2024 Apple Crop Protection Research Review

A Fuji apple fruitlet infected with Fireblight located at the Washington State University test orchard in Orondo, Washington.

Photo Source: Paige Beuhler

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Hybrid Format
Wenatchee, WA

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

Report Type: Continuing Project Report – No Cost Extension

Primary PI: William Walker
Organization: USDA-ARS
Telephone: 509-454-6566
Email: william.walker@usda.gov
Address: Temperate Tree Fruit and Vegetable Research Laboratory
Address 2: 5230 Konnowac Pass Rd
City/State/Zip: Wapato, WA, 98951

Co-PI 2: Lisa Neven
Organization: USDA-ARS
Telephone: 509-454-6570
Email: lisa.neven@usda.gov
Address: Temperate Tree Fruit and Vegetable Research Laboratory
Address 2: 5230 Konnowac Pass Rd
City/State/Zip: Wapato, WA, 98951

Cooperators: Dr. Johannes Jehle, Julius Kühn-Institut, Darmstadt, Germany (Scientific Advisor, CpGV expert); Dr. Anne Nielsen, Rutgers University, New Jersey, USA (Scientific Consultant and Potential Collaborator); River Bioscience, Port Elizabeth, South Africa (CrpeNPV supplier); BioTepp Inc., Lévis, Quebec, Canada (CpGV supplier); Certis Biologicals, Columbia, MD, USA (CpGV supplier)

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: Awarded
Funding Duration: 2024 - 2024
Amount: $20,000
Agency Name: Washington Commission on Integrated Pest Management
Notes: Application submitted to fund further exploratory research on viral control of codling moth, with specific objectives related to but not covered in this project. Proposed project title: “Efficacy testing of novel viral pesticide CrpeNPV against codling moth and other agricultural pest insects”; funding decision pending.

WTFRC Collaborative Costs: None

Budget 1
Primary PI: William Walker
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

2021	2022	2023	2024
$40,089.00	$41,425.00	$42,762.00	
$14,031.00	$14,499.00	$14,967.00	
$4,076.00	$4,076.00	$4,075.00	
$58,196.00	$60,000.00	$61,804.00	$0.00

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 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 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. Cultivars of CpGV and CrpeNPV would be combined and exposed to codling moth larvae and then screened for efficacy. 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

- Dose-response and survival time assays with CrpeNPV against our colony codling moth insects, which was obtained from local orchards, demonstrated that the CrpeNPV virus was effective in killing local codling moth larvae.
- Compared to CpGV applied alone, CrpeNPV applied alone was less lethal than CpGV against our codling moth colony insects.
- When CrpeNPV was combined with CpGV at a ratio of one-to-one, enhanced lethality was not observed compared to when either virus was applied individually.

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 survival time 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: Due to delays in obtaining different strains of CpGV, initiation of research for this objective has been delayed. However, different strains representing genome groups A and E, which are representative of the two main commercially available products in USA. During the coming final year of the project, initial experiments will be conducted aimed at testing co-infection of codling moth larvae with both strains of CpGV with the ultimate aim of 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. Furthermore, specimens from lab colonies known to be resistant to CpGV (Fan et al., 2022) will be tested 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, 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 infect codling moth larvae.

Injection of codling moth larvae with the CpGV engineered bacmid is sufficient to cause infection of the larvae (Hilton et al., 2008) from which whole-virus specimen can be recovered and purified. Purified virus can then be used to directly inoculate codling moth larval diet (artificial diet or apple leaves) for the larval mortality bioassays. These bioassays will be conducted using CpGV 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, survival time 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 past year, a *Hvt* expression construct has been designed for transformation and of the CpGV bacmid. Due to the synthetic nature of this construct, it is necessary for its generation to be commercially outsourced to a gene synthesis company; due to delays in obtaining an acceptable agreement with the chosen company, the synthetic construct has not yet been obtained. It is expected that this will soon be resolved, and thus, during the last year of the project, the CpGV-Hvt bacmid will be generated, and initial dose-response and survival time larval infection experiments will be conducted with an in-house codling moth colony that has no known resistance to CpGV. Additionally, specimens from lab colonies known to be resistant to CpGV (Fan et al., 2022) will be tested 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 survival time 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.

One 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 (Fan et al., 2022) will assayed 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, CpGV strains commercially available in USA 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 mixtures of equal 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 survival time 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 most recent year of the project, optimization of CpGV and CrpeNPV co-infection parameters have been assessed with dose-response and survival time experiments conducted on 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 final year of the project, using optimally determined ratios and dosages as determined during the past year's experiments. specimens from lab colonies known to be resistant to CpGV (Fan et al., 2022) will be assayed 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, and experimentation according to the objectives of the project have been ongoing continuously since then.

For Objective 1, we have decided to focus our research on CpGV genome groups that are commercially available in the USA, namely genome group A (Cyd-X) and genome group E (ViroSoft). Baseline parameters have been established in our laboratory for the genome group E strain. For this, a dose-response curve was generated using five dosages on a ten-fold dilution series, compared to distilled water as a negative control. (Figure 1; Table 1) A clear dose-response effect was observed, with LC50 values of 9.43 x 10^5 and 2.11 x 10^5 at days 7 and 10 post infection exposure, respectively. At the highest dose (1 x 10^7 CpGV occlusion bodies (OBs) per mL), 100% larval mortality was observed by day 10, and 100% larval mortality was observed at the highest two dosages by day 14.

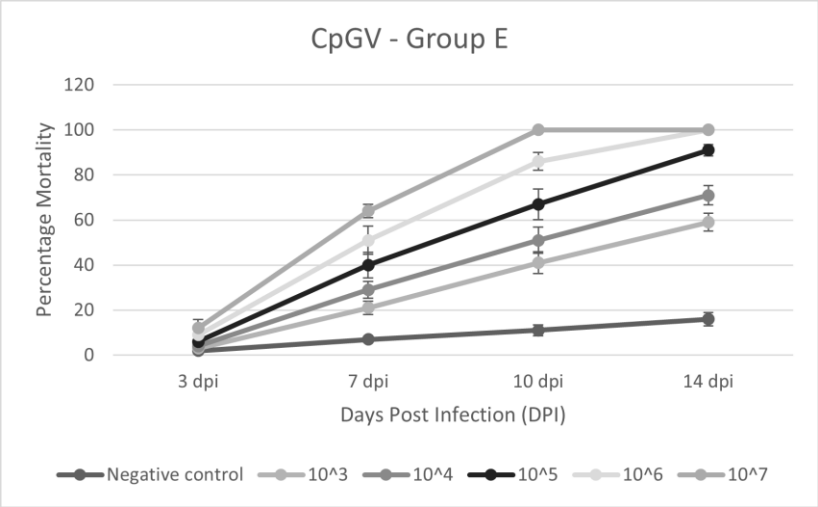

Figure 1. Dose-response mortality assay for codling moth neonate larvae exposed to CpGV - Group E. Twenty larvae tested per replicate (n=20), with five replicates for each treatment. 10 microliters of virus solution or distilled water control were applied at specified concentrations for all treatment groups at the specified dosage, ranging from 1 x 10^7 CpGV occlusion bodies (OB)/mL down to 1 x 10^3 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 (dpi). Error bars indicate standard error values.

A Genome Group A strain has recently been obtained and assays will be conducted in the coming months to establish similar baseline parameters. Subsequent to this, co-expression assays with both group A and E virus specimen will be conducted to evaluate against either strain individually.

For Objective 2, a construct has been designed that will enable expression of the *Hvt* spider-toxin in codling moth larvae (Figure 2). Production of this construct has been commercially outsourced, and it has not yet been generated and provided to us yet. Once we obtain the construct, we will commence with its transfer to the CpGV Bacmid and begin codling moth larvae infection experiments to produce the CpGV-*Hvt* virus and assess effects of the *Hvt* toxin.

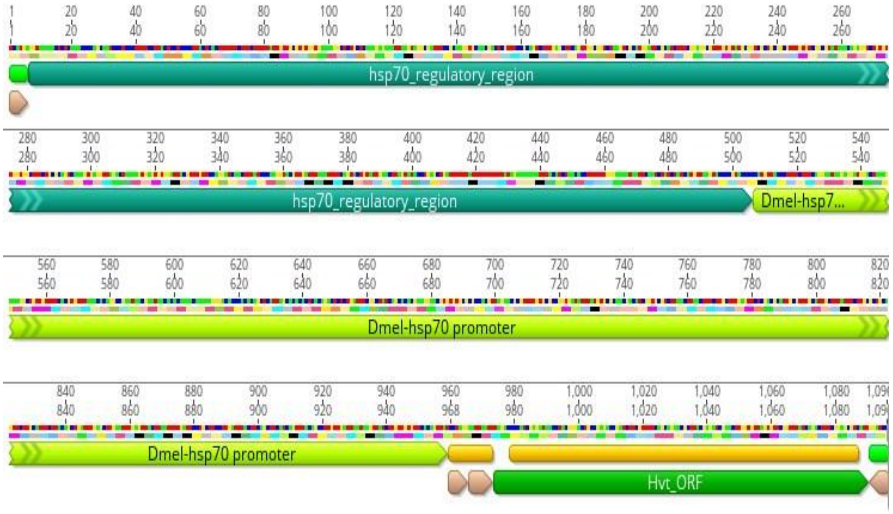

Figure 2. Expression construct for the Hvt toxin gene. Relevant features include the Hvt spider-toxin gene open reading frame (ORF) positioned downstream of the *Drosophila melanogaster* (Dmel) heat shock protein 70 (hsp70) gene expression driver, which includes the regulatory region and promoter sequences. The Dmel hsp70 promoter has previously been shown to drive constitutive gene expression across different insects including moths (Uhlírová et al., 2002).

For Objective 3, after establishing baseline parameters for CpGV (Genome Group E) and CrpeNPV, co-infection experiments have been conducted with these two specimen. Initial assays were first conducted with CrpeNPV alone, to generate a dose-response curve, as with CpGV (Group E) using the same five dosages on a ten-fold dilution series, compared to distilled water as a negative control. For CrpeNPV infections alone, maximum larval mortality was observed to be 81% for the highest dose (1×10^7 OBs/mL) on day 14 (Figure 3). Compared to infections with CpGV (Group E) for which 100% larval mortality was observed with the highest dose as early as day 10 (Figure 1), overall larval mortality effects caused by CrpeNPV were lower than larval mortality effects caused by CpGV (Group E) at all dosages and time-points examined. These observations are consistent with results obtained in the initial report on efficacy of CrpeNPV against codling moth (Wennmann et al., 2019), which compared efficacy of CrpeNPV against codling moth to CpGV strains representative of genome groups A and B, but not E. When both viruses were combined at equal ratios, mortality rates were lower early in the period of infection, 3 dpi and 7 dpi, more similar to when CrpeNPV was presented alone; later in the infection, 10 dpi and 14 dpi, larval mortality rates were higher, more similar to when CpGV was presented alone (Figure 4, Table 1). These results are similar to findings of a previous report, in which it was observed that co-infection of cutworm larvae (*Agrotis segetum*) with both an NPV (AgseNPV-B) and GV (AgseGV) did not result in changes in mortality rates when either virus was presented alone (Wennmann et al., 2015). These findings may be attributable to the principle of super-infection exclusion, by which infection of individual cells by one virus precludes simultaneous infection by other viruses (Beperet et al., 2014).

Table 1. Codling moth percent larval mortality range across all viral dosages at each time-point days post infection (dpi), from lowest to highest dosage

	3 dpi	**7 dpi**	**10 dpi**	**14 dpi**
CpGV (Group E)	3% - 12%	21% - 64%	41% - 100%	59% - 100%
CrpeNPV	2% - 3%	10% - 22%	22% - 51%	48% - 81%
CpGV (E) + CrepeNPV	0% - 7%	5% - 29%	37% - 81%	65% - 100%

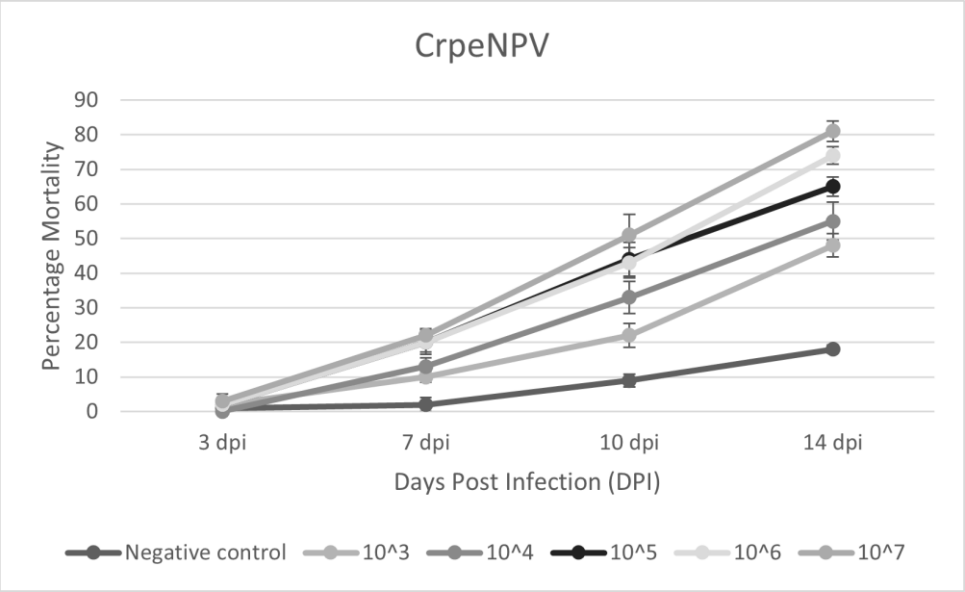

Figure 3. Dose-response mortality assay for codling moth neonate larvae exposed to CrpeNPV. Twenty larvae tested per replicate (n=20), with five replicates for each treatment. 10 microliters of virus solution or distilled water control were applied at specified concentrations for all treatment groups, ranging from 1 x 10^7 CpGV occlusion bodies (OB)/mL down to 1 x 10^3 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 (dpi). Error bars indicate standard error values.

Finally, survival time analyses were conducted, in which larval mortality rates were measured twice daily across 3 dpi through 7 dpi for both CpGV (Group E) and CrpeNPV presented individually; results for survival time CpGV and CrpeNPV co-infection assays are pending. For these assays, a single concentration was assessed for each virus, 3.7 x 10^2 OB/mL. This dosage, which is lower than the range of concentrations applied in our dose-response assays, was chosen to best assess how/when the course of infection causes larval mortality. This was done because differences in larval mortality rates for CpGV (Group E) and CrpeNPV were already apparent by 7 dpi at all higher doses tested (1 x 10^3 OB/mL through 1 x 10^7 OB/mL). For the dosage tested, a marked difference in survival rates was observed during the early phase of the infection, from 96 to 120 hours post infection (4 to 5 days after initiation of the experiment). These results are consistent with observations from the dose-response assays conducted at higher concentrations. Subsequently, we observed similar survival time rates for both CpGV (Group E) and CrpeNPV, with a substantial decrease in survival occurring between days 5 and 6 (from 126 to 144 hours post infection), compared to the no-treatment control in which only autoclaved distilled water was applied to the larval diet. These results suggest that infections by both viruses have similar time-course metrics with apparent higher lethality to codling moth larvae caused by CpGV.

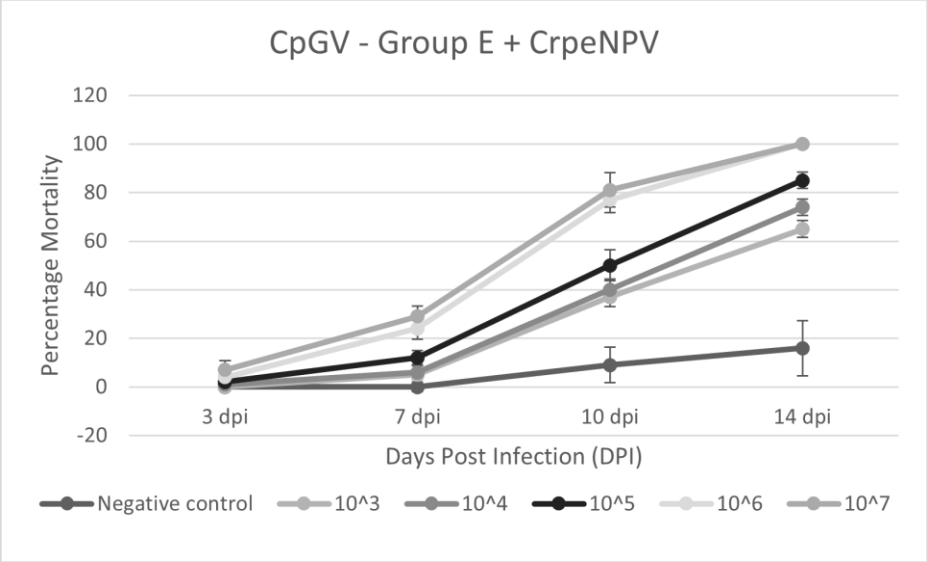

Figure 4. Dose-response mortality assay for codling moth neonate larvae exposed to CpGV (Group E) and CrpeNPV. Twenty larvae tested per replicate (n=20), with five replicates for each treatment. 10 microliters of virus mixture or distilled water control were applied at specified concentrations for all treatment groups, ranging from 1 x 10^7 occlusion bodies (OB)/mL down to 1 x 10^3 OB/mL. Dilutions for each virus were made in autoclaved distilled water, which was also used as the negative control, then 1 mL of each virus at same concentration was combined before application to larval diet. 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 (dpi). Error bars indicate standard error values.

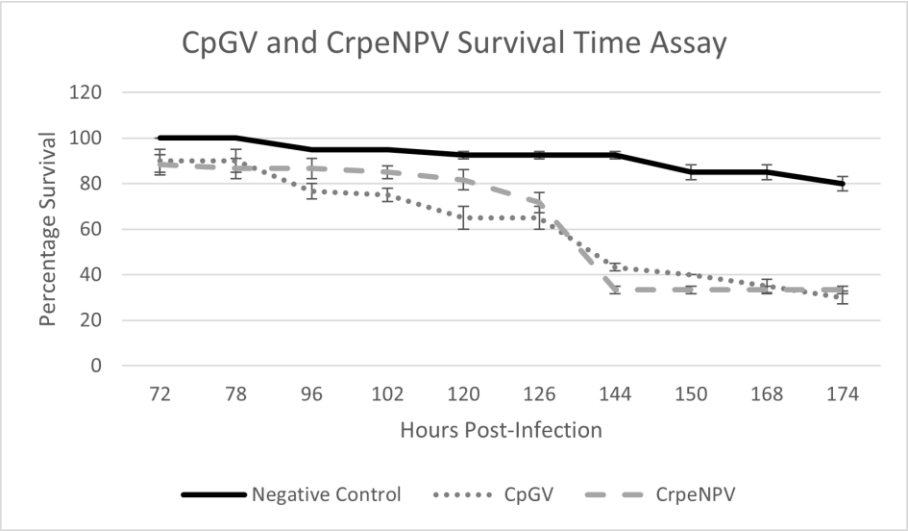

Figure 5. Survival time assays for codling moth neonate larvae exposed to CpGV (Group E) or CrpeNPV. Twenty larvae tested per replicate (n=20), with three replicates for each treatment. 10 microliters of virus mixture or distilled water control were applied at specified concentrations for all treatment groups, at a single concentration of 3.7 x 10^2 occlusion bodies (OB)/mL. The dilutions for each virus was 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 twice daily on days 3 through 7 after initiation of experiments (dpi). Error bars indicate standard error values.

Project Title: Novel control of Codling Moth with RNA interference

Report Type: Continuing Project Report

Primary PI: William Walker
Organization: USDA-ARS
Telephone: 509-454-6566
Email: william.walker@usda.gov
Address: Temperate Tree Fruit and Vegetable Research Laboratory
Address 2: 5230 Konnowac Pass Road
City/State/Zip: Wapato, WA, 98951

Co-PI 2: Rodney Cooper
Organization: USDA-ARS
Telephone: 509-454-4463
Email: rodney.cooper@usda.gov
Address: Temperate Tree Fruit and Vegetable Research Laboratory
Address 2: 5230 Konnowac Pass Road
City/State/Zip: Wapato, WA, 98951

Cooperators: Dr. Alex Flynt, University of Southern Mississippi, Hattiesburg, Mississippi, USA (Scientific consultant and collaborator on RNA interference in insects)

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

Budget 1
Primary PI: William Walker
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	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

- No RNAi phenotype observed with multiple genes when long double-stranded RNA was overlayed on or mixed into larval diet for feeding uptake by neonate larvae.
- Small RNA transcriptome sequencing of larval tissues reveals candidate “trigger” sequences that may be exploited to induce RNAi effect through an alternate RNAi pathway, called piRNA that may be more viable for codling moth.

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; Walker et al., 2023). 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

For Objective 1, whole transcriptome RNA-sequencing has been conducted on neonate and fifth instar whole larvae to facilitate identification of candidate genes for the canonical long double-stranded RNA (dsRNA) RNAi pathway. Initial targets have been identified, including the IAP gene, which has severed as a "model" gene for RNAi in other insects (Pridgeon et al., 2008; Walker and Allen, 2011), and also the *chitin synthase A* (CHSA) gene, which has recently been demonstrated to be a good RNAi target with a larval mortality phenotype across multiple Lepidoptera Families (Rana et al., 2020), though it has not been examined as an RNAi effector in any Tortricidae.

In addition to whole transcriptome messenger-RNA (mRNA) sequencing, small RNA transcriptome sequencing has been conducted on neonate, third instar and fifth instar larvae to

identify "trigger" sequences that would direct effector dsRNA molecules into an alternative RNAi cellular pathway, known as the piRNA pathway (Flynt 2020). This approach has been pursued in collaboration with Dr. Alex Flynt after initial observations of no long dsRNA mediated RNAi phenotype, as described below with regards to Objective 2.

For Objective 2, thorough experimental feeding assays attempting RNAi against codling moth larvae by exposing the larvae to dsRNA targeting the IAP and CHSA genes and compared to saline buffer (in which the dsRNA is diluted) and dsRNA of the non-insect gene, green fluorescent protein (GFP). Several different approaches have been taken including: 1) overlaying a standard large dose of target gene dsRNA (500 ng/µL) once on top of the larval diet; 2) applying two large doses on top of the larval diet several days apart (as reported in Wang et al., 2015) 3) mixing in a lower dose (final mixed concentration at 50 ng/µL) of target gene dsRNA directly into the larval diet; 4) testing neonate larvae; 5) testing 3rd or 5th instar larvae that were first reared on untreated larval diet and then transferred to dsRNA treated diet. For all of these approaches taken, at least 20-50 larvae were tested per replicate, with 2-3 replicates tested per condition. Regardless of the approach taken, however, no mortality effect was observed when IAP or CHSA were targeted compared to the saline buffer and GFP dsRNA controls.

Lack of a mortality phenotype may occur for several reasons. It is possible that the genes targeted (or sequence regions of these genes) are not good targets in codling moth. This is possible, however unlikely. Both genes have proven to be effective long dsRNA targets for RNAi in other species. Furthermore, dsRNA has been generated sufficiently long to generate a breadth of cleaved small RNA molecules across the length of the gene to initiate the sequence-specific mRNA degradation pathway, and in the case of CHSA, the dsRNA was derived from the exact region of the gene used effectively in RNAi assays against several other moth species (Rana et al., 2020).

It has alternatively been reported that some insect species may be insensitive to RNAi uptake through feeding due to degradation of the dsRNA effectors in the oral track (Walker et al., 2012) or midgut (Luo et al., 2013). In codling moth larvae, this may be controlled for through injection of dsRNA effectors directly into the hemolymph. These injection experiments will be conducted during the coming year with the same IAP and CHSA dsRNA molecules used for the feeding bioassays. If these experiments are successful in inducing an RNAi-mediated mortality phenotype, it may indicate that larval feeding of long dsRNA is not a viable approach to trigger the RNAi effect in codling moth larvae.

A further cause of no RNAi-mediated phenotype could be attributed to the phenomena of reduced long-dsRNA cellular transport and processing, which has observed in some lepidopteran species, resulting in poor RNAi response in these species (Shukla et al., 2016). The piRNA pathway is one potential alternative to the long-dsRNA pathway that may be viable for inducing RNAi in codling moth, as there are indications that this pathway can be a viable approach to induce RNAi effect in lepidopteran species (Flynt, 2020).

As mentioned above, for Objective 1, an alternative RNA-sequencing approach, aimed at sequencing only small RNAs has been conducted, to identity trigger sequences that activate the piRNA-mediated RNAi pathway. As such, small RNA transcriptomes have been generated across several codling moth larval stages, and several different piRNA trigger sequences have been identified. These sequences will be incorporated into novel dsRNA constructs to target IAP, CHSA and other genes for mRNA degradation. Feeding and injection experiments aimed at the piRNA pathway will be conducted during the coming year.

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

Primary PI: David Crowder
Organization: WSU Entomology
Telephone: 509-335-7965
Email: dcrowder@wsu.edu
Address: 166 FSHN Building
City/State/Zip: Pullman, WA, 99164

Co-PI 2: Betsy Beers
Organization: WSU Entomology/TFREC
Telephone: 509-293-8755
Email: ebeers@wsu.edu
Address: 1100 N Western Ave
City/State/Zip: Wenatchee, WA 98801

CO-PI 3: Tobin Northfield
Organization: WSU Entomology/TFREC
Telephone: 509-293-8789
Email: tnorthfield@wsu.edu
Address: 1100 N Western Ave
City/State/Zip: Wenatchee, WA 98801

Cooperators: Louis Nottingham, WSU Entomology/NWREC

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:

Primary PI: David Crowder
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 (2023)

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

In the first year (2022), Robert Curtiss, a postdoctoral scholar in Entomology, led the sampling effort for the project. Our goal was to sample codling moth populations across orchards that reflected variability in production conditions and management practices used in Washington (i.e., across gradients from North to South, and East to West, with variable elevations, weather, etc.). We achieved our goals except we were unable to identify any orchard blocks that had no mating disruption or early-season insecticides; while our study thus lacked a true "untreated control", we felt confident our sites reflected practices used in Washington orchards. During the growing season of 2022, our team conducted weekly sampling of codling moth in each orchard with a total of ten pheromone traps that were placed both along block edges and towards the center of the block (Fig. 1). We used Orange Pherocon VI delta traps baited with a PHEROCON® CM-DA COMBO™ Lure + AA Lure (Trécé, Inc.) to attract both male and female moths. Traps were placed within the top 1/3 of pre-marked trees and lures were changed every eight weeks, and traps were monitored every 7 days. 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 this first year of data, codling moth were rare across our 7 orchard blocks, with only 360 total moths collected across all sites; only 20% of the moths collected were wild type moths and 80% sterile moths. These data provided a basis for modeling, but more data were needed.

Figure 1. Example of sampling design in a Washington apple orchard. Traps were placed throughout the block and were checked weekly to assess adult codling moth population dynamics.

Data Collection in 2023. To supplement our field work conducted in 2022, in the past year we worked with commercial growers in Washington and the OK-SIR program in British Columbia (Fig. 2) to gather a considerable amount of additional trap data from fields managed with variability in sterile insect releases and insecticides. We were able to gather data from 237 sites with weekly sampling of at least 10 pheromone traps to complement our field survey. Our datasets represented areas with variation in trap density (Fig. 2), which could affect the probability that we are able to accurately estimate abundance of codling moth populations. When we combined field data from our own trials with data from our commercial partners, we were able to graph how variable codling moth trap catch actually is within fields (Fig. 3). In Fig. 3, we show both the phenology model for codling moth (blue line) as well as a model fit to the trap catch data (black line). This shows that in real-world orchards it is common for the phenomenon that growers will see a lag between when the phenology model predicts moths are initially active and when they are actually caught.

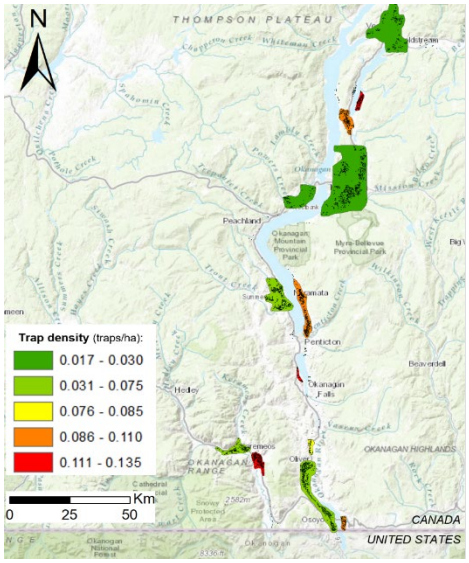

Fig. 2. Location of trap sites in British Columbia where data was collected to complement data from orchards we sampled in Washington State

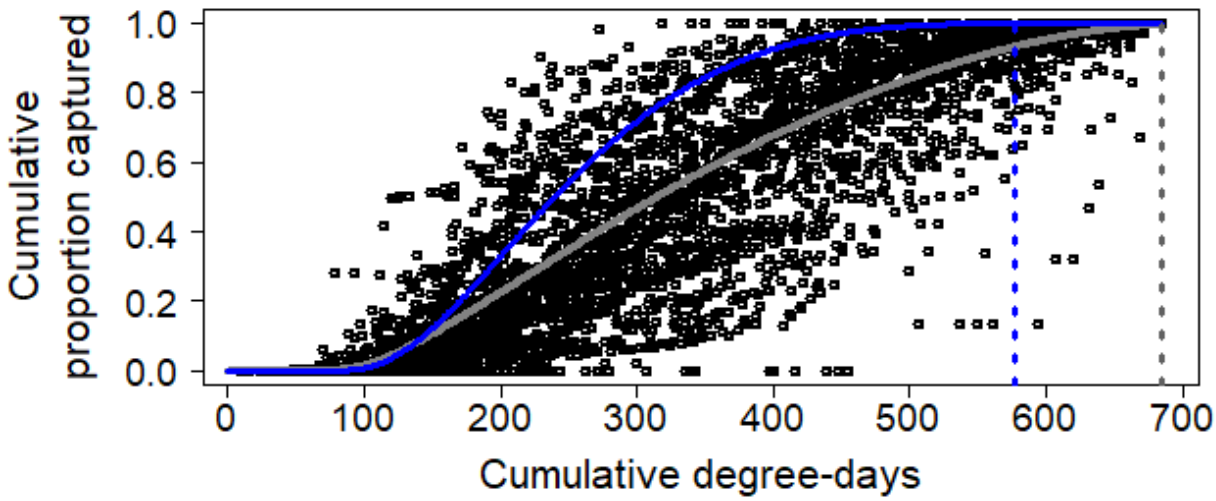

Figure 3. Data from over 240 sites showing trap catch of codling moth adults over the first generation. The blue line shows the phenology model used on the WSU DAS (Jones and Wiman 2012), and the gray line shows a population dynamics curve fit to the data. The difference between the lines shows the typical lag in when adults are captured in traps compared to when a phenology model predicts they are emerging.

(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 spring of 2023 we brought on Diego Rincon, a postdoctoral scholar in the Crowder lab, to work full time on the modeling and data collection parts of the project, as former postdoc Curtiss moved onto other projects. Our goal was to use data from the field sampling to determine if we could make projections of future codling moth population size from trap-catch data. If so, we would plan to incorporate both phenology and population dynamics into future moth models.

Using the field data, we compared parameters associated with the phenology model for codling moth used on DAS with those from a new model describing field-level population dynamics (Table 1, Fig. 3). One of the main takeaways is that growers or consultants may see up to a 100DD lag in when adults are collected compared to when they may emerge. This reflects variability in our ability to trap codling moth in orchards; from our data we were able to estimate we catch far fewer than 1% of total moths in orchards, even with high trap density (Fig. 4)

Table 1. Coefficients used in the Johnson SB distribution function to predict codling moth adult emergence and capture in pheromone traps as a function of cumulative degree-days.

Model	Parameters				Reference
	γ	δ	ξ	λ	
Phenology model	1.073	1.239	69	577.2	(Jones and Wiman, 2012)
Pheromone capture model	0.460 (SE = 0.170)	0.867 (SE = 0.094)	69.220 (SE = 7.573)	662.6 (SE = 33.366)	This study

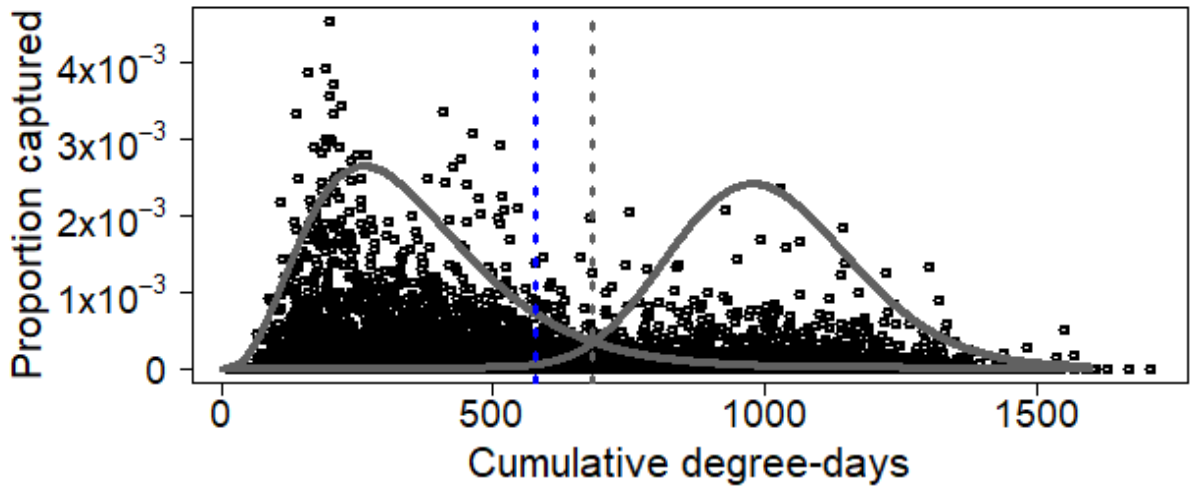

Fig. 4. Estimated proportion of codling moth caught in traps across the first and second adult generations

Predictive modeling. In 2023 we build a series of models to attempt to predict future codling moth abundance based on degree days and cumulative trap catch (Fig. 5). In our approach, trap catch data can be input into a model and an estimate of the future phenology and population dynamics is generated (blue and red lines the Fig. 5, respectively). We then use variance in the trap catch data to estimate a 95% confidence interval for the future growth of the population (dashed lines in Fig. 5). In areas with greater trap density, or where codling moth populations are more abundant, our ability to catch early-emerging moths increases (Fig. 4). In such areas, the confidence intervals should shrink compared to sites with lower trap density. Regardless, our approach can be used to model population dynamics by combining phenology and trap catch.

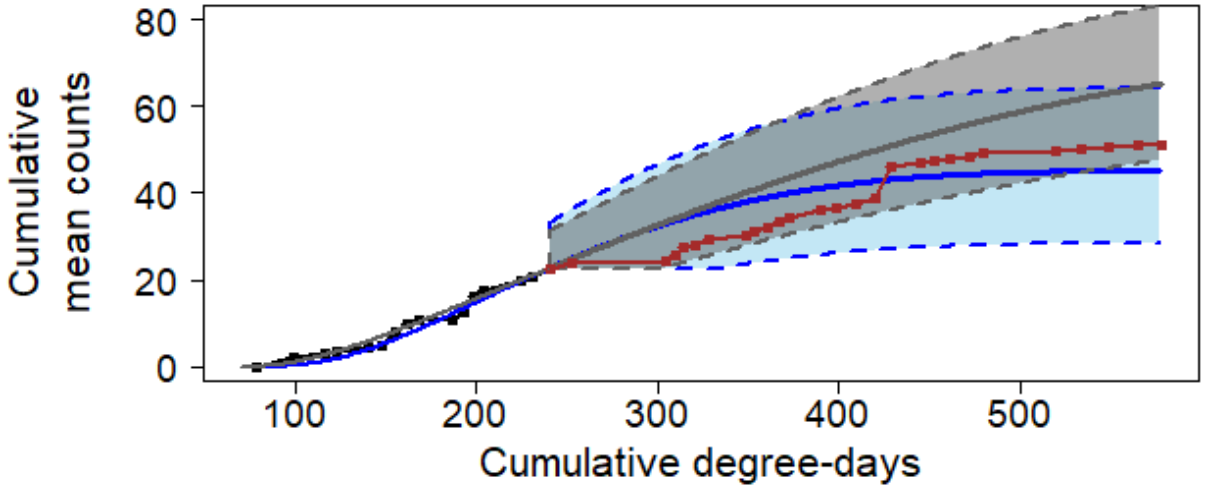

Fig 5. Example of our modeling approach to predict future codling moth abundance based on phenology and ongoing sampling. In this example, data is collected in traps up to 250 degree days (black squares), and a cumulative count of moths is made. At this point, both a phenology (blue line) or population dynamics (red line) models are fit to the trap data, and variance in the trap catch is used to estimate future 95% confidence intervals. After this point, future trap catch can be monitored to validate the model.

Validation of predictions. Sensitivity analyses showed that the model was robust for a wide range of sample sizes and codling moth densities, with prediction accuracy for long-term outputs (prediction lengths >120 degree-days) ranging from 69 to 94% and from 63 to 95% for models that used the phenology-based and the moth capture functions (Fig. 6). The phenology-based function had greater accuracy when the prediction was made as early as 250 cumulative degree-days, and the moth capture function produced slightly better predictions for other prediction lengths (Fig. 6). Short-term outputs of the model (< 120 degree-days) were between 90 to 100% accurate in model validation, and the phenology-based model (mean accuracy = 98%, SD = 0.03) had similar accuracy to the moth capture model (mean accuracy = 97%, SD = 0.02). Overall, our approach suggests we may be able to accurately predict future codling moth densities.

In 2024 we plan to extend our approach and conduct further validation. We will also attempt to build our models into the WSU DAS. This would allow users of the DAS system to enter their trap catch data and get a prediction on future population dynamics. This will allow users to better link phenology models with trap catch to make decisions on codling moth.

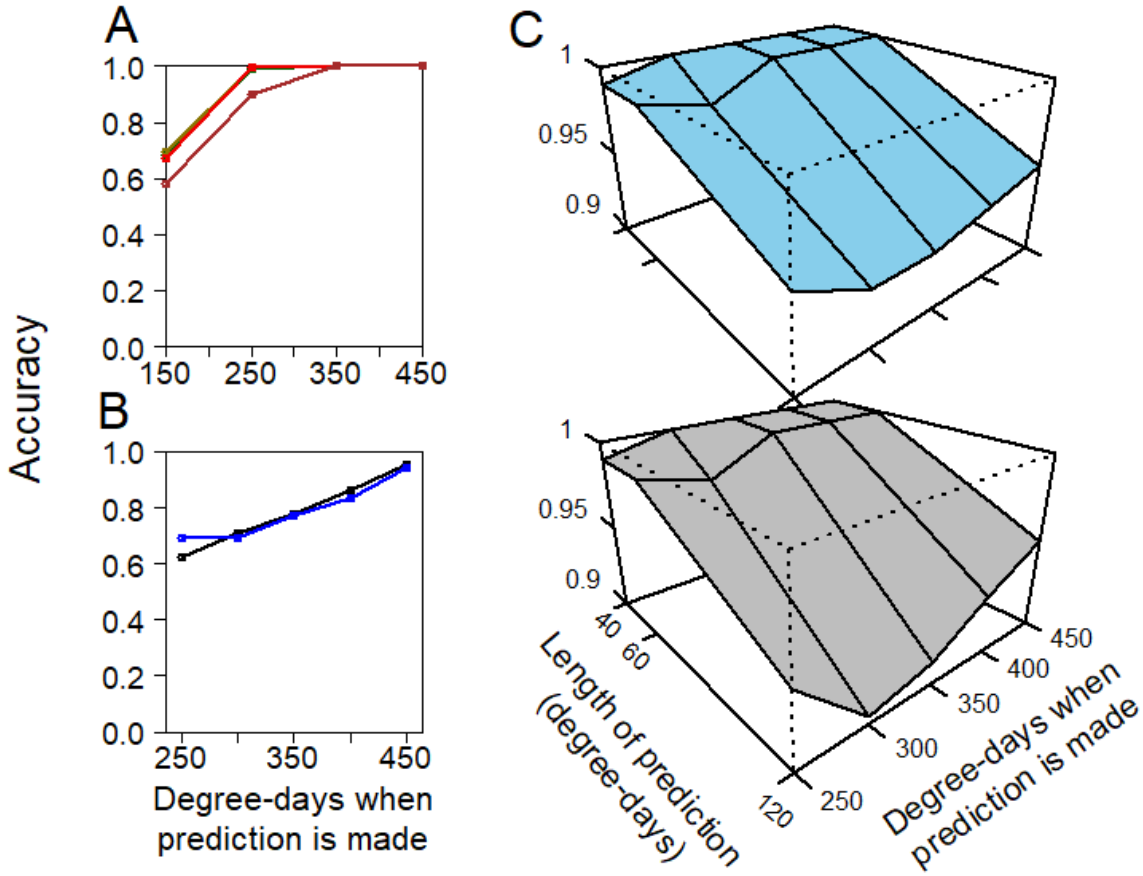

Fig 6. Accuracy (the proportion of predictions that fall within the prediction band) of (A) the phenology and (B) moth capture models. Model accuracy of the model is presented as a function of degree-days when predictions are made; each line represents a different sample size. (C) Blue and grey surfaces represent the accuracy of predictions for phenology-based and moth capture models, respectively.

(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, including at the 2023 Hort Show, the largest industry conference of the year. Crowder and the DAS team also offered two codling moth workshops in spring 2023 that were attended by over 50 individuals. Diego Rincon, the lead postdoctoral scientist on the project, also gave a talk at the 2023 Entomological Society of America talk.

In 2023 we also provided weekly updates to the industry, which focused largely on codling moth in the late spring and early summer months, Liesl Oeller, the coordinator of the WSU DAS and a member of PI Crowder's lab, wrote up weekly reports that were shared with a list serve of over 200 individuals. We also provided updates through the tree fruit extension team.

In 2024 we hope to expand our outreach and showcase ways that our models can benefit growers and other members of the tree fruit industry to manage codling moth.

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 work in 2022 and 2023 established a sampling program across commercial orchards in Washington and British Columbia, and our team began a process of collecting data from a larger set of commercial orchards for modelling. From these data we made considerable progress on showing how phenology models can be used to accurately predict codling moth population dynamics (i.e., abundance) Our project is supporting one postdoctoral scholar that is working to compile all the data from the field objectives and conduct the modeling. Our team also is highly involved in outreach to ensure that progress on codling moth models for management are shared widely with the industry.

Proposal Title: Crop Protection Product Efficacy Testing for Codling Moth - Laboratory
Report Type: Continuing Project Report

Primary PI: **RT Curtiss**
Organization: Washington State University - TFREC
Telephone: 917-685-1546
Email: rcurtiss@wsu.edu
Address: 1100 N. Western Ave
City/State/Zip: Wenatchee, WA 98801

Co-PI 2: Louie Nottingham
Organization: Washington State University - NWREC
Telephone: 540-798-2044
Email: louis.nottingham@wsu.edu
Address: 16650 State Route 536
City/State/Zip: Mount Vernon, WA 98273

CO-PI 3: Tobin Northfield
Organization: Washington State University - TFREC
Telephone: 509-293-8789
Email: tnorthfield@wsu.edu
Address: 1100 N. Western Ave
City/State/Zip: Wenatchee, WA 98801

Cooperators: Robert Orpet (WSU-TFREC), Mike Doerr (Wilbur Ellis), Alix Whitener (FMC), John Aigner (UPL), Dan Snipes (Corteva), Travis Schoenwald (Valent), Sean McDonald (Terramera), Dan Peck (Vestaron).

Project Duration: 2-Year

Total Project Request for Year 1 Funding: $42,500
Total Project Request for Year 2 Funding: $37,500

Other related/associated funding sources: Requested:
Funding Duration: 2023
Amount: $20,460
Agency Name: WSCPR
Notes: Wages for time-slip

WTFRC Collaborative Costs: NONE

Budget 1
Primary PI: RT Curtiss
Organization Name: Washington State University
Contract Administrator: Anastasia Mondy
Telephone: 509-335-9661
Contract administrator email address: ORSO@wsu.edu
Station Manager/Supervisor: Chad Kruger
Station manager/supervisor email address: cekruger@wsu.edu

Item	2023	2024	
Salaries	$8,500.00	$8,840.00	
Benefits	$2,754.00	$2,864.00	
Wages	$15,600.00	$16,224.00	
Benefits	$1,592.00	$1,655.00	
RCA Room Rental			
Shipping			
Supplies	$11,062.00	$4,806.00	
Travel			
Plot Fees			
Miscellaneous			
Total	$39,508.00	$34,389.00	$0.00

Footnotes: [1]RT Curtiss, Project lead, Salary+Benefits (@0.1 FTE); [2]Time-slip employee Wages+Benefits (@$20/hr, 15hr/week); [3]Supplies (year 1 equipment: Autoclave ($5000), Shelves, Rearing cages, Diet heater and mixer, Air purifiers; year 1 and 2 supplies: artificial diet, exposure arenas, misc. consumables, and lab supplies). Tobin Northfield does not require salary for this project, but will provide research space and guidance at TFREC, assistance with analysis and WSU processes.

Louie Nottingham
*Do not award separately

Salaries1	$2,260.00	$2,350.00	
Benefits	$732.00	$761.00	
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies			
Travel			
Plot Fees			
Miscellaneous			
Total	$2,992.00	$3,111.00	$0.00

Footnotes: [1](Louie Nottingham was RT Curtiss' postdoc advisor and provides project guidance on WSU processes for obtaining funds from insecticide companies) Salary+Benefits (@0.02 FTE)

ORIGINAL PROJECT OBJECTIVES:

1) Establish a codling moth colony available for use in new crop protection product testing
2) Lab-test current and new conventional and organic materials and strategies' effectiveness as CM control tactics
3) Supply moths to researchers and companies to sustain outside funding (prevent future requests to WTFRC)
4) Update extension resources to include new product information

SIGNIFICANT FINDINGS:

Objective 1 – 2023 Key Findings

- Of the 2190 cardboard bands placed in apple trees on commercial farms in 2022, 599 final instar larvae and pupae were extracted in the spring of 2023 and emerged as adults in the laboratory shortly thereafter.
- No mating occurred in the mating arenas we constructed based on previous literature, and the adults slowly died apparently without mating or laying eggs.
- We changed tactics and began extracting larvae from infested apples collected on commercial farms in Chelan, Douglas, Okanagan, and Grant Counties. Between June and September 2023, we extracted a total of 1546 larvae from infested apples.
- The adults that emerged from June and July collections, like those that overwintered, failed to mate and no eggs were produced.
- In Mid-July, PI Curtiss designed a new mating arena to test, and by the end of July, reared adults were mating and laying eggs in the new arenas.
- By September, 302 F1 Generation larvae were produced in the colony. They all entered diapause in the cold chamber, and will have diapause broken beginning in late January
- In addition, several thousand cardboard bands were placed on commercial apple farms and collected for pupa/larva extraction. Most have high numbers of individuals.

Objective 2 – 2023 Key Findings

- Due to difficulty inducing mating in the laboratory early in the season, limited numbers of F1 Generation moths were produced. They were all reserved for mating and creation of the F2 generation.
- No new products were able to be tested in 2023.
- However, we will begin testing new products in early 2024 as mating and egg laying occurs in the new mating arenas.
- In addition, we will begin testing some insecticides on larvae being extracted from cardboard bands in late January 2024.

Objective 3 – 2023 Key Findings

- Now that we have solved the mating and egg laying issues, we are prepared to start producing enough moths to provide them for other research projects.

Objective 4 – 2023 Key Findings

- This objective will be addressed in 2024

METHODS

Objective 1: Establish a codling moth colony

Codling moths, sourced and aggregated from Washington State apple farms, will be reared in the laboratory on artificial codling moth diet (Frontier Agricultural Sciences product #F9370B) using well-established protocols to ensure that the colony will establish and grow to sustainable levels within one year. The research colony will be housed at WSU TFREC in Wenatchee, WA. Apple trees in commercial and research orchards were banded for final instar codling moth caterpillars to colonize in summer 2022. Bands will be collected in January-February 2023 (*14 Feb. 2023 update: Bands (ca. 550) from 5 locations (1 TFREC, 3 Grant County, 1 Chelan County) have been collected and are in WTFRC cold storage. We expect to recover several hundred codling moth pupae from these bands to establish the colony). Upon returning colonized bands to the laboratory, individuals will be prepared for a break in diapause and caged for emergence and mating. Eggs (F0 generation) from field-collected mated females will then be placed on commercial pre-mixed diet. Larvae will feed upon the diet until they are of sufficient size to pupate. Emerging F0 generation adults will then lay the eggs of the F1 generation, the first generation potentially available for research use. All generations will be reared at constant day length (16L:8D), temperature (24-28 °C (75-82 °F)), and RH (50-70%) to synchronize development.

Through the project we will continue banding in other WA locations to maintain the colony's genetic diversity. There will be a quarantine process much like initial colony establishment before they are incorporated into the main colony to ensure we are not introducing diseases into the colony. Beyond the end of the project, we will also periodically re-collect from the initial sites and new sites to maintain genetics.

Although past WSU codling moth colonies were maintained, this proposed codling moth colony will not be managed exactly the same way. Previous WSU colonies used a codling moth pinto bean diet from made from scratch, and instead, we'll be using a commercial premixed diet because it requires less space, equipment, and personnel. We may be using some of the same growth rooms as past WSU colonies, but we will be following the rearing protocols for codling moth recommended by the diet company (Frontier Agricultural Sciences).

Potential problem: Occasionally colonies crash and many of the individuals will die. There are often simple explanations for a colony crash, including poor genetics, and proliferation of disease through the colony. Though there is a possibility that we will experience a colony crash, we will take steps, such as sterilizing equipment, limiting entry into the growth rooms, and treating egg surfaces with dilute bleach to minimize the risk. We are prepared to field collect new individuals if necessary to replenish the colony.

Objective 2: Lab-test current and new conventional and organic materials and strategies

As the Washington representative codling moth colony grows to a large enough size to accommodate removal of individuals, we will begin testing new and current insecticides' efficacies. We will employ two test methods that are well-established and accepted by the scientific community to measure efficacy against first instar larvae, 1) a larval diet incorporation study, and 2) direct contact assays. Against eggs, ovicidal compounds will be tested using residual assays or topical assays.

The larval diet incorporation study will follow IRAC Susceptibility Test Methods (Method no. 20, Version 3.2 https://irac-online.org/content/uploads/Method_020_v3.2.pdf) for incorporating insecticides into artificial diet. These methods are well-established and provide guidelines for acceptable insecticide assays. Direct spray assays will use a Potter Spray Tower, or similar small droplet application device to test insecticide contact efficacy. The Potter Spray Tower is the standard

of reference for insecticide spraying techniques in the laboratory and is used to study the effects of contact and residual insecticides on organisms (Potter, 1952; Roychoudhury et al. 2016). Codling moth survival following exposure to insecticides will be monitored for up to 10 days.

Residual ovicidal compounds will be tested by applying residues to egg deposition substrates (waxed paper sheets) with the Potter Spray Tower or similar device. Residues' aging will be tested by delaying egg deposition on sprayed egg deposition substrates. Topical ovicides will be tested by directly spraying eggs laid on egg deposition substrates with a Potter Spray Tower or similar device. Egg hatch will be monitored after exposure to insecticides for up to ten days.

Beginning in 2023, once the colony is established, we have new codling moth products from Vestaron and Terramera that we will be testing using the above methods. Studies like these will not be able to proceed without first establishing a codling moth laboratory colony at WSU-TFREC. Funds obtained from industry for both studies will contribute to the long-term viability of the colony as well as the salaries of personnel tasked with maintaining the colony.

Beyond 2024, when this proposed project ends, the codling moth colony will be continued. The primary source of funds for this colony will be from chemical company contributions for testing new products. Laboratory-based product testing experiments typically generate between $500-750 of revenue per treatment, while those that will occur in the field typically generate $2,500 per treatment. Secondary sources of funding will come from research projects (both our research and sales of moths to other researchers' projects). In addition, the colony will be scaled up or down as funds/needs dictate. If there are times where demand lags, it can easily be scaled down without elimination. There is usually a several month negotiation with insecticide companies before moths are needed (i.e., discussions with chemical companies began in August 2022 for spring 2023 trials). We do not intend for the colony to be dissolved while we are at WSU. In addition, our process for quarantine of new genetic strains will allow us to collect and grow the colony quickly from new field-collected individuals when needed.

Objective 3: Supply moths to researchers and companies to sustain outside funding

When the colony is established, and there are enough moths produced for our research needs, we will provide excess moths to other labs for research use. Because there is a cost associated with production of moths, we will establish a per moth cost structure for researchers. Eventual costs from a robust colony may be as low as $0.02/moth, though initially it will cost as much as $0.10 to produce each moth. Sterile moths produced by the Okanagan Kootenay Sterile Insect Release facility will cost researchers $0.02/moth in 2023, and the only stage available is adult, so our target cost is comparable, and our facility will be able to provide all life stages to researchers. This codling moth colony at WSU-TFREC will become an invaluable resource for discovery of new management tactics and techniques and understanding codling moth biology and behavior in Washington orchards.

The colony will be large enough within six months for us to begin product testing, and we expect that by 2024 we will have enough excess production to begin providing moths for other researchers' projects.

Objective 4: Update Extension resources to include new product information

As new products and solutions are tested, approved for use, and found to be effective as management tools, we will incorporate suggestions for their use in extension talks and online resources. We will develop strategies for incorporating new chemistries into the management recommendations found in the crop protection guide and on the Decision Aid System. In addition, we will publish our findings in peer reviewed journal articles, on the Tree Fruit Website, and in other fruit industry publications.

RESULTS AND DISCUSSION

Objective 1: Establish a codling moth colony available for use in new crop protection product testing

Trees that were cardboard banded in fall 2022 in Grant and Chelan Counties were used to obtain codling moth larvae in puparia. 2,115 cardboard bands from four Grant County locations yielded 215 codling moth larvae, while approximately 75 cardboard bands from Chelan County yielded 384 moth larvae (Fig. 1). Diapause was broken for these overwintering larvae beginning in May, and by June 20 all larvae had been extracted from bands, allowed to pupate, and placed in mating arenas. Unfortunately, no successful mating occurred, and no viable eggs were laid from either the Grant or Chelan County collections.

Figure 1. Checking cardboard bands for CM larvae

Figure 2. CM larva to be collected from infested apple

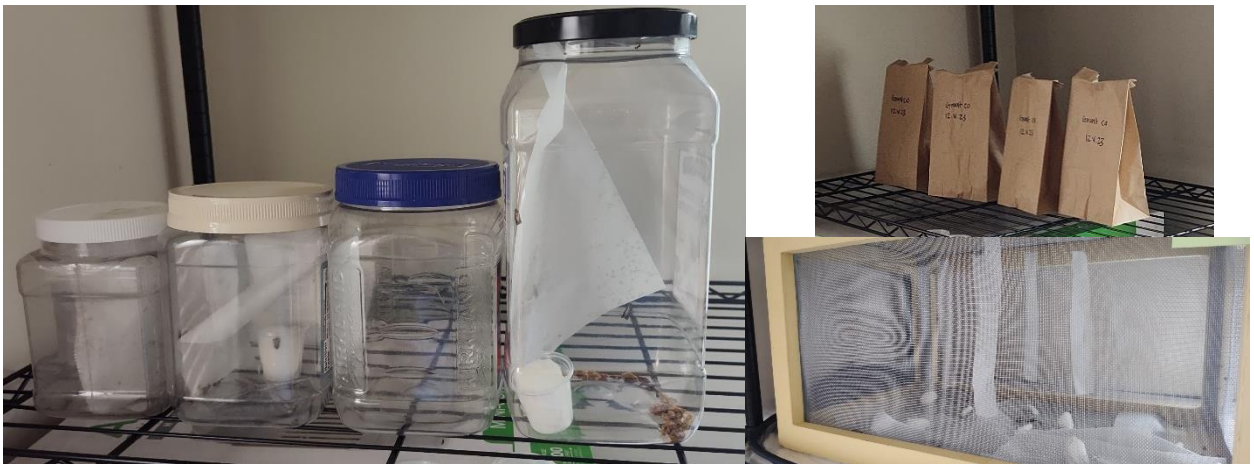

Figure 3. Some mating arena set-ups tested

Because it was possible that the cardboard band process negatively impacted moth fitness, beginning in July 2023, we changed tactics and began extracting codling moth larvae from infested apples collected on farms in Chelan, Douglas, and Grant Counties, while placing cardboard bands on farms in Chelan, Douglas, Grant, Yakima, and Okanagan Counties (Fig. 2). Larvae extracted from apples and cardboard bands, 766, were fed on artificial diet, pupated, and emerged as adults. The first several rounds of adult moths were once again placed into the mating arenas, and neither mating nor

egg laying occurred. The mating arena design that our colony failed to mate in was based on descriptions found in White and Hutt 1970, 1971; Hathaway et al. 1971, 1972, 1973; Howell and Clift 1972; Bathon et al. 1991; Toba and Howell 1991; Dyck 2010. Again, we changed tactics and tested several new and modified mating arena setups (Fig. 3). By the end of July, one of the tested mating arenas successfully allowed mating, egg deposition, and produced viable larvae (Fig. 4,5). From these mating individuals we obtained 302 F1 generation larvae by the middle to end of September. The majority of these individuals entered diapause and are housed in cold storage and will not be used until late January 2024. Cardboard bands from several on-farm locations will be processed through winter 2023/24.

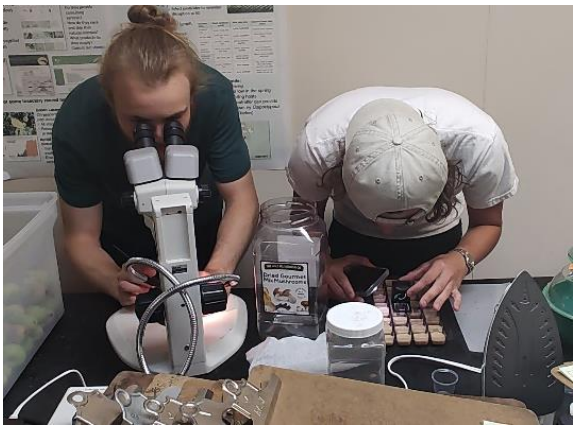

Figure 4. Transferring F1 Generation CM larvae from mating arena to artificial diet

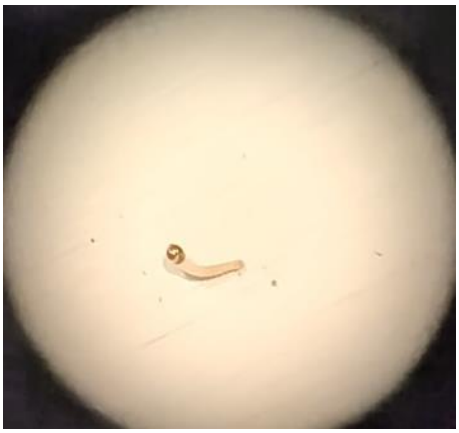

Figure 5. First successful F1 generation CM larva

Objective 2: Lab-test current and new conventional and organic materials and strategies' effectiveness as CM control tactics

Due to the lack of mating in the first mating arena tested, much of the first half of the year yielded no larvae upon which to test insecticides. However, now that we have successful rearing occurring in the colony, we will be able to begin testing new materials in 2024. In addition, we will utilize individuals from cardboard bands in some insecticide tests.

Objective 3: Supply moths to researchers and companies to sustain outside funding (prevent future requests to WTFRC).

As described, we only produced 302 F1 Generation final instar larvae in 2023, however, when they break diapause, they will be used to produce enough eggs and larvae to provide to other projects.

Objective 4: Update extension resources to include new product information

No new updates were produced in 2023 due to the previously described issues.

References:

Bathon, H, et al. "Rearing Methods." Tortricid Pests: Their Biology, Natural Enemies and Control. , vol. 5, World Crop Pests, The Netherlands, Amsterdam, 1991, pp. 283–293.

Dyck, V. A. “Collecting, Holding, Marking and Sexing Adults.” Rearing Codling Moth for the Sterile Insect Technique, Food and Agriculture Organization of the United Nations, Rome, 2010, pp. 75–79.

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Hathaway, D. O., L. G. Schoenlfber, et al. “Codling moths: Plastic pellets or waxed paper as oviposition substrates.” Journal of Economic Entomology, vol. 65, no. 6, 1972, pp. 1756–1757, https://doi.org/10.1093/jee/65.6.1756.

Hathaway, D. O., L. V. Lydin, et al. “Monitoring mass rearing of the Codling Moth.” Journal of Economic Entomology, vol. 66, no. 2, 1973, pp. 390–393, https://doi.org/10.1093/jee/66.2.390.

Howell, J. F., and A. E. Clift. “Rearing codling moths on an artificial diet in trays.” Journal of Economic Entomology, vol. 65, no. 3, 1972, pp. 888–890, https://doi.org/10.1093/jee/65.3.888.

Toba, H H, and J F Howell. “An improved system for mass-rearing codling moths.” J. Entomol. Soc. British Columb, vol. 88, 1991, pp. 22–27.

White, L. D., and R. B. Hutt. “An inexpensive transparent holding cage for insect oviposition and longevity studies.” Journal of Economic Entomology, vol. 64, no. 2, 1971, pp. 551–552, https://doi.org/10.1093/jee/64.2.551.

White, L. D., and R. B. Hutt. “Codling Moth catches in sex and light traps after exposure to 0, 25, or 40 krad of gamma irradiation.” Journal of Economic Entomology, vol. 64, no. 5, 1971, pp. 1249–1250, https://doi.org/10.1093/jee/64.5.1249.

White, L. D., and R. B. Hutt. “Effects of gamma irradiation on longevity and oviposition of the codling moth.” Journal of Economic Entomology, vol. 63, no. 3, 1970, pp. 866–869, https://doi.org/10.1093/jee/63.3.866.

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

Report Type: Continuing Project Report

Primary-PI: RT Curtiss
Organization: Washington State University - TFREC
Telephone: (917) 685-1546
Email: rcurtiss@wsu.edu
Address: 1100 N. Western Ave
City/State/Zip: Wenatchee, WA 98801

CO-PI 2: Louie Nottingham
Organization: Washington State University - NWREC
Telephone: 540-798-2044
Email: louis.nottingham@wsu.edu
Address: 16650 State Route 536
City/State/Zip: Mount Vernon, WA 98273

CO-PI 3: Tobin Northfield
Organization: Washington State University - TFREC
Telephone: 509-293-8789
Email: tnorthfield@wsu.edu
Address: 1100 N. Western Ave
City/State/Zip: Wenatchee, WA 98801

Cooperators: Teah Smith (Zirkle), Matt Jeffery (McDougall & Sons), Torrey Hansen (Auvil), Randy Brown (Gebbers), Nick Stephens, M&M orchards, Warren Morgan Orchards LLC, and Jeff Pheasant and Nathan Wash (AgriMACS).

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:
Funding Duration:
Amount: **$**
Agency Name:
Notes: *After Western SARE preproposal was invited to submit a full-proposal for cost off-sets, it was rejected in 2023 for the ca. $345,000 request.*
WTFRC Collaborative Costs: none

Budget 1
Primary PI: RT Curtiss
Organization Name: Washington State University
Contract Administrator: Anastasia Mondy
Contract administrator email address: arcgrants@wsu.edu
Telephone: 503-335-4564
Station Manager/Supervisor: Chad Kruger
Station manager/supervisor email address: cekruger@wsu.edu

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 – 2022-2023 key findings

- 192 total releases in 2022-2023 resulted in variable capture by lure and mating disruption (MD) type
- Early spring capture is again poor with all lures
- Passive mating disruption (hand applied reservoir dispensers) again suppressed capture for 4 out of 5 lures
- The CMDA+AA lure again had the most consistent capture across the three MD schemes
- More replication in year 3 is needed to accurately estimate traps/acre and population densities

Objective 2

- PI Curtiss has presented preliminary findings at 4 grower meetings in 2022 and 3 in 2023
- 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 contain 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. 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 (Figure 1).

Figure 1. 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 2). 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.

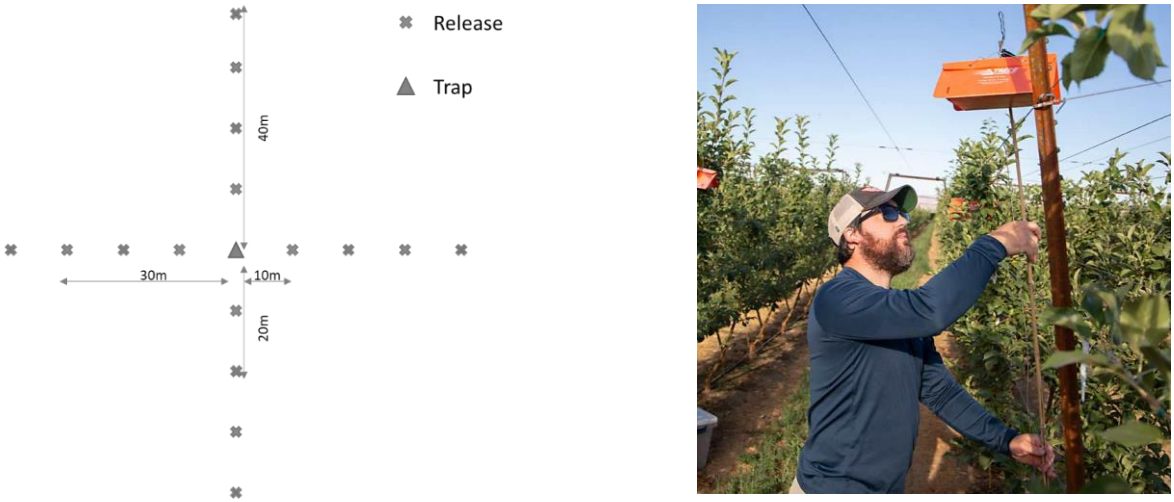

Figure 2. Cardinal-direction mark-release-recapture with a single central trap experimental layout. RT Curtiss is shown hanging a trap in the orchard canopy (R. Courtney, Good Fruit Grower Magazine).

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 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 3).

		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 3. 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 were 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., 2017). 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.

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

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 were conducted in 45 commercial orchards in 2022–2023. Orchards were divided into three geographically distinct blocks corresponding to latitudes and longitudes 46-47°N and 119–121°W (Royal city region), 47–48°N and 119–121°W (Quincy Region), and 48–49°N and 119–121°W (Okanogan Region). 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, unless moth supply issues interfered.

There were 100 total releases performed over 20 weeks of the summer 2022, and due to moth supply issues only 92 releases were performed in 2023. Each orchard (lure × mating disruption combination) received at least two releases, resulting in 9–15 replications of each combination across the three geographic blocks and the two years of releases. There were no statistical differences in moth capture due to geography or treatment type, likely due to the low overall replication of only two years' releases. 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 wild moth 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 had the most consistent capture across the three mating disruption schemes and provided the overall highest combined capture (see Figure 4).

Preliminary population density estimates based on the 2022-2023 replications also show some trends (Figure 5). The CMDA+AA and Megalure 4k lures both appear to detect codling moth at low population levels across management schemes. 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.

Although the results presented in this continuing report are only from two seasons, and thus the replication is lower than the total planned, 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 year will be returned many-fold to the industry through more precise codling moth spray decisions.

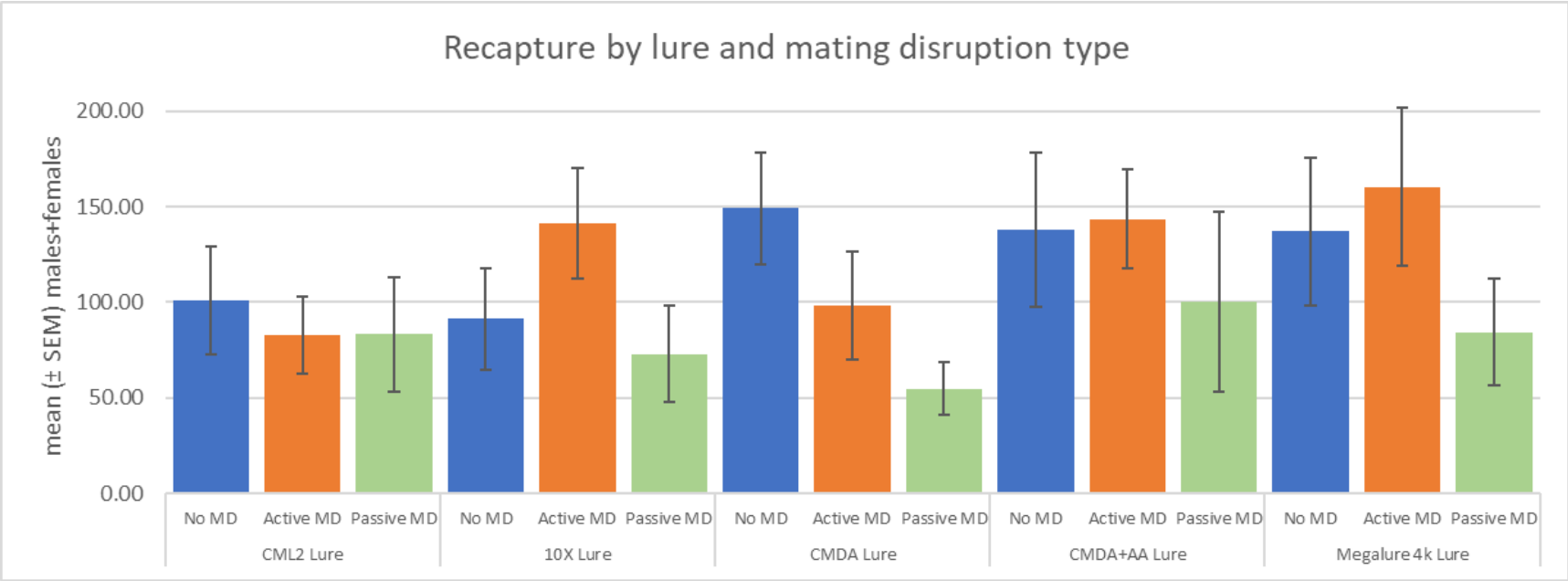

Figure 4. Preliminary results on mean (± SEM) combined recapture of males and females by lure × mating disruption type (2022 – 2023). The data sets are (L to R): CML2 Lure, 10xLure, CMDA Lure, CMDA+AA Lure, and Megalure 4K Lure. The data bars within each data set are (L to R): No Mating Disruption (MD), Active MD, and Passive MD.

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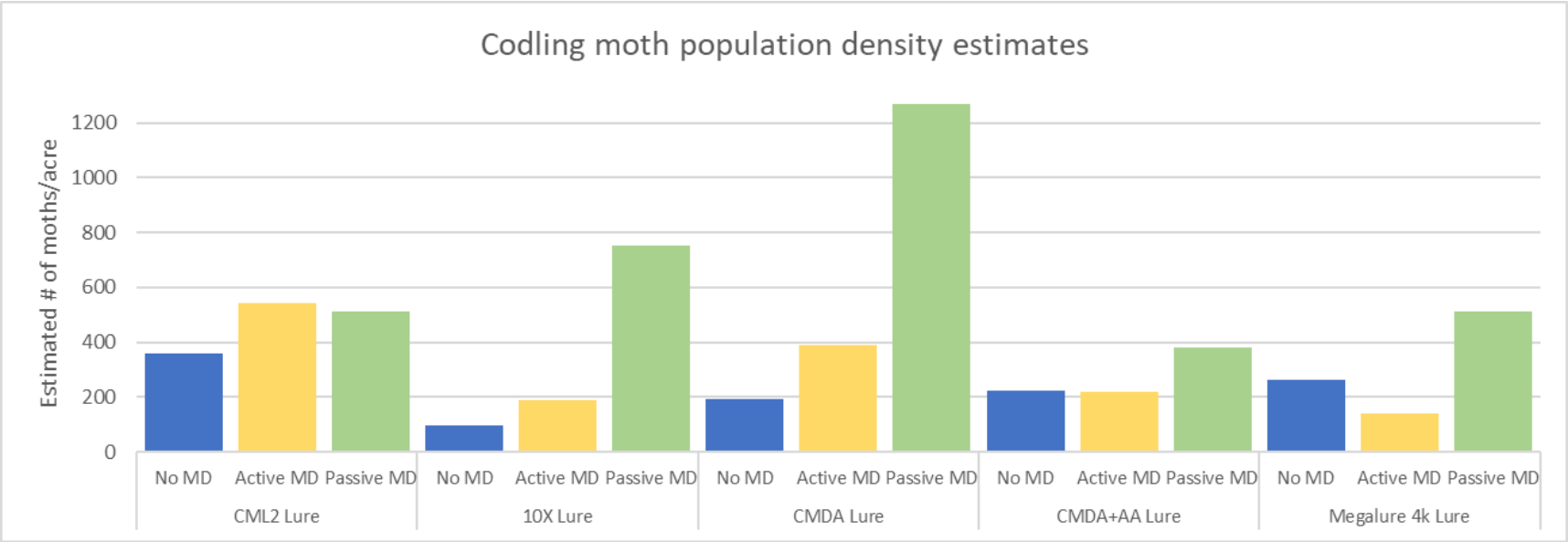

Figure 5. Preliminary results on codling moth population density estimates when one moth is captured in a trap (2022 – 2023) in orchards with three different pheromone treatments. There are no Standard Error measures for this estimate, thus there are no error bars on this figure. The data sets are (L 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. Smaller bars indicate less disruption of lures by pheromone treatments.

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

PI RT Curtiss has presented preliminary project findings at four grower meetings in 2022 and three in 2023. At least 150 growers and decision-makers were present collectively at these meetings. Although the decision matrix is in development (See Preliminary table 1), it will not be completed until after field season 2024 when enough replications are accomplished to provide meaningful results for analysis. As of the writing of this report, the project webpage and project-related fact sheets are in development. Project fact sheets will be completed by project end.

In addition to project-specific activities, we 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 that was ultimately rejected. The Western SARE proposed project would have allowed us to expand the scope of this project, cover unanticipated cost increases, and fund additional personnel. Unexpected cost increases are primarily for sterile moths which have increased considerably since our original quote in summer 2021 (quoted at $24/unit in 2021, cost $30/unit in 2022, increased to $38/unit in 2023) when this project was in preparation. The increased costs of sterile moths caused us to not have sufficient funds for hiring hourly staff. Despite the lack of staff, we were able to complete all the releases for which we received moths, but we had to postpone work on the project fact sheets and attend fewer grower meetings in 2023. Those same financial issues are likely to continue in 2024 when moth costs may increase again.

References:

Adams, C.A.; Schenker, J.H.; McGhee, P.S.; Gut, L.J.; Brunner, J.F.; and Miller, J.R. 2017. Maximizing Information Yield from Pheromone-Baited Monitoring Traps: Estimating Plume Reach, Trapping Radius, and Absolute Density of *Cydia pomonella* (Lepidoptera: Tortricidae) in Michigan Apple. Journal of Economic Entomology 110 (2): 305-318.

Curtiss R.T. 2021. Factors influencing sterile codling moth (*Cydia pomonella* L.) recapture, dispersion, and effectiveness as a control tactic in apple orchard systems. PhD. Dissertation, Michigan State University Press, East Lansing, MI.

Curtiss R.T., Nottingham L., and Gut L.J. 2023. Estimating plume reach and trapping radii for male and female *Cydia pomonella* (Lepidoptera: Tortricidae) captured in pheromone–kairomone baited traps in Washington State apple orchards under mating disruption. J. Econ. Ent. 116(5): 1592–1603. https://doi.org/10.1093/jee/toad167

Miller, J.R.; Adams, C.G.; Weston, P.A.; and Schenker, J.H. 2015. Trapping of Small Organisms Moving Randomly: Principles and Application to Pest Monitoring and Management. Springer Briefs in Ecology. pp 114.

		LURE TYPE				
		CML2	**CM 10x**	**CMDA**	**CMDA+AA**	**Megalure 4k**
MATING DISRUPTION TYPE	**Passive**	Recapture: 0.385% n=13 Dispersive Distance: 86m Population Est.: 510/ha Trap area: 5.73ac # Traps / 10 ac: 1.74	Recapture: 0.314% n=12 Dispersive Distance: 87m Population Est.: 754/ha Trap area: 5.88ac # Traps / 10 ac: 1.70	Recapture: 0.255% n=12 Dispersive Distance: 87m Population Est.: 1270/ha Trap area: 5.88ac # Traps / 10 ac: 1.70	Recapture: 0.432% n=13 Dispersive Distance: 85m Population Est.: 382/ha Trap area: 5.61ac # Traps / 10 ac: 1.78	Recapture: 0.400% n=10 Dispersive Distance: 87m Population Est.: 511/ha Trap area: 5.88ac # Traps / 10 ac: 1.70
	Active	Recapture: 0.356% n=13 Dispersive Distance: 91m Population Est.: 544/ha Trap area: 6.42ac # Traps / 10 ac: 1.56	Recapture: 0.608% n=15 Dispersive Distance: 87m Population Est.: 190/ha Trap area: 5.88ac # Traps / 10 ac: 1.70	Recapture: 0.471% n=9 Dispersive Distance: 89m Population Est.: 389/ha Trap area: 6.15ac # Traps / 10 ac: 1.63	Recapture: 0.618% n=11 Dispersive Distance: 88m Population Est.: 218/ha Trap area: 6.00ac # Traps / 10 ac: 1.67	Recapture: 0.739% n=14 Dispersive Distance: 85m Population Est.: 142/ha Trap area: 5.61ac # Traps / 10 ac: 1.78
	None	Recapture: 0.434% n=11 Dispersive Distance: 90m Population Est.: 359/ha Trap area: 6.28ac # Traps / 10 ac: 1.59	Recapture: 0.433% n=10 Dispersive Distance: 95m Population Est.: 305/ha Trap area: 7.02ac # Traps / 10 ac: 1.42	Recapture: 0.642% n=14 Dispersive Distance: 90m Population Est.: 194/ha Trap area: 6.28ac # Traps / 10 ac: 1.59	Recapture: 0.593 n=14 Dispersive Distance: 91m Population Est.: 222/ha Trap area: 6.42ac # Traps / 10 ac: 1.56	Recapture: 0.590% n=13 Dispersive Distance: 90m Population Est.: 263/ha Trap area: 6.28ac # Traps / 10 ac: 1.59

Preliminary table 1. Codling moth monitoring decision table. The first line in each cell (average treatment recapture and n) are the field findings and number of replications to date from the current study. Dispersive distances, population density estimates when one moth is captured, trap area, and recommended number of traps needed per acre are calculated from recapture data. Lower population density estimates indicate more accuracy in lure/mating disruption combinations' capture in traps.

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

Report Type: Final Project Report

Primary PI: Rebecca Schmidt-Jeffris
Organization: USDA-ARS
Telephone: 509-454-6556
Email: rebecca.schmidt@usda.gov
Address: 5230 Konnowac Pass Rd
City/State/Zip: Wapato, WA 98951

Co-PI 2: Louis Nottingham/Robert Orpet
Organization: Washington State University
Telephone: 509-293-8756
Email: louis.nottingham@wsu.edu, robert.orpet@wsu.edu
Address: 1100 N Western Ave
City/State/Zip: Wenatchee, WA 98801

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), Teah Smith (Zirkle Fruit), Greg Newman (NWFM) [note: pear grower cooperators will be specified in pear report]

Project Duration: 2-Year, 3 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, Artemia (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 expanded the efforts in this grant by providing support

to conduct grower input sessions and a needs assessment survey. The WIPMC grant was also used to start a grant team and stakeholder advisory group that submitted the WSARE grant (below).

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

Requested

Funding Duration: June 2024 – May 2027
Amount: $350,000
Agency Name: Western Sustainable Agriculture Research and Education (WSARE)
Notes: This project proposal used the data gathered from "Tactics to improve natural enemy releases in tree fruit" to develop targeted questions that will allow for the creation of best management practices for lacewing releases in tree fruit.

Funding Duration: June 2024 – May 2027
Amount: $81,139
Agency Name: Washington Tree Fruit Research Commission (ACP)
Notes: The WSARE proposal above includes funding for one lead technician's salary and extension activities. Due to budget limitations, we were unable to request salary for additional research support. Therefore, this funding request is for an assistant for the lead technician so that the research can be completed. We will be informed of the funding decision in March.

Funding Duration: 2024-2026
Amount: >$15,000
Agency Name: BioBee
Notes: In-kind match for the above WSARE project; commercial insectary lacewings (Awarded: will receive if the above is funded)

Funding Duration: 2024-2026
Amount: ~$7,500
Agency Name: Zirkle Fruit
Notes: In-kind match for the above WSARE project; commercial insectary lacewings and drone pilot labor/fees (Awarded: will receive if the above is funded)

Funding Duration: June 2024 – May 2027
Amount: $109,581
Agency Name: Washington Tree Fruit Research Commission (ACP) & Fresh and Processed Pear Committee Research
Notes: New funding request to pursue research on whirligig mite releases and conservation. In addition to unrelated work in potatoes, this proposal was brought about by results from Obj. 1 of this project and other projects in pears.

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

Note: This report contains apple-related content only. Pear results will be presented in the pear report. Findings from preliminary work (2020-2021) are also included to provide context for the chosen treatments and results of the 2022-2023 research.

OBJECTIVES

Obj. 1. Improve retention of released natural enemies. We tested whether commercially available food supplements (Artemia cysts on tape, *Ephestia* eggs on cards) and lures (methyl salicylate) increased retention of released natural enemies and also examined whether they recruited resident natural enemies and decreased pest populations. Only Artemia cysts were used in 2023 (*Ephestia* eggs were dropped). All fieldwork and pest/natural enemy counts are completed and analyzed for this project, but the molecular work is not yet complete. Several factors caused significant delays, including a move to a new lab space (which needed repairs before use) and the need to change our gut content protocols; we determined that neither pear psylla nor orchard aphid pests amplify well with COI universal primers. To overcome this, a colleague (B. Ohler) designed a pear psylla primer and we adapted aphid primers from another lab – these must be run as a separate PCR from the COI primers, increasing the number of samples we are running. Finally, the need to identify lacewings using molecular techniques (see below) added many additional samples to our workflow. The molecular work will be completed before the project term date (June 2024).

Obj. 2. Determine cost-effectiveness and efficacy of natural enemy release by drone. In 2022, 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. We determined that the 0.25-acre plot trials were not an adequate method for testing drone releases and instead focused entirely on various ground-based methods for releasing lacewings in 2023. An objective specifically testing lacewing releases by drone at a large scale was included in the proposed WSARE project (see other/related funding sources).

SIGNIFICANT FINDINGS

Mealybug destroyer releases are likely not a viable tactic for controlling mealybugs in orchards. They were marginally successful in the 2020 trial, but in the three following trials (2021-2023), they did not lower mealybug counts and rapidly dispersed from the orchard. Because this insect costs $680-950/acre to release, the low chance of success means that fruit growers should avoid using this insect and other options should be explored for mealybug control.

Convergent ladybeetles tested as part of the 2023 mealybug destroyer study (as a comparison treatment) also rapidly dispersed from the orchard and did not lower mealybug counts. This provides preliminary evidence that adult ladybeetles are not suited for orchard releases, but should be investigated further.

Lacewing identification became a critical component of this project. We determined that the "*Chrysoperla carnea*" we purchased for trials in 2021 were actually *C. externa* (purchased as larvae) and *C. plorabunda* (purchased as eggs). *Chrysoperla externa* can be separated from other lacewings visually under magnification, but to distinguish between "resident" lacewings and the released *C. plorabunda*, we had to develop molecular methods. We determined that the COI gene, which we are using in our gut content analysis, can also be used to separate resident from released lacewings. It is important to note that the lacewing species present in orchards that is often referred to as "*C. plorabunda*" is likely *C. johnsoni* and therefore a different species that what is commercially available. However, *C. plorabunda* is native to Washington (found outside of orchards) and therefore likely to be a better climate match that *C. rufilabris*.

Lacewing releases varied in efficacy. In 2021, releases of *C. plorabunda* eggs or *C. rufilabris* larvae reduced aphid abundance by 57% and 43%, respectively. In the following two years,

none of the lacewing release treatments reduced aphid abundance. We attributed this to very high initial aphid counts in 2022 and poor survival of released lacewings in 2023, potentially due to the use of organic pesticides. In general, low numbers of released lacewings were recovered, but recovery was very poor in 2023 compared to 2021-2022. Therefore, when determining efficacy of beneficial releases, scouts should focus on pest numbers, not necessarily natural enemy recovery; however, presence or absence of the released natural enemy can give some sense of survival. We determined that releases need to be conduced earlier in the season than anticipated (mid-April in southern Washington) to get an adequate head-start on aphids.

Tactics for retaining and recruiting natural enemies had highly variable results between sites and years. In general, methyl salicylate lures showed some promise for recruiting lacewings and *Stethorus*. Food supplements may have increased *O. insidiosus* retention.

***Orius insidiosus* releases** were performed as part of the retention experiments, but data from these trials also allowed us to access the efficacy of this predator for pest control. One release of *O. insidious* (2,000/acre) reduced adult thrips on sticky cards by 50% in both apple trials. Evaluations of thrips damage did not occur as part of this work, but should be included in future studies. More frequent releases (at lower rates) may be more effective and economical.

Whirligig mite was found in abundance on beat trays in some of our study locations. The role of this predator in North American orchards has received little attention, but research from Ireland and preliminary work from other projects suggest that it may be an important orchard natural enemy. It recently became available for purchase in the U.S. (Oregon only).

Grower survey and discussion, 2021-2022. Leveraged funding from the Western IPM Center allowed us to conduct a grower survey and a series of listening sessions (in collaboration with Tianna DuPont and Ashley Thompson). 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. Information from the survey and sessions was used to support the pending WSARE grant application to expand the work on lacewings.

RESULTS AND DISCUSSION

Obj. 1. Improve retention of released natural enemies

The study was conducted two commercial organic apple orchards (Wapato, WA in 2022 and Benton City, WA in 2022). The Wapato orchard had a very high ant population, so we changed locations in 2023 in case the ants were affecting our release treatments. The Wapato orchard primarily had rosy apple aphid (RAA) with some green apple aphid (GAA), whereas the Benton City orchard had primarily RAA with some woolly apple aphid (WAA). The Benton City orchard was chosen in part because it typically has serious WAA infestations, but overall aphid abundance was uncharacteristically low in 2023.

There were five treatments consisting of combinations of lure use (Predalure, methyl salicylate), food supplements (Artemia, brine shrimp cysts on tape Fig. 1 + *Ephestia* eggs on cards), and releases (100,000 "*C. carnea*" lacewing eggs + 2,000 *Orius insidiosus* per acre): (1) Predalure + Foods + Release, (2) Predalure + Release, (3) Food + Release, (4) Release only, and (5) No-release control. In

Fig. 1. Ladybeetle feeding on Artemia tape

2022, the "Food" treatment only used Artemia tape (the *Ephestia* eggs were dropped). Rates for the food treatments and lures were: 1 lure/plot, 50 m Artemia tape/plot, and 35,000 *Ephestia* eggs/plot (1 card/30 tags). Each combination was replicated in the orchard 5 times in 0.25-acre plots. Aphids and lacewings were counted prior to release and then once weekly after release. Aphids were counted in the field by counting the number of infested leaves (GAA, RAA) or number of colonies (WAA) per 3 shoots each on 9 trees in the center of the plot. Beat tray samples were collected from the 9 center trees of each plot and all natural enemies from the tap counts were collected and stored in ethanol for identification and use in molecular gut content analysis. Two sticky cards were also hung in each plot to monitor adult natural enemies. The "*C. carnea*" have been tentatively identified as *C. plorabunda* (see lacewing results in Obj. 2).

We conducted an additional study using a similar design in the USDA research orchard in Moxee, WA. Because of the size of the orchard, we removed the no-release treatment. In both years, we only tested the Artemia tape (no *Ephestia* eggs). In 2022, we only released *O. insidiosus*, while in 2023, we also released *C. carnea* eggs.

In the six trials (2 commercial apple, 2 research apple, 2 pear), only 8 *O. insidious* were recovered. However, the consistent decrease in thrips counts in plots where *O. insidiosus* were released indicates that this predator remained in plots long enough to reduce pest populations. Although it was most commonly found 1-2 weeks post-release, in the 2022 commercial apple and 2023 research apple orchards, *O. insidiosus* were found over a month after release. This species is not native to Washington and has never been found in an area where it was not recently released, therefore all recovered *O. insidiosus* are from that year's releases. Of the few *O. insidiosus* found, 75% of them were recovered from plots with supplementary foods. The two individuals recovered from plots without foods were found one month post-release, when the foods were likely completely consumed/decayed. Therefore, there is some evidence that the Artemia tape increased retention of *O. insidiosus* in the field. In future studies examining efficacy of *O. insidiosus* for thrips control in apples, the use of releases in combination with Artemia tape should be explored.

Molecular identification of the *carnea*-group lacewings recovered from the retention trials is ongoing (see lacewing release results in Obj. 2 for more information on lacewing identification). All samples have been processed and sequenced. Sequences have been aligned and we are currently constructing computationally-intensive phylogenetic trees to determine which collected individuals "match" the controls directly removed from insectary bottles. This analysis is anticipated to be completed in February 2024. Based on preliminary analysis, no treatment increased

	Commercial apple			
	Lures		Foods	
	2022	2023	2022	2023
Aphids	increase	not abundant	no effect	not abundant
Thrips	increase	no effect	no effect	slight decrease
Campylomma	no effect	increase	no effect	no effect
Lacewings	slight increase	no effect	no effect	no effect
Ladybeetles	no effect	no effect	no effect	no effect
Stethorus	increase	no effect	no effect	decrease
Syrphids	no effect	not abundant	no effect	not abundant
Whirligigs	not abundant	slight decrease	not abundant	no effect
Spiders	no effect	no effect	no effect	no effect

	Research apple			
	Lures		Foods	
	2022	2023	2022	2023
Aphids	not abundant	decrease	not abundant	no effect
Thrips	decrease	no effect	no effect	no effect
Brown mites	decrease	no effect	no effect	no effect
Apple rust mite	no effect	no effect	no effect	no effect
Campylomma	not abundant	not abundant	not abundant	not abundant
Lacewings	increase	no effect	no effect	no effect
Ladybeetles	no effect	no effect	no effect	no effect
Stethorus	increase	no effect	no effect	decrease
Syrphids	not abundant	increase	not abundant	no effect
Whirligigs	not abundant	no effect	not abundant	increase
Spiders	increase	no effect	no effect	no effect

Results summary from retention trials.

retention of released lacewings. However, applications of methyl salicylate lures timed for approximately when released lacewings become adults (as opposed to during the release) may increase the likelihood that the adults remain in the orchard.

In the commercial apple orchard in 2022, releases of *O. insidiosus* and *C. plorabunda* decreased green apple aphids by ~50% compared to the no-release control (Fig. 2). Rosy apple aphids were also present but appeared to be unaffected by our treatments. Seasonal thrips counts were reduced by 30% and counts immediately after release were reduced by 50% (Fig. 3). 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. 2). 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. 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.

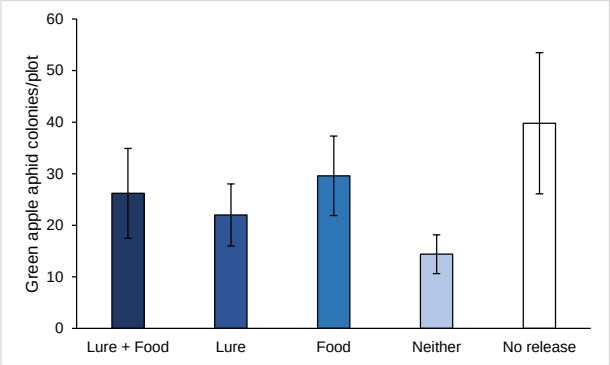

Fig. 2. Releases decreased GAA, but foods and lures increased GAA. 2022 seasonal sums.

In the 2023 commercial apple orchard, aphid populations were too low to discern differences between treatments. Pest thrips seemed to increase in the "release only" treatment, but this effect was not seen in the treatment with releases in combination with food (Fig. 4). Lure treatments had slightly more *Campylomma* and fewer whirligig mites. In this trial, *Stethorus* did not increase in the lure treatments, but instead decreased in the food treatments.

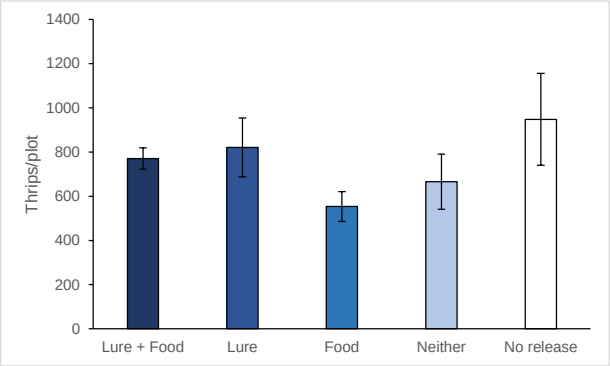

Fig. 3. Releases decreased thrips, especially in the food treatment. 2022 seasonal sums.

In the 2022 retention trial in the Moxee research orchard, lure treatments had fewer brown mites (Fig. 5). Thrips were 48% less abundant in plots with lures. Because this trial was conducted in July, aphids were scarce and the effects of treatments on aphids could not be assessed. Lures increased abundance of lacewings by 100% and spiders by 50%. Surprisingly, *Stethorus* populations were 45% lower in lure plots – they appeared to just "follow" where brown mites were higher.

In 2023, the Moxee trial was conducted earlier in the season. Aphid counts were halved in plots with lures (Fig. 6). Lacewing and *Stethorus* abundance did not differ between treatments, but syrphid counts were higher in the lure treatments; syrphids may have been responsible for the decrease in aphid abundance in the lure treatments. Whirligig mites were more abundant in the food treatments.

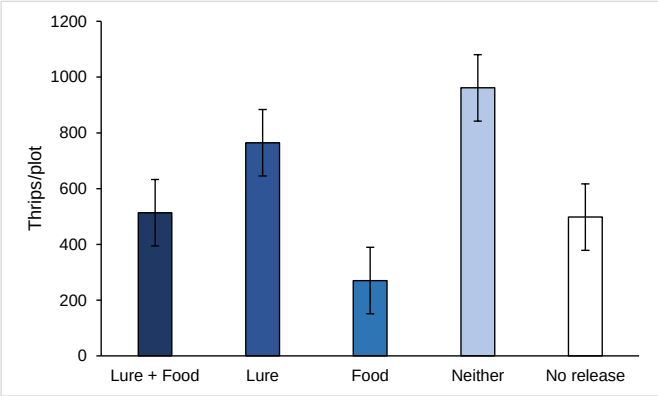

Fig. 4. Releases decreased thrips, but only in the food treatment. 2023 seasonal sums.

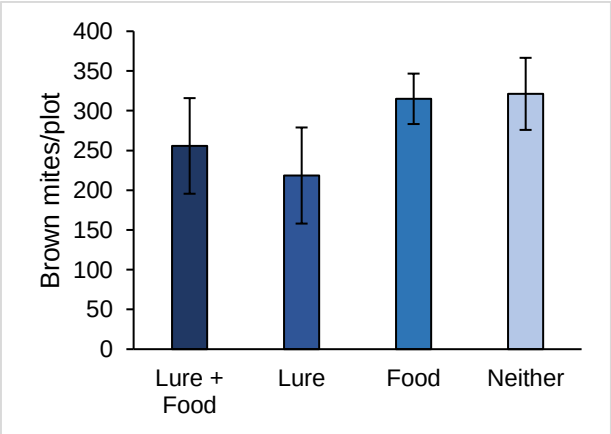

Fig. 5. Lures decreased brown mites. 2023 seasonal sums.

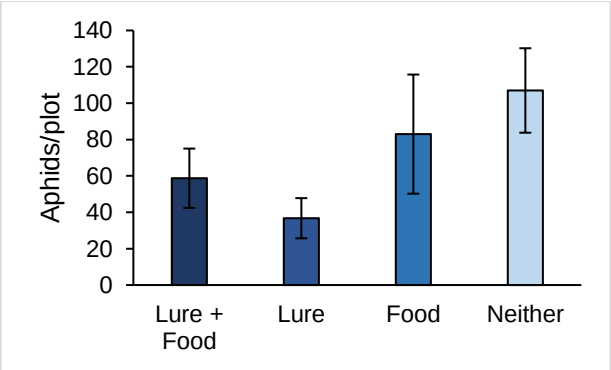

Fig. 6. Lures decreased aphids. 2023 seasonal sums.

Between sites and across years, there was very little consistency in the effects of the treatments. Taken in combination with the pear data, *Stethorus* generally increased in plots with lures and may exert control on mites while rapidly moving between plots. Lacewings also showed a similar, although weak, trend. Because natural enemies interact with each other and pests over time, it is difficult to discern if changes in natural enemy abundance due to treatments are due to predation amongst themselves or changes in pest densities. The gut content work, which should be completed by June 2024, may provide additional information about these relationships.

Obj. 2. Determine cost-effectiveness and efficacy of natural enemy release by drone

Mealybug destroyers, 2020-2023. All trials were conducted in organic commercial apple orchards. In 2020, mealybug destroyer adults released by hand in either May or June caused a numerical ~3× decrease in mealybug populations compared to the no-release control, but this effect was highly variable between plots. The drone release did not cause a decrease. Recovery of the mealybug destroyers was moderate 1 and 2 weeks after the early season release: ~3 per plot, then ~1 per plot, respectively (27 then 9 per acre). After this period, only 1 mealybug destroyer was found across all plots each week for the rest of the sampling period (0.5 mealybug destroyers/acre). The plots in this trial were small (0.11 acres), so we sought to conduct the work at a larger scale the following year.

	Mealybug Destroyer Treatments
2020	5,000 adults/acre by ground May 14
	5,000 adults/acre by ground June 17
	2,000 adults/acre by ground June 17
	5,000 adults/acre by drone June 17
	No release control
2021	1,000 adults/acre by ground on May 27
	1,000 adults/acre by drone on May 27
	No release control
2022	2,000 adults/acre by ground May 20
	2,000 larvae/acre by ground May 18
	2,000 adults/acre by ground May 20
	No release control
2023	2,000 adults/acre by ground on June 22
	"Grower standard" = 20,000 convergent ladybeetle adults/acre by ground on June 23
	No release control

In 2021, we examined mealybug destroyer releases in one-acre plots (five replicates/treatment), 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. The 1-day recovery was lower in the drone (0.4/acre) compared to the hand-release treatment (3.3/acre). There were no differences between treatments in mealybug counts.

In 2022, mealybug destroyers released as larvae or adults were never recovered after release, although there were enough mealybugs in plots for them to feed on. There were no differences in mealybug counts between treatments. A series of organic fire blight and mildew sprays made during the releases may have negatively affected the mealybug destroyers: Serenade+Previsto on May 19, ProBlad Verde+Merivon+FireLine+FireWall on May 20, and Serenade+Previsto on May 23. The effects of these pesticides on natural enemies are not well described. In a preliminary lab trial, we found that Serenade at field rate did not cause any mortality in adult mealybug destroyers after 24 h, but follow-up studies are needed.

In 2023, we sought to compare mealybug destroyers to a "grower standard": releases by the grower of convergent ladybeetles (20,000/acre). No mealybug destroyers were recovered post-release. One convergent ladybeetle was found pre-release (indicating that they were already present in the orchard) and three were found throughout the entire four-week post-sampling period. These may have been released or "resident" ladybeetles. The treatments did not differ in mealybug counts. 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 (~$0.30/insect).

	Lacewing Treatments	Release Date
2021	100,000 "*C. carnea*" eggs/acre sprinkled	May 5th
	20,000 "*C. carnea*" larvae/acre sprinkled	
	100,000 *C. rufilabris* eggs/acre sprinkled	
	20,000 *C. rufilabris* larvae/acre sprinkled	
	No release control	
2022	100,000 "*C. carnea*" eggs/acre sprinkled	May 12th
	100,000 *C. rufilabris* eggs/acre sprinkled	May 4th
	100,000 *C. rufilabris* eggs/acre cards	May 4th
	100,000 *C. rufilabris* eggs/acre drone	May 5th
	20,000 *C. rufilabris* larvae/acre sprinkled	May 4th
	No release control	
2023	100,000 "*C. carnea*" eggs/acre sprinkled	May 11th
	100,000 "*C. carnea*" eggs/acre cards	
	100,000 *C. rufilabris* eggs/acre sprinkled	
	100,000 *C. rufilabris* eggs/acre cards	
	20,000 *C. rufilabris* larvae/acre sprinkled	
	No release control	

Lacewing release efficacy, 2021-2023. All trials were conducted in organic commercial apple orchards. In 2021, we purchased *Chrysoperla rufilabris* (Fig. 7) and "*Chrysoperla carnea*". We found that the *C. carnea* larvae (which came from a different insectary than the "*C. carnea*" egg order) were actually *C. externa*. Both *C. externa* and *C. rufilabris* can be visually distinguished from other lacewings under magnification. Species of *Chrysoperla* lacewings in the "*carnea*-group" cannot be separated using visual characteristics. This includes the insectary "*C. carnea*" sold as eggs and our native *Chrysoperla* lacewings (*C. johnsoni* is the most common). The most reliable method of identification is

Fig. 7. A released *C. rufilabris* seen in the orchard in post-release sampling

mating song analysis, which requires multiple, live specimens of both sexes. We determined that the COI gene (although it cannot be used to definitively identify a lacewing to species) can be used to "match" DNA sequences of field-collected lacewings to those taken directly from the shipping container; this method can therefore distinguish "released" and "resident" lacewings. A collaborating lacewing biologist (K. Taylor, University of Maryland) will conduct song analysis on specimens from this insectary in 2024 to provide a definitive identification. Preliminary comparison of genetic sequences from other insectaries indicates this is likely *C. plorabunda*.

In 2021, the *C. plorabunda* egg and *C. rufilabris* larvae releases reduced aphid counts compared to the control (Fig. 8), but the other treatments did not. *Chrysoperla externa* ("*C. carnea*" larvae) is likely a poor climate match for our area because it is native to the southeastern U.S. Low numbers of larvae of the released lacewing species were found throughout the trial (1-5 per treatment, across 8 weeks of sampling) (Fig. 9). Therefore, when determining efficacy of beneficial releases, scouts should focus on pest numbers and the presence/absence of the released natural enemy, not necessarily the abundance of the released natural enemy. We also found several species of native, non-released *Chrysopa* lacewings. *Chrysopa* larvae were not found until three weeks after our releases and then in lower numbers than our released lacewings (Fig. 9). 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 trial were *Chrysopa*, therefore we did not find evidence that the released lacewing larvae fully developed and reproduced.

In 2022, none of the treatments caused a reduction in aphids (Fig. 8). Pesticide applications were made much more frequently in this orchard compared to the orchard used in 2021. Initial aphid counts were 13-times higher in this trial than in 2021 (Fig. 8). However, recovery of lacewing larvae was higher than in 2021 (Fig. 9). None were recovered from the drone treatment or the control (Fig. 9). There were also resident populations of *Chrysoperla* lacewings, which also would have contributed to biological control. 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, potentially with larvae. Two adult *C. rufilabris* were found during the trial, indicating that this species can complete development in Washington orchards. Additionally, the *C. rufilabris* larvae found 8-weeks post-release (Fig. 9) is likely a second generation, indicating that the adults reproduced in the orchard. Genetic analysis of the *carnea*-group adults is still being conducted to determine if any of the released insectary *C. plorabunda* reproduced in the orchard. These results indicate that early season organic spray programs should be evaluated for effects on lacewings.

In 2023, applications of organic pesticides (including spinosad) were made more frequently than the prior two trials. Genetic analysis of the *carnea*-group adults is still being conducted to determine if any of the released insectary *C. plorabunda* reproduced in the orchard. None of the treatments differed from the control in aphid counts (Fig. 8) and recovery of released lacewings was

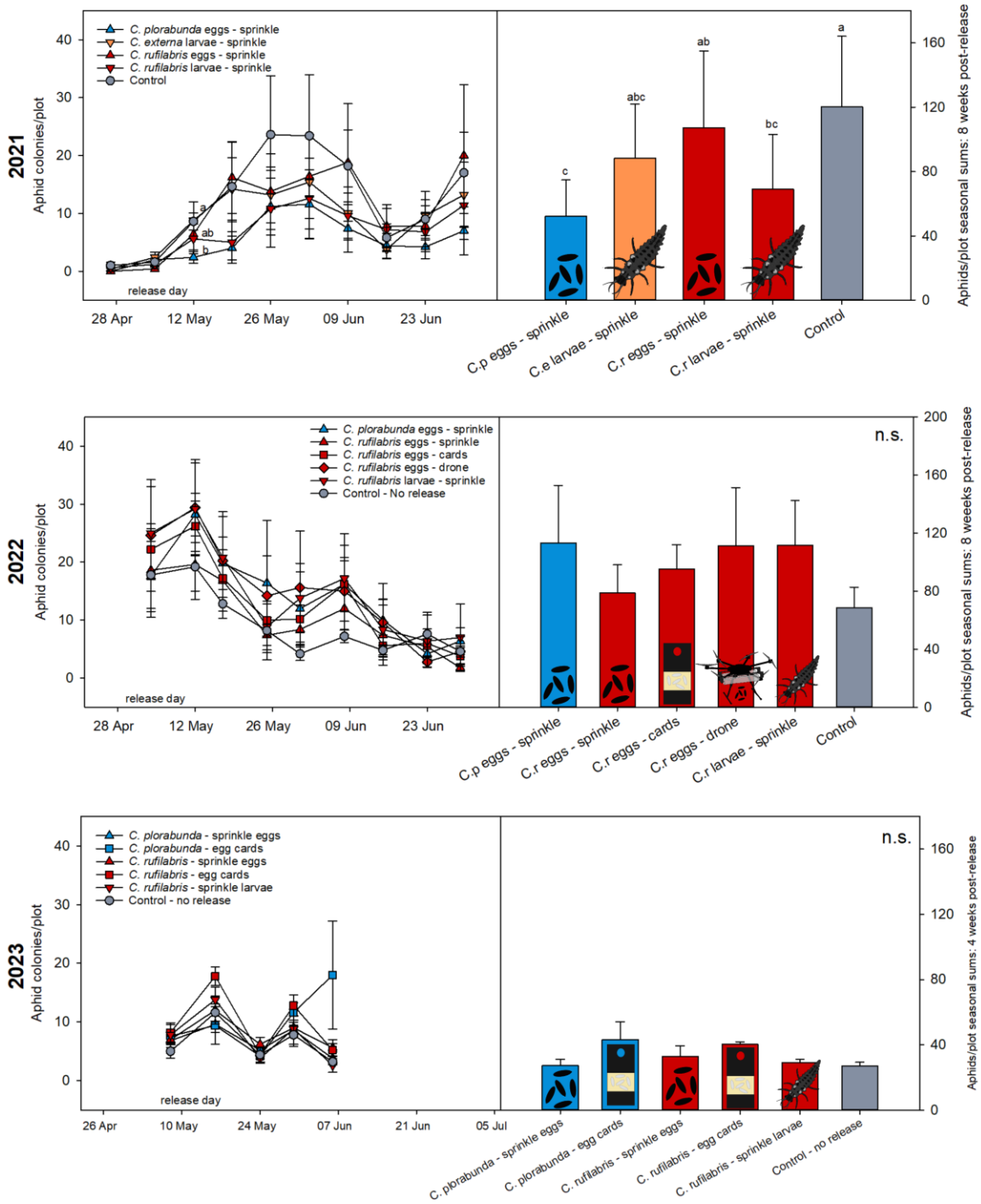

Fig. 8. Aphid counts (all species) by date and seasonal sums following single releases of lacewings released at a rate of 100,000 eggs/acre or 20,000 larvae/acre.

very low compared to the two previous years (Fig. 9). Collectively, the 2022-2023 results highlight the importance of using a selective spray program when conducting releases and releasing as early as

	Lacewing spp.	Released stage	Release method	Lacewing larvae/plot									
				Pre-release	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Total
2021	*C. plorabunda*	eggs	sprinkled	0	0.2	0	0	0.2	0	0	0	0	0.4
	C. externa	larvae	sprinkled	0	0.6	0	0.4	0	0	0	0	0	1
	C. rufilabris	eggs	sprinkled	0	0	0.4	0	0	0	0	0	0	0.4
	C. rufilabris	larvae	sprinkled	0	0.8	0	0.4	0.2	0	0	0	0	1.4
	Resident *Chrysoperla*	-	-	0	0	0.04	0	0	0	0	0	0	0.04
	Resident *Chrysopa*	-	-	0	0	0	0.04	0.04	0.04	0	0	0	0.12
2022	*C. plorabunda*	eggs	sprinkled	0	0	0.2?	0	0	0	0	0	0	0.2?
	C. rufilabris	eggs	sprinkled	0	0	0	0.2	0	0	0	0	0	0.2
	C. rufilabris	eggs	card	0	0.4	1.6	0.2	0	0	0	0	0	2.2
	C. rufilabris	eggs	drone	0	0	0	0	0	0	0	0	0	0.0
	C. rufilabris	larvae	sprinkled	0	0.2	0.6	0.2	0	0	0	0	0.2	1.2
	Resident *Chrysoperla*	-	-	0.04	0.04	0.20	0.08	0.04	0.04	0	0	0	0.4
	Resident *Chrysopa*	-	-	0	0	0.10	0.03	0.07	0.27	1.20	0.53	0.17	2.4
2023	*C. plorabunda*	eggs	sprinkled	0	0.8?	0	0.2?	0					1?
	C. plorabunda	eggs	card	0	0.2?	0	0	0					0.2?
	C. rufilabris	eggs	sprinkled	0	0	0	0	0					0.0
	C. rufilabris	eggs	card	0	0.2	0	0	0					0.2
	C. rufilabris	larvae	sprinkled	0	0.2	0.2	0	0					0.4
	Resident *Chrysoperla*	-	-	0	0.30	0.15	0.05	0.10					0.6
	Resident *Chrysopa*	-	-	0	0	0	0.30	0.33					0.6

Fig. 9. Captures of released lacewings post-release and comparison to resident populations of *Chrysoperla* and *Chrysopa* (using control plots). The date of first capture is indicated in red (lighter shading in gray scale). "?" indicate a *carnea*-group lacewing was recovered, but identification as a resident or released individual has not yet been complete. The *C. rufilabris* larvae found on Week 8 in 2022 is likely a second generation.

possible. Early releases provide both a "head start" on resident natural enemies and make it more likely that aphid populations are at levels that lacewings can suppress. All molecular work for this project is anticipated to be completed by June 2024, including molecular identification of captured adult lacewings (2022-2023 samples) and gut content analysis of captured larvae (2021-2023 samples).

Of the natural enemies we have tested, lacewings appear to be the most promising and cost-effective. This project has allowed us to identify areas where more information is needed to provide best practice recommendations for releasing lacewings in orchards. Most importantly, these lacewing efficacy trials were conducted in one orchard each year, leaving results susceptible to factors unique to that particular location (spray programs, pest pressure, regional differences). Our pending proposal with Western SARE will address the remaining questions needed to develop best practice recommendations for releasing lacewings in orchards. This will include testing different release rates, organic pesticide compatibility, multi-site trials, and large-scale drone release assessments.

EXECUTIVE SUMMARY

Project title: Tactics to improve natural enemy releases in tree fruit

Key words: lacewing, mealybug destroyer, *Orius insidiosus*, lures, supplementary foods

Abstract:

Growers have experimented with releases of natural enemies to control pests in organic apples, but there are currently no best practice recommendations for releases in orchards. The purpose of this project was to determine which natural enemies and release methods showed the most promise for controlling orchard pests. We also examined the potential of lures and supplementary food products for recruiting resident natural enemies and retaining released natural enemies. Releases of mealybug destroyers (2,000 or 5,000/acre) showed promised in a preliminary trial (2020), but in the three following trials (2021-2023), they did not lower mealybug counts and rapidly dispersed from the orchard. Because this insect costs $680-950/acre to release, the low chance of success means that fruit growers should avoid using this insect and other options should be explored for mealybug control. Lacewing releases varied in efficacy. In 2021, releases of *C. plorabunda* eggs (100,000/acre) or *C. rufilabris* larvae (20,000/acre) reduced aphid abundance by 57% and 43%, respectively. In the following two years, none of the lacewing release treatments reduced aphid abundance. We attributed this to very high initial aphid counts in 2022 and poor survival of released lacewings in 2023, potentially due to the use of organic pesticides. Across all years, releases of lacewing larvae resulted in higher recapture than releases of eggs. Low numbers of released lacewings were recovered in all trials, even in treatments where aphid abundance decreased. Therefore, when determining efficacy of beneficial releases, scouts should focus on pest numbers, not necessarily natural enemy recovery; however, presence or absence of the released natural enemy can give some sense of survival. Releases should be conducted earlier in the season than anticipated (mid-April in southern Washington) to get an adequate head-start on aphids. Multi-site studies are needed to fine tune recommendations, but early season (mid- to late April) releases appear to be critical for success. Tactics for retaining and recruiting natural enemies had highly variable results between sites and years. In general, methyl salicylate lures showed some promise for recruiting lacewings and *Stethorus*. Food supplements may have increased retention of released *O. insidiosus* and subsequently reduced thrips abundance. The use of lures after a lacewing release should be investigated to determine if they encourage released lacewings to remain in the orchard after they develop into adults.

WTFRC INTERNAL PROJECT – BUDGET SHARED FOR INFORMATIONAL PURPOSES ONLY

CONTINUING REPORT **PROJECT LENGTH (CROP YEARS)**: 2023-2025

Project Title: Pesticide residues of WA apples

Primary PI: Tory Schmidt
Organization: WA Tree Fruit Research Commission
Telephone: (509) 669-3903
Email: tory@treefruitresearch.com
Address: 1719 Springwater Ave.
City/State/Zip: Wenatchee, WA 98801

Cooperators: Gerardo Garcia (WTFRC), Northwest Horticultural Council, Pacific Agricultural Labs (Sherwood, OR), Cameron Burt, WSU Sunrise Research Orchard

Project Duration: 3 Years

Total Project Request for Year 1 Funding: $ 6600
Total Project Request for Year 2 Funding: $ 6825
Total Project Request for Year 3 Funding: $ 7050

Other related/associated funding sources: Most chemical products donated by registrants

Primary PI: Tory Schmidt
Organization Name: WTFRC
Contract Administrator: Paige Beuhler
Telephone: (509) 665-8271
Contract administrator email address: paigeb@treefruitresearch.com

Item	2023	2024	2025
Salaries			
Benefits			
Wages1	$1,500.00	$1,600.00	$1,700.00
Benefits1	$800.00	$850.00	$900.00
RCA Room Rental			
Shipping2			
Supplies	$300.00	$300.00	$300.00
Travel3	$1,500.00	$1,525.00	$1,550.00
Plot Fees			
Miscellaneous			
Analytical lab fees	$2,500.00	$2,550.00	$2,600.00
Total	$6,600.00	$6,825.00	$7,050.00

Footnotes:
Schmidt estimates 8% 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 and driving samples to analytical lab in OR

2023 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 seventeen insecticide/acaricides, 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 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 7 and delivered the next day to Pacific Agricultural Labs (Sherwood, OR) for chemical residue analysis.

TRIAL DETAILS

- 16th 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 1.17 inches of rain fell on the trial block after the initial application of Penncozeb (May 10), with 0.71 inches occurring after August 6 (39 days before harvest)

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 2023.

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*
mancozeb	Penncozeb 75DF	128 (8 lbs)	PF (May 10)	<0.49	0.6	3	0.6 (Mex)
ethephon	Ethephon 2SL	32	June 16	<0.1	5	0.01*	0.1 (Can)
flutianil	Gatten	8	35	0.014	0.15	0.01*	0.15 (many)
abamectin	AgriMek SC	4.25	35	<0.01	0.02	0.01*	0.01 (many)
benzovindiflupyr	Aprovia	7	35	0.043	0.2	0.01*	0.2 (many)
pydiflumetofen	Miravis	3.4	35	0.071	0.2	0.01*	0.2 (many)
tolfenpyrad	Bexar	27	35 & 21	0.61	1	0.01*	0.01 (Twn,Tha)
indoxacarb	Avaunt	6	35 & 21	0.15	1	0.01*	0.1 (Can)
flupyradifurone	Sivanto prime	14	35 & 21	0.39	0.7	0.01*	0.5 (Twn)
cyflufenamid	Torino	6.8	30	0.033	0.06	0.01*	0.01 (Tha)
acequinocyl	Kanemite	31	30	<0.025	0.4	0.01*	0.01 (Chn,Tha)
lambda-cyhalothrin	Warrior II	2.56	30	0.036	0.3	0.01*	0.2 (many)
flonicamid	Beleaf 50SG	2.8	30	0.069	0.2	0.01*	0.2 (many)
cyflumetofen	Nealta	13.7	30 & 14	0.23	0.3	0.01*	0.3 (Can,Mex)
sulfoxaflor	Transform	2.75	30 & 14	0.13	0.5	0.01*	0.3 (many)
chlorantraniliprole	Altacor eVo	2.2	30 & 14	0.20	1.2	0.01*	0.4 (many)
cyprodinil	AXIOS Cion	9	30 & 14	<0.01	1.7	0.01*	0.05 (Idn)
ipflufenoquin	AXIOS Cion	9	30 & 14	0.041	0.15	0.01*	0.01 (Tha)
afidopyropen	Versys	3.5	30 & 14	<0.05	0.02	0.01*	0.02 (many)
buprofezin	Centaur WDG	34.5	21	0.043	3	0.01*	1 (Twn)
phosmet**	Imidan 70-W**	92	14	1.8	10	0.01*	2 (Twn)
mefentrifluconazole	Cevya	5	14	0.12	1.5	0.01*	0.01 (Tha)
cyclaniliprole	Verdepryn	11	14	0.056	0.3	0.01*	0.2 (many)
cyfluthrin	Baythroid XL	2.8	14	<0.05	0.5	0.01*	0.1 (many)
fenazaquin	Magister	36	14	0.40	0.6	0.2	0.01 (Tha)

[1] Top markets for WA apples with established MRLs; 14 November 2023. 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 2023.

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*
mancozeb	Penncozeb 75DF	128 (8 lbs)	70 (June 29)	1.8	0.6	3	0.6 (Mex)
benzovindiflupyr	Aprovia	7	35	0.034	0.2	0.01*	0.2 (many)
pydiflumetofen	Miravis	3.4	35	0.064	0.2	0.01*	0.2 (many)
acequinocyl	Kanemite	31	35 & 21	0.032	0.4	0.01*	0.01 (Chn,Tha)
abamectin	AgriMek SC	4.25	30	<0.01	0.02	0.01*	0.01 (many)
lambda-cyhalothrin	Warrior II	2.56	30 & 21	0.053	0.3	0.01*	0.2 (many)
flonicamid	Beleaf 50SG	2.8	30 & 21	0.029	0.2	0.01*	0.2 (many)
tolfenpyrad	Bexar	27	30 & 14	0.049	1	0.01*	0.01 (Twn,Tha)
flupyradifurone	Sivanto prime	14	30 & 14	0.18	0.7	0.01*	0.5 (Twn)
indoxacarb	Avaunt	6	21 & 14	0.082	1	0.01*	0.1 (Can)
flutianil	Gatten	8	21 & 14	0.019	0.15	0.01*	0.15 (many)
chlorantranliprole	Altacor eVo	2.2	21	0.11	1.2	0.01*	0.4 (many)
cyclaniliprole	Verdepryn	11	21 & 7	0.11	0.3	0.01*	0.2 (many)
cyflumetofen	Nealta	13.7	21 & 7	0.25	0.3	0.01*	0.3 (Can,Mex)
phosmet**	Imidan 70-W**	92	21 & 7	4.5	10	0.01*	2 (Twn)
cyflufenamid	Torino	6.8	14	0.043	0.06	0.01*	0.01 (Tha)
buprofezin	Centaur WDG	34.5	14	1.1	3	0.01*	1 (Twn)
afidopyropen	Versys	3.5	14 & 7	<0.05	0.02	0.01*	0.02 (many)
ethephon	Ethephon 2SL	48	14 & 7	0.90	5	0.01*	0.1 (Can)
sulfoxaflor	Transform	2.75	14 & 7	0.13	0.5	0.01*	0.3 (many)
cyprodinil	AXIOS Cion	9	14 & 7	<0.01	1.7	0.01*	0.05 (Idn)
ipflufenoquin	AXIOS Cion	9	14 & 7	0.052	0.15	0.01*	0.01 (Tha)
fenazaquin	Magister	36	7	0.37	0.6	0.2	0.01 (Tha)
cyfluthrin	Baythroid XL	2.8	7	<0.05	0.5	0.01*	0.1 (many)
mefentrifluconazole	Cevya	5	7 & 1	0.37	1.5	0.01*	0.01 (Tha)

[1] Top markets for WA apples with established MRLs; 14 November 2023. 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

With the exception of the mid-summer application of Penncozeb 75DF, no material produced a residue that exceeded the tolerance level set by the US Environmental Protection Agency; mancozeb products are rarely used on apples in WA, and usually applied in the spring. We included Penncozeb 75DF in these apple studies as a proxy for pears, where the use of mancozeb products are more common. No residues of mancozeb could be detected when it was applied at its more typical petal fall timing. Other than the late use of Penncozeb 75DF, 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. Several products we tested did produce **residues which exceed Maximum Residue Levels** (MRLs) set in important export markets for Washington apples: **Penncozeb 75DF, Kanemite, Bexar, Avaunt, Imidan 70-W, Torino, Centaur WDG, Ethephon 2SL, AXIOS Cion, Magister, and Cevya.** 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 would potentially violate India's standards.

Results from this year's study produced no detectable residues of ethephon when applied to promote return bloom (June 16), but did find residue levels which could be problematic for apples to be exported to Canada when Ethephon 2SL was applied 14 & 7 days before harvest. This was the first year we applied ethephon at this preharvest timing and plan to repeat that treatment in 2024.

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: Assessing Barriers to and Benefits of AMF Colonization in Apple

Report Type: Continuing Report

Primary PI: Dr. Tracey Somera
Organization: USDA ARS Tree Fruit Research Laboratory
Telephone: (858) 344-9750
Email: tracey.somera@usda.gov
Address: 1104 N. Western Ave
City/State/Zip: Wenatchee, WA 98801

Cooperators: Dr. Lee Kalcsits; Dr. Loren Honaas

Project Duration: 3-Year

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
Primary PI: Dr. Tracey Somera
Organization Name: USDA ARS Tree Fruit Research Laboratory
Contract Administrator: Chuck Meyers & Sharon Blanchard
Telephone: 510.559.5769 (CM), 509.664.2280 (SB)
Contract administrator email address: chuck.myers@usda.gov, sharon.blanchard@usda.gov
Station Manager/Supervisor: David Rudell
Station manager/supervisor email address: david.rudell@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 pathogenic root fungi and tolerance to water stress.

SIGNIFICANT FINDINGS:

- Phylogenetic (tree-based) taxonomic assignments of sequence data greatly improved our ability to assign taxonomy to AMF sequences and supported the detection of a new, undescribed lineage of AMF.

- The use of phylogenetic-based tools by industry producers would improve quality control in terms of AMF product composition/consistency.

- Pre-established (nursery-derived) AMF *strongly* influence AMF community structure after planting and limit effective colonization by mycorrhizal inoculants. However, depending on the species/rootstock present, commercial inoculants can effectively alter and/or compete with pre-existing AMF communities.

- Optimal mycorrhizal colonization of apple root systems occurs in a rootstock genotype-AMF species specific manner.

- Among AMF, *Rhizophagus* and *Claroideoglomus* spp. appear to colonize apple root tissue rapidly and maintain strong associations over time.

- Faster growing (i.e., more vigorous) rootstocks were associated with faster rates of initial AMF establishment.

- Matching host genetics with compatible AMF species has the potential to enhance agricultural practices in nursery and orchard systems.

METHODS:

Development of an in-house, up-to-date tool for taxonomic classification of AMF (required for Objective 1): Obtaining accurate taxonomic assignments of DNA sequences from arbuscular mycorrhizal fungi (i.e., Phylum Glomeromycota) using currently available reference databases (e.g., UNITE, Gen Bank) is challenging (Stefani et al., 2020). This is because the eukaryotic nuclear ribosomal internal transcribed spacer (ITS) gene, which is the standard "barcode" for molecular identification of fungi, does not provide adequate resolution of AMF taxa. As a rule, in order to describe arbuscular mycorrhizal fungi at the genus and species-level, the scientific community generally sequences the V4 region of the 18S rRNA gene (Stefani et al., 2020). At present, there are no web-based, AMF-specific (18S rRNA) databases that are actively curated (i.e., up to date) (e.g. MaarjAM; https://maarjam.ut.ee/). Therefore, the

best way to infer taxonomic identify of AMF sequence data is via the placement of these sequences onto a phylogenetic tree.

In collaboration with Dr. Loren Honaas and Dr. Huiting Zhang (an expert taxonomist), a phylogenetic tree for Phylum Glomeromycota was successfully constructed based on over 140 high-quality (i.e., from well-identified AMF cultures) 18S rRNA gene sequences (Krüger, et al. 2012; Stefani et al., 2020). This includes 91 different AMF species from 24 different genera. In itself, the phylogenetic tree represents an important achievement and is currently being used in the Somera Lab to accurately assign high throughput sequencing data to the species level (i.e., the resolution required in order characterize AMF community structure and composition).

Tree-based assessment of AMF community structure in apple rootstocks inoculated with commercially available AMF (updated analysis for Objective 1): An experiment designed to assess the ability of commercially available AMF to compete with pre-existing/nursery-derived AMF contained in apple rootstocks was previously conducted (as described in the Year 1 Continuing Report). For each rootstock genotype (G.890, G.935, M.7, and M.26), DNA was extracted from 1) root tissue obtained pre-planting (i.e., the nursery-derived AMF community), 2) root tissue obtained following cultivation in pasteurized potting mix (PPM) inoculated with a commercially available "multi-species" AMF product and 3) root tissue obtained following cultivation in non-inoculated PPM. PacBio Sequel DNA sequence data was then generated using the Glomeromycota-specific primer set AML1/AML2 (Lee et al., 2008). This year, the AMF communities from these samples were reevaluated using a phylogenetic approach.

AMF product panel - Selection (Objective 1; for Experiment 1): Extensive research was conducted in order to identify commercially available AMF products readily available to growers in Washington State. Seven different**,** single-species AMF products designed for soil application which contained ecologically relevant AMF species expected to interact successfully with apple were selected for testing (Table 1). An additional 3 AMF products were obtained from the International Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM). The INVAM products included AMF species belonging to the genus *Paraglomus*, which has been repeatedly identified in apple roots cultivated in a variety of orchard soils in Central Washington (Van Horn, et al., 2021; Somera et al., 2021).

AMF product panel - Viability assessment (Objective 1; for Experiment 1): High spore/propagule viability is an essential prerequisite for any mycorrhizal product designed for use in agroecosystems. All products tested were purported to contain at least 100 spore/propagules per gram. We are currently in the process of confirming this. In addition, the Most Probable Number (MPN) method was used to confirm product viability in corn (Cochran, 1950). Corn is the standardized assay host because it is highly prone to forming associations with AMF, produces abundant roots in three weeks and fungal structures in the roots are easy to visualize. MPN assays were conducted under controlled environmental conditions in growth chambers. Briefly, two surface-sterilized corn seeds were sown into each 150 ml plastic cone-tainer (5 replicate cone-tainers per product) containing pasteurized growth medium (1:1 sand: vermiculite) diluted with AMF inoculum (1:10, 1:100 and 1:1000). Corn roots were harvested 4 weeks after plant emergence, fine root tissue (0.2 g per plant) was cleared and stained with trypan blue, and roots were scored as "positive" or "negative" for the presence of fungal structures using a dissecting microscope. The MPN per liter of inoculum was calculated according to MPN tables based on the number of "positive" plants per dilution.

AMF product panel - Assessment of root colonization: All 10 AMF products were screened for their ability to successfully colonize apple roots. An initial experiment was conducted in March of 2023 using 5g of inoculum per plant. The experiment was repeated in November of 2023 in accordance with the recommended application rates/procedures, which varied depending on the manufacturer (Table 1). Prior to planting into sterile growth medium (1:1 sand: vermiculite), the roots of 6-week old apple seedlings

(Gala) were sprinkled with a "coating" of AMF inoculum; any remaining material was then incorporated into the planting hole. The experiment included 10 biological replicates for each AMF product and the non-amended control treatment. After 6-weeks (Dec 2023), ½ of the plants were harvested (n = 5 per treatment). The extent of mycorrhizal colonization in each treatment is currently being assessed via trypan blue staining (Fig. 1).

Identification of compatible AMF/rootstock genotype associations (for Objective 2): Two separate experiments designed to assess the effects of rootstock genotype and arbuscular mycorrhizal fungal (AMF) species on colonization of apple were completed in July of 2022 and in August of 2023. In both of these experiments, we directly tested the ability/efficacy of four different "ecologically relevant" AMF species to colonize four commercially available apple rootstock genotypes. The AMF species used in these experiments 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). Single species inocula were obtained from MycoInTech, Tarragona, Spain. Nursery-derived apple rootstocks come with pre-existing AMF communities. Therefore, micro-propagated dwarfing (G.11, G.41) and semi-dwarfing (G.210, G.890 and G.969) plantlets of uniform size were used (North American Plants, McMinnville, OR).

As described in the Year 1 Continuing Report, micro-propagated plantlets were either inoculated with individual species of AMF or were not inoculated. In the first experiment (July 2022), pasteurized potting mix was used as the growth medium. Due to the excessive level of phosphorous in the pasteurized potting mix (P = 186 mg/kg), the experiment was repeated (August 2023) using pasteurized orchard soil (P = 15 mg/kg). The rootstock genotypes G.11, G.41, G.890, and G.969 were crossed with five different AMF treatments (uninoculated control, *R. irregularis*, *S. deserticola*, *C. claroideum*, and *C. etunicatum*). Root mycorrhization and plant growth were assessed after 2 weeks, 5 weeks (an agriculturally relevant time frame sufficient for colonization to occur) and 8 weeks.

RESULTS AND DISCUSSION:

Tree-based assessment of AMF community structure in apple rootstocks inoculated with commercially available AMF (updated analysis for Objective 1): Previously, AMF taxonomy was determined using the AMF-specific reference sequence database MaarjAM (Opik, et al., 2010) (Year 1 Continuing Report). However, as noted above, this database is no longer actively curated. Tree-based (phylogenetic) taxonomic assignments of sequence data greatly improved our ability to accurately assign taxonomy to AMF sequences. Phylogenetic classification revealed that all AMF sequences detected in the commercial mixture (CM) either branched from Clade 2 (*Funneliformis/Glomus*) or Clade 4 (*Claroideoglomus* species) (Fig. 2). In Clade 2, the amplicon sequence variants (ASVs) identified in the commercial mixture were most closely related to either *Funneliformis mosseae or Funneliformis coronatus*. Further, *F. monosporum* (= *Glomus monosporum*, *F. mosseae;* https://invam.ku.edu/mosseae) was one of the AMF species purported to be contained in the CM. Therefore, in contrast to previous web-based sequence analyses, tree-based taxonomic assignments provided evidence that *F. mosseae* was in fact present in the mixture. It should be noted, this AMF species was only detected in the commercial inoculum and did not appear to successfully colonize apple roots. This result was unexpected considering that *Funneliformis* species including *F. mosseae* have been identified in the apple orchards of Washington State and Italy (Van Horn et al., 2021; Turrini, et al., 2015).

The commercial inoculum also contained a handful of ASVs in Clade 4 (*Claroideoglomus*) (Fig.2). Of these, ASV 32 (most closely related to *Claroideoglomus luteum* and *Claroideoglomus claroideum*) was also present in M.26 and M.7 (1X) samples. In addition, ASV 79 and 96 (most closely related to *Claroideoglomus claroideum*) were detected in M.26 1X samples. These taxa represented ~3%

of the total AMF community in both M.26 (1X) and M.7 (1X) treatments but were *not* detected in apple roots pre-planting or in non-inoculated controls. Therefore, it is likely that the *Claroideoglomus* species contained in the commercial inoculum successfully colonized both Malling rootstocks. In fact, ordination analysis of relative abundance data indicated that M.26 and M.7 did not differ significantly in AMF community composition in the 1X CM treatment (Fig. 3). Notably, no ASVs associated with the CM were detected in the Geneva rootstocks used in this study (G.890 and G.935).

Rootstock genotype appears to be an important factor in the assembly of AM fungal communities. However, rootstock genotype/AMF associations are not always differentiated by rootstock type (e.g., Geneva vs. Malling). In this experiment, clear differences in compatibility between *Rhizophagus* species and a variety of apple rootstock genotypes were observed. Within Clade 1, ASV 1 and 2 were the dominant taxa (Fig. 2). ASV 1 was most closely related to the references sequences for *Rhizophagus irregularis* and *R. vesciculiferous*, while ASV 2 appeared to be most closely related to *Rhizophagus fasciculatus* **(=***Glomus fasciculatum*). ASV1 was present in both G.935 and M.7 pre-planting at relatively high abundance (~25%). Therefore, the association between G.935 and M.7 with ASV1 (*R. irregularis* or *R. vesciculiferous*) was most likely formed at the nursery. The relative abundance of ASV1 in these rootstocks remained unchanged (~25%) after planting, regardless of treatment (Control vs. 1X CM). The ability of these rootstocks to maintain a strong association with ASV1 suggests that the symbiosis was highly compatible. Similar to ASV1, ASV 5 and 6 (*R. irregularis/ R. vesciculiferous*) were also consistently associated with G.935 regardless of treatment. One-way ANOSIM analysis of relative abundance data also indicated that G.935 and M.7 did not differ significantly in AMF community composition in any treatment (Fig. 3).

In comparison, ASV2 (*R. fasciculatus*) was the most abundant AMF taxa (~25%) identified in G.890 pre-planting. ASV2 was also the only *Rhizophagus* species detected in M.26 (5-8%) at this time. In G.890, the relative abundance of ASV2 remained relatively unchanged after planting, regardless of treatment (Control vs. 1X CM; ~20-25%). In M.26, the relative abundance of ASV 2 greatly increased in the non-inoculated control samples (~25%) but was not detected in the roots of plants cultivated in the 1X CM treatment. This indicates that, unlike G.890, M.26 did not maintain its initial association with ASV2 in the presence of the commercial inoculum. One-way ANOSIM analysis of relative abundance data also reflected differential responses by M.26 and G.890 to the 1X CM treatment (Fig. 2). Although *Rhizophagus clarum* was purported to be in the commercial mixture, no *Rhizophagus* species were detected in the CM samples.

It should be noted that, in general, genetically different nuclei can coexist within individual AMF spores (i.e., there is high within-spore variation in rDNA sequences) (Harrison, 1999; Hijri and Sanders, 2005; Mathieu et al., 2018). In particular, the intraspecific sequence variability for *R. irregularis* is known to be relatively high (Chen et al., 2018). This may explain the high number of ASVs (27) associated with only three different species of *Rhizophagus* (Fig. 2; Clade 1). Along these same lines, Clade 5 represents AMF in the Order Archaeosporales. Similar to Clade 1, the large number of ASVs likely represents a limited (albeit undetermined) number of AMF species. Within Clade 5, for example, ASVs 4, 11 and 3 (all terminal branches connected to a single node) likely represent sequence variation within a single species. All three ASVs became elevated in root tissue after planting (10-30% relative abundance) regardless of rootstock genotype but persisted in the presence of the commercial inoculum in M.26 and M.7 only (~35% relative abundance).

Another a group of closely related sequences likely to represent a single species included ASVs 14, 28, and 16 (Clade 5). Taken together, these ASVs accounted for >25% of the total AMF community in M.26 non-inoculated control samples. Unlike ASVs 4, 11 and 3, this association was not maintained in the presence of the commercial inoculum (1X M.26). A similar pattern was seen in M.26(1X) with ASV 2

(*R. fasciculatus*). AMF community shifts/losses in M.26 may have been partly due to colonization by a *Claroideoglomus* sp. from the commercial mixture.

Finally, phylogeny-based classification indicated the existence of a unique, well-supported clade of AMF (Clade 3) of unknown taxonomy which represented >10% and ~1-2% relative abundance in M.26 and G.935 pre-planting, respectively. Although comparisons of ASV sequences with those deposited in the NCBI 18S rRNA sequence (SSU) database (blastn) identified *F. mosseae* as the closest match (≥ 92% identity for ASV 23, 85, 19, 49, 41 and 139), it is clear that Clade 3 represents a distinct phylogenetic group (Fig. 2). Most importantly, this result suggests that we detected a new, undescribed lineage of AMF and highlights the need for additional studies which utilize phylogenetic-based classification (as opposed to web-based databases) to characterize indigenous AMF communities, including those adapted to the orchard/nursery systems of Washington State. A better understanding of what "real" AMF communities look like will be important for the successful transfer of compatible rootstock/AMF combinations from lab to field.

Taken together, the results of this analysis show that predicting AMF community dynamics in apple rootstocks is complex (even in an AMF-free growth medium). In this experiment, a number of different rootstock/AMF interactions which were established pre-planting persisted, regardless of treatment. These included G.935/M.7 x *R.irregularis/R. vesciculiferous* and G.890 x *Rhizophagus fasciculatus* . This result supports the hypothesis that nursery-derived and/or pre-established AMF communities can strongly influence AMF community structure in apple roots after planting. However, because AMF species and rootstock genotype are both factors which determine compatibility, commercial inoculants may effectively alter and/or compete with pre-existing AMF communities, depending on the species/rootstock present. In this experiment, a *Claroideoglomus species* (*C. claroideum* or *C. luteum*) derived from the commercial inoculant preferentially colonized Malling vs. Geneva rootstocks.

Considering the context dependency of AMF community structure in response to commercial AMF formulations, it is important to point out that even if commercial inoculants do not effectively colonize plant roots, they can still impact AMF community dynamics. In this study, for example, an AMF species within Clade 5 (Order Archaeosporales), became elevated in all 4 rootstock genotypes after planting into non-inoculated control soil (10-30% relative abundance). In the presence of the commercial inoculum, however, the nursery derived Archaeosporales species persisted in M.26 and M.7 only (~35% relative abundance).

The lack of AMF species purported to be contained in the CM is highly concerning. The product used in this experiment was indeed selected on the grounds that it contained "a diverse mix of AMF species." While this is concerning, it is not surprising, given the issues associated with the use of web-based databases for determining AMF identity. As interest in the use of AMF in orchard systems seems to be growing, industry producers should consider the use of phylogenetic-based tools to ensure quality-control in terms of product composition/consistency.

The development of a phylogenetic tree for the analysis of mycorrhizal fungal communities is expected to benefit future projects within the Somera Lab. This tree will be a critical resource for subsequent experiments designed to further assess the ability of commercially available AMF products to compete with pre-existing AMF (from the nursery where they are produced) as well as with native AMF/fungi in "live" orchard soil. This work is planned for Spring 2024.

AMF product assessment (required for Objective 1): The MPN method was used to confirm the viability of the commercially available AMF products in corn (which is highly prone to forming associations with AMF). So far, 4/10 products have effectively colonized corn (Table 1). Colonization

was not detected in corn roots for 2/3 AMF products obtained from INVAM (*P. brasilianum* and *P. occultum)*. Although corn is known to host both of these species (https://invam.ku.edu/brasilianum; https://invam.ku.edu/occultum), mycorrhizal structures in *Paraglomus* spp. typically stain weakly (or not at all) in trypan blue. Thus, the MPN results for *Paraglomus* do not necessarily indicate non-viable inoculum and are unlikely to reflect actual colonization patterns. The remaining 4 products are currently being tested.

At the same time, all 10 AMF products were screened for their ability to successfully colonize apple roots. In December of 2023, ½ of the experimental plants were harvested (6-weeks post inoculation). The extent of mycorrhizal colonization in apple roots by each product is currently being assessed via staining/microscopy. None of the inoculants resulted in significant increases in total plant biomass or shoot height relative to the no-AMF control treatment (One-way ANOVA followed by Dunnett's multiple comparisons test). The remaining plants will be harvested 12-weeks post inoculation. Based on these results, 2 different "highly promising" commercially available products will be selected for use in an experiment designed to assess the ability of commercially available AMF to compete with native AMF and other indigenous fungi in "live" orchard soil (i.e., Experiment 1 as described in the original proposal). This experiment will also further explore the influence of nursery-derived AMF (pre-existing in root tissue) on AMF community structure. The apple rootstocks required for this experiment (G.11) were ordered in November of 2023 (from TRECO and Cameron Nurseries); the experiment is planned for Spring of 2024.

Identification of compatible AMF/rootstock genotype associations (in preparation for Objective 2): In the repeat experiment conducted in pasteurized orchard soil (low phosphate), rootstock genotype and AMF species were both found to significantly influence % AMF colonization ($p=0.002$ and $p=0.0001$, respectively) with 33 and 21% of the total variation coming from the main effects of AMF species and rootstock genotype, respectively. At 5-weeks post inoculation, the highest levels of mycorrhization were observed with G.11 and G.41 x *C. etunicatum* and G.11 x *C. claroideum* (Fig. 4). Although, G.11 x *C. etunicatum* was the only rootstock/AMF combination identified as being significantly higher than the associated no-AMF control treatment ($p=0.04$), no AMF structures were detected in control roots (i.e., the controls were free from infection). G.11 x *C. etunicatum* also showed significantly higher mean colonization than G.11 x *S. deserticola* ($p=0.02$) at this time (Fig. 4).

Initially, *R. irregularis* and *C. etunicatum* appeared to colonize apple roots more readily than *S. deserticola* or *C. claroideum* (2 week timepoint; Fig. 4). This was true for all rootstock genotypes with the exception of G.11. At 2 weeks, the highest colonization rates were observed in G.969 x *C. etunicatum* (9.60%) and G.969 x *R. irregularis* (9.56%). Mean colonization rates at 2 weeks were significantly lower in dwarfing (G.11, G.41 = 0.16 %) than in semi-dwarfing rootstocks (G.969, G.890 = 1.4 %) (Unpaired t test; $p = 0.009$). In addition, the effect of rootstock vigor (i.e., dwarfing vs semi-dwarfing) on colonization efficacy was statistically significant (Unpaired t-test; $p = 0.005$). Together, these results indicate that faster growing (i.e. more vigorous) rootstocks may be associated with faster rates of initial AMF establishment.

By 5-weeks post-inoculation, *R. irregularis* and *C. etunicatum* remained the best colonizers overall (Fig. 4). However, *C. claroideum* had also successfully colonized all rootstock genotypes with the exception of G.969 with % colonization rates ranging from 6-25%. In comparison, little to no colonization by *S. deserticola* was detected. Interestingly, after 5 weeks, the highest mean colonization rates were observed in G.11 x *C. etunicatum* (41%), G.41 x *C. etunicatum* (29%) and G.11 x *C. claroideum* (25%). This was surprising, considering that G.11 was the only rootstock with little to no colonization at the 2-week timepoint.

By 8 weeks post-inoculation *S. deserticola* had successfully colonized G.890 (G.969 was excluded from statistical analyses at this timepoint due to mycorrhization in the control plants) Colonization rates detected in G.890 x *C. etunicatum* (43%) and *C. claroideum* (37%) were among the highest observed over the course of the study. By comparison, little/no colonization was detected within G.11 plants despite the high levels of colonization observed in similarly treated plants at 5 weeks post-inoculation. In combination with the delayed colonization in G.11 (which became apparent 2 weeks post-inoculation), the marked reduction in colonization may suggest that G.11 is more conservative than some other rootstocks regarding it's outsourcing to AMF. In general, dwarfing rootstocks grow more slowly, do not grow for as long during the season, and may also have less leaf area relative to scions on more vigorous rootstocks (Atkinson and Else, 2001). Thus, dwarfing rootstocks may be more sensitive to the carbon costs associated with AMF colonization and may be less likely to maintain the symbiosis over time. In terms of the effects of specific rootstock genotype/AMF combinations on plant growth over time, no significant differences in plant fresh weight were detected between the no-AMF control and any of the specific treatments at either 5 or 8 weeks post-inoculation (Kruskal-Wallis test followed by Dunn's multiple comparisons test). However, the goal of this experiment was not to assess benefits of specific apple rootstock/AMF associations.

In summary, we have shown that, in apple, AMF colonization efficiency depends on both rootstock genotype and AMF species. Among AMF species, *Rhizophagus* and *Claroideoglomus* spp. appear to colonize apple root tissue rapidly and effectively, while *S. deserticola* is less effective. The ability of an AMF to *rapidly* colonize a host is likely to benefit it's competitive ability over time. Pre-established and/or AMF-rootstock combinations that lead to rapid establishment of a relationship (i.e., within 4-6 weeks) show the most promise for successful use in natural field settings with existing AMF communities (esp. post fumigation).

The final year of the project is expected to provide clear evidence of AMF species directly functioning in beneficial roles with commercially available apple rootstock genotypes. Select apple rootstock/AMF associations identified in the compatibility experiments (described above) will be used in the experiments designed to explore functional benefits of AMF colonization. Specifically, we plan to test the ability of *C.etunicatum* and *C. claroideum* to enhance plant defense against infection by the fungal replant pathogen *Rhizoctonia solani* AG-5 in G.41 and G.890.

In in collaboration with Dr. Lee Kalcsits, we will also be conducting an experiment designed to test *R. irregularis*-mediated tolerance to water stress in 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 indicates that *R. irregularis* effectively colonized G.11 after 5 weeks. This experiment will also allow us to assess whether *R. irregularis* benefits plants in terms of nitrogen uptake. Isotopic labeling of Nitrogen represents a powerful addition to the current toolkit with which to analyze the functional benefits of AMF symbioses.

TABLES AND FIGURES:

Table 1. List of commercially available AMF products tested for their ability to successfully colonize apple roots.

Manufacturer	Product/AMF Species	Recommended Application Rate*	Product Viability ‡
Symborg	MycoUp (*Glomus iranicum*)	0.5 g (diluted in water)	TBD
Lallemand	LALRISE (*Rhizophagus irregularis*)	0.1 g (diluted in water)	TBD
RTI-Ag	*Rhizophagus intraradices*	1 tsp per plant	Viable
RTI-Ag	*Funneliformis mosseae*	1 tsp per plant	Viable
RTI-Ag	*Claroideoglomus etunicatum*	1 tsp per plant	Viable
RTI-Ag	*Claroideoglomus claroideum*	1 tsp per plant	TBD
RTI-Ag	*Septoglomus deserticola*	1 tsp per plant	TBD
INVAM···	*Diversispora eburnea*	1 tsp per plant	Viable
INVAM···	*Paraglomus brasilianum*	1 tsp per plant	Not Viable§
INVAM···	*Paraglomus occultum*	1 tsp per plant	Not Viable§

**All products purported to contain spore concentrations ≥ 100 per gram*
‡ The Most Probable Number (MPN) method was used confirm product viability in corn; TBD refers to products which are currently under testing
INVAM; International Collection of Vesicular Arbuscular Mycorrhizal Fungi; University of Kansas
§ Likely due to weak staining

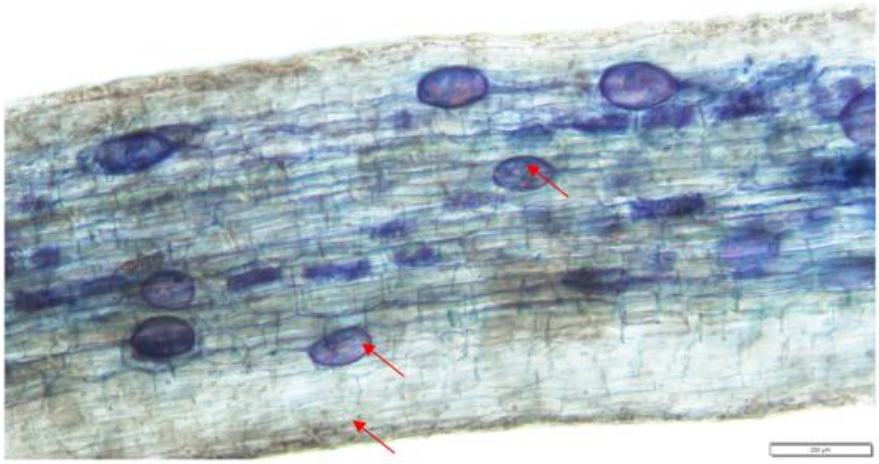

Figure 1: Microscopic visualization of arbuscular mycorrhizal fungi stained with trypan blue in apple root. Red arrows indicate from top to bottom: intracellular fungal hyphae, arbuscules, and vesicles. Scale bar = 200µm.

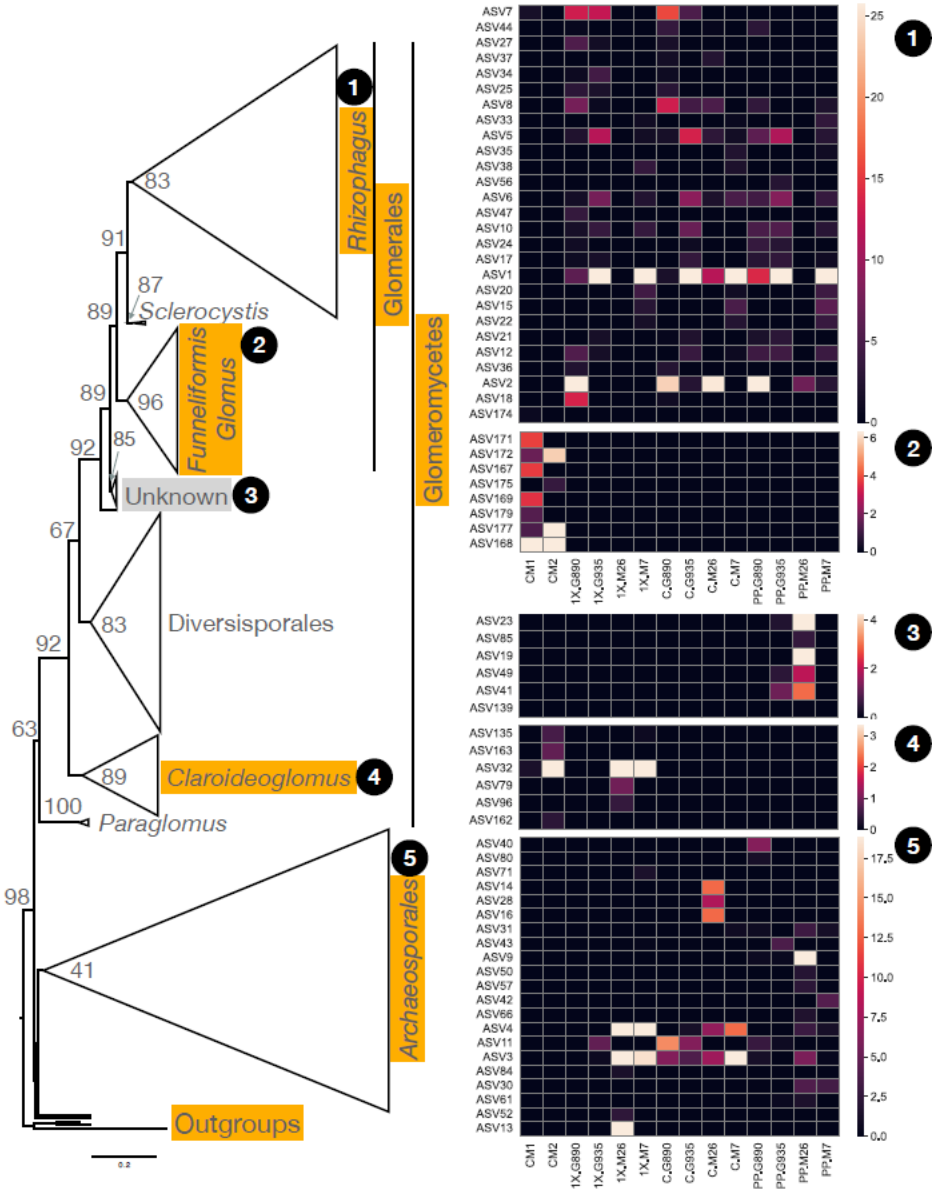

Figure 2. (left) A simplified version of the full phylogenetic tree for Phylum Glomeromycota. Triangles represent clades (i.e., groups of related sequences) that have been "collapsed" in order to visualize tree structure. Triangle length represents sequence divergence within a clade; triangle height represents the number of sequences in the clade. Numbers at each node represent "bootstrap" values (support for each branch). Clades highlighted in orange contain amplicon sequence variants (ASVs) recovered from our sequencing analysis along with reference sequences. Clades highlighted in grey only contain ASVs from our sequencing analysis that are not taxonomically annotated (i.e., no reference sequences). Clades with no background contain only reference sequences. **(right)** Heatmaps showing relative abundance of ASVs within the major clades shown on the left (labeled 1-5). Experimental treatments are listed along the x-axis of the heat map; CM = commercial mixture; DNA extracted directly from the AMF material; 1X = DNA extracted from rootstocks inoculated with CM; C (control) = DNA extracted from non-inoculated rootstocks; PP (pre-planting) = DNA extracted from roots prior to use in the experiment. The left y-axis lists ASVs identified in the experimental samples; the relative abundance of each ASV is depicted on the right y-axis along a color gradient (the lighter the color, the more abundant the ASV). Values represent the mean of 5 biological replicates for each treatment; CM1 and 2 represent individual samples.

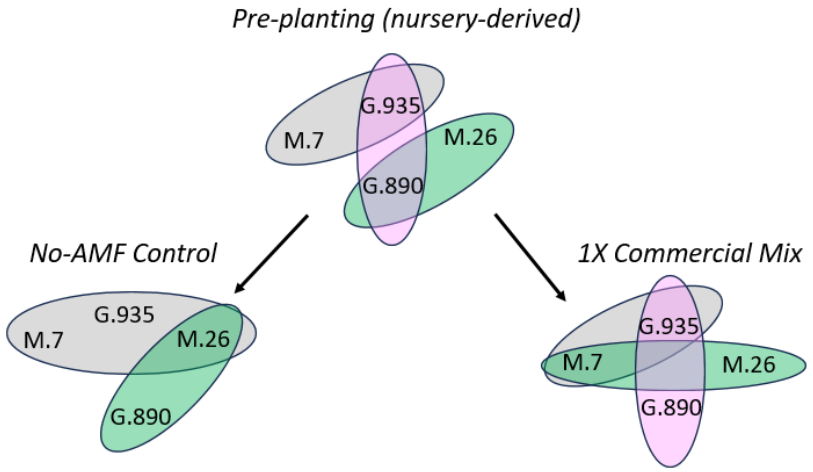

Figure 3. Venn diagrams reflecting how nursery-derived AMF communities diverged after planting into pasteurized potting mix (left), and into pasteurized potting mix containing a commercially available AMF mixture (right). One-way ANOSIM of relative abundance data was conducted using the Bray-Curtis dissimilarity coefficient. Pre-planting, significant differences were found between M.7 and M.26 (p=0.004), M.7 and G.890 (p=0.04), and G.935 and M.26 (p=0.02). In the No-AMF control treatment, significant differences were found between G.935 and G.890 (p =0.01) and between M.7 and G.890 (p=0.02). In the 1X CM treatment containing AMF, significant differences were identified between M.26 and G.890 (p =0.02), M.7 and G.890 (p =0.02) and between M.26 and G.935 (p=0.02).

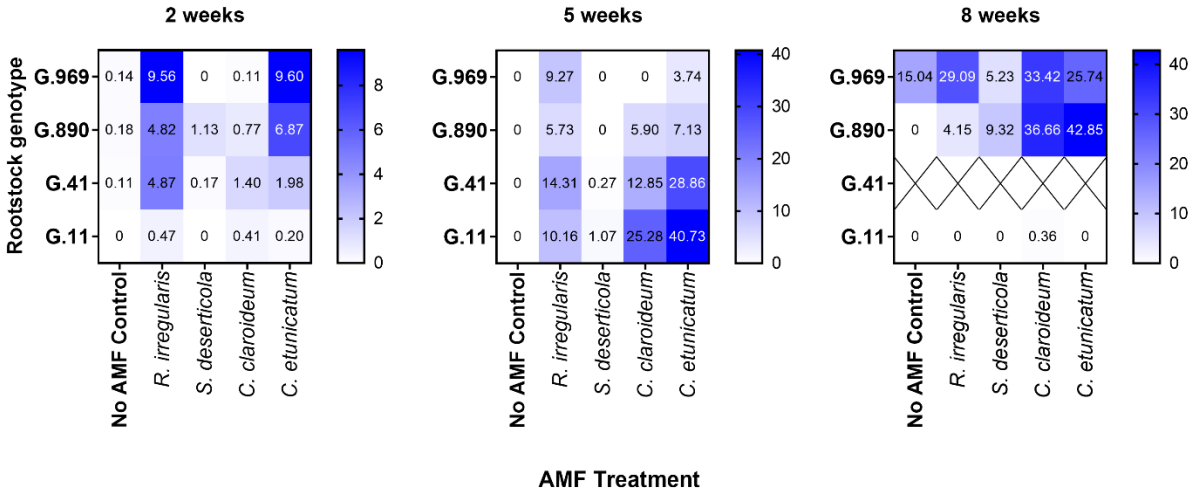

Figure 4: Mean percentage of root colonization in 4 different apple rootstock genotypes inoculated with 4 different species of AMF in Exp 23. Heatmaps are shown for 2-, 5- and 8-weeks post AMF inoculation, respectively. Data represents the mean of three biological replicates (x 3 technical replicates each) per rootstock x AMF combination per timepoint. G.41 could not be included in the 8-week analysis due to insufficient plantlets. G.969 was also excluded from statistical analyses at 8-weeks due to AMF colonization in the No AMF Control treatment.

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Project Title: Comprehensive monitoring and mapping antibiotics resistance in orchards

Report Type: Continuing Project Report/NCE

PI: Youfu "Frank" Zhao
Organization: WSU-IAREC
Telephone: 509-786-9284
Email: youfu.zhao@wsu.edu
Address: 24106 N. Bunn Rd.
City/State/Zip: Prosser, WA 99350

Cooperators: Tianna Dupont, WSU-TFREC, Wenatchee, WA;

Project Duration: 2-Year

Total Project Request for Year 1 Funding: $77,898
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Funding Duration: 2020-2025; 2023-2026
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Agency Name: USDA-NIFA-SCRI and WSDA-SCBG

WTFRC Collaborative Expenses: None

Budget

Organization Name: WSU-IAREC
Contract Administrator: Jamie Meek
Telephone: (509) 786-9231
Email address: jamie.meek@wsu.edu or prosser.grants@wsu.edu
Center Director: Naidu Rayapati
Email address: naidu.rayapati@wsu.edu

Item	2022	2023	2024
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**	**0**

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:

- No *Erwinia amylovora* isolates exhibited resistant to streptomycin and oxytetracycline in both 2022 and 2023;
- 38 *Erwinia amylovora* isolates exhibited resistant/tolerant to kasugamycin in 2023;
- Among them, 68% were isolated from pear samples and 32% from apple samples;
- Minimum inhibition concentration (MIC) for these resistant/tolerant isolates was determined;
- No mutation was found in the kasugamycin target *ksgA* gene in *E. amylovora* isolates;
- Genome sequence of non-*Erwinia amylovora* isolates, which exhibited resistance to all three antibiotics at high levels, showed a plasmid containing both streptomycin and oxytetracycline resistant genes.

Methods

Procedures and Methodology. Like last year, we either collected symptomatic samples in central Washington by our own field trips to local area growers or samples were sent to us via mail by growers or consultants. We also collected asymptomatic blossom samples. Samples were placed in plastic bags and held on ice or in a refrigerator until they were processed. Samples were processed by cutting into small pieces with a sterile knife, washed briefly with sterile water, soaked in 900 µl 10 mM PBS, vortexed, and streaked for isolation onto five types of media: LB, CCT, LB + Sm 100 µg/mL, LB + Kg 100 µg/mL, LB + Tc 20 µg/mL and incubated at 28 °C for 48 - 72 h. Colonies that appeared purple in color on CCT media, smooth, slightly raised and nonfluorescent were suspected to be *E. amylovora*. Screening for resistance was performed by observing the presence of individual colonies on antibiotic media(Figure 1). Isolates of known resistant *E. amylovora* strains were obtained from culture collections for use as positive controls. Isolates were then confirmed by PCR using *E. amylovora* specific primers G1-F and G2-R.

Spot dilution test was performed for selected resistant/tolerant strains (Figure 2). Bacteria were grown on LB plates and a single colony was inoculated in LB broth and grown for 24 hr with shaking at 250 rpm. Bacterial suspensions were adjusted to an absorbance of OD_{600} = 1 in PBS and 10-fold serial dilution was made in PBS. For each dilution, 5 µL was spotted onto plates: LB and LB + Kg 100 µg/mL and incubated at 28°C for 48 - 72 h. Bacterial growth was visually observed on plates with or without antibiotics. Growth on plates without antibiotics was used as a control to compare to the plates with antibiotics (Figure 2). In addition, the minimum inhibitory concentration (MIC) for each resistant/tolerant isolate was determined. Bacteria were grown on LB plates and a single colony was inoculated in LB broth with shaking at 250 rpm. Overnight bacterial suspensions were adjusted to an initial concentration of OD600 = 0.1 and 2-fold serial dilutions were performed, starting with LB + Kg1000 µg/mL and ending with LB + Kg 0.976 µg/mL. IC_{50} was defined as the concentration of antibiotics at which growth of the bacterium was 50 % less of that of the control without antibiotics. IC_{95} was defined as the concentration of antibiotics at which growth of the bacterium was 95 % less of that of the control without antibiotics.

Selected resistant/tolerant *E. amylovora* isolates were used to amplify the kasugamycin target *ksgA* gene by primers KsgA-F and KsgA-R. PCR products were then sequenced by Eton Biosciences Inc, San Diego, CA and compared to those of known sensitive strains. Furthermore, whole genome sequence was obtained for non-*Erwinia amylovora* isolates, which exhibited high levels of resistance to all three antibiotics by using nano-pore sequence technology.

Types and timing of anticipated results: The anticipated results are that we determined whether the *E. amylovora* population in surveyed orchards exhibits resistance to streptomycin, oxytetracycline, and kasugamycin, at what levels, and understood the mechanism of resistance (mutation of target gene or enzymatic degradation of antibiotics). We then made 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. Our results were immediately conveyed to growers within the funding year by email or text messages.

Potential problems or limitations that may be encountered: We did not encounter any problems and limitations as the method is technically straightforward.

Results and Discussion:

Findings during the current year of the project. Samples from 45 locations in central Washington were collected, including 15 apple and 5 pear varieties, i.e. Gala, Jazz, Pink Lady, Fuji, Crips Pink, Granny Smith, Cosmic Crisp, Honey Crisp, Envy, Ambrosia, Golden Russet, Macintosh and Sweet Tango; Bosc, Anjou, Bartlett and Star Krimson. A total of 192 *E. amylovora* isolates were obtained and confirmed by PCR. Among them, 38 isolates collected in 2023 were shown to be resistant or tolerant to kasugamycin at 100 ppm (Table 1), where 68% were isolated from pear samples and 32% from apple samples. Among the pear and apple samples, 58% were varieties Bosc or Bartlett and 58% were Pink Lady or Jazz, respectively. However, no isolates were found to be resistant to streptomycin or oxytetracycline.

Among the resistant/tolerant isolates, colony size was significantly smaller as compared to growth of the same isolate on LB medium (Figure 1) and spot dilution assay showed similar growth for resistant/tolerant isolates at LB with antibiotics and without antibiotics (Figure 2). MIC data showed that the majority of isolates had an IC_{50} value above 60 µg/mL, with the highest reaching 117.1 µg/mL, whereas IC_{95} values ranged from 167.8 µg/mL to 1081 µg/mL based on 95 % growth inhibition for these isolates (Table 1). However, sequence comparison of the *ksgA* gene showed no difference among the 29 isolates, which had an IC_{50} value above 70 µg/mL, as compared to known type strains (data not shown). Based on previous studies, 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. Our results indicate that resistance to kasugamycin of *E. amylovora* isolates is not due to mutations in the *ksgA* gene.

On the other hand, whole genome sequencing of non-pathogenic resistant isolates revealed a plasmid, containing both *strAB* and *tetA* genes, which are responsible for streptomycin and oxytetracycline resistance, respectively (data not shown).

Significance to the industry, and potential economic benefits. Since the identification of streptomycin-resistant strains of *E. amylovora* by Loper et al. in 1991, there has been limited data evaluating the status of antibiotic resistance throughout the central Washington regions. The significance of this research to the industry lies in two aspects. First, this is the first report of kasugamycin resistant/tolerant *E. amylovora* isolates in Washington or elsewhere and the isolates were from orchards in 10 different locations, including Mattawa, Prosser, Cashmere, Wenatchee,

Malaga, and Entiat. These results suggest that resistance may be more widespread than this study has indicated. Growers should take immediate actions in terms of how to and what antibiotic to use for controlling fire blight disease. Based on our findings, growers should take precautions in only applying kasugamycin to treat fire blight or should mix it with oxytetracycline if still want to use kasugamycin for fire blight control.

Second, our preliminary whole genome sequencing data indicates the presence of *strAB* and *tetA* genes in non-*Erwinia amylovora* bacterial isolates, which exhibit resistance to all three antibiotics in orchards at very high levels. In terms of streptomycin resistance mechanisms, the most common mechanism is a point mutation in the *rpsL* gene, which confers high levels of resistance with MIC up to 2000 ppm. The most dominant type of streptomycin resistance for *E. amylovora* isolates from the eastern US is the *strA*-*strB* associated transposon in plasmids, which confer relatively low levels of resistance with MIC at 500-750 ppm. Although we did find *strAB* genes in non-pathogenic isolates in WA orchards, we still have not found those genes in *E. amylovora* isolates. Therefore, continuing monitoring the distribution of the *strAB* genes will be critical to the industry, and to growers throughout the state, which would predict whether *E. amylovora* might develop another type of resistance or not to streptomycin in WA.

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 might shield light on whether *E. amylovora* will develop resistance or not, which will help us prepare if this indeed occurs to the fire blight pathogen in the future.

Table 1 List of *E. amylovora* isolates showing resistant/tolerant to kasugamycin

		Antibiotic resistance					
			Kg100[b]				
Sample	Tissue [a]	Kg100	IC_{50}[c]	IC_{95}[d]	R^2	Sm100[b]	OTc20[b]
K43	fruit	+	42.19	240.9	0.9973	-	-
K63	leaf, small	+	54.15	167.8	0.9909	-	-
K63	leaf, big	+	54.87	240.5	0.994	-	-
K4	stem	+	63.54	266.3	0.9953	-	-
K26	petioles, small	+	64.08	501.4	0.9914	-	-
K28	petioles, small	+	67.44	459	0.9922	-	-
K33	leaf	+	68.29	227.3	0.9975	-	-
K2	stem	+	69.38	296.8	0.9943	-	-
K28	leaf	+	69.6	483.5	0.993	-	-
K26	petioles, big	+	70.15	707.7	0.9913	-	-
K39	leaf	+	71.66	338.9	0.9917	-	-
K26	leaf	+	72	488.8	0.99	-	-
K2	stem	+	73.33	478.6	0.9913	-	-
K56	petioles	+	74.58	550.2	0.9933	-	-
K4	stem, big	+	75.69	388.7	0.9914	-	-
K32	leaf	+	78.56	533.6	0.9953	-	-
K36	petioles	+	79.04	388.1	0.992	-	-
K34	petioles	+	80.55	632.3	0.9918	-	-
K24.4	leaf	+	81.57	321.1	0.9901	-	-
K24.3	petioles	+	82.29	394.2	0.996	-	-
K31	fruit	+	82.55	614	0.9905	-	-
K30	fruit	+	82.74	287.9	0.9927	-	-
K24.2	ooze	+	83.3	287.5	0.9984	-	-
K27	leaf	+	83.87	374.5	0.9921	-	-
K27	fruit	+	85.77	401.5	0.9924	-	-
K24.1	leaf	+	86.63	374.8	0.9936	-	-
K24.5	stem	+	87.11	305.1	0.9971	-	-
K25.1	ooze	+	87.31	347.6	0.9947	-	-
K25.2	leaf	+	88.5	321.4	0.9958	-	-
K37	fruit	+	89.09	614.6	0.9908	-	-
K29	leaf	+	91.81	1081	0.9924	-	-
K41	fruit	+	93	495.3	0.9933	-	-
K4	stem, small	+	93.02	387.7	0.9914	-	-

K30	leaf	+	96.2	395.3	0.9948	-	-
K28	petioles, big	+	96.27	400.2	0.9925	-	-
K59	leaf, small	+	110.5	287	0.995	-	-
K54	fruit, small	+	113.9	305.7	0.9978	-	-
K28	fruit	+	117.1	416.5	0.9936	-	-

Tissue source: leaf, petioles, fruit, stem, ooze. Isolates were grown on LB + streptomycin (Sm) 100 µg/mL, LB + kasugamycin (Kg) 100 µg/mL, LB + oxytetracycline (Tc) 20 µg/mL. + symbol indicates growth, – symbol indicates no growth.
[a] Small vs. big represent colony size differences. [b] Measured in µg/mL.
[c] IC_{50} was defined as the concentration of antibiotic required for 50% inhibition.
[d] IC_{95} was defined as the concentration of antibiotic required for 95% inhibition.

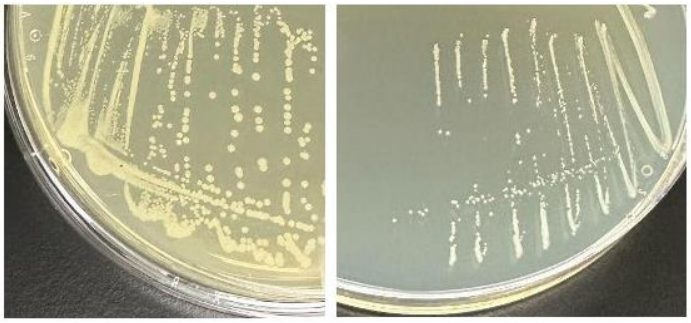


Figure 1 Growth of *E. amylovora* isolates on LB (left) and LB + kasugamycin 100 µg/ml (right) medium.

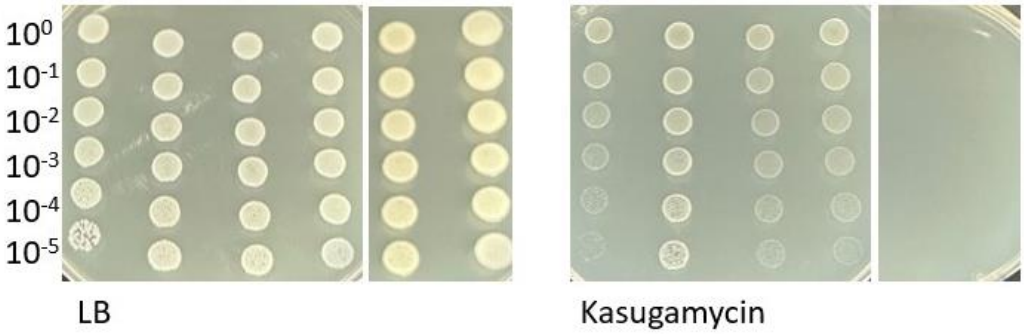


Figure 2 Spot dilution assay for representative resistant/tolerant isolates in 2023. Serial 10-fold dilutions were made in PBS. For each dilution, 5µL was spotted on LB plates containing no antibiotics or kasugamycin 100 µg/ml. Pictures were taken 48 hours post inoculation.

Project Title: Phase 3 New Biocontrol Strains against Fire Blight
Report Type: Continuing Project Report

Primary PI: Sharon L. Doty
Organization: University of Washington, Seattle
Telephone: (206) 616-6255
Email: sldoty@uw.edu
Address: School of Environmental and Forest Sciences
Address 2: UW Box 352100
City/State/Zip: Seattle, WA 98195

Co-PI 2: Tianna DuPont
Organization: Washington State University
Telephone: (509) 293-8758
Email: tianna.dupont@wsu.edu
Address: 1100 N. Western Ave.
Address 2:
City/State/Zip: Wenatchee, WA 98801

Cooperators: None

Project Duration: 2-Year

Total Project Request for Year 1 Funding: $ 17,751 (original request)
Total Project Request for Year 2 Funding: $12,321 (original request)

Item	2023	2024
Salaries	$10,968.00	$7,046.00
Benefits	$3,137.00	$2,193.00
Wages		
Benefits		
RCA Room Rental		
Shipping		
Supplies	$350.00	$250.00
Travel	$332.00	$332.00
Plot Fees	$2,500.00	$2,500.00
Miscellaneous	$464.00	
Total	$17,751.00	$12,321.00

Footnotes: See revisions to Year 1 and Year 2 in the individual budgets

Budget 1
Primary PI: Sharon L. Doty
Organization Name: University of Washington
Contract Administrator: Carol Rhodes
Telephone: 206-543-4043
Contract administrator email address: osp@uw.edu

Item	2023	2024
Salaries	$0.00	$10,768.00
Benefits	$0.00	$2,626.00
Wages		
Benefits		
RCA Room Rental		
Shipping		
Supplies	$0.00	$915.00
Travel	$0.00	$200.00
Plot Fees		
Miscellaneous		$464.00
Total	$0.00	$14,973.00

Footnotes: Due to an overhaul of the UW financial system that caused severe delays, none of the Year 1 funds came to the UW part of the project. Therefore, the funds were shifted to 2024 as indicated above. Though Professor Sharon Doty, serving as PI, provided the inoculum for the 2023 field trials, those costs were absorbed. For Year 2 of the project, she will commit 12 months at 1.5% FTE to the project for a total cost of $2,957. She will lead the project, prepare the reports, deliver the inoculum to Wenatchee, and write a manuscript on the project. Postdoctoral researcher, Robert Tournay, will commit 3 months in Year 2 at 28% FTE for the bioinformatics part of the project for a total cost of $5,210. Research Scientist 3, Andrew Sher, will commit a total of 2 months at 20% FTE in Year 2 for the microbiological work of the project for a total cost of $2,575. He and Tournay will also assist in writing the reports and manuscript. Travel to the WSU Tree Fruit Research Center in Wenatchee to deliver the microbial inoculum in Year 2 will require a total of $200. The project budget requires a total of $915 for the microbiology supplies, genomic DNA preparation, and analysis. In the Miscellaneous category, genomic sequencing by NovoGene will cost $116 per strain for a total of $464 for 4 strains.

Budget 2
Co PI 2: Tianna DuPont
Organization Name: Washington State University-Wenatchee
Contract Administrator: Darla Ewald/Stacy Mondy
Telephone: 509-293-8800
Contract administrator email address: dewald@wsu.edu arcgrants@wsu.edu
Station Manager/Supervisor: Chad Kruger
Station manager/supervisor email address: cekruger@wsu.edu

Item	2023	2024
Salaries	$3,750.00	$3,750.00
Benefits	$1,299.00	$1,299.00
Wages		
Benefits		
RCA Room Rental		
Shipping		
Supplies		
Travel		
Plot Fees	$2,500.00	$2,500.00
Miscellaneous		
Total	$7,549.00	$7,549.00

Footnotes: Technician salary for one month at base rate $3,750 benefits at 34.6% $1,299. The technician will be responsible for running fire blight efficacy trials under the supervision of the PI including application of biological controls, inoculation of the pathogen, efficacy rating, enumeration of pathogen cells in flowers, data entry and summary, statistical analysis and report writing. This request is for one month of salary. The overall project will include twenty products tested occupying 5 months of the Post Doc's time.

Objectives
1) Repeat the field trial with #UW 58 (4RDLA) and two new strains #UW 42 (4RSC) and #UW 90 (3ThL1) that inhibited *E. amylovora in vitro* (Year 1).
2) Genomic sequencing and analysis of strains #42 and #90 as well as two additional strains for subsequent testing as Aim 3 (Years 1 and 2)
3) In Year 2, repeat the field trial with strains #42 and #90 if they performed well, or the additional sequenced strains
Deviations: The University of Washington underwent a massive revision to its financial system in 2023, causing significant delays. Doty did not receive any of the Year 1 funds. Therefore, the genomic sequencing and analysis proposed in Year 1 will be performed in Year 2. The field trial proceeded on schedule in Year 1. Since the two new strains had not performed well, they will not be sequenced. Instead, 4 new strains from the original *in vitro* screens will be chosen.

Significant Findings
A field trial was conducted in spring 2023 for the first three strains. The three UW treatments applied alone the day before inoculation, the morning after inoculation and 4 days after inoculation (petal fall) resulted in 39.5, 41.2 and 38 infections per 100 clusters, none of them significantly different than the water-treated control. The biological standard Blossom Protect + Buffer Protect, with 32 infections per 100 clusters, was also not significantly different than the water-treated control (19.2% relative control[1]). The streptomycin standard provided significant control with 7.2 infections per 100 clusters (81.9% relative control). No treatments resulted in commercially important fruit skin marking of 3 or greater.

Methods
Objective 1 Methods
For the 2023 trial, Doty prepared the inoculum as concentrated, cryogenically-stored stocks delivered on dry ice to co-PI DuPont. We will prepare the cultures for the spring 2024 trials the same way.
Field trial:
A 0.25 ha research block of 3-yr-old Gala apples at WSU Columbia View Orchard 48 Longview Rd. East Wenatchee, WA was used for this trial. The experiment was arranged in a randomized complete block with five single tree replicates. Products were applied to the whole tree according to manufacturer recommendations using a Stihl SR420 mist blower backpack sprayer. Products were applied to wet, near dripping at 0.2-0.4 gal/tree (100 gal/A). Application dates were: 30 Apr (1), 2 May (2), 3 May (3, full bloom), 4 May (4), 5 May (5), 7 May (6, petal fall), 10 May (7). At 100% bloom (of the king blooms), on 3 May 2023, *Erwinia amylovora* was applied at $5x10^6$ CFU ml^{-1} (verified at $4.2x10^6$ CFU ml^{-1}) to lightly wet each cluster. Trees were visually evaluated for flower cluster infection weekly from when symptoms became visible, 9 days after inoculation, for 2 weeks and infection counts summed across all dates. Fruit were evaluated for fruit skin marking from an average of 25 fruit per tree on a 0 to 15 scale, where ratings below 3 indicate no commercial downgrades. Statistical analysis was performed with SAS v 9.4 using general linear mixed models (GLIMMIX) analysis of variance ANOVA and multiple means comparison (LSD) for infections, and nonparametric analysis (Kruskal-Wallis) followed by pairwise two-sided multiple comparison analysis (Dwass, Steel, Critchlow-Fligner Method) for the fruit skin marking.

Environmental conditions during bloom (28 Apr – 7 May 2023) were warm and ranged from an average maximum temperature of 78.2 °F to minimum of 51.5 °F with 47.4% average humidity. During the following week of petal fall (8 May – 14 May 2023), temperature ranged from an average maximum of 79.5 °F to a minimum of 49.6 °F with 45.5% average humidity. Three precipitation

[1] 'relative control' (S_{rc}) $S_{rc} = (1 - I_t \div I_c) \times 100$ where I_t and I_c are incidence of diseases flower clusters for a treatment and the water-treated control respectively.

events occurred after the inoculation of *Erwinia amylovora*, one on 5 May (0.04 in), approximately 31 h after inoculation (17 to 20 h after the full bloom + 1-day sprays), and the other two on 8 May 20 to 23 h after petal fall sprays (0.01 in) and 28 to 31 h after petal fall sprays (0.06 in). All applications were made under fast drying conditions.

Objective 2 Methods

Selection of strains Due to delays in the UW financial system, this objective was postponed until Year 2. Since UW-42 and 90 did not perform well in the 2023 field trial, they will not be sequenced as planned in the original proposal. Seven strains from the original screens activity against *E. amylovora* (Figure 2) will be identified by 16S rRNA gene sequencing. Four of these will be selected for full genomic sequencing based on these results. For example, although strains 82 and 89 exhibited strong activity (Fig 2), they were identified as *Enterobacter sp.*, some of which are clinical isolates, so they were not chosen.

De Novo whole genome assembly and annotation of newly sequenced strains. Genomic DNA will be extracted from the 4 chosen bacterial strains. The purified DNA will be submitted to Novogene Co. Ltd. for sequencing by Illumina's Novaseq 6000 platform, after which de novo genomes assembled from the trimmed and normalized paired-end reads assembled in the Geneious Prime assembler (www.geneious.com). The draft assemblies will be screened for completeness using CheckM and annotated via the Rapid Annotation Subsystems Technology (RAST). Functional annotation and KEGG Ortholog (KO) assignment will be performed using KEGG KofamKOALA and whole-genome-based taxonomic classification and phylogenetic analysis with the Type (Strain) Genome Server (TYGS).

Genomic analysis of antimicrobial traits. The assembled genomes of the strains will be analyzed for the presence of biosynthetic gene clusters (BGC) associated with antimicrobial activity using the antiSMASH pipeline [8]. Candidate BGC will be cross-referenced with the literature to identify potential biocontrol mechanisms and inform future lab experiments.

Genomic analysis for human pathogenicity. The proteomes of the endophytes will be analyzed using the PathogenFinder pipeline to predict human pathogenicity based on the presence of gene clusters associated with either pathogen or non-pathogen gene families. Strains with indeterminant PathogenFinder pathogenicity scores will be screened for the presence of known pathogenicity islands via whole genome alignments using the IslandViewer 4 pipeline. Predicted pathogens, or strains harboring known pathogenicity islands, will be eliminated from subsequent experiments

Objective 3 Methods

We will repeat the field trials as done in 2023 but with strains #UW 58 (4RDLA) that performed well in 2022, and two new strains chosen from the *in vitro* assays performed in Phase 1 of this project (Figure 2) that also passed the genomic analysis screening (Objective 2).

Results and Discussion

2023 field trial results: The three UW treatments applied alone the day before inoculation, the morning after inoculation and 4 days after inoculation (petal fall) resulted in 39.5, 41.2 and 38 infections per 100 clusters, none of them significantly different than the water-treated control (Table 1). The biological standard Blossom Protect + Buffer Protect, with 32 infections per 100 clusters, was also not significantly different than the water-treated control (19.2% relative control[2]). The streptomycin standard provided significant control with 7.2 infections per 100 clusters (81.9% relative control). No treatments resulted in commercially important fruit skin marking of 3 or greater (Table 1, Appendix 3).

The UW42 treatment reduced *Erwinia amylovora* populations at 1 and 4 days after inoculation, although not significantly different than the water-treated control (Fig. 1). The other two UW

treatments, as well as the biological standard Blossom Protect + Buffer Protect did not show a reduction of the population size of the pathogen compared to the water-treated control. The streptomycin standard significantly reduced *Erwinia amylovora* populations at all time points analyzed (1, 4 and 7 days after inoculation) (Fig. 1).

Further directions. It may be necessary to apply the biocontrol strains sooner so they have more time to colonize plant tissue and express the antimicrobial compounds. We submitted a new proposal that will explore the hypothesis that endophyte strains will perform better if allowed to pre-colonize the plant.

Table 1. Effect of UW treatments applied to apple, cv. Gala on infection of *Erwinia amylovora* in apple blossoms in Wenatchee, WA in 2023 [z]

Treatment	Amount per 100 gal	Timing [y]	Infections per 100 clusters [x]				Fruit skin marking [w]	
Streptomycin standard (Firewall 50WP) [v]	8 oz	3,4,6	7.2	±	1.6	a [u]	0.02	a
Blossom Protect + Buffer Protect	1.25 lb + 5 lb	2,3	32.0	±	2.0	b	0	a
UW42 [t]	12.8 fl oz	2,4,6	39.5	±	6.0	b	0	a
UW58 [t]	12.8 fl oz	2,4,6	41.2	±	6.2	b	0	a
UW90 [t]	12.8 fl oz	2,4,6	38.0	±	4.5	b	0	a
Water-treated control	NA	3,4,6	39.6	±	4.9	b	0	a

[z] Inoculation was conducted on the evening of 3 May 2023 at full bloom (of king blooms) using a suspension of freeze-dried cells of *Erwinia amylovora* strain Ea153 (streptomycin and oxytetracycline sensitive strain) prepared at $5x10^6$ CFU ml^{-1} (verified at $4.2x10^6$ CFU ml^{-1}).
[y] Timings, 1: first bloom, 2: 70-90% bloom, 3: morning before evening inoculation (full bloom), 4: morning after inoculation, 5: 2 days after inoculation, 6: 4 days after inoculation (petal fall), 7: 7 days after inoculation
[x] Transformed sqrt(x + 1) prior to analysis of variance; non-transformed means are shown.
[w] Fruit skin marking is rated from an average of 25 fruit per tree. Rated on a 0 to 15 scale where ratings below 3 indicate no commercial downgrades.
[v] Amended with Regulaid: 16 fl oz per 100 gallons. Buffered to 5.6 pH.
[u] Treatments followed by the same letter are not significantly different at P=0.05 Fisher's T test (LSD).
[t] Amended with Regulaid: 16 fl oz per 100 gallons.

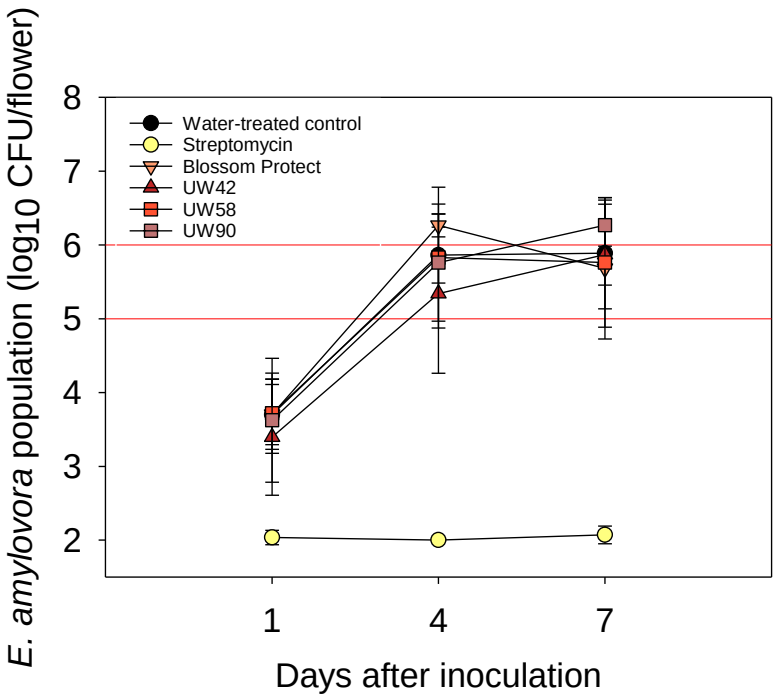


Figure 1. Effect of the biological products from the UW applied to Gala apple trees on the population size of *E. amylovora* strain 153N on flowers 1 day after inoculation (full bloom + 1 day), at petal fall (full bloom + 4 days) and 3 days post petal fall (full bloom + 7 days).

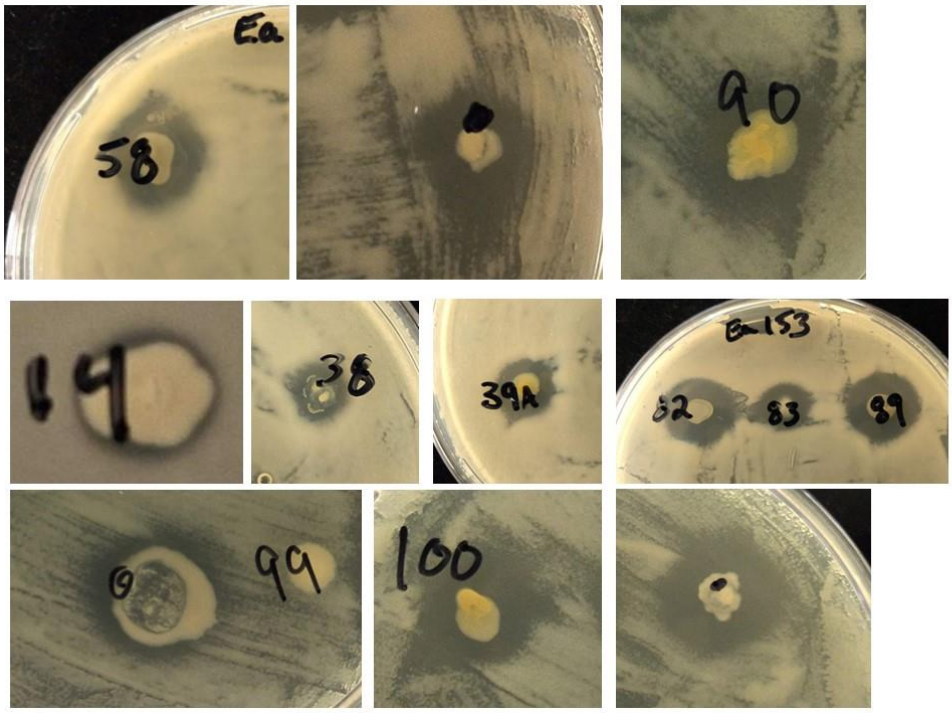
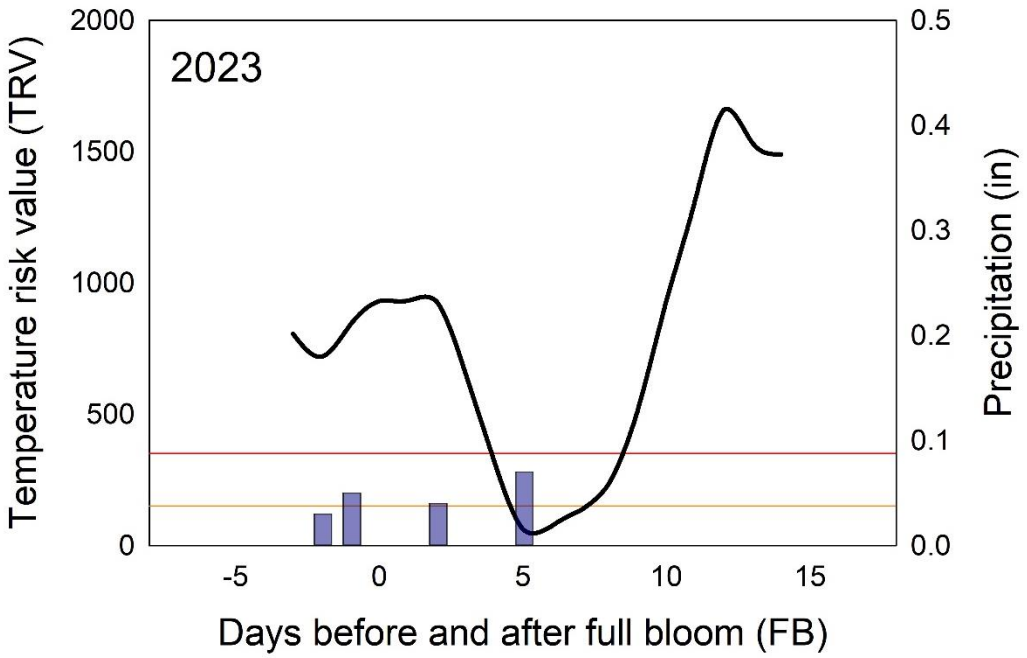


Figure 2. Photos of *in vitro* screening results against *E. amylovora* from Phase 1 of this project. A lawn of the pathogen was spread onto agar plates, and candidate biocontrol strains were spotted onto the lawn. Upper panel, UW-58 (performed well in 2022 field trial); UW-29 and 37 were included in the 2023 field trial. Lower two panels: Seven additional strains to be tested first by 16S sequencing will be 14, 38, 39A, 83, 98, 100, and 102. Of these, 4 will be selected for genomic DNA sequencing. Along with UW-58, two will be chosen for the 2024 field trial based on lack of animal or plant pathogenicity genes in their genomes.

Appendix 1. Environmental conditions during bloom.

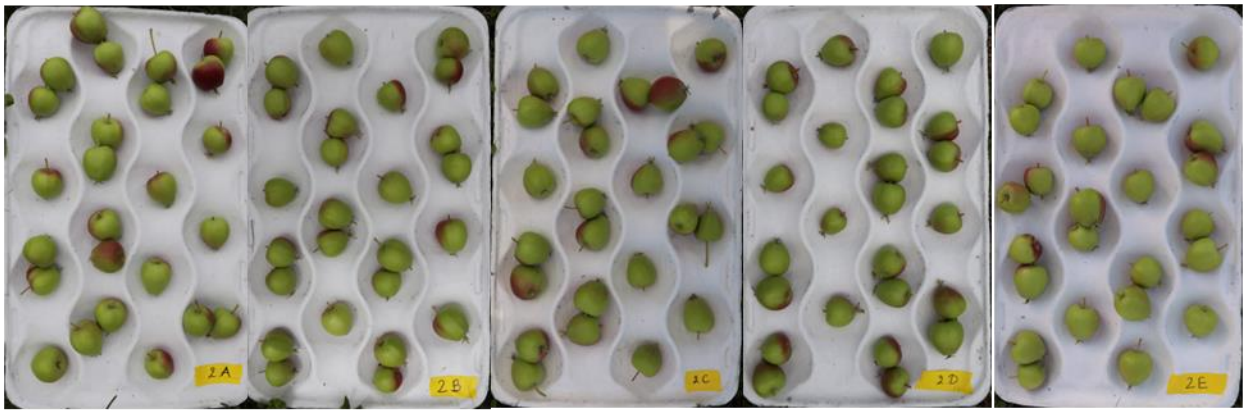
Date	Min Air Temp (°F)	Max Air Temp (°F)	Humidity (%)	Leaf wetness (h)	Precipitation (in)	Wind speed (mph)	Total Solar Radiation (MJ/m^2)
04/27/2023	51.6	77.9	35.1	0	0	4.2	23.21
04/28/2023	46.4	81.4	36.4	0	0	2.9	24.50
04/29/2023	48.2	85.6	36.4	0	0	2.7	24.03
04/30/2023	54.5	85.1	39.6	0	0	4.3	23.28
05/01/2023	52.7	65.6	59.8	1	0.03	3.6	10.51
05/02/2023	52.4	86.2	56.1	4	0.05	3.9	22.22
05/03/2023	58.5	88.8	45.4	0	0	2.9	23.84
05/04/2023	56.5	85.8	45.8	0	0	4.3	21.41
05/05/2023	51.6	61.6	63.8	7	0.04	2.3	5.14
05/06/2023	49.4	68.9	45.6	0	0	3.6	17.18
05/07/2023	44.9	73.2	44.9	0	0	3.6	23.11
05/08/2023	48.9	66.9	60.5	1	0.07	3.7	17.13
05/09/2023	48.7	71.1	54.2	0	0	2.8	19.40
05/10/2023	43.8	76.6	51	1	0	3.2	25.98
05/11/2023	46.9	80.6	45.2	0	0	3.1	24.90
05/12/2023	49.6	85.5	39.2	0	0	2.8	26.49
05/13/2023	51.6	88.3	34.4	0	0	4.6	26.29
05/14/2023	57.9	87.6	33.7	0	0	7.1	25.91
05/15/2023	62	90.1	38.1	0	0	5	19.75
05/16/2023	59.7	78.5	55.9	0	0	3.6	17.42
05/17/2023	56.3	86.1	58.7	8	0	3.1	23.06

Appendix 2. Fire Blight Temperature Risk Values (TRV) During Bloom in East Wenatchee, WA. Blue bars indicate rainfall events.

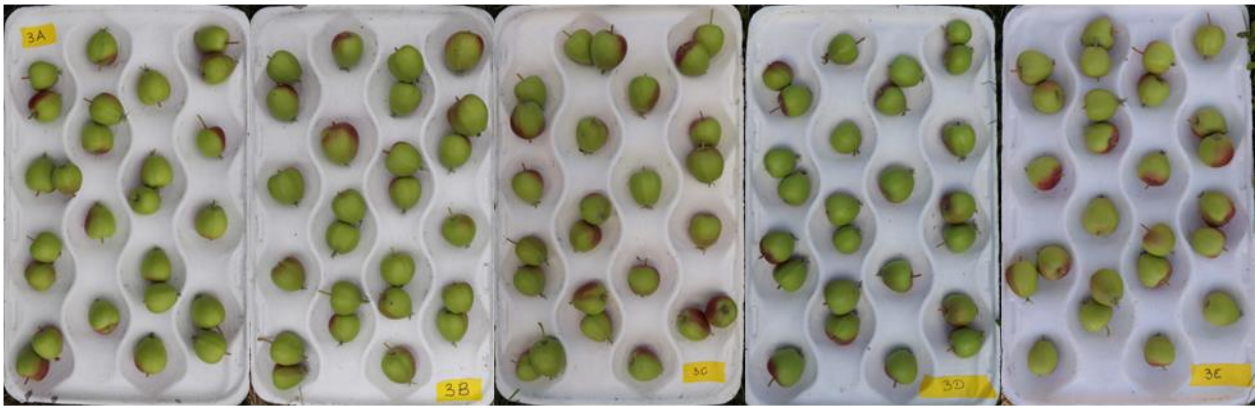


Appendix 3. Effect of UW treatments applied to apple, cv. Gala on fruit skin marking in Wenatchee, WA in 2023.

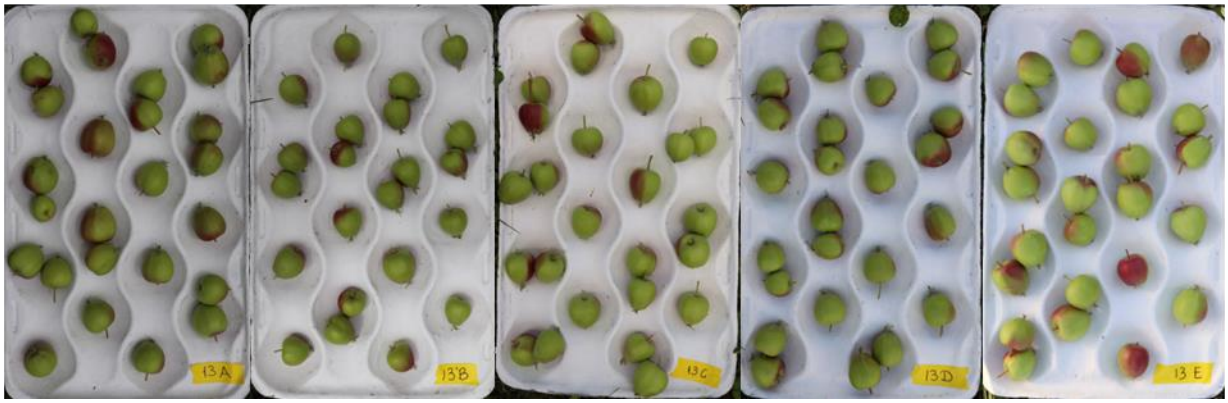
Streptomycin standard (Firewall 50WP)



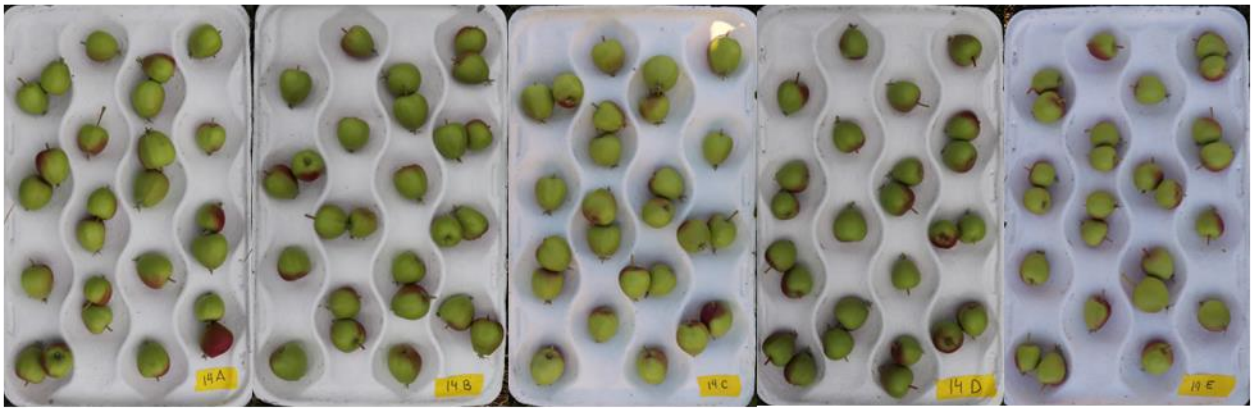
Blossom Protect + Buffer Protect



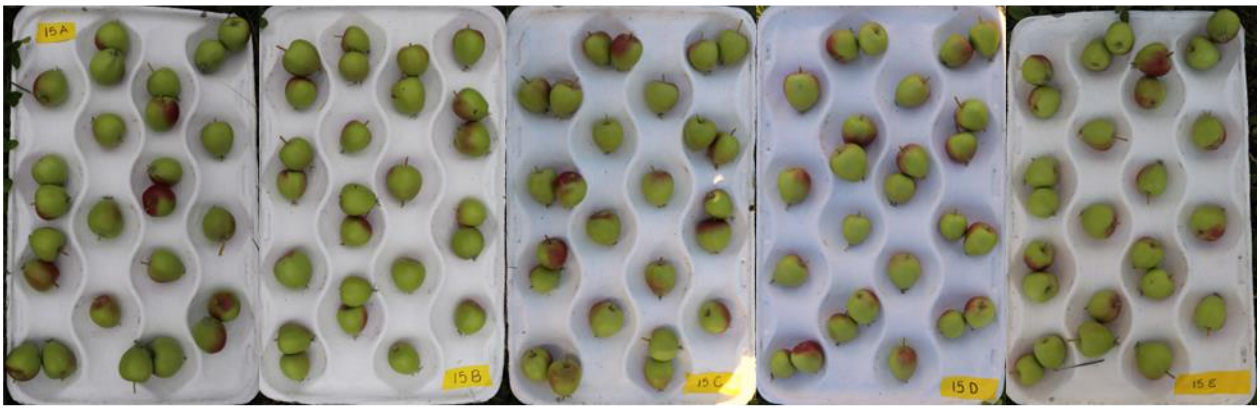
UW42



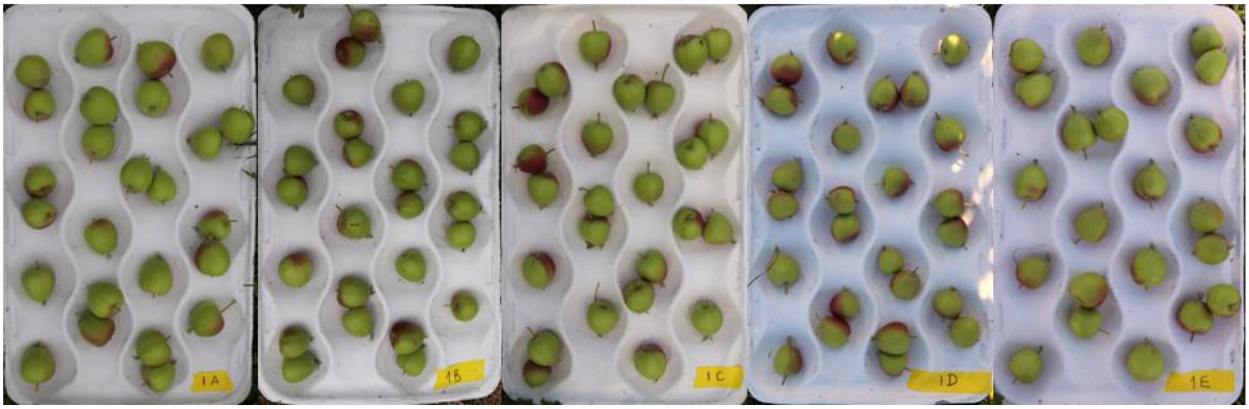
UW58



UW90



Water-treated control



Project Title: Functional peptides as new tools for the control of fire blight

Report Type: Continuing Project Report

Primary PI: Aina Baro Sabe
Organization: Washington State University
Telephone: (509) 293 8792
Email: aina.barosabe@wsu.edu
Address: 1100 N Western Ave
City/State/Zip: Wenatchee, WA, 98801

Co-PI 2: Tianna DuPont
Organization: Washington State University
Telephone: (509) 293 8758
Email: tianna.dupont@wsu.edu
Address: 1100 N Western Ave
City/State/Zip: Wenatchee/WA/98801

Cooperators: University of Girona (Girona, Spain)

Project Duration: 2-Year

Total Project Request for Year 1 Funding: $14,964
Total Project Request for Year 2 Funding: $15,030

Other related/associated funding sources: Comprehensive Fire Blight Management Systems for the United States. PD: Sundin, G., PI: Adaskaveg, J., Cox, K., **DuPont, S.T.**, Gallardo, K., Johnson, K., Kon, T., Khan, A., Rothwell, N., Villani, S., Youfu, Z. (2020-2024) (**$418,722**). *SCRI grant is supporting post doc salary.*

WTFRC Collaborative Costs: None

Budget 1
Primary PI: Aina Baro Sabe
Organization Name: Washington State University
Contract Administrator: Darla Ewald | Stacy Mondy
Telephone: (509) 293-8802
Contract administrator email address: dewald@wsu.edu | arcgrants@wsu.edu
Station Manager/Supervisor: Chad Kruger
Station manager/supervisor email address: cekruger@wsu.edu

Item	1-Mar-23	1-Mar-24
Salaries		
Benefits		
Wages		
Benefits		
RCA Room Rental		
Shipping	$250.00	$250.00
Supplies	$13,150.50	$13,150.00
Travel	$232.00	$232.00
Plot Fees	$1,331.50	$1,398.00
Miscellaneous		
Total	$14,964.00	$15,030.00

Footnotes:

Travel: Travel to Columbia View Research Orchard 25 mi x 16 trips at $0.58 per mile

Plot fees: 0.5 acres at $2,663 per acre 2023, $2,796 per acre 2024.

Supplies include synthesis of the experimental compound ($12,000), and field trial supplies: $500 for Personal Protective Equipment and spray supplies, $500 for laboratory supplies, $150 for sprayer services after the trial.

ORIGINAL PROJECT OBJECTIVES

The objective of this grant is to test and optimize the application of the novel compound bifunctional peptide BP178 for the control of fire blight infections in the field. Spring 2023 was the first year of data collection, and spring 2024 will be the second one. Multiple years are necessary to obtain efficacy results for the control of fire blight since year-to-year variability has been observed in the field.

The specific objectives are:

1. Determine the efficacy of the bifunctional peptide BP178 in controlling fire blight by means of (i) evaluating flower cluster infections weekly for three weeks starting from when symptoms become visible, (ii) enumerating the population levels of *Erwinia amylovora* 1, 4, and 7 days after inoculation, and (iii) evaluating the severity of shoot blight infections by measuring the length of the canker.
2. Evaluate fruit marking.
3. Compare results with the ones obtained using either streptomycin (antimicrobial activity), Actigard (induction of plant systemic acquired resistance) or water (water-treated control).

SIGNIFICANT FINDINGS

<u>Objective 1</u>

- The bifunctional peptide BP178 applied alone the day before inoculation, the morning after inoculation (full bloom) and 4 days after inoculation (petal fall) did not show significant differences in the number of flower cluster infections compared to the water-treated control.
- The bifunctional peptide BP178 reduced *Erwinia amylovora* populations at 1 and 4 days after inoculation, showing significant differences compared to the water-treated control 1 day after inoculation.

<u>Objective 2</u>

- The bifunctional peptide BP178 did not show fruit skin marking in any of the 25 fruit evaluated. None of the other treatments resulted in commercially important fruit skin marking of 3 or greater.

<u>Objective 3</u>

- Both streptomycin (Firewall 50WP) and Actigard applied three times during the bloom period significantly reduced number of flower cluster infections compared to the water-treated control, while Blossom Protect did not.
- The reduction of *Erwinia amylovora* population obtained with the bifunctional peptide BP178 1 day after inoculation was not significantly different than the one obtained with streptomycin. At 4 and 7 days, populations were only significantly reduced by the streptomycin standard.

METHODS

<u>Synthesis of the bifunctional peptide (University of Girona)</u>

The peptide BP178 was synthesized manually by the solid-phase procedure as previously described (Badosa et al., 2013). Briefly, a PAC-ChemMatrix resin (0.66 mmol/g) was used for the synthesis of BP178, and once the peptidyl sequence was completed, the resulting resins was treated with trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (TIS) (95:2.5:2.5) for 2 h at room temperature. Following TFA evaporation and diethyl ether extraction, the crude peptide was dissolved in H_2O,

lyophilized, analyzed by HPLC, and characterized by mass spectrometry to determine its purity.

Since the manual synthesis of peptides is a long process and fire blight trials happen during bloom, only 2 g of the bifunctional peptide BP178 were provided for the season 2023. Adjustments to the protocol were made in order to reduce the amount of peptide needed in the field trials.

Field trials (Washington State University)

Site: A 0.6 acre research block of 250 third leaf apple trees cultivar Gala rootstock G41 planted on a 4 ft spacing at the WSU Columbia View Orchard 48 Longview Rd. East Wenatchee, WA 98802-8283 was used for the trial. Soils are a Cashmont Gravely Sandy Loam with a 3-8% slope. The site has good air drainage and some wind protection.

Plots: The experiment was arranged in a randomized complete block with five single tree replicates. Experimental blocks were spaced from one another by two buffer trees.

Treatments: Treatments included the bifunctional peptide BP178, as well as positive and negative controls. Positive controls included the antibiotic streptomycin as a control for bactericidal activity and the systemic acquired resistance inducer acibenzolar-S-methyl (Actigard) as a control for plant defense activation. A water-treated control was applied as a negative control treatment. The experimental product (peptide) was applied by tree to the area of the tree to be inoculated (flower clusters) according to manufacturer recommendations using a manual pump 4-gallon backpack sprayer. All the other products were applied to the whole tree according to manufacturer recommendations using a Stihl SR420 mist blower backpack sprayer; products were applied to wet, near dripping previously calibrated to equal 100 gal/A. Application dates were: 30 Apr (1), 2 May (2), 3 May (3, full bloom), 4 May (4), 5 May (5), 7 May (6, petal fall), 10 May (7). All applications were made under fast drying conditions.

Inoculation: At 100% bloom of the king bloom (on 3 May 2023) *Erwinia amylovora* strain EA153 (streptomycin and oxytetracycline sensitive and nalidixic acid resistant strain) was applied at $5x10^6$ CFU ml^{-1} (verified at $4.2x10^6$ CFU ml^{-1}) to lightly wet each cluster using freeze dried inoculum. A 4-gallon backpack sprayer (solo) will be used to lightly wet clusters.

Evaluation: Trees were visually evaluated for flower cluster infection weekly from when symptoms became visible, 9 days after inoculation, for 2 weeks and infection counts summed across all dates. Infected flower clusters were removed in each evaluation.

Erwinia amylovora was enumerated 1, 4, and 7 days after inoculation from a bulk sample of 10 flowers per replicate (2 flowers from 5 different clusters). Flowers were sonicated in sterile DI water for 3 minutes and a 10-µl sample of the wash and two 1:100 dilutions were spread on nutrient agar amended with nalidixic acid (50 µg/ml) and cycloheximide (50 µg/ml) to selectively enumerate *Erwinia amylovora* (Ea153N).

Fruit was evaluated for fruit skin marking before fruit colored over. 25 fruit per replicate were rated. Russet ratings are on a 1 to 15 scale with individual values lower than 3 considered insignificant for commercial packing.

Environmental conditions were tracked on an hourly basis including temperature, humidity, leaf wetness, solar radiation and windspeed.

Statistical analysis: Statistical analysis was performed with SAS v 9.4 using general linear mixed models (GLIMMIX) analysis of variance ANOVA and multiple means comparison (LSD) for

infections and *Erwinia amylovora* populations, and nonparametric analysis (Kruskal-Wallis) followed by pairwise two-sided multiple comparison analysis (Dwass, Steel, Critchlow-Fligner Method) for the fruit skin marking.

RESULTS AND DISCUSSION

Environmental conditions during bloom (28 Apr – 7 May 2023) were very conducive to fire blight disease (Appendix 1 and 2). Temperatures were warm and ranged from an average maximum of 78.2 °F to minimum of 51.5 °F with 47.4% average humidity. During the following week of petal fall (8 May – 14 May 2023), temperature ranged from an average maximum of 79.5 °F to a minimum of 49.6 °F with 45.5% average humidity. Three precipitation events occurred after the inoculation of *Erwinia amylovora*, one on 5 May (0.04 in), approximately 31 h after inoculation (17 to 20 h after the full bloom + 1-day sprays), and the other two on 8 May, 20 to 23 h after petal fall sprays (0.01 in) and 28 to 31 h after petal fall sprays (0.06 in).

The bifunctional peptide BP178 applied alone the day before inoculation, the morning after inoculation and 4 days after inoculation (petal fall) resulted in 40.4 infections per 100 clusters, not significantly different than the water-treated control (Table 1). The biological standard Blossom Protect + Buffer Protect, with 32 infections per 100 clusters, was also not significantly different than the water-treated control (19.2% relative control[1]). The streptomycin standard applied the morning before evening inoculation (full bloom), the morning after inoculation and 4 days after inoculation (petal fall) provided significant control with 7.2 infections per 100 clusters (81.9% relative control). The systemic acquired resistance inducer Actigard applied at first bloom (3 days before inoculation), the morning before evening inoculation (full bloom) and 4 days after inoculation (petal fall) resulted in 16.4 infections per 100 clusters (58.7% relative control), significantly different than the water-treated control and not significantly different than the streptomycin standard. No treatments resulted in commercially important fruit skin marking of 3 or greater (Table 1, Appendix 3).

[1]'relative control' (S_{rc}) $S_{rc} = (1 - I_t \div I_c) \times 100$ where I_t and I_c are incidence of diseases flower clusters for a treatment and the water-treated control respectively.

Table 1. Effect of the bifunctional peptide BP178 and controls applied to apple, cv. Gala on infection of *Erwinia amylovora* in apple blossoms in Wenatchee, WA in 2023 [z]

Treatment	Amount per 100 gal	Timing [y]	Infections per 100 clusters [x]				Fruit skin marking [w]	
Streptomycin standard (Firewall 50WP) [v]	8 oz	3,4,6	7.2	±	1.6	a [u]	0.02	a
Blossom Protect + Buffer Protect	1.25 lb + 5 lb	2,3	32.0	±	2.0	b	0	a
Actigard [t]	2 oz	1,3,6	16.4	±	5.2	a	0	a
Bifunctional peptide BP178	4.3 oz	2,4,6	40.4	±	3.8	b	0	a
Water-treated control	NA	3,4,6	39.6	±	4.9	b	0	a

[z] Inoculation was conducted on the evening of 3 May 2023 at full bloom (of king blooms) using a suspension of freeze-dried cells of *Erwinia amylovora* strain Ea153 (streptomycin and oxytetracycline sensitive and nalidixic acid resistant strain) prepared at $5x10^6$ CFU ml^{-1} (verified at $4.2x10^6$ CFU ml^{-1}).
[y] Timings, 1: first bloom, 2: 70-90% bloom, 3: morning before evening inoculation (full bloom), 4: morning after inoculation, 5: 2 days after inoculation, 6: 4 days after inoculation (petal fall), 7: 7 days after inoculation
[x] Transformed sqrt(x + 1) prior to analysis of variance; non-transformed means are shown.

[w] Fruit skin marking is rated from an average of 25 fruit per tree. Rated on a 0 to 15 scale where ratings below 3 indicate no commercial downgrades.
[v] Amended with Regulaid: 16 fl oz per 100 gallons. Buffered to 5.6 pH.
[u] Treatments followed by the same letter are not significantly different at P=0.05 Fisher's T test (LSD).
[t] Amended with Regulaid: 16 fl oz per 100 gallons.

The bifunctional peptide BP178 reduced *Erwinia amylovora* populations at 1 and 4 days after inoculation, showing significant differences compared to the water-treated control 1 day after inoculation. The streptomycin standard significantly reduced *Erwinia amylovora* populations at all time points analyzed (1, 4 and 7 days after inoculation), while none of the other standards/treatments showed a reduction of the population size of the pathogen compared to the water-treated control (Fig. 1).

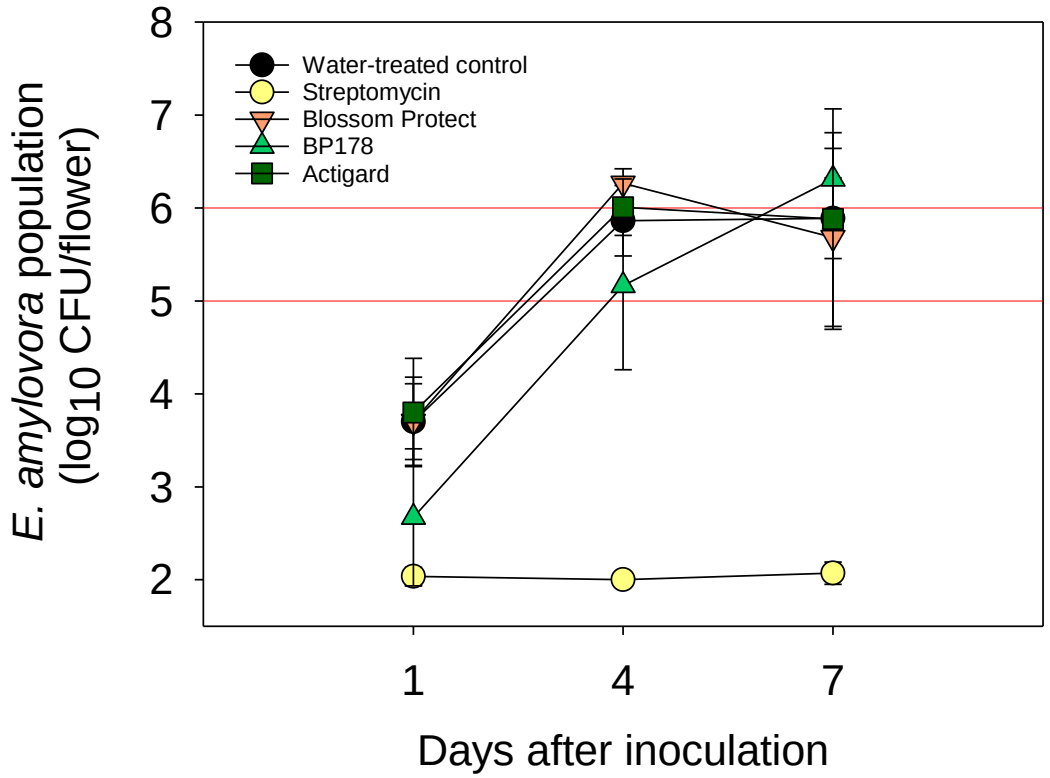


Figure 1. Effect of the bifunctional peptide BP178 and controls applied to Gala apple trees on the population size of *Erwinia amylovora* strain 153N on flowers 1 day after inoculation (full bloom + 1 day), at petal fall (full bloom + 4 days) and 3 days post petal fall (full bloom + 7 days).

Results from this year, which has been of exceptionally high-risk regarding fire blight conducive weather conditions, showed no control of fire blight with the experimental peptide applied in young susceptible Gala apple trees the day before inoculation, the morning after inoculation and 4 days after inoculation (petal fall) at a concentration of 4.3 oz/100 gal. However, a reduction of *Erwinia amylovora* population size 1 and 4 days after the pathogen inoculation was observed when using the peptide, indicating its bactericidal activity against *Erwinia amylovora* strain Ea153. Lack of correlation between reductions in colony size and disease incidence is probably due to an insufficient reduction of the

pathogen population and environmental conditions favorable for the development of fire blight: high temperatures after the pathogen inoculation followed by three rain events, one on 5 May (0.04 in), approximately 31 h after inoculation (17 to 20 h after the full bloom + 1-day sprays), and the other two on 8 May, 20 to 23 h after petal fall sprays (0.01 in) and 28 to 31 h after petal fall sprays (0.06 in) (Appendix 1 and 2). Clear and sunny spraying days (22.22, 21.41 and 23.11 MJ/m^2 respectively) could also have impacted the activity of the peptide since proteins and peptides are generally known for being vulnerable to UV light (Gammelgaard et al., 2019). Additionally, and due to the peptide availability for this year's season, we had to apply the peptide by tree to the area of the tree to be inoculated (flower clusters) using a manual pump 4-gallon backpack sprayer, and this could also have reduced the potential systemic acquired resistance induction activity of the peptide, since not all the tree was exposed to it.
Due to the high susceptibility of the young Gala trees used in this trial and this year's high-risk conditions for fire blight development, no evaluation of the severity of the shoot blight was conducted.

Improvements for 2024 trial

Due to the peptide availability for the 2023 season, we also had to apply the peptide at a lower concentration than the one initially proposed, so the first thing to modify for next year's trial is increasing the peptide concentration applied to at least 5.4 oz/100 gal (125 µM).

Because the peptide is an experimental product, this year the peptide was applied in the field by itself since no combinations with acidifiers or surfactants have been tested. Because the addition of these adjuvants to antibiotics and other types of pesticides improves their efficacy in the field, we believe that this can also be the case with the peptide, since it has already been discussed the enhance of their activity at lower pH (Huan et al., 2020; Kacprzyk et al., 2007; Malik et al., 2016). A small-scale *in vitro* test with these adjuvants will be performed before the field trial to see how they influence the bactericidal activity of the peptide and how we can combine them to obtain better results in the field.

Application timings of the peptide in the field will also be adjusted such that there is no more than two days elapse between applications under conducive environmental conditions. Application under low UV exposure of the peptide (before sunrise or evening) will also be considered for all the sprays.

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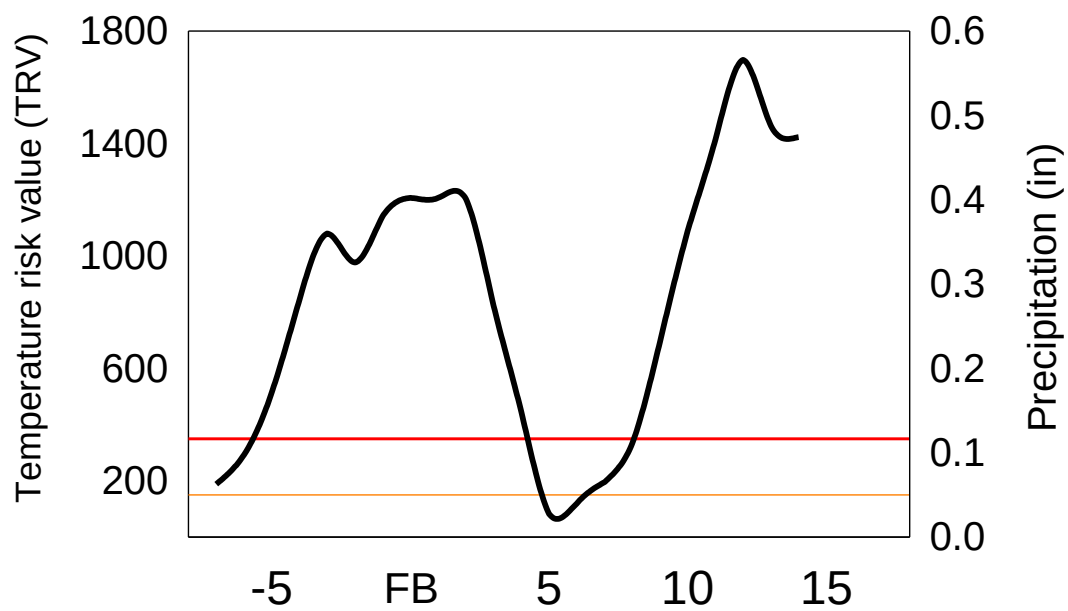
Appendix 1. Environmental conditions during bloom in East Wenatchee, WA.

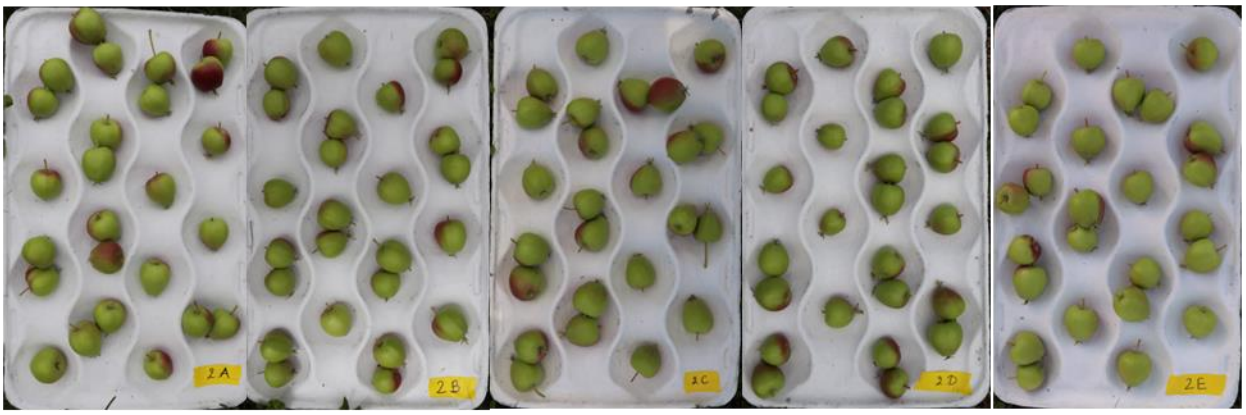
Date	Min Air Temp (°F)	Max Air Temp (°F)	Humidity (%)	Leaf wetness (h)	Precipitation (in)	Wind speed (mph)	Total Solar Radiation (MJ/m²)
04/29/2023	48.2	85.6	36.4	0	0	2.7	24.03
04/30/2023	54.5	85.1	39.6	0	0	4.3	23.28
05/01/2023	52.7	65.6	59.8	1	0.03	3.6	10.51
05/02/2023	52.4	86.2	56.1	4	0.05	3.9	22.22
05/03/2023	58.5	88.8	45.4	0	0	2.9	23.84
05/04/2023	56.5	85.8	45.8	0	0	4.3	21.41
05/05/2023	51.6	61.6	63.8	7	0.04	2.3	5.14
05/06/2023	49.4	68.9	45.6	0	0	3.6	17.18
05/07/2023	44.9	73.2	44.9	0	0	3.6	23.11
05/08/2023	48.9	66.9	60.5	1	0.07	3.7	17.13
05/09/2023	48.7	71.1	54.2	0	0	2.8	19.40
05/10/2023	43.8	76.6	51	1	0	3.2	25.98
05/11/2023	46.9	80.6	45.2	0	0	3.1	24.90
05/12/2023	49.6	85.5	39.2	0	0	2.8	26.49
05/13/2023	51.6	88.3	34.4	0	0	4.6	26.29
05/14/2023	57.9	87.6	33.7	0	0	7.1	25.91
05/15/2023	62	90.1	38.1	0	0	5	19.75
05/16/2023	59.7	78.5	55.9	0	0	3.6	17.42
05/17/2023	56.3	86.1	58.7	8	0	3.1	23.06

Appendix 2. Fire Blight Temperature Risk Values (TRV) during bloom in East Wenatchee, WA. Blue bars indicate rainfall events, and the orange and red lines are the risk thresholds for fire blight, created based on observations of more than 30 years of infection events in Washington and Oregon. The orange line is the high-risk threshold (150 TRV), and the red line is the extreme risk threshold (350 TRV). Inoculation occurred at full bloom (FB).

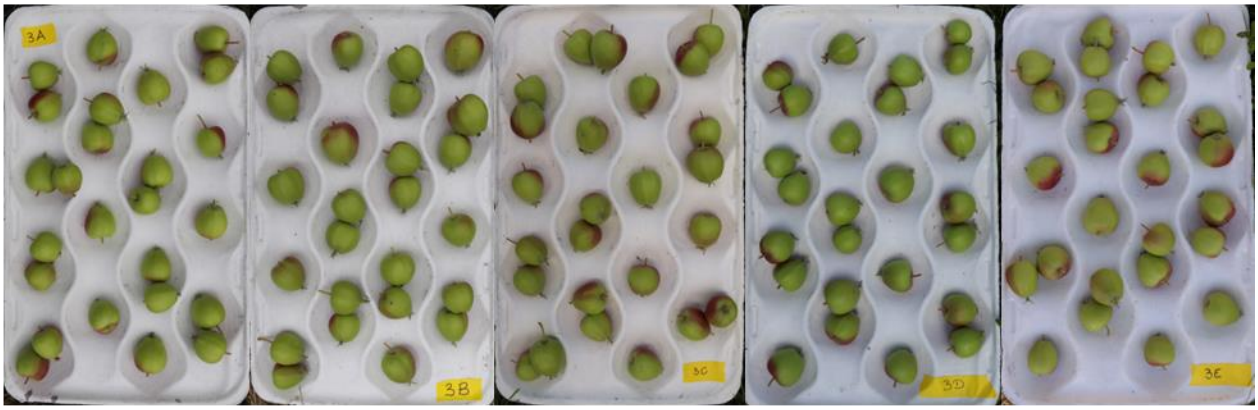


Appendix 3. Effect of the bifunctional peptide BP178 and controls applied to apple, cv. Gala on fruit skin marking in 2023.

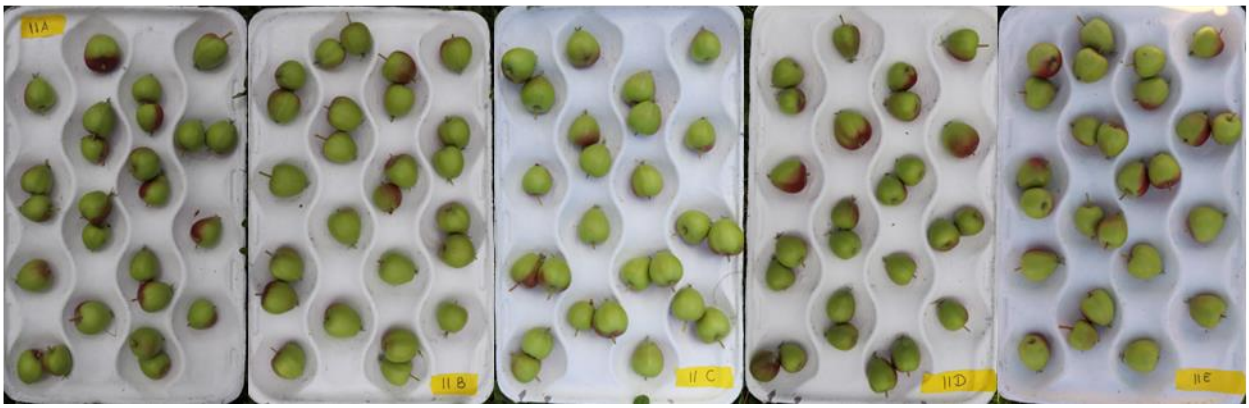
Streptomycin standard (Firewall 50WP)



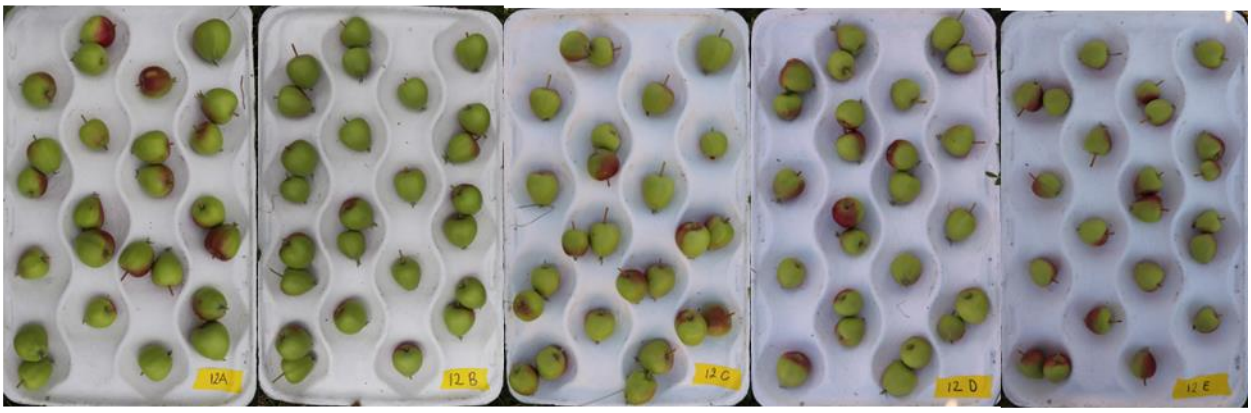
Blossom Protect + Buffer Protect



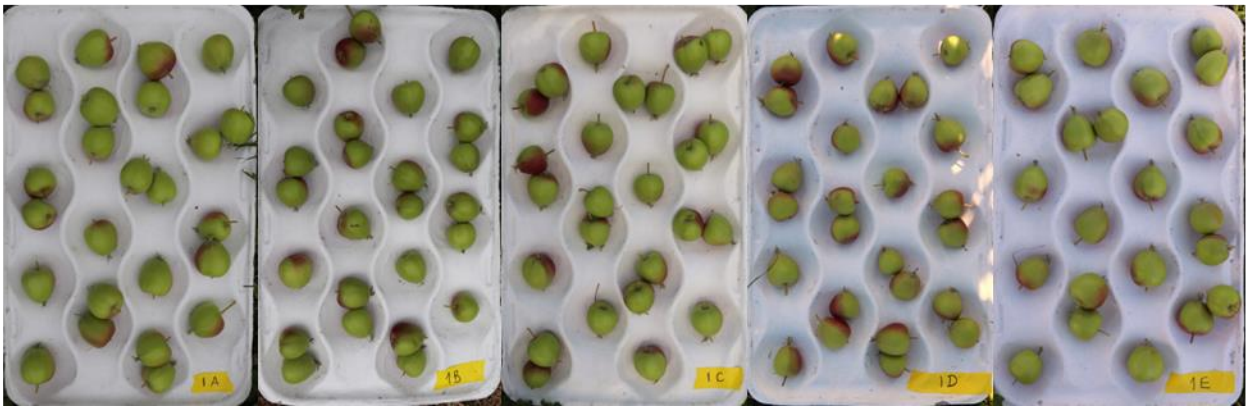
Bifunctional peptide BP178



Acibenzolar-S-methyl (Actigard)



Water-treated control



Proposal Title: New products for the prevention and control of shoot trauma blight

Report Type: Continuing Project Report

Primary PI: **Ricardo Delgado Santander**
Organization: Prosser-WSU-IAREC
Telephone: 845-905-2309
Email: r.delgadosanatnder@wsu.edu
Address: WSU Irrigated Agriculture Research and Extension Center
Address 2: 24106 N Bunn Rd
City/State/Zip: Prosser, WA 99350

Co-PI 2: **Youfu 'Frank' Zhao**
Organization: Prosser-WSU-IAREC
Telephone: 509-786-9284
Email: youfu.zhao@wsu.edu
Address: WSU Irrigated Agriculture Research and Extension Center
Address 2: 24106 N Bunn Rd
City/State/Zip: Prosser, WA 99350

Co-PI 3: **Aina Baro Sabe**
Organization: Wenatchee-WSU-TFREC
Telephone: 509-293-8758
Email: aina.barosabe@wsu.edu
Address: WSU Tree Fruit Research & Extension
Address 2: 1100 N Western Ave
City/State/Zip: Wenatchee, WA 98801

Co-PI 4: **Tianna DuPont**
Organization: Wenatchee-WSU-TFREC
Telephone: 509-293-8758
Email: aina.barosabe@wsu.edu
Address: WSU Tree Fruit Research & Extension
Address 2: 1100 N Western Ave
City/State/Zip: Wenatchee, WA 98801

Cooperators: **Joel Van Ornum**, president of Dungeness Environmental Solutions Inc.
Ricki Schneider, Agricultural Business Development & Research, TidalVision

Project Duration: 2-Year

Total Project Request for Year 1 Funding: $ 39,642
Total Project Request for Year 2 Funding: $ 41,227

Other related/associated funding sources: None

Budget 1

Primary PI: Ricardo Delgado Santander
Organization Name: Prosser-WSU-IAREC
Contract Administrator: Jamie Meek, Samantha Bridger, and Stacy Mondy
Telephone: 509-786-9231
Contract administrator email address: jamie.meek@wsu.edu | samantha_bridger@wsu.edu | arcgrants@wsu.edu
Station Manager/Supervisor: Naidu A. Rayapati
Station manager/supervisor email address: naidu.rayapati@wsu.edu

Item	4/1/2023	4/1/2024
Salaries	$20,064.00	$20,867.00
Benefits	$9,000.00	$9,361.00
Wages		
Benefits		
RCA Room Rental		
Shipping		
Supplies	$10,328.00	$10,749.00
Travel	$250.00	$250.00
Plot Fees		
Miscellaneous		
Total	$39,642.00	$41,227.00

Footnotes: 4% inflation for year 2. [1]Salary 4.5 months; [2]Benefits rate 44.9%; [3]Lab and field supplies include the tested compounds, apple trees, substrate and fertilizers, culture media and reagents for qPCR.

Objectives:

1. *In vitro* screening for antibacterial activity, dosage, and potential additive and/or synergistic effects in different products (e.g., soluble chitosan derivatives, rhamnolipids, lauric acid, caprylic acid) or mixtures with already used antimicrobials.
2. Elaborate a methodology to screen materials for shoot blight prevention and control, mimicking natural infection conditions after wind and hail damage.
3. Evaluate *in planta* efficacy of products and/or product combinations in greenhouse and field conditions.

Significant Findings

- Chitosan lactate showed the most significant inhibitory in vitro activity among all assayed products.
- Developed a reproducible wounding and inoculation method to mimic trauma blight symptoms in crabapple and apple.
- Sodium caprylate is highly phytotoxic when applied at high concentrations.
- Tidal Grow SPECTRA provided the best disease control (around 64%) and significantly reduced the disease severity on potted, 2-year-old 'Pink Lady' trees.
- Preliminary in vitro tests indicate that rhamnolipids and sodium caprylate co-applied with streptomycin, kasugamycin and oxytetracycline synergistically enhance the antibiotic activity.

Methods

1. **Tested materials**

The compounds we tested are SAR inducers and/or possess bactericide properties not yet tested on the apple-*E. amylovora* pathosystem: Chitosan from snow crab (ChitoLytic), chitosan lactate from snow crab (ChitoLytic), chitosan oligosaccharides from mushrooms (ChitoLytic), chitosan oligosaccharides from snow crab (ChitoLytic), rhamnolipids (Sigma-Aldrich), sodium laurate (Sigma-Aldrich), and sodium caprylate (Sigma-Aldrich). We also tested *in planta* efficacy of two chitosan-based commercial products approved for use in Washington State, TidalGrow SPECTRA (Tidal Vision) and Armour-Zen 15% (Botry-Zen), as well as a test product, ChitoAg-80, developed by Dungeness Environmental Solutions Inc. (Everett, WA) (see cooperators). None of these products have been tested before against fire blight in experimental plots.

2. ***In vitro* testing of antimicrobial activity and potential synergistic effects between different compounds**

To assess the bactericidal dosage of the compounds, we determined their antimicrobial activities *in vitro* using miniaturized assays in 96-well plates. Compounds were dissolved in 0.5x Lysogeny Broth (LB), followed by filter sterilization (0.22 m pore diameter). *E. amylovora* strain Ea4 (isolated in Washington State) was inoculated at 10^7 CFU/mL in medium containing 2-fold increasing concentrations of the compounds. Plates were incubated at 28ºC (82.4ºF), and bacterial growth was measured spectrophotometrically after 18 h with a 96-well plate reader.

With this information we calculated the IC_{50} and IC_{95}, which tells us the amount of a substance needed to reduce bacterial growth by 50% and 95%, respectively. These values help us understand how effective the substances are against the bacteria.

We also checked if some of the studied compounds enhance antibiotic activity by the diagonal drug interaction method. After incubating again for 18 hours, we measured bacterial growth and calculated the FIC (Fractional Inhibitory Concentration) to understand how well the substances and antibiotics

work together.

3. Greenhouse experiments

3.1. Initial evaluation of product phytotoxicity and initial product efficacy testing using 'Fuji' apple plantlets

'Fuji' plantlets were obtained by seed germination on damp paper towels. Germinated seeds were transferred to seedling pots with seedling substrate mixed 5:1 with perlite and transferred to the greenhouse with natural illumination and temperature regulated at 82ºF until 8-10 leaf stage.

We tested different products (shown in **Table 1**) on these plants. The concentrations we used were chosen based on lab tests, the instructions from the manufacturers, and advice from collaborators. For the commercial products, we tried the highest recommended doses or, in some cases, even higher amounts. Since chitosan-based commercial products had low pH levels (between 3.22 and 5.15) and were prepared in diluted acetic acid, we also prepared the remaining products in 0.5% glacial acetic acid.

Table 1. Spray treatments applied in initial tests on 'Fuji' plantlets.

Treatments	Product	Product dilution used	% Active compound in sol.	pH [a]
	Commercial products for agriculture			
1	Tidal Gro SPECTRA (5.75% chitosan)	22 fl oz/2 gal	0.49	3.77
2	ChitoAg-80 (4% chitosan)	4 qt/100 gal	0.04	3.85
3	Armour-Zen 15% (15% chitosan)	8 qt/50 gal	0.60	5.22
	Other products			
4	Chitosan Lactate	0.3% [b]	0.3	3.17
5	Chitosan Oligos. Mushroom	1%	1	3.24
6	Chitosan Oligos. Crab	1%	1	3.72
7	Rhamnolipids	0.4%	0.4	3.69
8	Sodium caprylate	0.4%	0.4	3.9
9	Chitosan	0.3%	0.3 [b]	3.7
10	Water + 0.5% Acetic acid (pH adjusted with K_2HPO_4)	NA	NA	3.17
11	Untreated control	NA	NA	NA

[a] The pH values of the commercial products was measured after dilution in water. In the remaining products, the reported pH results after dissolving the products in 0.5% glacial acetic acid.

[b] Although the IC95 of this product was 6 times lower, most chitosan-based products are used in similar concentrations, and because the assay goal was to detect potential phytotoxicity of the products, we opted for a higher concentration.

[c] We did not test this product in vitro due to the high viscosity and poor solubility. This concentration was chosen to compare results with chitosan lactate.

We sprayed different treatments on the plants on days 0, 3, and 6. On day 8, we infected the plants with *E. amylovora* Ea4 by cutting the five youngest leaves with scissors dipped in a bacterial solution at 10^7 CFU/mL. Then, on days 9 and 12 (3 and 6 days after the inoculation), we sprayed the plants again with the treatments. Each treatment was tested in 3-6 plantlets placed on a bench in a completely randomized design.

To see how the treatments affected the plants, we looked at the development of symptoms (necrosis) over time. We used a severity index to measures how much necrosis spreads on the leaves, and compared the differences statistically.

3.2. Standardization of a methodology to reproduce trauma blight symptoms

Before we tried to replicate trauma blight symptoms in apple trees, we experimented with two-year-old crabapple trees in the greenhouse to find the best conditions. Crabapple trees were fertilized every two weeks with all-purpose fertilizer 24-8-16 N-P-K. Trees were maintained in the greenhouse with the maximum temperature threshold set at 82ºF, with natural illumination. The trauma blight treatments tested were:

A. Three cuts were performed on each of the 5 youngest leaves on one shoot/tree in 2 trees, and sprayed the shoots with *E. amylovora* Ea4 at 10^6 CFU/mL in distilled water.

B. Leaves were cut as in treatment A but sprayed with a higher concentration of *E. amylovora* Ea4 (10^7 CFU/mL).

C. First sprayed the shoots with *E. amylovora* Ea4 at 10^6 CFU/mL and then cut the leaves as in treatment A.

D. Sprayed the shoots with a higher concentration of *E. amylovora* Ea4 (10^7 CFU/mL) and then cut the leaves as in treatment A.

We measured the incidence and width of the necrotic lesions to see how well each method replicated trauma blight. After comparing the results statistically, we chose treatment A as it provided the most consistent and realistic outcomes. This method was then used in the following greenhouse experiments with potted 'Pink Lady' trees.

3.3. Evaluation of different products for prevention and control of trauma blight in 'Pink Lady' apple trees under controlled conditions

We conducted experiments with two-year-old potted 'Pink Lady' trees in a greenhouse. The trees were planted in a mix of peat moss and sandy loam soil and were kept at a maximum temperature of 82ºF with natural light. We started the treatments about a month and a half after planting, when the new shoots were around 7.5 – 9 inches long. Throughout the experiments, we regularly watered the trees, but no fertilizers were used.

The concentrations of the treatments are listed in **Table 2**. We chose these concentrations based on in vitro tests, recommendations from collaborators, and the phytotoxicity checks with 'Fuji' apple plantlets. For comparison, since all the commercial products had the same active compound, we set treatment dosages to cover a range of chitosan concentrations (0.02%, 0.15%, and 0.49%). To achieve this range, we used five times the recommended dosage for TidalGrow SPECTRA compared to standard leaf spraying.

Table 2. Spray treatments applied on 2-year-old potted 'Pink Lady' trees.

Treatment	No. Trees	Active compound	Application concentration	% Active compound	Application Pattern
Actigard 50 WG	5	Acibenzolar-S-methyl (50%)	2 oz/100 ga	0.007	A
FireWall 50 WP	4	Streptomycin sulfate (65.8%)	8 oz/100 ga	0.039	B
Fire Line 45 WP	4	Oxytetracycline hydrochloride (48.8%)	9 oz/100 ga	0.033	B
Cueva	5	Copper octanoate (10%)	4 qt/100 ga	0.100	B
Armour-Zen 15%	4	Chitosan (15%)	4 qt/100 ga [a]	0.150	A
ChitoAg-80	5	Chitosan (4%)	2 qt/100 ga	0.020	A
TidalGrow SPECTRA	4	Chitosan (5.75%)	22 fl oz/ 2 ga	0.494	A
Rhamnolipids [b]	5	Rhamnolipids (90%)	0.40%	0.400	A
Sodium caprylate [b]	5	Sodium caprylate (98%)	0.08% [c]	0.080	A
Chitosan Oligos. [b]	5	98.2% Deacetylated chitin (mushroom)	0.20%	0.200	A
Untreated control	5	NA	NA	NA	A

[a] Because of potential phytotoxicity at the highest recommended concentration, we used a lower one.

[b] Products adjusted to pH 4.0 with glacial acetic acid.

[c] Due to high phytotoxicity, we used a concentration 5 times lower than the original one (0.4%), which showed no phytotoxicity in apple plantlets.

[d] **A**, for products with recognized or potential plant-defense inducers: treatment application on 7-29-23, 8.01.23; tree wounding and inoculation on 8.03.23; treatment application 8.04.23 (9-11h post-inoculation) and 8.07.23. **B**, for products with bactericidal activity only: treatment application 8.02.23; tree wounding and inoculation on 8.03.23; treatment application on 8.04.23 (9-11h post-inoculation) and 8.07.23.

The commercial products were prepared according to the manufacturer's instructions. For other compounds, we diluted them in water and adjusted the pH to 4.0 using glacial acetic acid. All treatments were sprayed to drip. The untreated, inoculated plants were sprayed with distilled water. Control treatments, including Cueva, FireWall, FireLine, and Actigard, were also prepared according to the manufacturer's recommendations (**Table 2**).

The timing of the spray application depended on the product characteristics, such as whether they were plant defense inducers or bactericides. The application of treatments and inoculation patterns is detailed in **Table 2**.

For *E. amylovora* inoculations, we made cuts at the base of 5 leaves per shoot, 4 shoots per tree (totaling 20 leaves per tree), and used 4-5 tree replicates for each treatment. We sprayed *E. amylovora* at a concentration of $4x10^6$ CFU/mL to drip. We monitored fire blight symptoms over time, regularly measuring the maximum necrosis width perpendicular to the cut.

Results and Discussion

1. Most of the assayed materials possess bactericidal activity against *E. amylovora*

Of all the tested products, chitosan lactate demonstrated the strongest *in vitro* activity against *E. amylovora*. It significantly reduced *E. amylovora* populations by 50% and 95% at very low concentrations of 0.03% and 0.05%, respectively (**Figure 1C**). The next most effective products were sodium caprylate, with IC_{50} and IC_{95} values of 0.17% and 0.45%, respectively (**Figure 1A**), and rhamnolipids (**Figure 1B**). Rhamnolipids produced a biphasic growth inhibition curve, indicating two concentrations (IC^1_{50} at 0.10% and IC^2_{50} at 0.40%) causing a 50% decrease in populations in each phase.

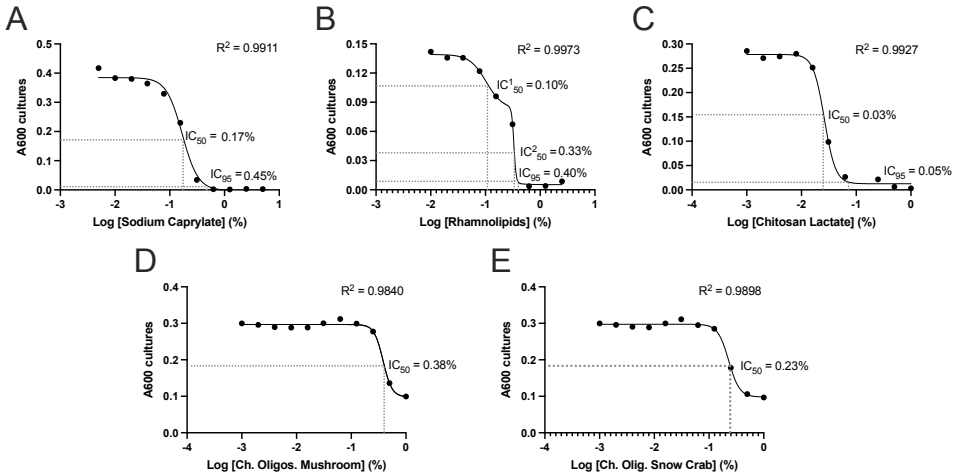


Figure 1. Effect of sodium caprylate (A), rhamnolipids (B), chitosan lactate (C), chitosan oligosaccharides from mushroom (D), and chitosan oligosaccharides from snow crab (E) on *E. amylovora* growth. Each chart shows the IC50 and IC95 values obtained in triplicate assays. Curves were adjusted by non-linear regression after log-transformation of the assayed compound concentrations. The effect of rhamnolipids on the pathogen's growth fitted better a biphasic curve. Accordingly, two IC50 values are provided, one for each phase. Only in this case, the IC95 value in the chart was obtained by manual interpolation, using as a reference the A_{600} nm values in the absence of rhamnolipids. In the remaining charts,

both the IC50 and IC95 values were calculated with GraphPad Prism software. The R^2 values indicate the goodness of fit of the curves to the obtained data.

Chitosan oligosaccharides from mushroom and crab had certain bactericide activity but did not completely stop *E. amylovora* growth under the test conditions, showing low effectiveness against the pathogen (**Figure 1D** and **1E**, respectively).

Raw chitosan could not be included in this assay due to incompatibilities with the experimental conditions. Chitosan solutions are extremely viscous and require acidic conditions for solubilization. Sodium lactate was soluble, but precipitated during incubation, making it impossible to measure bacterial growth (**Table 3**).

In summary, all the tested substances demonstrated antimicrobial properties that could be beneficial in managing fire blight in the field. A noteworthy observation is that we specifically designed the assay to determine if the compound itself, rather than just the pH, had inhibitory effects on *E. amylovora*. We conducted the test under controlled pH conditions in a medium that favors pathogen growth. This is important because many agricultural products are initially prepared at low pH levels before being sprayed, and this low pH may also contribute to killing the pathogen cells when applied in the field. The activity of caprylic acid, rhamnolipids, and chitosan has been reported to persist or even increase at low pH levels, such as pH 4.0 or 5.0.

Table 3. Performance of different compounds inhibiting *E. amylovora* growth.

Compound	Concentration Range	IC50	IC95
Sodium Caprylate	0 - 5 %	0.17%	0.45%
Sodium Laurate [a]	0 - 5 %	ND	ND
Rhamnolipids [b]	0 - 5 %	0.10% 0.33%	0.40%
Chitosan [c]	0-1 %	NA	NA
Chitosan Lactate	0 - 1 %	0.03%	0.05%
Chitosan Oligos (Crab) [d]	0 - 1 %	0.38%	NA
Chitosan Oligos (Mushroom) [d]	0 - 1 %	0.23%	NA

[a] Although sodium laurate was soluble in water and culture medium, after overnight incubation, the salt precipitated making on the bottom of the wells, making it impossible to measure the A600 nm to quantify *E. amylovora* growth. ND, not determined.

[b] The effect of rhamnolipids on *E. amylovora* growth fitted a biphasic curve. Hence, the first IC50 value (0.10 %) corresponds to the first significant decrease in *E. amylovora* populations, and the second IC50 value (0.33 %). The IC95 value was calculated manually, interpolating the value directly from the chart.

[c] Due to the low solubility under the assayed conditions without extremely altering the pH, this product was removed from these assays. NA, not applicable.

[d] The assayed concentrations did not decrease *E. amylovora* populations a 95% with respect to the control medium without the chitosan derivatives. Higher concentrations were difficult to dissolve. NA, not applicable.

2. Caprylic acid dilution is required to avoid phytotoxicity on leaves

In our initial test with 'Fuji' plantlets and the concentrations specified in **Table 1**, we observed significant phytotoxicity of caprylic acid at the tested concentration based on *in vitro* assays (0.4%). All plants sprayed with this compound displayed abundant necrotic lesions on all leaves within 24 hours after the first spray. This finding was further confirmed in a follow-up experiment where decreasing 2-fold dilutions of caprylic acid (ranging from 0.4% to 0.16%) caused necrotic spots on the leaf surface shortly after application. Plants sprayed with 0.08% of this compound did not show signs of phytotoxicity, so we used this concentration in subsequent experiments with potted plants (see Results section 4).

Additionally, in one out of three/six plants sprayed with Armour-Zen 15% and chitosan oligos (mushroom) at the tested concentrations, isolated necrotic spots appeared on some leaves by the end

of the experiment. As a precaution, we reduced the concentrations of these products in the subsequent assay with potted trees (**Table 2**).

During this preliminary test with 'Fuji' plantlets, conducted before optimizing the inoculation method (see Results section 3), we also inoculated plants with *E. amylovora* using scissors contaminated with the pathogen. The only plants significantly different from the controls were those treated with Armour-Zen 15%, displaying significantly larger necrotic lesions at 10 days post-inoculation (data not shown). Plants treated with rhamnolipids and caprylic acid showed smaller necrotic lesions compared to untreated controls sprayed with water or water adjusted to pH 3.17 (control included to rule out the effect of acidic pH on infections), although these differences were not statistically significant (data not shown).

3. Trauma blight-like symptoms can be recreated artificially with high reproducibility

In **Figure 2**, we compare two methods to replicate trauma blight symptoms: cutting with sterile scissors followed by spray inoculation vs. spray inoculation followed by wounding with scissors. The first method mimics situations where plant tissues are damaged by strong winds or hail, allowing *E. amylovora* cells to reach the wounds via biological (e.g., insects) or physical vectors (e.g., wind-driven rain). The second method simulates a less likely scenario where *E. amylovora* is already present on the plant surface, and tissues are wounded by wind or hail, facilitating the pathogen's entry into internal plant tissues. Generally, *E. amylovora* is considered a poor colonizer of leaves and other plant organs besides flowers. Abundance *E. amylovora* cells on other plant surfaces than flowers is more likely in heavily damaged orchards with abundant ooze, necrotic lesions, or infected flowers.

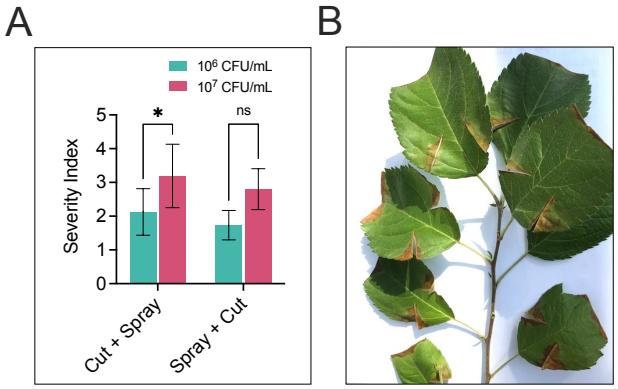


Figure 2. Optimization of an inoculation method to recreate trauma blight-like symptoms using white crabapple plants. We assayed two inoculation methods, cutting leaves plus spray inoculation with *E. amylovora,* and spray inoculation followed by cutting with sterile scissors. We tested both methods with two bacterial concentrations (10^6 and 10^7 CFU/mL). Results are average values of 5-8 replicate leaves per shoot in two different shoots. The asterisk indicates significant differences associated to the inoculum concentration when sprayed after cutting ($P \leq 0.05$), assessed by Šídák's multiple comparisons test (A). Representative illustration of the cut position and fire blight symptoms 7 days after incubation (B).

We tested both methods at two *E. amylovora* concentrations, 10^6 and 10^7 CFU/mL. Both methods were highly reproducible, with infection rates reaching 100% regardless of the method or pathogen concentration used. This suggests that even at 10^6 CFU/mL, the recreated conditions could match a scenario with high infection pressure. In the statistical analysis, the only factor significantly affecting symptom severity after 7 days was the pathogen concentration, accounting for 36.4% of the observed variability ($P = 0.0016$). Multiple comparisons tests revealed that *E. amylovora* concentrations had a significant effect when cuts were performed before spray inoculation ($P = 0.0185$) but not when the pathogen was sprayed before performing the cuts ($P = 0.0568$) (**Figure 2A**). Based on these results, and considering the first scenario seems more plausible, we selected the strategy of making cuts before spray inoculation as the preferred method for reproducing trauma blight symptoms in apples.

For the inoculations in this experiment, we made two cuts at the base of the leaf toward the central nerve and one transversal cut to the leaf tip (**Figure 2B**). While all cuts showed signs of infection, the ones at the base of the leaf exhibited highly reproducible symptom severities, unlike those on the leaf tip (**Figure 2B**). Therefore, for the primary greenhouse assay with potted 'Pink Lady' trees (see below), we only made cuts at the base of the leaves before *E. amylovora* spray inoculation.

4. Tidal Grow SPECTRA and rhamnolipids showed promising disease control and/or significant symptom severity reduction when applied in the assayed doses

The optimized inoculation method developed with crabapples was employed to evaluate the disease control efficacy and the ability to reduce symptom severity of selected treatments (**Table 2**) on two-year-old 'Pink Lady' apple trees. The treatments included Actigard as a plant defense-induction control, along with copper (Cueva), streptomycin (FireWall), and oxytetracycline (FireLine) as bactericide controls.

Results from the first greenhouse trial with potted plants are presented in **Figure 3**. Disease control percentage was calculated as the reduction in disease incidence in inoculated wounds compared to the untreated control. Out of the 10 treatments tested, only FireWall, TidalGrow SPECTRA, and FireLine provided significant fire blight disease control throughout the experiment, with median values of 76.8%, 63.9%, and 56.6% at the end of the experiment (33 days post-inoculation) ($P \leq 0.0117$) (**Figure 3A**). Rhamnolipids showed relatively good disease control of around 46.7% at 11 dpi (P = 0.0120) but decreased to 12.4% at the end of the experiment ($P = 0.0021$). Other treatments like Cueva and ChitoAg-80 provided similarly low but statistically significant disease control at 33 dpi of 12.4 and 13%, respectively ($P \leq 0.0482$) (Figure 3A). However, these values, calculated based on infection incidence, do not fully illustrate the activity of the tested products. **Figure 3B** shows that although Actigard, Cueva, and rhamnolipids provided low disease control efficacy percentages, the lesion size was 30-60% smaller than in untreated controls within the same period ($P \leq 0.0356$). On the opposite end of the spectrum, the other commercial chitosan-based compounds, Armour-Zen 15% and ChitoAg-80, performed poorly in reduction of disease symptoms (**Figure 3B**) and controlling the disease, especially Armout-Zen 15% (**Figure 3A**).

The suboptimal protection conferred by Actigard may be due to its application every 3 days (while the recommended frequency is weekly). This pattern was chosen to test the efficacy of all products similarly. However, the lower activity observed in Actigard and other tested products might be improved by extending the period between sprays, a consideration for future experiments. Another factor is the dosage; for example, rhamnolipids were used at 0.4%, but a commercial rhamnolipid-based fungicide, Zonix, recommends a dosage of 300 – 1000 ppm (i.e., 0.03 - 0.1%) to combat fungal diseases. These concentrations are 4 – 13 times lower than the concentration sprayed in this study. Rhamnolipids may exhibit better fungicidal than bactericidal activity, but also, the product might function better as a plant-defense inducer at lower concentrations than those used in this study. More experiments are required to test these hypotheses.

Comparisons between the three commercial chitosan-based products suggest that high chitosan concentrations are necessary for significant protection against fire blight. TidalGrow SPECTRA treatments were prepared to reach approximately 0.5% chitosan concentration, Armour-Zen 15% treatment contained 0.15% chitosan, and ChitoAg-80 treatment had 0.02% chitosan (**Table 2**). The most effective protection was achieved with TidalGrow SPECTRA. Interestingly, ChitoAg-80 applied at a low dosage provided better disease control than Armour-Zen 15%, containing 7.5 times more chitosan. Similarly, the symptom severity was slightly reduced in plants treated with ChitoAg-80 compared to those treated with Armour-Zen 15% (**Figure 3B**). These results suggest that additional ingredients in the commercial product formulation may contribute to the better activity of ChitoAg-80, despite the lower concentration in the treatment or the lower activity of Armour-Zen 15%, regardless of the higher dosage employed.

With some differences, these results with potted trees align with those observed in preliminary assays with 'Fuji' plantlets inoculated with *E. amylovora*-contaminated scissors (Results section 2), where Armour-Zen 15% provided poor protection against symptom development, even exacerbating fire blight symptoms in treated plants.

Regarding treatments with sodium caprylate, none of the trees sprayed at 0.08% showed signs of phytotoxicity on the leaves. Although the analysis did not reveal significant differences with the control, it is worth mentioning that, except for one tree, symptom severity was overall reduced compared to the control.

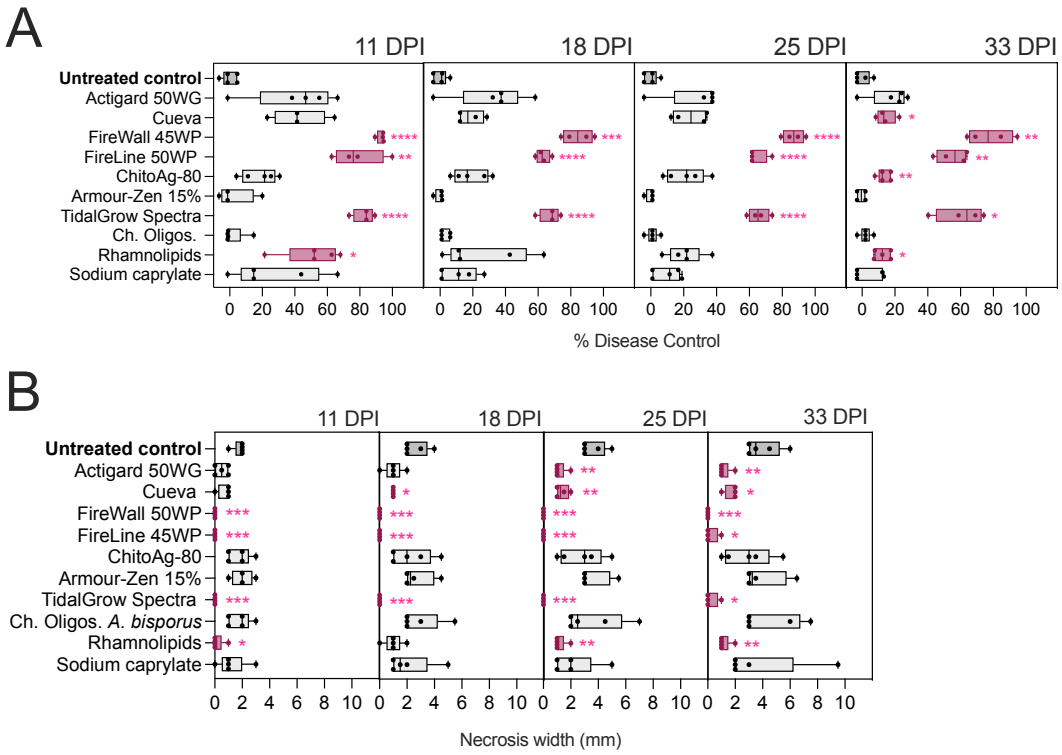


Figure 3. Efficacy of the assayed products in disease control and symptom severity reduction throughout time. The **%** disease control was calculated as the % disease incidence reduction with respect to the % disease in the untreated control, multiplied by 100. Asterisks show statistically significant differences between the indicated treatment and the untreated control, assessed by Dunnett's multiple comparisons test ($\alpha = 0.05$). Treatments providing significant disease control and/or symptom reduction are highlighted with pink color.

To sum up, considering both disease control and disease severity, the most effective compounds protecting against fire blight were Firewall, demonstrating the highest disease control and the smallest necrotic lesion sizes. Following closely were TidalGrow SPECTRA and FireLine, which exhibited comparable control and reduction of disease symptoms. Rhamnolipids and Cueva also showed similar results for the two analyzed parameters (Figure 3A,B). It's important to note that these outcomes were achieved under high infection pressure conditions and without optimizing application timing and concentrations. Hence, it is likely that some of the tested products may perform even better under less restrictive conditions.

5. **Preliminary assays indicate that rhamnolipids and caprylic acid could also be used in lower doses as coadjuvants to improve the activity of antibiotics**

To explore additional applications of the tested compounds, we evaluated the ability of two of them (rhamnolipids and sodium caprylate) to enhance the bactericidal activity of streptomycin (Sm), kasugamycin (Kg), and oxytetracycline (OTc) using the diagonal drug interaction method (see Methods, section 2). Preliminary results from assays performed in duplicate are presented in **Figure 4**. For all the tested compounds, the concentrations required to reduce *E. amylovora* populations when used alone were higher than when combined. This trend was observed for combinations of rhamnolipids (RL) and sodium caprylate (SC) (**Figure 4A**), as well as combinations of RL or SC with Sm (**Figure 4B**), Kg (**Figure 4C**), and OTc (**Figure 4C**).

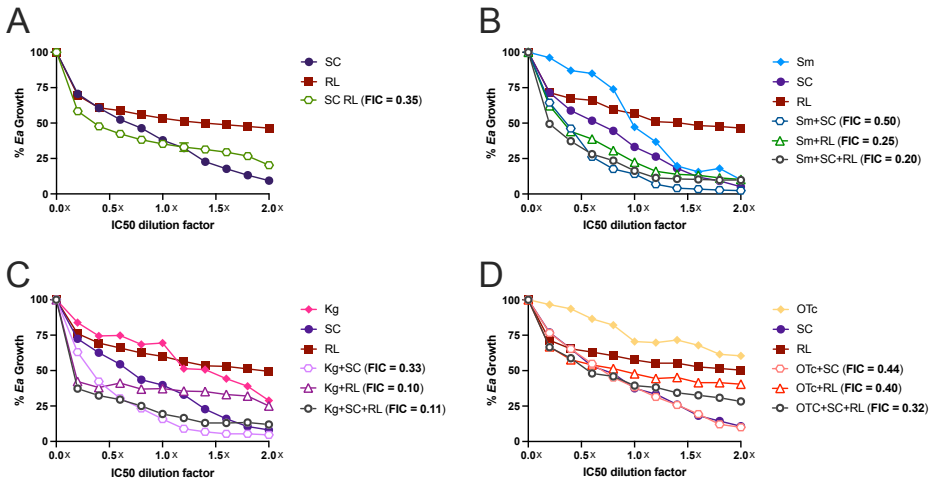


Figure 4. Interaction between rhamnolipids, sodium caprylate and antibiotics. Graphs show *E. amylovora* growth inhibition (expressed as a percentage) by concentrations of streptomycin (Sm), kasugamycin (Kg), oxytetracycline (OTc), rhamnolipids (RL) and sodium caprylate (SC) adjusted to 0 – 2x their IC50 calculated in a separate assay. As an example, values in the graph at 0.5x mean that the assayed concentration of the product, or products within a mixture, equals to half of the IC50 calculated for each product in a previous assay. In each graph, products are tested individually and combined.

In this type of assay, if the product interactions are additive, the mixture components will contribute to the mixture IC_{50} proportionally to their individual IC_{50}. This implies that the observed mixture IC_{50} will be close to the average of the IC_{50}s of each product alone, known as the estimated IC_{50}. To assess the type of product interaction, we use the FIC value (FIC = Observed IC_{50}/Estimated IC_{50}). In cases of additive interactions, the observed mixture IC_{50} aligns with the average IC_{50}, resulting in an FIC value of 1. Lower FIC values than 1 indicate that the mixture performs better than expected, considering the IC_{50} of the products acting separately (indicating a synergistic interaction).

All tested product combinations demonstrated synergistic interactions (FIC values below 1). In all cases, mixtures of antibiotics with rhamnolipids (RL) enhanced the bactericidal activity of the combinations (lower FIC values) better than with sodium caprylate (SC). This effect was particularly significant when mixing Kg+RL (**Figure 4C**). In this instance, the IC_{50} value of the mixture was 10 times lower than the expected average IC_{50} of Kg and RL assessed individually. Generally, all antibiotic interactions with RL showed FIC values closer to those of antibiotics plus RL and SC together (**Figure 4**). This indicates that RL potentiates the action of antibiotics almost the same as when combining them with RL+SC.

Our findings suggest that, mostly rhamnolipids, but also sodium caprylate could serve as effective coadjuvants, enhancing the action of antibiotics in the field. In future experiments, we plan to include assays to evaluate the efficacy improvement of antibiotics mixed with rhamnolipids and chitosan or chitosan-based products.

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

Report Type: Continuing Project Report Year 3 NCE

PI: Achour Amiri
Organization: Washington State University
Telephone: 509-293-8752
Email: a.amiri@wsu.edu
Address: WSU-TFREC
Address 2: 1100 N. Western Ave.
City/State/Zip: Wenatchee, WA, 98801

Co-PI 2: Hyun Jung Lee
Organization: University of Idaho
Telephone: 208-885-9703
Email: hlee@uidaho.edu
Address: UI-AVFS
Address 2: 875 Perimeter Dr. MS2312
City/State/Zip: Moscow, ID 83843

Co-PI (3): Dojin Ryu
Organization: University of Missouri
Telephone: 573-884-8742
Email: dryu@missouri.edu
Address: 1406 Rollins Street
City/State/Zip: Columbia, MO 65211

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: Washington State University
Contract Administrator: Kevin Rimes, Stacy Mondy
Telephone: 509-786-2226, 509-335-4563
Contract administrator email address: kevin.rimes@wsu.edu, arcgrants@wsu.edu
Station Manager/Supervisor: Chad Kruger
Station manager/supervisor email address: cekruger@wsu.edu

Item	2021	2022	2023	2024
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	0

Footnotes:
[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: Dojin Ryu
Organization Name: University of Missouri **Contract Administrator:** Lou Zimmerman
Telephone: 573-882-0095 **Email address:** zimmermanje@missouri.edu

Item	2021	2022	2023	2024
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	0

Footnotes:
[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

- Eleven and 13 apple fruit lots were surveyed in 2022 and 2023, respectively.
- Fungi associated with fruit decay were isolated from both symptomatic and asymptomatic apples from diverse apple cultivars including Autumn Glory, Gala, Fuji, Honeycrisp, WA38, Red Delicious, Spur Golden, Golden Delicious coming directly from orchards and processing facilities.
- The majority of recovered fungi belonged to *Penicillium* species, however fungi from 11 other fungal species were recovered.
- *Penicillium expansum* was the most recovered species but isolates from 7 other *Penicillium* species were isolated from rotten apples.
- 50 isolates including *Penicillium* and other species were tested for patulin production in 2022 and 50 other isolates were tested in 2023. All *P. expansum* isolates produced patulin and interestingly some other *Penicillium* species produced patulin as well.
- 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 the 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.

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. Sampling was carried out in 11 and 13 lots in 2022 and 2023, respectively, including sampling from decayed and asymptomatic apples. In 2022, asymptomatic and symptomatic fruit showing superficial bruises or not were collected from 5 cultivars and yielded 544 isolates from 12 different fungal genera were recovered with *Penicillium* spp. being the majority (Table 1).

Table 1. Frequency of major genera of fungi recovered from apples collected from processing facilities in 2022.

Lot	Cultivar	Number of Fruit	Source	Bruised fruit	Decay visiable	Number of isolates recovered	Frequency of major pathogens recovered					
							Penicillium	*Cladosporium*	*Alternaria*	*Fusarium*	Other minors	Unknown
1	Gala	50	Orchard	No	No	35	17.9	0.0	35.9	7.7	28.2	0.0
2	Spur golden	25	Orchard	No	No	16	0.0	0.0	23.5	0.0	58.8	11.8
3	Gala	52	Storage	Most	All	92	100	0.0	0.0	0.0	0.0	0.0
4	Gala/Golden delicious	134	Storage	Few	Few	121	60.3	24.8	5.0	1.7	0.0	8.3
5	Cosmic crisp	72	Storage	Most	Few	72	62.3	20.3	11.6	2.9	1.4	5.8
6	Honeycrisp	36	Storage	Most	Few	29	89.3	3.6	10.7	0.0	0.0	0.0
7	Gala	52	Storage	Few	Few	38	89.5	7.9	0.0	0.0	2.6	0.0
8	Gala	56	Storage	Few	No	23	94.4	5.6	5.6	0.0	22.2	0.0
9	Gala	56	Storage	Few	Few	41	59.0	35.9	7.7	0.0	0.0	2.6
10	Honeycrisp	52	Storage	Most	Most	48	88.6	0.0	4.5	0.0	2.3	13.6
11	Golden Delicious	54	Storage	Most	Most	29	93.1	0.0	0.0	3.4	0.0	3.4

In 2023, 13 lots including 5 fruit samples directly from orchards and 8 fruit samples from storage from 7 cultivars were surveyed. Bruises were seen in 5 lots of the 13 at incidence ranging from 0 to 50%, whereas visible decays were seen in 6 lots with an incidence between 19 and 100% (Table 2). In total, 773 isolates from 6 fungal genera were collected with *Penicillium* making up to 56.5% followed by *Alternaria* and *Cladosporium* with 16 and 15%, respectively.

Table 2. Frequency of major genera of fungi recovered from apples collected from processing facilities in 2023.

Lot	Cultivar	Number of Fruit	Source	Bruised fruit (%)	Fruit with Decay visiable (%)	Number of isolates recovered	Frequency (%) of major pathogens recovered					
							Penicillium	*Cladosporium*	*Alternaria*	*Fusarium*	Other minors	Unknown
1	Red Delicious	52	Orchard	50	100	100	40.0	0.0	21.0	3.0	33.0	3.0
2	Koru	51	Orchard	20	2	38	5.3	0.0	76.3	2.6	5.3	10.5
3	Fuji	50	Orchard	1	0	76	23.7	32.9	15.8	0.0	0.0	27.6
7	Fuji	50	Orchard	0	0	45	20.0	31.1	28.9	0.0	2.2	17.8
8	Fuji	56	Orchard	44	1	68	54.4	27.9	16.2	0.0	0.0	1.5
4	Autumn Glory	49	Storage	24	19	92	53.3	26.1	16.3	0.0	2.2	2.2
5	Sunrise Majic	56	Storage	1	50	49	85.7	10.2	4.1	0.0	0.0	0.0
6	Sunrise Majic	56	Storage	2	52	52	82.7	3.8	9.6	0.0	0.0	3.8
9	Honeycrisp	58	Storage	0	56	60	98.3	0.0	1.7	0.0	0.0	0.0
10	Gala	58	Storage	0	53	85	90.6	2.4	2.4	0.0	4.7	0.0
11	Honeycrisp	48	Storage	25	4	46	50.0	32.6	10.9	0.0	2.2	4.3
12	Gala	50	Storage	0	4	39	51.3	15.4	15.4	0.0	7.7	10.3
13	Gala	49	Storage	1	0	23	78.3	13.0	8.7	0.0	0.0	0.0

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).

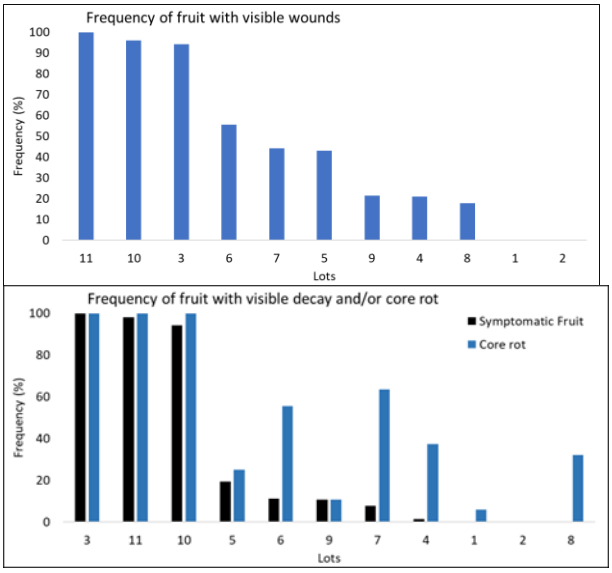


Figure 1. Frequency of fruits with visible wounds at sampling (a) and frequency of fruit with visible decay and core rot (b) in 2022.

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).

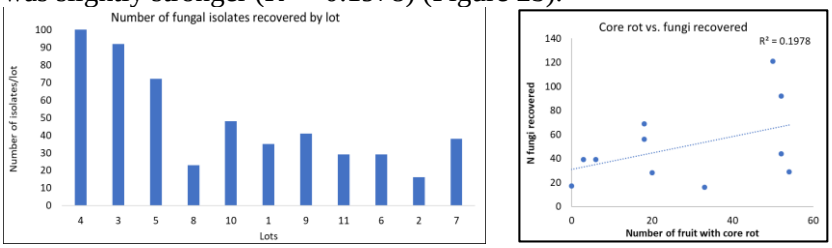


Figure 2. Number of fungal isolates recovered by lot in 2022 (left) and correlation between number of fungi recovered and incidence of core rots in the fruit (right).

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.

Molecular Characterization. Further, 180 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.

Sample preparation step – 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.

SPE clean up – 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.

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 – 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.

Sample preparation step – 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.

Extraction method – 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.

SPE clean up – 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 2024

- Complete characterization of isolates to species level
- 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.