positively correlated with the wounds (R^2 = 0.09) and visible decay (R^2 = 0.014) (data not shown), whereas when asymptomatic fruits were incubated at room temperature for > 21 days, the correlation was slightly stronger (R^2 = 0.1978) (Figure 2b).

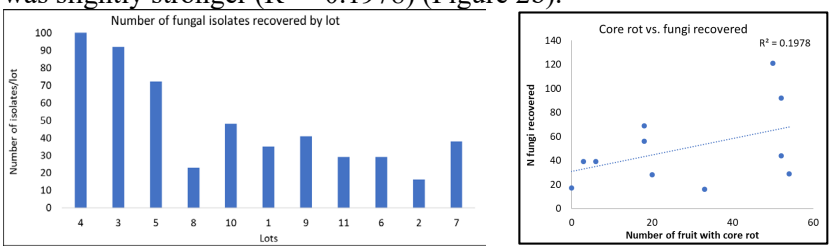

Figure 2. Number of fungal isolates recovered by lot in 2021-22 (a) and correlation between number of fungi recovered and incidence of core rots in the fruit (b).

Activity 1.2. Identification of fungal pathogens collected from commercial apples. 544 Fungal isolates were collected in 2021-22 and were first identified using morphological methods. Most fungi recovered belong to the genera of *Penicillium, Cladosporium, Alternaria* and *Fusarium.* A minor proportion of recovered isolates (<5) belong to genera of *Pestalotia, Neonectria, Aureobasidium, Phacidiopycnis, Trichoderma, Talaromyces, Mucor, Lambertella, Botrytis and* few unknowns which are being characterized (Table 2). Examples of fungi cultured from apples collected in 2021-22 are shown in Figure 3.

Table 2. Frequency of major genera of fungi recovered from apples collected from processing facilities in 2021-22.

Lot	Cultivar	Number of isolates	Frequency of major pathogens recovered					
			Penicillium	*Cladosporium*	*Alternaria*	*Fusarium*	Other minors	Unknown
1	Gala	35	17.9	0.0	35.9	7.7	28.2	0.0
2	Spur golden	16	0.0	0.0	23.5	0.0	58.8	11.8
3	Gala	92	100	0.0	0.0	0.0	0.0	0.0
4	Gala/Golden delicious	121	60.3	24.8	5.0	1.7	0.0	8.3
5	Cosmic crisp	72	62.3	20.3	11.6	2.9	1.4	5.8
6	Honeycrisp	29	89.3	3.6	10.7	0.0	0.0	0.0
7	Gala	38	89.5	7.9	0.0	0.0	2.6	0.0
8	Gala	23	94.4	5.6	5.6	0.0	22.2	0.0
9	Gala	41	59.0	35.9	7.7	0.0	0.0	2.6
10	Honeycrisp	48	88.6	0.0	4.5	0.0	2.3	13.6
11	Golden delicious	29	93.1	0.0	0.0	3.4	0.0	3.4

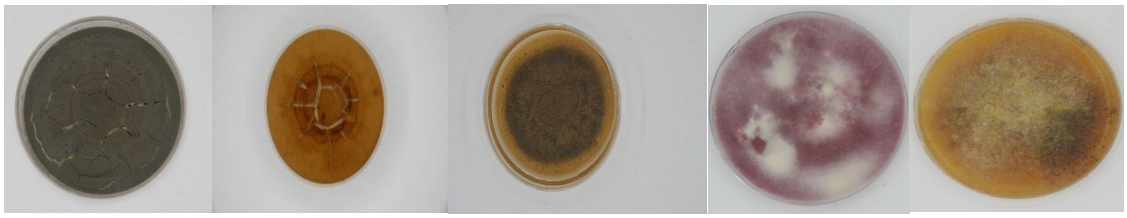

Figure 3. Examples of some of fungi cultured on Potato dextrose agar from apples collected in 2021-22

Molecular Characterization. Further, 90 representative isolates were characterized using partial sequences of four molecular markers, i.e., ITS, calmodulin (CMD), β-tubulin (Tub) and RPB2. Our sequencing analyses showed that most of the recovered isolates belonged to *Penicillium* species which correlates with initial morphological identification.

Objective 2. Characterize the ability of the different recovered species and subsamples of each species to produce patulin using biochemical and molecular methods. Year 1 and Year 2.

Activity 2.1. Selection of fungal strains to assess their ability to produce patulin (PAT).

Ten isolates were transported to Co-PI Ryu's laboratory and compared their patulin production potential in a solid media (malt extract agar, MEA). Isolates were grown on MEA for 7 days at room temperature, then three agar plugs were sampled using a diameter corkborer (5 mm diameter) and each plug was sonicated in 0.1% acetic acid, passed through 0.45 µm PVDF syringe filter, and the final extract was injected to HPLC. The isolate LF14 produced the highest amount of patulin (Table 3).

Table 3. Patulin concentration in mold strains (n=3)

Sample	Patulin (µg/plug)	Sample	Patulin (µg/plug)
LF14	76.50 ± 2.57	BR1	42.76 ± 4.12
LF25	65.30 ± 2.71	BR2	56.38 ± 5.43
LF26	53.22 ± 2.91	BR3	48.24 ± 8.13
LF31	48.04 ± 4.53	BR7	47.87 ± 12.42
LF35	49.53 ± 10.57	BR58	44.03 ± 1.34

Additional analysis is under way to test patulin production of other isolates and compare their patulin production at low temperatures (to mimic storage conditions).

OBJECTIVE 3. Develop and validate an efficient and quicker strategy for patulin detection in experimental and commercial fruit. [Lee & Ryu, Year 1-2]

Recent developments in analytical methods to detect and qualify patulin in apple and apple-based product were examined. New cleanup columns to improve the performance of HPLC-based method were tested as summarized below. A major reaction product that may be formed during processing, i.e., 5-hydroxy methyl furfural (5-HMF), was also considered during evaluation.

(a) MycoSep288 Aflapat Column – This one step cleanup column is supposed to provide a rapid cleanup and purification of apple juice as well as extracts from apple with reduced use of organic solvents. As patulin is unstable in alkaline solution, 1 drop of glacial acetic acid was added to the final 6 mL vial to help the stabilized the patulin. Due to the low recovery in juice sample, this method was determined not suitable or reliable in measuring patulin and not used for further test.

(b) SupelMIP SPE column – Molecularly imprinted polymer (MIP) is considered next generation cleanup column replacing traditional antigen-antibody based immunoaffinity column with lower cost and higher resistance to chemicals for more practical use. Nonetheless, this solid phase extraction (SPE) column still requires multiple steps of sample loading, washing and recovery of target analyte as traditional methods.

<u>*Sample preparation step*</u> – A10 g sample of apple was homogenized with 10 mL of distilled water and 150 µL of pectinase enzymes. Mixed sample was kept at 40°C for 2hr in a shaking incubator. After treatment, the sample was centrifuged at 3200 rpm for 5 min and supernatant filtered for the next step. This preparation step may be skipped for apple juice.

<u>*SPE clean up*</u> – SupelMIP SPE column (SupelMIP SPE-patulin, Supelco) was conditioned with 2 mL ACN followed by 1 mL water. And 5 mL of filtered sample was applied to column directly with a flow rate during sample load ≤ 1 mL/min. Column was washed by 1 mL of $NaHCO_3$, 2 mL water and 0.5 mL diethyl ether. Ethyl acetate (2 mL) was used for analyte elution at the final step, eluent was collected in 6 mL glass vial. Evaporated the collected solvents at 40°C under gentle stream of nitrogen to dryness. Added 500 µL water 0.1% acetic acid to the vial and closed with the screw cap. Vortexed the vial for at least 3 min to ensure that patulin is fully redissolved.

<u>*HPLC method*</u> – Analytical column, C18, 150 mm × 4.6 mm 5 µm; Flow rate, 1 mL/min; injection volume, 20 µL; detection, UV at 276 nm; mobile phase, 0.1% acetic acid in water:acetonitrile (95:5, v/v)

<u>*Results*</u> – Unlike the specification provided by the manufacturer, the recovery was less than adequate (i.e., up to 74% in apple juice and up to 27% in apple) under all conditions tested and was determined not suitable to measure patulin in apples even after enzyme treatment (i.e., pectinase to digest pectin and improve patulin recovery. However, this method may be considered a viable alternative of traditional SPE cleanup column in detecting and qualifying patulin in apple juice.

(c) Supelco Superclean LC-SI SPE column – This silica-based solid phase extraction (SPE) column has been considered as most reliable technique while it requires multiple steps of sample loading, washing and recovery of target analyte as traditional SEP based methods.

<u>*Sample preparation step*</u> – A 20 g sample of apple was mixed with 100 mL of distilled water and 450 µL of pectinase. Mixed sample was kept overnight at room temperature. The next day sample was centrifuged at 3200 rpm for 15 min and supernatant filtered for the next steps. In case of juice, preparation step could be skipped.

<u>*Extraction method*</u> – Into a centrifuge tube, 2 g sand, 15.0 g Na_2SO_4, and 2 g $NaHCO_3$ were added and mixed by shaking. Add 10 mL extraction solution to the prepared tube and close tightly. Filtered extract (10 mL) was transferred into a prepared centrifuge tube and vigorously shaken for 5 min on a mechanical shaker. Subsequently centrifuge the extraction mixture at a 2000 rpm for 90 sec to force layer separation.

<u>*SPE clean up*</u> – Added 50 µL acetic acid in ethyl acetate to a 6 mL glass vial and placed it under an unconditioned silica gel SPE column (Silica gel SPE, 500 mg, Supelco). Transferred 2.5 mL centrifuged extract onto SPE column and collected eluate in a 6 mL glass vial at 1 drop/s. Then immediately washed the SPE column with 3 mL ethyl acetate-hexane mixture to elute purified patulin from the column. When most of the washing solution has passed through, the remaining solvent from the column was pushed out using vacuum. Collected eluate was evaporated at 40°C under gentle

stream of nitrogen to dryness. Acidified water (1 mL, pH 4) was added to the vial, closed with screw cap, and mixed by vortex to ensure that patulin was fully redissolved.

HPLC method – Analytical column, C18, 150 mm × 4.6 mm 5 µm; flow rate, 1 mL/min; injection volume, 20 µL; detection, UV at 276 nm; mobile phase, 0.1% acetic acid in water:acetonitrile (95:5, v/v)

Results – In HPLC analysis, patulin peak appeared at 5.1-5.5 min. Recovery studies were carried out by spiking patulin to the non-contaminated apple and apple juice at different concentrations ranging 20 – 200 ppb. Recoveries ranged 85-93% and 78-109% from Gala apple and commercial apple juice, respectively (Table 7). Inter-/intra-day precision were also estimated with three different concentrations of patulin (20, 50, and 100 ppb) that were spiked to the samples. Linearity of response was determined by injecting extracts spiked at 20, 50, 100 and 200 ppb under identical conditions. The correlation coefficient (r^2) was 0.997. The limit of detection (LOD) and quantification (LOQ) for patulin were 1.88 ng/mL and 3.99 ng/mL, respectively.

Table 7. Recoveries of patulin spiked samples (n=3)

Spiked level (ppb)	Recovery of apple (%)	Recovery of juice (%)	Intra-day % RSD	Inter-day % RSD
20	89.10 ± 22.06	108.81 ± 17.86	5.13	3.97
50	85.37 ±12.11	89.14 ± 19.40	1.33	7.83
100	93.06 ± 6.58	77.81 ± 19.26	2.66	4.32
200	86.45 ± 23.07	99.06 ± 22.58	-	-

Activity 3.2. **Development and validation of strategy for applicable rapid patulin detection test.** Activities to fulfil this objective will continue in the second year. Rapid detection methods (i.e., commercially available ELISA test kits) will be evaluated and their performance will be compared with the HPLC method developed in this report. Suitability or applicability of a given method to the industry so that it can be used in day-to-day operation while providing reliable results to meet the needs.

Projected experiments/activities in 2023

- A second year survey of fungi associated with patulin contamination in in Washington State.
- Complete patulin detection in detached fruit assay in recovered isolates from 2022. Continue patulin detection in detached fruit assay for 2023 recovered isolates.
- Carry out genetic analysis of patulin genetic clusters fungicide sensitivity assays in isolates
- Carry out rapid detection methods using commercially available ELISA test kits and comparing their performance with the HPLC method developed in this report.
- Evaluate suitability or applicability of a given method to the industry so that it can be used in day-to-day operation while providing reliable results to meet the needs.

Presentations describing research data from this project

1. Jibrin, M.O. and Amiri, A. (2022). Survey and characterization of fungal populations causing patulin contaminations in processed apples in Washington State. *Research News Flash,* Washington State Tree Fruit Association Annual Meeting in Wenatchee, Washington State. December 6, 2022.
2. Jibrin, M.O. and Amiri, A. (2022). Addressing Emerging Patulin Concerns in Washington State Apple Processing Industry: Survey and Characterization of Associated Fungal Species. Plant Health 2022 *Oral Presentation* in the technical session *"Investigations into the Management of Toxin-Producing Fungal Pathogens"*. Pittsburgh, PA, August 6-10, 2022.