2026 Apple Crop Protection Research Review

Rosy Apple (RAA) and Green Apple Aphids (GAA) on an infested apple leaf with spotted lacewings at CRO in Rock Island. This project led by Rebecca Schmidt-Jeffris and currently funded by WTFRC is titled “Best Practices for Lacewings in Apples.”

Photo Source: Raesibe Kgaphola

January 28, 2026

Hybrid Format
Yakima, WA

Project Title: Survey for Codling Moth Virus Resistance in Washington State

Report Type: Continuing Project Report (NCE)

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Project Duration: 1-Year

Total Project Request for Year 1 Funding: $99,930
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Total Project Request for Year 3 Funding: $

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Amount: $178,805 (Rutgers portion)
Agency Name: WTFRC_**ACP**
Notes: *sub-award with Tobin Northfield (WSU) as PI*

Other related/associated funding sources: Awarded, (WTFRC_ACP to Tobin Nothfield, 2025-2028; WSARE to Tobin Northfield, 2025-2028)
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Amount: $180,803 (Rutgers portion)
Agency Name: Western SARE
Notes: *sub-award with Tobin Northfield (WSU) as PI*

WTFRC Collaborative Costs: none

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Item	2025	2026 (NCE)	(Type year start date of year 3 here if relevant)
Salaries		$64,000.00	
Benefits		$29,030.00	
Wages			
Benefits			
RCA Room Rental			
Shipping		$500.00	
Supplies		$4,400.00	
Travel		$2,000.00	
Plot Fees			
Miscellaneous			
Total	$0.00	$99,930.00	$0.00

Footnotes:

Salaries: Post-doctoral researcher at 100% FTE to conduct bioassays and mass crossings
Benefits: Rutgers negotiated fringe rate for post-doctoral for non-federal funding sources is 43.65%
Shipping: $500 for shipping of cardboard bands containing codling moth larvae from orchards with management failures
Supplies: Dispensable laboratory items for codling moth rearing and bioassays
Travel: Travel to Washington to visit infected orchards x2 and assist in the collection of codling moth larvae.

Objectives

1. Conduct mass crossings of a novel codling moth population, WA6, to guide management efforts.
2. Expand resistance survey to five additional orchards
    a. Collect and rear codling moth collected from Washington apple orchards in 2024.
    b. Conduct laboratory bioassays for five populations against first instar codling moth larvae on CpGV-M.
    c. Populations showing reduced susceptibility to CpGV-M will be assayed against two resistant breaking products.

Changes to objectives: The mass crossing experiment is currently underway. Populations demonstrating low susceptibility to CpGV were only assayed against the resistance breaking isolate CpGV-0017, Madex XLV, as the genetic make-up of the other isolate was determined not to be beneficial to evaluate.

Significant Findings

- Five codling moth populations were collected from organic apple orchards in Washington State experiencing management failures
- Three were successfully reared for bioassays and screened against CpGV-M (Cyd-X) and CpGV-S (Virosoft). Cyd-X is genetically the same as Cyd-X HP and Madex, and Madex HP.
- One population, WA6, was identified as resistant. A second population, WA7, showed reduced susceptibility, specifically to CpGV-S, and will be screened again.
- WA6 did have 100% mortality against the resistance breaking Madex XLV.

Methods

Obj 1. Conduct mass crossings of a novel codling moth population, WA6, to guide management efforts.

There is no reliable genetic marker to identify CpGV resistance so inheritance must be determined using inheritance predictions. Using the approach from Sauer et al. (2017) we will perform mass crossing between the LabS (susceptible strain) and the WA6 population. The two primary resistance types differ in that Type I is sex-linked on the female Z chromosome and Type II is autosomal so the expected survivorship of the resulting offspring differ between the two crosses. Males of LabS will be allowed to mate with WA6 females, and females of LabS with WA6 males in temperature cabinets at 25ºC 16:8 light:dark in BugDorm I cages containing oviposition substrate. Eggs will be held at 10ºC over the oviposition period to synchronize hatch and then moved to 25ºC, 16:8. Upon hatching, first instar larvae will be transferred onto diet with one of three treatments: UTC, CpGV-M (Cyd-X), and CpGV-S (Virosoft CP4), replicated three times, and mortality data recorded as in Obj 2b below. If it is Type I resistance, offspring will die on diet containing Virosoft. It the resistance is Type II, offspring will survive on both. The surviving larvae on the UTC of each new cross will be backcrossed with F1 males and WA6 females to confirm inheritance.

Obj 2. Expand resistance survey to five additional orchards.

Obj 2a. Collect and rear codling moth collected from Washington apple orchards in 2024

Diapausing codling moth will be collected from cardboard bands on trees by collaborator Tobin Northfield. No funds are requested at this time for his collaboration as they will be covered by the Tree Fruit Entomology Endowment. We plan to evaluate 5 populations in this initial screening using virus use patterns, the presence of management issues, and geographic location to guide the selection of sites. They will be sorted and then shipped to Rutgers Agricultural Research and Extension Center in Bridgeton, NJ by the Northfield lab. Proper permits are in place for shipping codling moth between WA and NJ. Larvae will be maintained under diapausing conditions (8ºC, dark) for 2-3 months. After which time they will be moved to 25 °C at 16:8 light:dark for rearing. Depending on the number of surviving larvae, the field-based populations may require 1-3 generations of rearing before large enough synchronized populations are present for testing.

Obj 2b. Conduct laboratory bioassays for five populations against first instar codling moth larvae on CpGV-M.

Populations from WA will be compared with a susceptible laboratory strain (LabS) derived from Benzon Research (Carlisle, Pennsylvania, USA) for all bioassays.

Virus stocks of different commercial products are stored at -20 °C. The OB concentration will be counted using a Petroff-Hauser chamber (depth 0.02 mm) under the dark field of light microscope (Nikon ECLIPSE E600). For resistance testing, virus occlusion bodies will be suspended in molecular grade water to a concentration of 3×10^6 OB/ml and a total volume of 1 ml was incorporated into 50 ml of semi-artificial diet, resulting in a final concentration of 6×10^4 OB/ml diet. At this diagnostic OB concentration, the mortality of susceptible codling moth larvae infected with CpGV-M is expected to be > 90% after 7 dpi and up to 100% after 14 dpi (Asser-Kaiser et al. 2007). After mixing the diet with OBs, 50 ml of diet was poured into 50-well plates (LICEFA, Bad Salzuflen, Germany) and allowed to dry before newly hatched codling moth first instars are individually transferred to the plates.

Larvae of the LabS strain and new WA strains will be evaluated using the same protocol. Thirty to fifty larvae will be evaluated for each replication with three replicates of each isolate. Dead individuals were recorded at 1, 7, 14, and 21 dpi. Larvae that die on day 1 because of handling, will be excluded from the experiment. If needed, the mean mortality at 7, 14, and 21 (pupae) dpi was corrected for control mortality according to Abbott´s formula. The same procedure was performed for the untreated control, with 1 ml of molecular grade water incorporated into the diet.

Obj 2c. Populations showing reduced susceptibility to CpGV-M will be assayed against two resistant breaking products.

Using the same methods as above, any colony indicating less than 90% susceptibility to Cyd-X at 14 dpi will further be bioassayed against CpGV-0017 (Madex XLV) and CpGV-S (Virosoft) at the diagnostic OB concentration. If needed, the mean mortality at 7, 14, and 21 dpi was corrected for control mortality according to Abbott´s formula. Data were analyzed with one-way ANOVA and Tukey HSD comparison for significant difference analysis. PI Nielsen will travel to infested orchards in Washington State to collect additional larvae and discuss

Results and Discussion

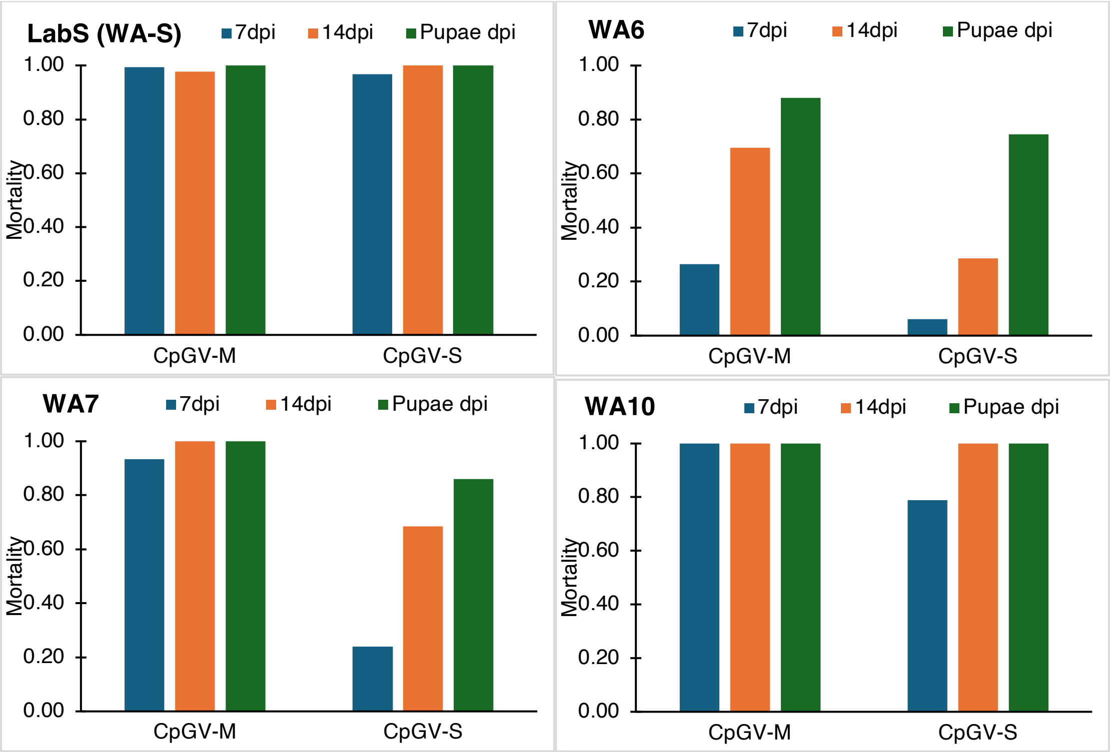
Rutgers completed screenings of 3 populations collected from organic orchards in Washington that demonstrated management failures. Only one showed reduced susceptibility, although another population will be screened again. The resistant population, WA6, was susceptible to the novel Madex XLV.

Obj 1. Mass crossings with WA6 are currently underway at Rutgers. We also have a field-collected susceptible population and will use that for comparisons of fitness between resistant and susceptible populations.

Objective 2a. Rutgers received overwintering larvae collected from 5 geographically distinct organic orchards in Washington State in late 2024. These populations are WA6, WA7, WA8, WA9, and WA10. They were kept in diapausing conditions until the late Spring when the process to break diapause and begin rearing codling moth for bioassays began. They were then reared under the same conditions as the laboratory susceptible colony, LabS, at 25°C and 16:8 light:dark. Colonies had to be reared for multiple generations (see below) prior to bioassays. WA6 was screened against the 5th generation, the sixth generation ofWA7 was bioassayed, and the first generation of WA10 were screened. Populations demonstrating resistance are reared under a lower rate of CpGV-M and CpGV-S to maintain virus exposure.

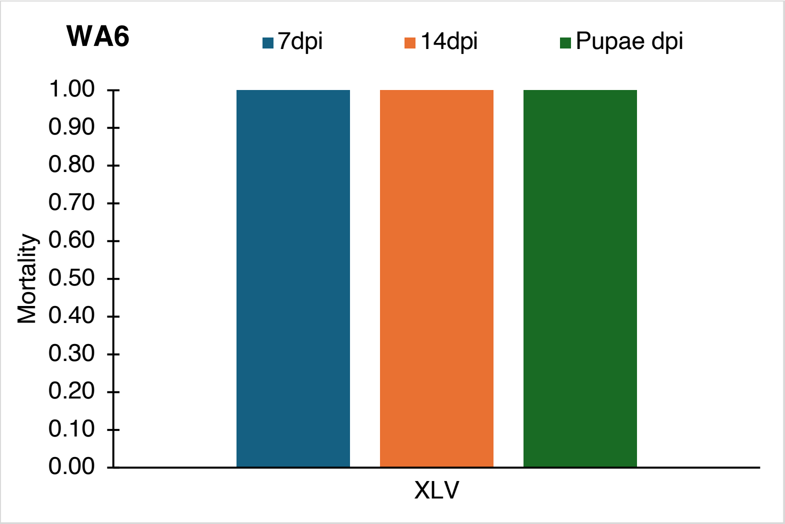
Objective 2b. We started assays in April, however there were significant issues with black mold and we lost two colonies, WA8 and WA9. These populations were collected again in 2025 and will be bioassayed after they are reared under laboratory conditions. The black mold issue has been resolved and bioassays began in early September 2025 with three replicates, each with 50 larvae for a total of ~150 larvae per population and treatment. Three replicates of an untreated control were also evaluated and data were corrected with Abbott's mortality factor. UTC data is not shown. Data was recorded at 7 dpi (days post infection), 14dpi, and pupae dpi. Of the three populations assayed against CpGV-M and CpGV-S, only one showed reduced susceptibility to the virus (Fig. 1). WA7 will be assayed again as it did show lower susceptibility at 93% 7dpi to CpGV-M and 235 for CpGV-S. We did see reduced susceptibility to CpGV-S, however after discussions with Andermatt Biologicals, we have new product will be evaluated in February 2026. It is also possible that high susceptibility occurred because it was the 6th generation without s=exposure to virus.

Figure 1. Results of laboratory bioassay screening of Codling moth colonies against CpGV-M (Cyd-X) and CpGV-S (Virosoft) in 2025.



Objective 2c. WA6 showed reduced susceptibility to the CpGV-M and CpGV-S (Cyd-X and Virosfot, respectively) and was screened against the novel resistance-breaking isolate Madex XLV. We saw 100% mortality at 7dpi indicting that this product is effective against resistant populations.

Figure 2.


WA6
7dpi
14dpi
Pupae dpi
Mortality
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
XLV

Project Title: Novel control of Codling Moth with RNA interference

Report Type: Continuing Project Report

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

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

OBJECTIVES

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

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

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

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

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

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

SIGNIFICANT FINDINGS

- 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.
- No RNAi phenotype observed with multiple genes when using multiple trigger sequences in attempt to knockdown genes with RNAi through feeding uptake via the piRNA pathway.

METHODS

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

Procedures: Whole transcriptome datasets will be generated and analyzed for several life stages of codling moth, including early and late embryo, early and late larval instars, pupae and adults. Lead-PI Walker has extensive experience with this approach in entomology research (Walker et al., 2016; Walker et al., 2019; 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 whole 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 synthetically, ordered from one of several commercial sources that supply dsRNA for RNAi experiments. dsRNA will also be generated corresponding to a jellyfish 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 jellyfish-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.

<u>Expected Results:</u> 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).

<u>Potential Problems and Contingencies:</u>

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., 2017). 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. If RNAi is proven to be functional in laboratory assays, 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, as well as hibernaculum-stage overwintering 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 chitin synthase genes, *chitin synthase A* (CHSA) and *chitin synthase B* (CHSB). CHSA 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. Additional targets have been identified for genes that are known in other species to be involved in sex determination.

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 2021). This approach has been pursued in collaboration with Dr. Alex Flynt (University of Mississippi) 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 jellyfish 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).

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, 2021).

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. Double stranded RNA sequences have been generated for IAP and CHSA as well as GFP (as a negative control), each with two different piRNA trigger sequences as leaders. dsRNA feeding experiments as described above with 500 ng/µL applied on top of larval diet and allowed to dry before placing larvae, or alternatively mixing in a lower dose of dsRNA directly into the larval diet. As of yet, no mortality effect has been observed for the target genes compared to negative controls.

Whether long dsRNA or the piRNA pathway have been used to induce RNAi, it has 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 (Allen and Walker, 2012) or midgut (Luo et al., 2013). In codling moth larvae, this may be tested for through injection of dsRNA effectors directly into the hemolymph of third to fifth instar larvae. Alternatively, embryonic injections targeting RNAi pathways with dsRNA molecules has also been reported to be efficacious in codling moth, with phenotypes subsequently observed in larval hatchlings (Pospíšilová et al., 2023).

As such, embryonic injection experiments have been initiated targeting CHSA and CHSB with long dsRNA molecules. Preliminary results indicate reduced neonate hatching and larval survival among larvae that do hatch, compared to saline and GFP injected controls. A full analysis of these experiments will be presented in the final report. Furthermore, additional injection and feeding experiments will be conducted during the final year with IAP and genes involved in sex determination and juvenile hormone pathways, which are respectively known to affect embryonic and larval development. If the injection experiments are determined to be fully successful in inducing an RNAi-mediated mortality phenotype, while the feeding experiments are not, it may indicate that larval feeding of neither long dsRNA nor piRNA trigger constructs are viable approaches to trigger the RNAi effect in codling moth larvae.

An additional approach that is being considered to overcome potential limitations related to either uptake of degradation of dsRNA delivered to codling moth via feeding involves the usage of nanoparticle materials to protect and deliver the dsRNA effector molecules to the cell. Such nanoparticles have been demonstrated to be effective for inducing RNAi in another lepidopteran species, the black cutworm moth, *Agrotis ypsilon* (Li et al., 2019). We have recently initiated discussions with Dr. Jinlong Han of Colorado State University, who is currently using this approach to induce RNAi in leafhoppers, to determine the best approach for incorporating nanoparticles into our RNAi experiments for temperate tree fruit insects including the codling moth.

In new trials conducted this year, similar mortality levels were observed for CHSA and GFP above levels observed for the control water treatment. Although it appears that mortality was slightly higher in larvae treated with CHSA dsRNA versus those treated with GFP dsRNA, high standard deviation values across these two treatment groups indicates statistical insignificance. These results suggest that non-specific effects of the dsRNA solutions themselves may be causing mortality as opposed to activation of RNAi specific pathways (Figure 1). More recently, however, research progress has been stalled due to an apparent infection of our codling moth research colony with the codling moth granulosis virus (CpGV). This infection is likely the result of cross-contamination from our ongoing CpGV research conducted elsewhere in our research facilities. Subsequently, the CpGV colony infection has resulted in high mortality in all RNAi assays, whether larvae are fed artificial diet treated with target gene dsRNA, control GFP dsRNA, or control water. Current efforts are underway to cure our colony through careful, focused individual insect rearing, and enhanced cleanliness protocols, across multiple generations to eliminate sick individuals and improve the overall health of the colony. We aim to resume experimentation in the coming months.

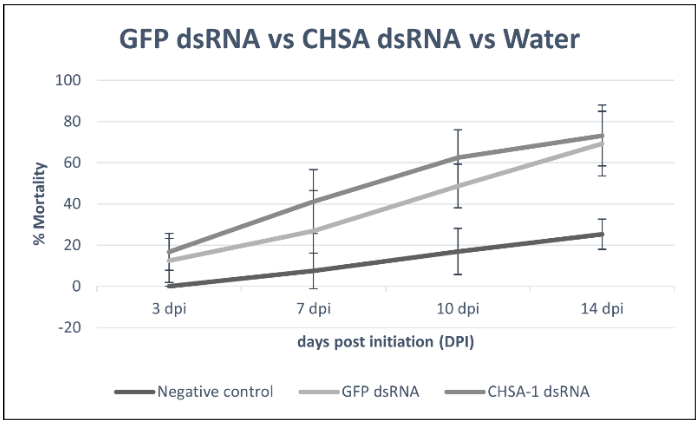

Figure 1 – Comparison of codling moth larvae exposed to artificial diet treated with water (negative control), GFP dsRNA (negative control), or CHSA-1 dsRNA (experimental treatment). For dsRNA treatments, 10 microliters of dsRNA at a concentration of 500 nanograms per microliter were overlaid on to artificial codling moth larval diet and after allowing the

solution to dry, a single neonate larvae was placed on top of the diet. For water treatment, four replicates were performed, for GFP dsRNA treatment, eight replicates were performed for CHSA-1 dsRNA treatment, fourteen replicates were performed; for all replicates, twenty individual codling moth larvae were tested. Error bars indicate standard deviation values. Larval mortality was assessed by assessment of larval motility.

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

Report Type: Final

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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
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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

A. 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.***

B. Objectives

Our project used a variety of data sources to build more effective tools to assess codling moth ecology in orchards. Our three complementary objectives were:

(1) Assess dynamics of codling moth populations across orchards with variation in management

(2) Improve predictive capacity of codling moth phenology models

(3) Conduct outreach related to codling moth population modeling

C. Progress on Objectives

(i) Summary. Our study focused on providing a better understanding of codling moth ecology to predict dynamics in orchards. To complete the project, we hired two postdoctoral scholars (Rob Curtiss and then Diego Rincon) who lead on research objectives with support for outreach and data science. Over the course of the project we were able to gather large field datasets on codling moth from commercial orchards through our own field sampling and through collaborations with producers and pest-control advisors. We used these data to validate and test phenology models and build new tools that assess codling moth flight. Our project can aid producers understand how phenology models can be validated with field trapping data.

(ii) Objective 1: Assess dynamics of codling moth populations across orchards with variation in management. From 2022 to 2025, we gathered data through our field work and by working with commercial producers and pest control advisors from the state of Washington and the OK-SIR program in British Columbia. The complete dataset is made of over 3 million records and was used to measure the population dynamics of codling moth across production orchards that vary considerably in management tactics. Using these data we assessed the lag that producers often see between predictions from the phenology model and moth captures in traps, and we then built a series of models to attempt to predict future codling moth abundance based on degree days and trap catch. In our approach, early trap catch data is input into a model and an estimate of the population dynamics is generated based on projections of phenology and past captures. We further tested models to estimate how codling moth flight dynamics might be impacted by weather variables; these models have been incorporated into the WSU Decision Aid System and are being used by producers and pest control advisors as part of their management tactics.

Field population dynamics assessments. 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 in Washington orchards. During 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 (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 and were then the focus of our project in 2023 to 2025 (see below).

Fig. 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 from 2023 to 2025. To supplement our field work conducted in 2022, in the past three years we hired a second postdoctoral scholar (Diego Rincon), who took over the project as a data scientist and Curtiss moved into a collaborator role as he took over other projects. Over these years we gathered more field data ourselves and by working with commercial producers in Washington and the OK-SIR program in British Columbia. Through these commercial partners we gathered a considerable amount of additional trap data from fields managed with variability in sterile insect releases and insecticides (Fig. 2). We were able to gather data from 287 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. This means that the phenology models is accurate, but that variability in sampling dynamics and population abundance affect the value of trap data in making population assessments based on real world data.

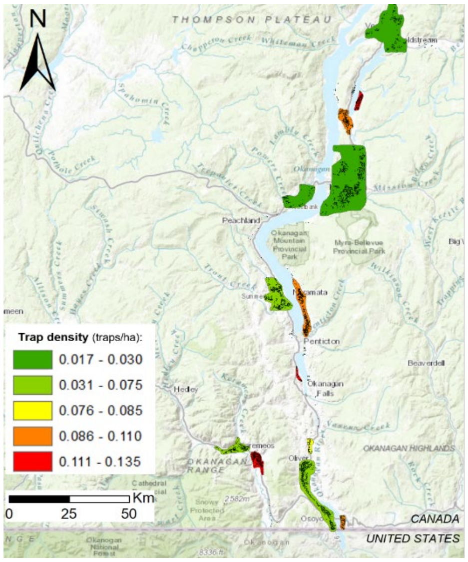

Fig. 3. Location of trap sites in British Columbia where data was collected to complement data from orchards we sampled in Washington State

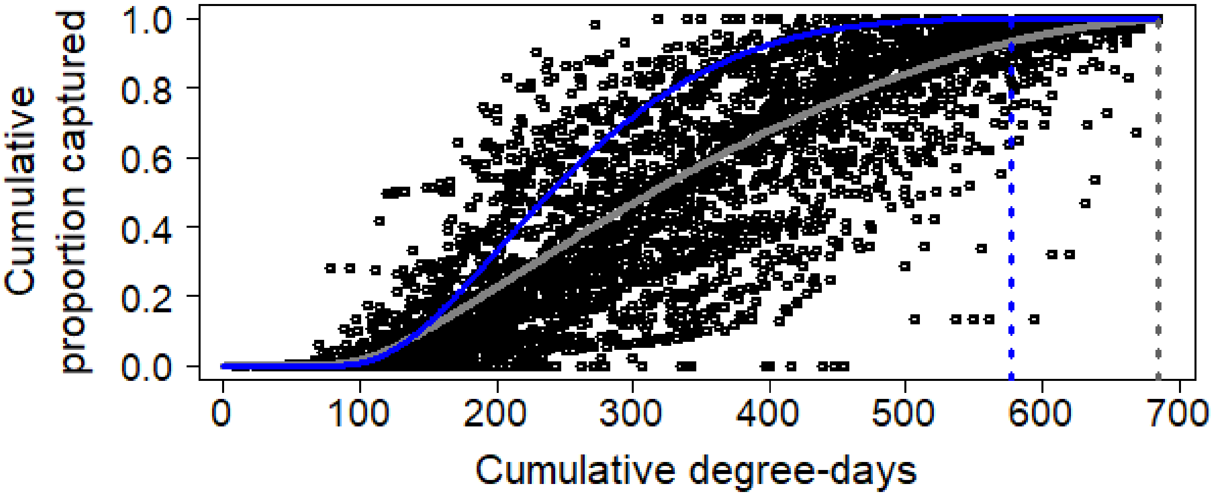

Figure 3. Data from over 280 sites showing trap catch of codling moth adults over the first generation. The blue line shows the phenology model used on the WSU DAS (Jones and Wiman 2012), and the gray line shows a population dynamics curve fit to the data. The difference between the lines shows the typical lag in when adults are captured in traps compared to when a phenology model predicts they are emerging.

Population modeling. In 2023 we brought on postdoctoral scholar Diego Rincon to work on the modeling parts of the project. 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. 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. 4). 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

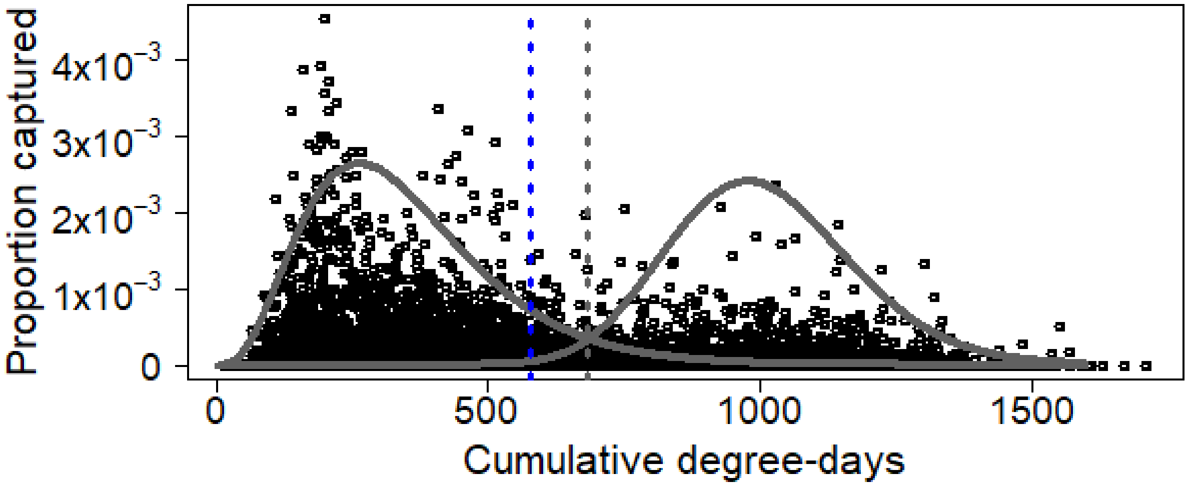

Fig. 4. Estimated proportion of codling moth caught in traps across the first and second adult generations

We build on our population dynamics analysis to 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 in Fig. 5). 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. 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.

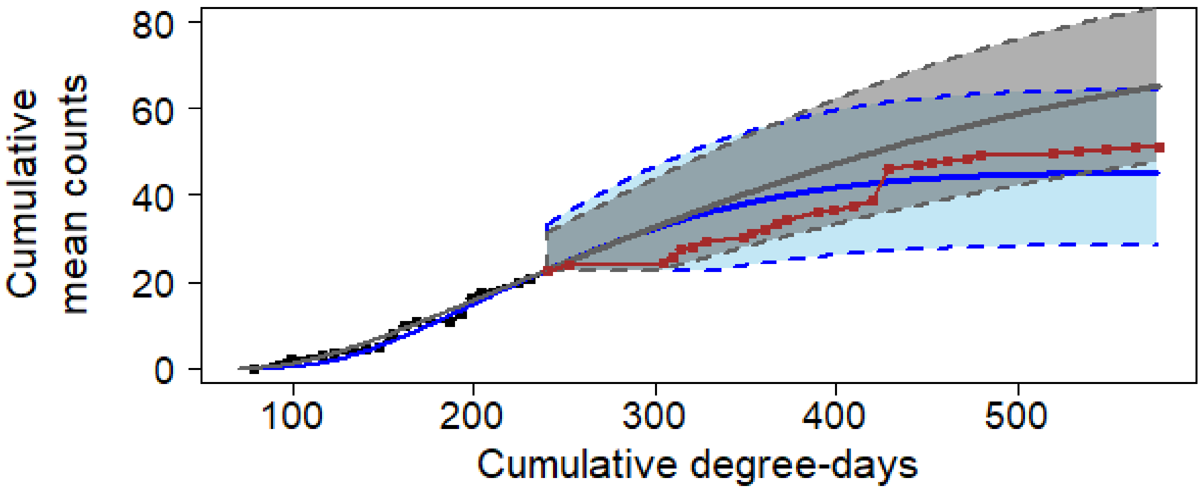

Fig 5. Example of our modeling approach to predict future codling moth abundance based on phenology and ongoing sampling. In this example, data is collected in traps up to 250 degree days (black squares), and a cumulative count of moths is made. At this point, both a phenology (blue line) or population 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 the model was robust for a wide range of sample sizes, codling moth densities, and management scenarios, with prediction accuracy for long-term outputs (prediction lengths >120 degree-days) ranging from 69 to 94% (Fig. 6). Overall, our approach suggests we may be able to predict future codling moth densities.

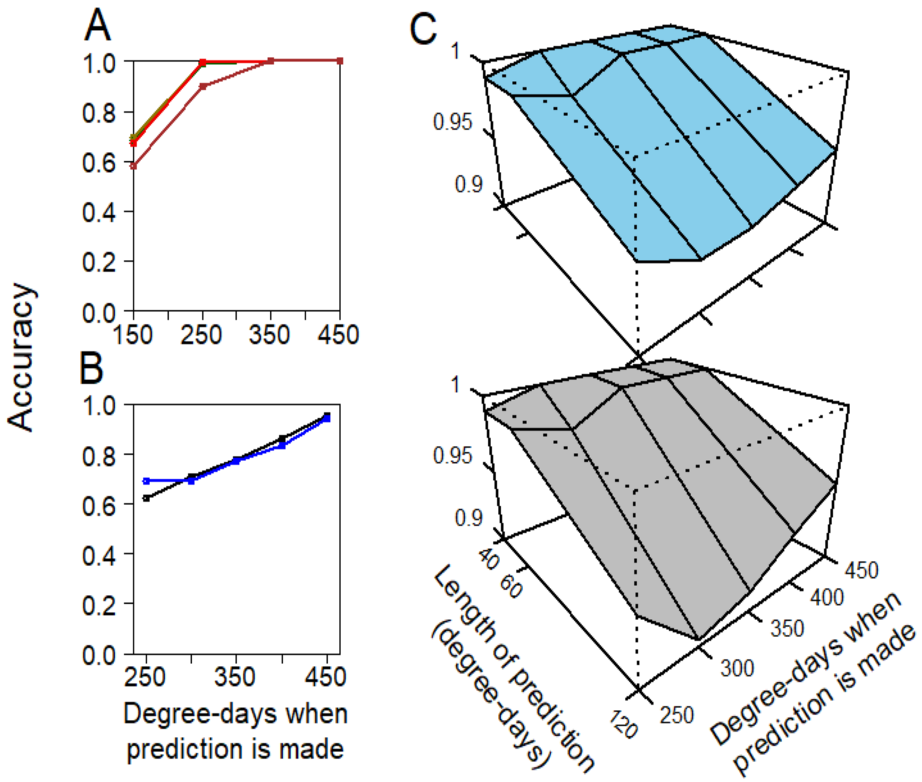

Fig. 6. In panels A and B, accuracy (the proportion of predictions that fall in a prediction band) of models used for codling moth. Model accuracy is shown as a function of degree-days when predictions are made; each line represents a sample size. In panel C, blue and grey surfaces represent the accuracy of predictions for two model types

We also gathered a dataset from seven orchards in WA with detailed information about trap location and density, with four years of weekly trap data, to further validate the model (Fig. 7). We found that the model closely resemble the data collected in Washington (Fig. 8). Also, after running a new validation analysis with the new data, we found our model maintains accuracy close to 80% (19% average error) when predictions are made at 350 degree days.

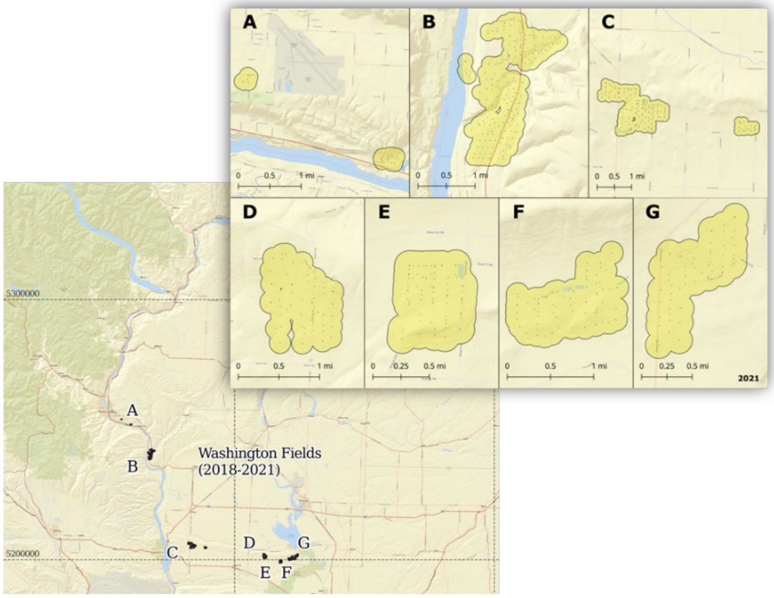

Fig. 7. Location of seven orchards from which new data was collected to further validate the codling moth forecasting models and evaluate the effect of trap density on moth capture efficacy and prediction. Sites varied both in configuration and the trap density. Data at each site was collected weekly for a four year period, and weather data from each site was also collected.

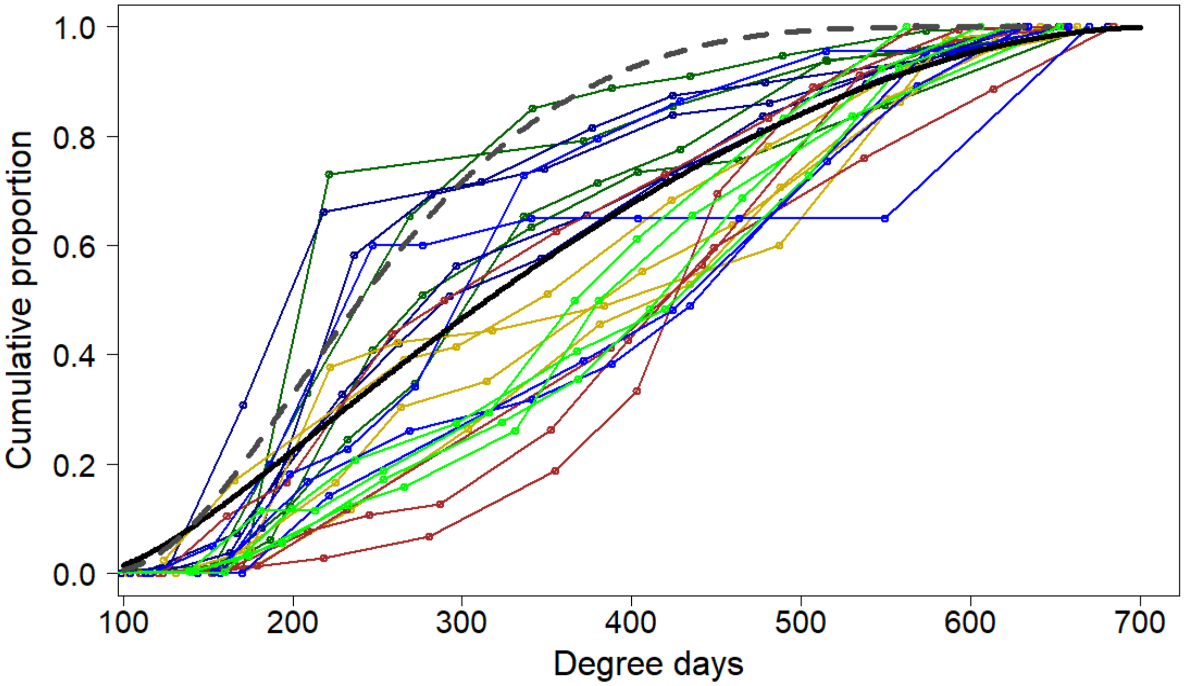

Fig. 8. Cumulative proportion of moth captures in pheromone traps for the first generation of the codling moth in seven WA orchards. Each broken line represents the average of captures in an orchard and year, and orchards are represented with different colors. The black solid curve represents the model fit to the OK-SIR dataset and the grey dashed curve is the phenology model used in WSU DAS.

(iii) Objective 2: Improve predictive capacity of codling moth phenology models

In the last two years we assessed how codling moth populations are affected by intensity of mating disruption and insecticide use (common programs in Table 2), and we built models to show when these treatments should affect population dynamics (Fig. 9).

Table 2. Treatment programs and expected efficacies used to assess the codling moth models

Treatment program	% survival per spray	% overall survival	No. of sprays	Timing (in degree-days)		
				First	Second	Subsequent
Conventional traditional	10, 10	35.5	2 larvicides	235	368 (14 days)	-
Delayed first cover	20, 10, 10	7.74	Oil and then 2 larvicides	210	290	423 (14 days)
Mating disruption †	-	75.49	-	-	-	-
Organic traditional ‡	30, 30, 30, 30	40.9	4 virus sprays	235	+7 days	+7 days

† The overall survival of mating disruption programs depends on temperature
‡ The organic traditional treatment program is deployed in addition to mating disruption

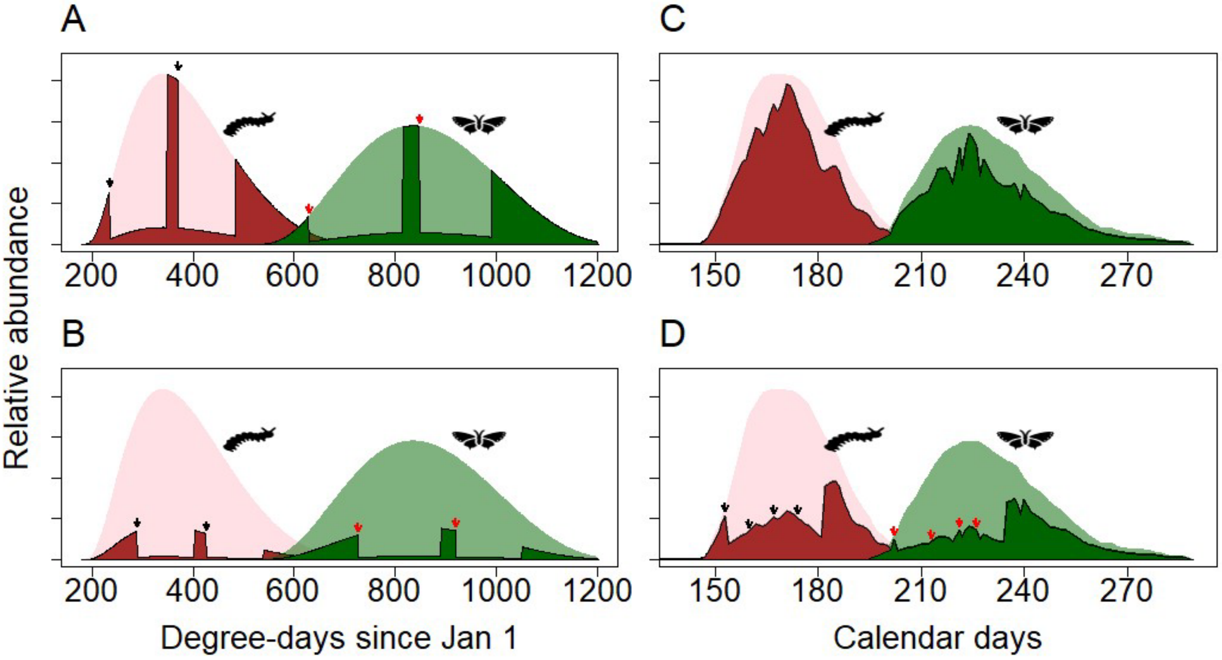

Fig. 9. Theoretical effect of four treatment programs on the codling moth larvae (brown for treated vs. pink for untreated) and adults (dark green for treated vs light green for untreated) for the first summer generation: (A) conventional traditional, (B) delayed first cover, (C) mating disruption, and (D) mating disruption plus organic traditional. The black arrows indicate the timing of insecticide treatments and the red arrows the corresponding start of the effect on adult emergence.

The models about the theoretical effect of insecticide applications and mating disruption on codling moth phenology were used to assess the effect of control treatments on codling moth populations. Results show that our model can distinguish pest populations that have been treated with control treatments that are >50% effective from pest populations treated with less effective controls. We also found that it is more challenging to detect efficacy of mating disruption programs compared with pesticide-based programs.

We built on our analysis by gathering weather data associated with all of our sites, and began to build and validate a model that assesses how codling moth flight varies based on temperature, wind, and rain. Trap captures may be delayed, even when moths have emerged, if the conditions in an orchard on a particular day are not suitable. The flight model has been built into the DAS and is being further validated based on data from our users (Fig. 10).

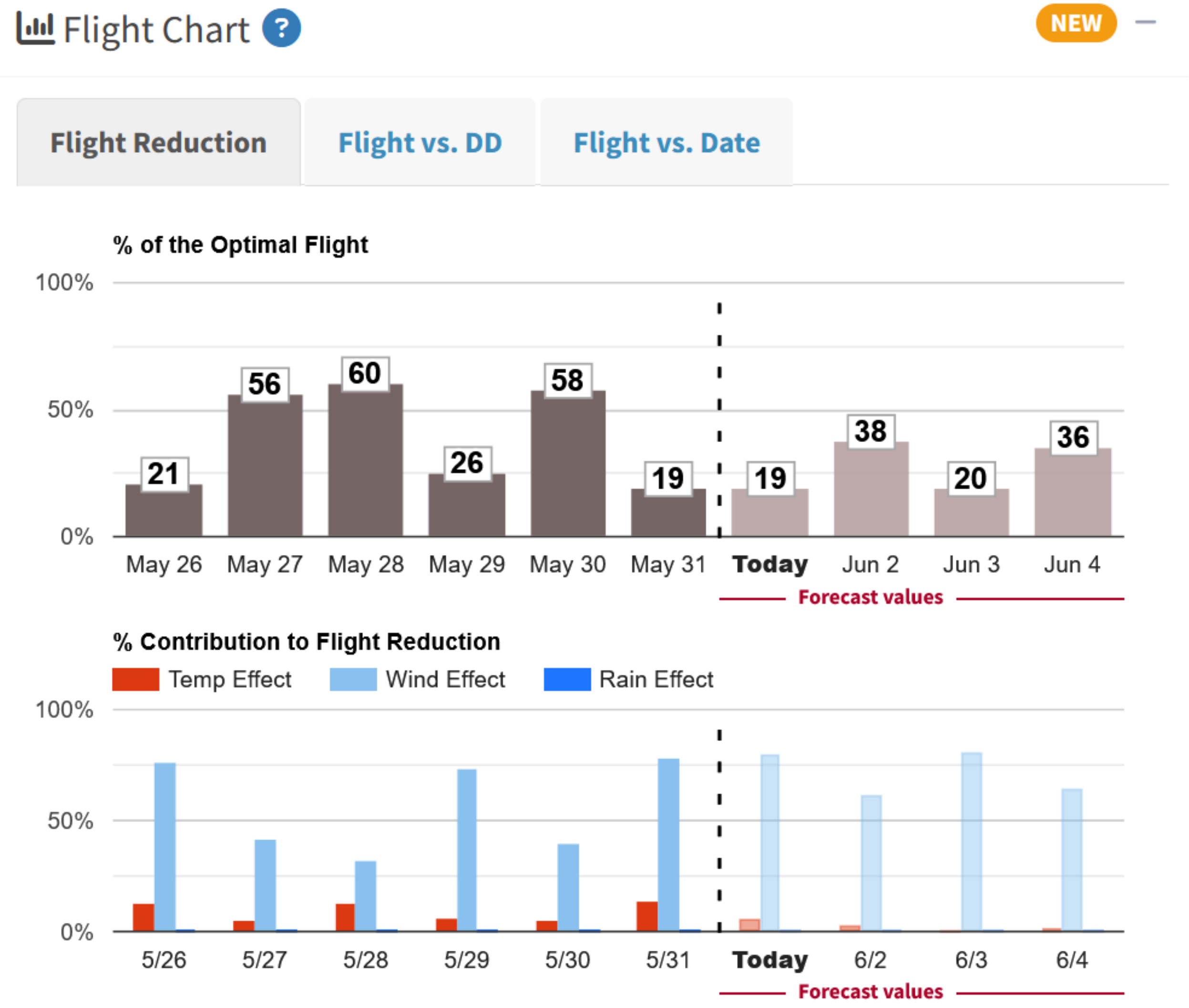

Fig. 10. Output of codling moth flight model shown on the WSU Decision Aid System, which uses site-specific data on temperature, wind, and rain to predict flight parameters.

(vi) Objective 3: Conduct outreach related to codling moth population modeling. Over the entire project we ran workshops, both in person and digital, primarily from January to April to talk about how codling moth models are run and interpreted. These workshops were attended by approximately 100 individuals in total, who spent 20+ hours with our team discussing codling moth models. Our team members also traveled regularly to production orchards in each year of the project to learn from pest control advisors and producers on their practices and how we can better build models to guide management. The flight model in fact came out of conversations we had with members on industry. We also gave talks at the meeting of the Entomological Society of America in 2024 and 2025.

Executive Summary

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

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

Abstract: This project was designed to provide information about how producers and pest control advisors can better 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 codling moth management, it is often unclear how growers should integrate trap catch data with models to make spray decisions. Our project provided data on the variability observed in codling moth populations across realistic Washington growing conditions, and showed how trap catch data may not always mirror predictions of phenology models. We showed that effective early season management using mating disruption, insecticides, or sterile insect releases may actually cause observed trap catch to lag considerably from what is predicted from models. We also found that weather variables like temperature, wind, and rainfall affect the flight conditions of codling moth, and we used these data to build better codling moth models. We integrated this information into the WSU Decision Aid System to provide better models that allow growers to conduct more responsive management that links real-time trap data with models.

Our work from 2022 to 2025 used data from field sampling in Washington orchards along with data from commercial orchards in Washington and British Columbia. 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) based on variable management scenarios. Our project supported two postdoctoral scholars, undergraduates, and permanent staff who conducted the field work, modeling, and outreach. Our team also continued to build a more informed user base for the digital tools built into the WSU Decision Aid System.

Proposal Title: DEVELOPING THE TOOLKIT FOR CODLING MOTH HOTSPOT MANAGEMENT

Report Type: Continuing Report

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Cooperators: Jeff Allen, Jenna Voelker (GS Long), Nathan Wash (Pheasant Orchards), Dani Gray (WSU ITT)

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $176,940
Total Project Request for Year 2 Funding: $161,030
Total Project Request for Year 3 Funding: $161,030

Other related/associated funding sources: Applied, not funded
Funding Duration: 2024 - 2027
Amount: $600,000 each
Agency Name: Washington Department of Agriculture Specialty Crop Block Grant in 2024 (Rejected, $250,000), Western SARE Grant in 2024 (Rejected, $350,000)
WTFRC Collaborative Costs: none

Budget 1
Primary PI: RT Curtiss
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Item	**2024**	**2025**	**2026**
Salaries	$68,490.00	$71,230.00	$74,079.00
Benefits	$29,279.00	$30,450.00	$31,669.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$13,835.00	$35,017.00	$30,696.00
Travel	$10,000.00	$10,000.00	$10,000.00
Plot Fees			
Miscellaneous			
Total	$121,604.00	$146,697.00	$146,444.00

Footnotes: Salaries for project technician (1@ 1 FTE), and postdoc (1 @ 0.3337 FTE); Benefits @ 38%; Supplies: computer, printer/software; lab/office supplies, electronics; video camera/accessories, sterile moths, traps and sticky bottoms, lures, cardboard bands, etc… Travel to plots, motor pool rental, fuel, per diem, other related travel.

Robert Orpet

Item	**2024**	**2025**	**2026**
Salaries	$8,713.00	$4,420.00	$4,597.00
Benefits	$3,713.00	$1,883.00	$1,959.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies			
Travel	$1,000.00		
Plot Fees			
Miscellaneous	$19,000.00	$2,000.00	$2,000.00
Total	$32,426.00	$8,303.00	$8,556.00

Footnotes: Salaries and Benefits: R.J. Orpet RAP yr 1: 0.1 FTE; yr 2-3: 0.05 FTE. Travel: to conduct in-person industry interviews. Miscellaneous: computer, printer/software; lab/office supplies, audio recorder; video camera/accessories hourly interview transcription costs, analysis costs.

S. Tianna DuPont

Item	2024	2025	2026
Salaries	$13,615.00	$1,127.00	$1,127.00
Benefits	$4,789.00	$397.00	$397.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$2,986.00	$2,986.00	$2,986.00
Travel	$1,520.00	$1,520.00	$1,520.00
Plot Fees			
Miscellaneous			
Total	$22,910.00	$6,030.00	$6,030.00

Footnotes: Salaries and Benefits: yr 1: 24.15%FTE postdoc (Staff time study circles: 2 days organizing/marketing, 1 day executing, 1 day follow up per event (2% time). Consult with 15 clients (doing spray calibration test, coverage test, spray evaluator to add to interviews conducted by Orpet) with codling moth challenges. Includes sprayer calibration, spray evaluator of previous year's applications, scouting of area for codling moth sources, additional research as necessary. Of 15 clients 4 are expected to provide good case studies to illustrate common challenges to clientele. (3 days per client, + 4 days case studies) (13% time).**; yr 2: 2%FTE postdoc (**Staff time study circles: 2 days organizing/marketing, 1 day executing, 1 day follow up per event (2% time)**. Travel: Travel to extension events and farm visits (**Speaker travel: 200 mi x $0.65 per mi x 2 events per year + lodging $120 x 2 events per year. Case study/consultation mileage 8 trips per year avg 150 mi x $0.65 per mi). **. Supplies:** Study circle supplies pear year: photo copies 360 x $1.7, folders 60 x $2, workshop supplies $400, speaker mike rental $50 x 2, video conference AV rental $100 x 2, participant dinner $26 x 60.

PROJECT OBJECTIVES:

1) Establish an advisory group and conduct industry interviews to better define hotspots' and problem blocks' current management needs.
2) Understand on-farm sources of hotspot infestations with on-farm damage assessments and a replicated mark-release-recapture/trapping/band study in vs. out of hotspots (moths collected in bands may be used in insecticide resistance assessments).
3) Develop new approaches to codling moth management, including conventional tactics that target late instar larvae, pupae, and adults, refinement and deployment of new organic control strategies, and variable SIT as a hotspot safety-valve treatment.
4) Establish SOPs and step by step how-to guide for managing hotspots and problem areas and provide Extension education in a series of study circles in fruit growing regions throughout the state each year.

SIGNIFICANT FINDINGS

Objective 1: Establish an advisory group, and conduct industry interviews to better define hotspots' and problem blocks' current management needs (Co-PI Orpet led objective, with contributions from Co-PIs DuPont and Northfield, and PI Curtiss)

- Interview questions were drafted in 2024
- The advisory group was engaged, and we met as a group once in 2025
- Although WSU Institutional review board approval of the interview questions was delayed, and this objective could not begin until Spring 2025, we conducted several interviews that will be analyzed in 2026.
- Approximately10 interviews were conducted by the end of 2025. Interviews followed a mixed-method design with simple quantitative and open-ended questions that were developed by the team in 2024 (and approved by the review board). Interviews documented how growers define hotspots, the extent of their problem, their opinions of reasons for the problems and what they have done to try to solve it.

Objective 2: Understand on-farm sources of hotspot infestations with on-farm damage assessments, a replicated mark-release-recapture/trapping/band study in vs. out of hotspots, fruit damage assessments, and insecticide resistance assays. (PI Curtiss and CoPI Northfield led objective)

- Years one and two of mark-release-recapture field experiments were conducted
- Years one and two of high-density trapping, banding, and fruit damage field studies were conducted
- Insecticide assays were conducted in spring 2025 as final instar caterpillars were extracted from overwintering bands

Objective 3: Develop new approaches to codling moth management including conventional tactics that target late instar larvae, pupae, and adults, refinement and deployment of new organic control strategies, and variable SIT as a hotspot safety-valve treatment. (PI Curtiss and CoPI Northfield led objective)

- In 2024 and 2025 we performed assays testing the effects of insecticides on final instar larvae and pupae.
- Few available insecticides were found to be acutely toxic to these stages
- Among those that did cause mortality, Malathion was the most toxic, but it was very slow to cause significant mortality

- Also, in 2024 and 2025, we tested a high-density tree banding treatment, but those findings are pending removal from cold storage and evaluation - results will be available in early 2026

Objective 4: Establish SOPs and step by step how-to guide for managing hotspots and problem areas and provide outreach education in a series of study circles in fruit growing regions throughout the state each year. (CoPI DuPont led objective with contributions from all other project team members)

- Two study circles were conducted in fall 2024 – 121 orchardists and consultants participated
- 58% of respondents reported learning a good or great deal from the event
- 98% of survey respondents plan to apply what they learned

- Three study circles were conducted in fall 2025 – 119 orchardists and consultants participated
- Study Circles were in Yakima and Tonasket Nov 20, Dec 2 and 3. Participants heard from speakers about new research including lures, hot spots and new biological insecticides, and the impacts of temperature and weather on codling moth outbreaks in 2025.
- The format allowed for in-depth discussion with WSU TFREC entomologists Northfield and Curtiss and other participants on key management considerations for 2026.
- 67% of participants surveyed said they learned a good or great deal (N=43).
- 97% of participants planned to apply their new knowledge (N=35).

- From interviews conducted, more intensive assessments were conducted at four of the sites. Assessments included sprayer application evaluation to determine coverage, spray record evaluation and assessment of mating disruption and trapping deployment.
- For example, through the interview and on-site evaluation at one site, outside sources appear to be the primary problem, while at another site spray timings appeared to be the primary problem. The team is working with these growers to help plan and deploy solutions in the following season. Additional follow-up is planned for remaining sites. From these assessments 2 case studies are in the works.

METHODS:

OBJECTIVE 1:

An advisory group of 3-5 representative apple farmers and consultants from throughout Washington will be established. The advisory group will include stakeholders from different farm scales, growing regions and economic gradients. This group will meet on an annual basis to help refine research and extension objectives and provide a feedback loop to improve application and extension. They will also provide knowledge on the potential causes of hotspots, as well as share strategies in identifying and managing the problem.

An interview-based study of apple orchard codling moth hotspot management will be conducted to define current problems, perceptions, and practices. All interview protocols will be reviewed by Washington State University's Institutional Review Board. The interview protocols are expected to be determined exempt from federal regulations on human-subject research due to low risk to participants. Interview questions and topics will be developed by all members of the project team in consultation with the to-be-established 3–5-member farmer/consultant advisory group.

First, a sample population of apple growers and consultants will be identified for the study. An article written for WSU's Fruit Matters Newsletter will be shared in Spring 2024 to describe our project and invite stakeholders with codling moth hotspot concerns to e-mail the project director if they are interested in being interviewed and having research conducted on their farms. Other networking by the project team will be used to identify more interviewees as needed to represent geographic and operational diversity of the industry. The project director will identify at least fifteen growers or consultants to interview from the resulting respondents for the study.

Following consultation with the advisory group, interviews will occur during summer–winter of 2024 and follow a mixed-method design, meaning they will have a combination of simple quantitative questions and open-ended qualitative questions. Interviews will be designed to document the extent of the codling moth problems experienced by the interviewee, their opinions on the reason(s) for the problem, what they have done to try and solve it, and what if anything they plan to try next. Interviews will last about an hour and will be recorded, transcribed, and analyzed for common themes across individuals. Interviewee's identities will not be linked with public presentations of data, and transcriptions will not be shared with anyone other than investigators on this proposal. In addition to this analysis, the results will also help the project team identify research locations and methods for objectives 2 and 3 of this proposal.

At least ten interviewees will receive follow-up farm visits conducted by this project's combined interview/research/extension team. Interviews will be led by CoPI Orpet, while CoPI DuPont will compliment interviews with evaluation of spray coverage, calibration, and previous years' application using the "WSU Spray Record Evaluator."

Interview questions will be developed in Spring-summer 2024 in consultation with the advisory group and Washington State University's Institutional Review Board. Interview findings from those conducted in summer-winter 2024 will be collated by Spring of 2025 and prepared for publication/distribution if appropriate. A limitation of these methods is the time needed to conduct the interviews, transcriptions, and analysis; relatively few interviews can be conducted, but those interviews will contain high quality information with more detail than surveys can gather.

OBJECTIVE 2:

Following identification of hotspot areas on commercial farms, we will conduct a series of controlled and replicated participatory research experiments to identify the sources of the infestations. Codling moth hotspots on commercial farms may be either a sink or a source of infestations, and they are not exclusive to any one management strategy. A replicated mark-release-recapture study will clarify codling moth movement into and out of hotspots. That study, coupled with high-density trapping and banding studies will contribute to a greater understanding of how hotspots develop. In addition, insecticide resistance may be contributing to increases in codling moth populations in hotspots, so screening of moths collected from hotspots needs to be conducted with select products.

MARK-RELEASE-RECAPTURE FIELD EXPERIMENTS:

Using replicated color-marked sterile codling moth releases and recapture in CMDA+AA baited orange delta traps, we will study movement of adult male and female codling moths into and out of hotspots. Hotspots identified from interviews will be used in this experiment. We will use three experimental release patterns (Fig. 1, see proposal). Each release location will receive approximately 800 sterile moths. The three designs combined will inform moth dispersal into and out of hotspots. The first design, pattern 1 in Fig. 1 (see proposal), replicates conditions where moth populations are uniform throughout a block and the hotspot and will feature interactions between moths marked in orange and blue. Release designs 2 and 3 represent conditions with uneven population densities in the blocks and hotspots. Pattern 2 represents a hotspot acting as a sink, while pattern 3 represents the hotspot acting as a source. Spatial analysis of moth captures in baited traps will be used to elucidate moth dispersal patterns.

HIGH-DENSITY TRAPPING, BANDING, AND FRUIT DAMAGE FIELD STUDIES:

Using hotspots identified from interviews, we will perform replicated high density trapping and cardboard banding experiments to understand the movement and dispersion of wild codling moths into and out of hotspots. Traps and/or bands will be placed in trees along transects that begin outside of the hotspot, traverse the hotspot, and terminate again outside of it (Fig. 2, see proposal). Numbers of transects, and density of traps/bands will depend on the size of the hotspot. For comparison, control orchard blocks will be used that do not have a hotspot, will be in the same region or on the same farm as hotspot blocks, will have similar management and conditions, and will be selected to receive transects of traps/bands. Finally, we will perform damage assessments comparing damage levels within and outside of hotspots. Damage assessments will analyze 600 fruit per block (300 on the exterior of the block and 300 on the interior) (Fig. 2, see proposal). Trees receiving fruit damage assessment will be selected randomly and will have 20 fruit assessed. Spatial analysis of moth dispersal within trap areas will further identify how codling moths enter or leave hotspots, and if hotspots are self-perpetuating.

INSECTICIDE RESISTANCE ASSAYS:

Using individuals collected from hotspots in cardboard band studies and apples, and the WSU codling moth colony will be used to test for resistance to several commonly used insecticides in Washington conventional and organic apple production. Targeting those insecticides moths from hotspots are commonly exposed to, we plan to test as many of the following 13 insecticides commonly used in commercial apple production as will be possible with the moths collected from field sites: Chlorantraniliprole (i.e., Altacor), Acetamiprid (i.e., Assail), CM granulosis virus (Cyd-X), Spinetoram (i.e., Delegate), Spinosad (i.e., Entrust), Pyriproxyfen (i.e., Esteem), Cyantraniliprole (i.e., Exirel), Phosmet (i.e., Imidan), Methoxyfenozide (i.e., Intrepid), Novaluron (i.e., Rimon), Carbaryl (i.e., Sevin), λ-Cyhalothrin (i.e., Warrior II), and Oil. Additional assays if enough moths are available will explore levels of resistance to other products such as Madex, Carpovirisine, Virosoft, Rango, and Spear-Lep, as well as comparing susceptibility to Washington functionally naïve populations sourced from abandoned and unsprayed orchards, and the impact of residue aging on insecticide resistance.

DIET INCORPORATION ASSAYS: We will utilize the IRAC Susceptibility Test Methods (Method no. 20, Version 3.2) for incorporating insecticides into artificial diet at several concentrations to measure larval resistance in codling moth populations. Commercial grade insecticides will be dissolved in water at their maximum allowable field concentrations to create stock solutions. Five serial dilutions of each insecticide will then be made to create more dilute concentrations. Insecticide dilutions will be incorporated into artificial diet for larval feeding assays to expose larvae to several concentrations of insecticides. Test larvae, from each region-specific colony, will be placed individually on insecticide impregnated artificial diet and allowed to feed for at least seven days. Control larvae will be placed on artificial diet without insecticide. Mortality of test individuals will be evaluated daily. Assays will include 10 individual larvae per replicate and will be replicated at least three times. Thus, at least 30 larvae from each hotspot and naive colony will be used to test each insecticide concentration and control.

DIRECT APPLICATION ASSAYS: For direct application assays (eggs, larvae, and adults), technical grade insecticides will be dissolved in solvent to create stock solutions with a 1000 mg L−1 concentration, and five serial dilutions (50:50) will be made to create more dilute concentrations. Control moths from each colony will be exposed to solvent alone. Adults and larvae will be exposed by directly applying a 1.0 µL drop to their ventral thorax. Eggs, sprayed with a Potter Spray Tower or similar device, will be monitored daily for seven days, and the number hatching will be recorded. Dosed larvae will be placed on artificial diet and their survival monitored daily for seven to ten days. Exposed adults will be placed individually in observation arenas, given a honey water-soaked cotton wick, and observed daily for seven days for survival. As in the previous experiment, assays will include 10 individuals (eggs, larvae, or adults) per replicate and will be replicated at least three times; 30 eggs, larvae, or adults from each hotspot tested and naive colony will be used to test each insecticide concentration and control.

ANALYSIS: Percent mortality in assays at each dose will be corrected with Abbott's formula (Abbot, 1925), and 95% confidence limits based on the binomial distribution for percentages will be estimated. Data will be analyzed to determine lethal concentration values (LC50 and LC90) as compared to the naïve laboratory colony. The naïve colony, for comparison purposes will be assigned a ratio of 1.0. The significance of differences in LD50 values among population responses to each insecticide concentration will be calculated using a lethal concentration significance test (i.e., Robertson et al., 2007). The lethal concentration (LC50 and LC90) values of the region-specific populations will be considered significantly different from those of the naïve population if the 95% confidence limits of their corresponding lethal concentration ratios do not include a value of 1.0 (α= 0.05).

OBJECTIVE 3:

HIGH-RATE STERILE INSECT TECHNIQUE EXPERIMENT:

On farms identified in objective 1, using a paired plot design replicated three times per treatment, with farmer participation on their farms, we will select at least three hotspot/non-hotspot blocks to receive high-density sterile insect releases (i.e., 3200 sterile moths/ac), no sterile moths (negative control), or the standard release density of 800 sterile moths/ac; positive control plots will be areas not considered by the farmer to be hotspots (Fig. 4, see proposal). Treatment impacts will be measured by performing fruit damage assessments like those described in objective 2. These methods may be modified based on findings in previous experiments, i.e., testing different strategies for different scenarios.

HIGH-DENSITY MATING DISRUPTION EXPERIMENT:

Another paired plot will be used to measure the impact of high-density mating disruption dispensers on hotspot infestation levels. Like Fig. 4 (see proposal), hotspot plots will be treated with passive dispensers at the currently recommended rate of 300-400/ac as the positive control, and the test treatment will be at densities of 500/ac and 700/ac. Mid- and end-of-season fruit damage assessments will be used to evaluate program effectiveness (e.g., Fig. 3, see proposal). These treatments will each be replicated at least 3 times, and participating farmers will be asked to contribute to the costs of mating disruption dispensers.

HIGH-DENSITY TREE BANDING EXPERIMENT:

Again, this experiment will utilize paired plots to measure the impacts of several tree banding densities on fruit damage and captures in traps the season after banding occurs. As in Fig. 4 (see proposal), treatments will include, no banding, 1 band every third tree, 1 band per tree, and 2 bands per tree deployed in orchards with and without hotspots. Bands will be deployed in orchard plots by the late summer in 2024 and collected that winter and number of larvae captured will be recorded. In 2025, at least 1 trap/2.5 acres will be used to monitor populations in banded plots, and mid-and end-of-season fruit damage assessments will be used to measure treatment impacts. In late summer 2025, bands will again be deployed into the same plots at the same densities and collected that winter for comparison with 2024 captures. In 2026, trapping and fruit damage assessment will once again occur in treated plots.

TOXIC TREE BANDS EXPERIMENT:

In a laboratory experiment, we will investigate toxicants that can be applied to tree bands for effectiveness at causing mortality to final instar larvae and pupae. Much like the insecticide treated netting used to capture and kill brown marmorated stinkbug, pyrethroids (with and without piperonyl butoxide), may be promising compounds to impregnate bands and kill codling moth larvae while they search for pupation sites. Additionally, there may be other compounds currently approved for tree fruit that can be applied to bands once they've been deployed to minimize survival before their removal. We will also test impregnating bands with biological compounds for use in organic farms.

NEW INSECTICIDE TRIALS:

Using the WSU Washington representative codling moth colony, we will test new, and rarely used insecticides' efficacies. We will employ two test methods that are well-established and accepted by the scientific community to measure efficacy against first and last instar larvae and pupae , 1) a larval diet incorporation study, and 2) direct contact assays. We will pursue cost share with insecticide companies.

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 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 ten days.

OBJECTIVE 4:

SOPs AND HOW-TO-GUIDE:

Using research findings from this project, a Standard Operating Procedures factsheet (available at treefruit.wsu.edu and pushed out through Fruit Matters newsletter to approx. 2,240 subscribers) will be developed to help growers identify and remediate hotspots on commercial farms. The guide is expected to include procedures growers can use to identify potential hot spot causes and standard operating procedures for hotspot treatments.

STUDY CIRCLES:

Study Circle discussion groups will be hosted in North-central Washington (e.g., Omak), Central Washington (e.g., Quincy), and/or South-central Washington (e.g., Yakima) and available via zoom to allow geographically dispersed participants to access in-depth information. At least 2 study circles will be conducted each year (2024, 2025 and 2026) to discuss project findings, suggestions for managing hotspots, and discussing farmer experiences. Each study circle meeting will include a 30 min presentation by members of the research team and 2 hours of facilitated discussion. A meal break provides informal networking time. In addition, extension activities will occur throughout the project. Ideally, interview participants, and those farmers adopting hotspot treatment suggestions will participate in the study circles, if they do not, the study circle format will be slightly less effective for all participants.

CONSULTATIONS/ CASE STUDIES:

Interviews of 10-15 growers with hotspots (objective 1) will be complimented with sprayer calibration, spray evaluator of previous year's applications, and scouting of the surrounding area for codling moth sources in order to give participating growers well-rounded diagnostics of potential codling moth management limitations. Of 10-15 growers participating in interviews/evaluations 4 are expected to provide good case studies to illustrate common challenges. Case studies will be elaborated with photographs and data to provide educators with concrete examples to share during winter presentations and as newsletter information. Case studies are effective as they enable adult learners to examine real-world situations, applying their own experiences with acquired knowledge, to determine pragmatic solutions (Baugher et al. 2017). As such individualized attention to a relatively small group will leverage outreach to 600 participants in winter presentations and 2,240 newsletter subscribers.

RESULTS AND DISCUSSION

As this report is only from the first two years of this project, results from many studies are incomplete. Several of the studies were conducted into the late fall and samples are still being processed as of the writing of this report.

OBJECTIVE 1:

In 2024 we drafted an interview script and eventually received approval from the WSU Institutional Review Board to conduct interviews. The approval was delayed, preventing us from beginning interviews during a time subjects were available. This part of this objective began to be conducted in 2025. Approved interview script follows:

1. What is your role in the tree fruit industry?
    a. Job title
    b. Number of acres of what crops
        i. What geographic area(s)?
    c. Conventional, organic, or a mixture?
2. What is your role in codling moth pest management decision making?

What is the codling moth problem?

3. Tell us about how important a pest codling moth is to your farm.
4. Do you have codling moth “hotspots”?
5. How do you define a hotspot? What is a hotspot?

Why do you think problems are happening?

6. What do you think causes your hotspot(s)? [we will go over a list of follow-ups we are specifically interested in if any of the following are not brought up]
    a. insecticide resistance
    b. resistance to mating disruption
    c. external sources of moth flight
    d. others?
    e. Is that in organic, conventional, or both?

What are you currently doing about the problems?

7. How do you manage codling moth in your hotspot(s)? Tell us about organic management first if you grow organic and then conventional.
    a. Follow-ups: do you use ____ on none, some, or all apple acreage? Please answer separately for Conventional & Organic. Tell us if you find it effective or not and what challenges are associated with each tactic.
        i. Mating disruption. What type of dispenser and rate?
        ii. Codling moth degree-day model? What is the source of your degree-days?
        iii. What pesticides do you use for coding moth?
        iv. Banding for codling moth removal?
        v. Removing all fruit at the end the season?
        vi. Proximity to bin piles?
        vii. Search for rogue trees and proximity to other sources?
8. Have you talked to other people for advice on your hotspot? Who? What did they say?
9. What resources have you used to get information about managing a hotspot?

Basic Demographics

Year born, education level

Spray records

Could you provide a spray record for your hotspot(s) areas for the last several years? Can the record include when mating disruption was deployed and what rate?

OBJECTIVE 2:

Years one and two of mark-release-recapture field experiments and high-density trapping (Fig. 1), banding, and fruit damage field studies were completed. However, as of the writing of this report, data are still being processed. Reporting of an incomplete data set at this time would be premature and potentially misleading. In addition, many bands must be evaluated from the banding experiment.

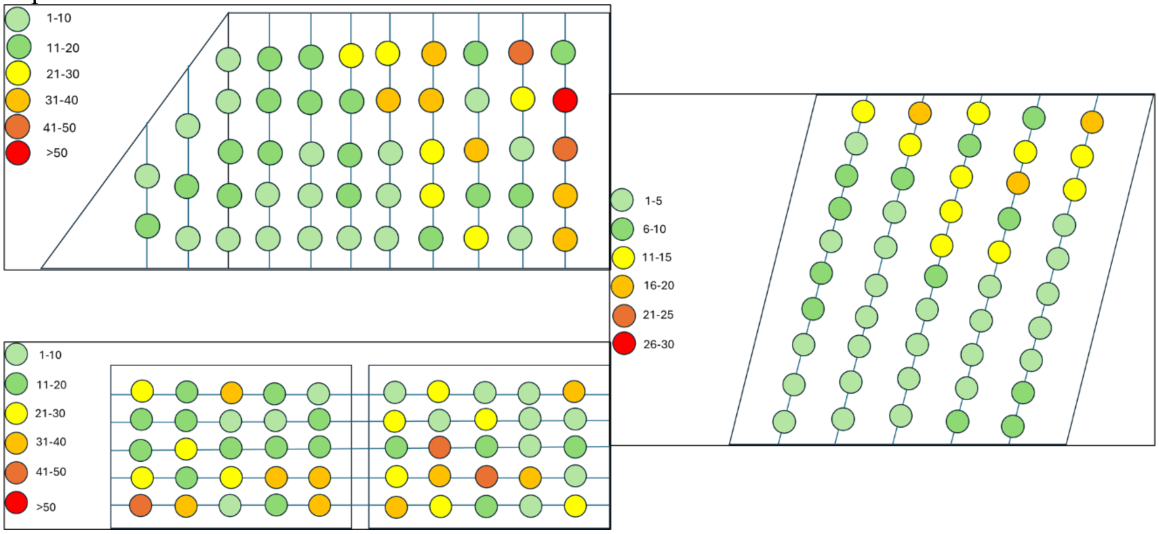

Figure 1. *Example capture patterns in three orchards as revealed by high density trapping.*

Insecticide assays were conducted in spring 2025 from final instar caterpillars that were extracted from overwintering bands.

OBJECTIVE 3:

As reported above in the bullet point summary, in 2024-25 we performed assays testing the effects of insecticides on final instar larvae and pupae but found few of the tested insecticides to be acutely toxic to these stages. Although not surprising, our positive control, Malathion was the most toxic, but it was very slow to cause significant mortality. Typical bioassays of this type evaluate impacts after 1-3 days, but in our studies, we found low mortality from any tested active ingredient at that timing. Further study is needed to test new active ingredients impacts on these stages, and if more mortality, or significant sub-lethal effects can be induced.

As with other field experiments from 2024-25 reported herein, the high-density tree banding tactic we tested does not yet have reportable results. Those data are pending removal of bands from cold storage and evaluation - results will be available in early 2026. Bands are being stored so the codling moth larvae in them can be used in other experiments included in this project in 2026.

OBJECTIVE 4:

On December 3 and 4, 2024 CoPI DuPont and Dani Gray (added to this project in 2024 as a cooperator) hosted Codling Moth Study Circles for 121 participants in Yakima and Omak (Fig. 1). Orchardists, researchers and consultants shared their knowledge and experience managing codling moth. The study circles each consisted of a 30-minute presentation by PI Curtiss followed by 1.5 hours of discussion broadly about managing codling moth in hotspots. More than half of participants (58%, N=59) reporting learning a good or great deal from the event including: importance of trap number and placement, lure effect on trap counts, potential of drapenet to exclude codling moth, sterile insect release information, and information on lures. A high proportion of attendees, 98% of survey respondents, planned to apply what they learned (N=43) including adding mating disruption, adapting SIR use, moving trap placement / adding density, experiment with netting, and reconsidering lures used.

One hundred and nineteen industry professionals participated in Codling Moth Study Circles in Yakima and Tonasket Nov 20, Dec 2 and 3, 2025. Participants heard from PI Curtiss about new research including lures, hot spots and new biological insecticides and other WSU entomologists about the impacts of temperature and weather on codling moth outbreaks in 2025. The study circle format encouraged in-depth discussions between WSU TFREC entomologists and other participants on key management considerations upcoming for 2026. Sixty-seven percent of participants surveyed said they learned a good or great deal. Participants learned about the 'importance of first-generation timing', 'codling moth spray timings,' and 'trap catch values and how they relate to population totals'. Ninety-seven percent of growers/consultants, and industry participants reported that they plan to apply their new knowledge (N=35). They plan to 'add more traps [per acre] with different lures,' 'make sure growers rotate [virus] by generation', and they 'may use BT for codling moth'.

Additional Extension activities include:

- Codling Moth topics organized by Dani Gray as part of Tree Fruit Association Annual Meeting including presentations by Rob Curtiss and Tobin Northfield (approx. 170 participants).
- Codling Moth session organized by Tianna DuPont at North Central Washington Apple Day Jan 23, 2025 including presentations: Codling Moth Management Reminders, Dani Gray, WSU Extension; Avoiding Resistance in Organic Codling Moth Management, Tobin Northfield, WSU Entomology (anticipated 200 participants).
- Codling Moth session organized by Tianna DuPont at Okanogan Horticultural Society Annual Meeting February 6, 2025 including presentations: Codling Moth Management Reminders, Dani Gray, WSU Extension; Avoiding Resistance in Organic Codling Moth Management, Tobin Northfield, WSU Entomology (anticipated 100 participants).
- Codling Moth Reminders presentation Jan 15, GS Long annual meeting, Spanish. Presenter Tianna DuPont, WSU Extension. (anticipated 500 participants)

Figure 2. December 3, 2025 Study Circle in Tonasket, WA. One hundred and nineteen industry professionals participated in Codling Moth Study Circles in Yakima and Tonasket Nov 20, Dec 2 and 3, 2025. Participants heard from PI Curtiss about new research including lures, hot spots and new biological insecticides and other WSU entomologists about the impacts of temperature and weather on codling moth outbreaks in 2025.

Project Title: Resistance management for codling moth in organic pome fruit

Report Type: Continuing Project Report

Primary PI: **Tobin D. Northfield**
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Cooperators: R.T. Curtiss, William Walker

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 70,283
Total Project Request for Year 2 Funding: $ 149,730
Total Project Request for Year 3 Funding: $ 35,413

Other related/associated funding sources: Funded
Funding Duration: 2025 - 2028
Amount: $349,763
Agency Name: Western Sustainable Agricultural Research and Education
Notes:

Budget 1
Primary PI: Tobin Northfield
Organization Name: Washington State University, Tree Fruit Research and Extension Center

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Station Manager/Supervisor: Lee Kalcsits
Station manager/supervisor email address: lee.kalcsits@wsu.edu

Item	2025	2026	2027
Salaries[1]	$7,800.00	$8,112.00	
Benefits[2]	$2,744.00	$2,853.00	
Wages[3]	$19,200.00	$19,968.00	
Benefits[4]	$1,933.00	$2,011.00	
RCA Room Rental			
Shipping			
Supplies	$2,000.00	$2,000.00	$2,000.00
Travel	$2,500.00	$3,000.00	$500.00
Plot Fees			
Miscellaneous			
Total	$36,177.00	$37,944.00	$2,500.00

Footnotes:

[1] Laboratory manager at 10% full time

[2] Benefit rate for laboratory manager at 35.2%

[3] Two hourly employees full time for 3 months

[4] Benefit rate for hourly employees at 10.1%

(*Complete the following budget tables if funding is split between organizations,* ***otherwise delete*** *extra tables.)*

Budget 2
Co PI 2: Anne Nielsen
Organization Name: Rutgers University
Contract Administrator: Chrissa Papaioannou
Telephone: (848) 932-4002
Contract administrator email address: cp847@research.rutgers
Station Manager/Supervisor: Daniel Ward
Station manager/supervisor email address: danward@njaes.rutgers.edu

Item	**2025**	**2026**	**2027**
Salaries [1]	$12,800.00	$66,240.00	$17,139.00
Benefits [2]	$5,806.00	$30,046.00	$7,774.00
Wages [3]	$8,000.00	$8,000.00	$8,000.00
Benefits [4]	$0.00	$0.00	$0.00
RCA Room Rental			
Shipping			
Supplies	$5,000.00	$5,000.00	
Travel	$1,000.00	$2,500.00	
Plot Fees			
Miscellaneous [5]	$1,500.00		
Total	$34,106.00	$111,786.00	$32,913.00

Footnotes:

[1] Post-doctoral researcher at 20%, 100%, and 25% full-time in Years 1, 2, and 3 respectively with a 3.5% annual increase to conduct bioassays and rear codling moth

[2] Benefit rate (non-federal) for post-doctoral researcher at 45.36%

[3] One hourly student employee for 20h/week at $16/h for 25 weeks to assist with colony maintenance and data collection

[4] Rutgers student employees have 0% benefits rate

[5] Publication fees for publication of project results in a peer-reviewed journal

Objectives

1) Evaluate codling moth resistance in organic blocks.

2) Evaluate resistance spillover in conventional blocks near organic blocks.

Note: in widespread discussions, growers and consultants have said conventional blocks near organic blocks do not seem to have problems, suggesting little spillover. Indeed, one grower has had success surrounding organic blocks with conventionally managed blocks, and at the codling moth taskforce meeting described this as an important part of resistance management.

3) Develop and disseminate a resistance management plan to slow the spread of virus resistance.

Significant Findings

- With grower cooperators, we collected bands from six sites in Washington: 3 in the Tri-Cities region from 3 different growers, 1 in the Quincy region, 1 in Tonasket, and 1 in the Royal City region.
- We are sorting and overwintering 5 sites in preparation for testing.
- The sixth site, in Royal City had insufficient numbers for testing from 1,000 bands, suggesting that resistance is not present at that site.

Methods

Resistance testing. Cardboard bands were wrapped around tree trunks in organic blocks with concerns of resistance, either in hotspots or across the block. The date on which they were deployed depended on location and grower preference, but occurred from August through September. Bands were either deployed by WSU or by grower cooperators, depending on grower capacity. The number of bands ranged from 500 to 3,000 per site, and either a single block or multiple nearby blocks managed by the same grower with the same virus program was focused on for each site. Bands were collected at the end of October to early November, and brought back to WSU Wenatchee TFREC to be placed in a cold growth room. They are currently being sorted to remove codling moth larvae. Sorted larvae will spend 3 months in cold temperatures before rearing separately by population and tested with bioassays at Rutgers University, following Fan et al. (2022) and Asser-Kaiser (2007).

With grower cooperators, we collected bands from six sites in Washington: 3 in the Tri-Cities region from 3 different growers, 1 in the Quincy region, 1 in Tonasket, and 1 in the Royal City region. We are sorting and overwintering 5 sites in preparation for testing. The sixth site, in Royal City had insufficient numbers for testing from 1,000 bands, suggesting that resistance is not present at that site. Sites were selected based on likelihood of resistance, based on discussions with the grower and/or consultant, and to improve geographic coverage.

Resistance management plan. The codling moth taskforce met on 27 February 2025 and 18 November 2025, and in each meeting, members discussed experiences with organic management using different products to develop a resistance management plan. A key priority is to identify methods of control for early season to deploy against the first generation to reduce numbers late in the year. To this end, the Northfield lab group conducted a field trial evaluating Celite applied only during the first generation, but it did not reduce damage. We are discussing other currently available products to use during this first generation, or applied to treat hibernaculum at the end of the season.

Results and Discussion

We are currently sorting larvae from bands, and do not have results from bioassays yet. Discussions on resistant management plan strategies have not been conclusive yet and are ongoing.

References

Asser-Kaiser, S.; Fritsch, E.; Undorf-Spahn, K.; Kienzle, J.; Eberle, K.E.; Gund, N.A.; Reineke, A.; Zebitz, C.P.; Heckel, D.G.; Huber, J.; et al. Rapid emergence of baculovirus resistance in codling moth due to dominant, sex-linked inheritance. Science **2007**, 317, 1916–1918.

Fan, J.; J. A. Jehle; A. Rucker; A. L. Nielsen. First Evidence of CpGV Resistance of Codling Moth in the USA. *Insects* **2022** 13, 533.

Project Title: Net Gains: Balancing Codling Moth and Aphid Control under Drape Nets

Report Type: Continuing Project Report

Primary PI: Elizabeth H. Beers
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Cooperators: DrapeNet North America

Project Duration: 3 Years

Total Project Request for Year 1 Funding: $59,341
Total Project Request for Year 2 Funding: $61,345
Total Project Request for Year 3 Funding: $0

Other related/associated funding sources: N/A

WTFRC Collaborative Costs N/A

Footnotes: Year 3 will be used to process any remaining frozen samples and conduct final data analysis, no funding request.

Budget 1
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Station manager/supervisor email address: kimi.lucas@wsu.edu

Item	**2025**	**2026**	**2027**
Salaries1	$18,295.00	$19,027.00	$0.00
Benefits2	$7,501.00	$7,801.00	$0.00
Wages3	$5,932.00	$6,169.00	$0.00
Benefits4	$597.00	$621.00	$0.00
RCA Room Rental	$0.00	$0.00	$0.00
Shipping	$0.00	$0.00	$0.00
Supplies5	$5,500.00	$5,500.00	$0.00
Travel6	$3,000.00	$3,000.00	$0.00
Plot Fees	$0.00	$0.00	$0.00
Miscellaneous	$0.00	$0.00	$0.00
Total	$40,825.00	$42,118.00	$0.00

Footnotes: [1]Salary for 0.3 FTE Scientific Assistant. [2]Benefits at 41%. [3]Wages $22.47/hr. [4]Benefits @10.1% [5]Field sampling supplies [6]Mileage @ $0.67/mile.

Budget 2
Co PI 2: Rebecca Schmidt-Jeffris
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Station Manager/Supervisor: Rodney Cooper
Station manager/supervisor email address: rodney.cooper@usda.gov

Item	**2025**	**2026**	**2027**
Salaries[1]	$12,069.00	$12,795.00	$0.00
Benefits[2]	$2,647.00	$2,832.00	$0.00
Wages	$0.00	$0.00	$0.00
Benefits	$0.00	$0.00	$0.00
RCA Room Rental	$0.00	$0.00	$0.00
Shipping	$0.00	$0.00	$0.00
Supplies[3]	$3,800.00	$3,600.00	$0.00
Travel	$0.00	$0.00	$0.00
Plot Fees	$0.00	$0.00	$0.00
Miscellaneous	$0.00	$0.00	$0.00
Total	$18,516.00	$19,227.00	$0.00

Footnotes: [1]Salaries: Two Biological Science Technicians: GS-4, Step 1 Limited Appointment and GS-7 Step 1 then 2 TERM both at 30% for 6 months per year, with 4% COLA. [2]Fringe Benefits 8% for LA and 32% for TERM. [3]Supplies: predators for release, marking/sampling supplies.

Objectives

1. ***Determine if predator-exclusion effects of nets can be mitigated without loss of codling moth control by*:**

    a. *Delaying net deployment*. Growers typically deploy nets after petal fall to allow for pollination. We will compare net deployment timings (relative to codling moth adult emergence) to determine if a delay allows for buildup of early season aphid predators on the trees, without compromising codling moth control.

    b. *Leaving nets partially opened*. Drape nets are typically tightly closed around the tree trunk, but codling moth prefer the upper canopy. Thus, there may be no effect on codling moth from a partially opened net, exposing the trunk and the underside of the lowest limbs. This may allow access by ground-dwelling and winged predators to aphid colonies inside the net.

2. ***Determine if predator releases can mitigate aphid pressure under netting.*** Purchased natural enemies tend to disperse out of the orchard. Releasing under nets may keep them where they belong. However, netting may disrupt the normal behavior of flying natural enemies and reduce their efficacy. We will test releases of lacewings and ladybeetles for their compatibility with netting.

3. ***Determine the level of reduction in spray penetration/efficacy caused by netting.*** Nets can prevent penetration of codling moth, but they may also reduce penetration of sprays. Nets may eliminate the need for codling moth sprays, but horticultural sprays (crop load management, nutrition) and plant pathology sprays will still need to be applied. Does the mesh size used in Washington significantly reduce the penetration of spray materials, and can we compensate with speed and nozzle output?

Significant Findings

- High populations of woolly apple aphid built up under the nets in late summer/fall.
- Where colored nets were compared, woolly apple aphids were 7x higher under the black net than the grey net.
- All of the fully netted plots, whether deployed at petal fall or the beginning of the 2nd codling moth flight, had high levels of woolly apple aphid; the late (Gen. 2) deployment reduced the peak only slightly.
- The partially netted (3 ft above the ground) plot had woolly apple aphid densities similar to the unnetted plots, which was low to moderate throughout the season.
- The addition of predators did not reduce woolly apple aphid populations relative to the comparable treatment without predators.
- An unusually high population of rosy apple aphid occurred before net deployment, and attracted large numbers of coccinellid predators
- Codling moth trap catch went to near zero once the nets were deployed, whether it was PF, Gen. 2, or partially netted.
- Codling moth damage was lowest in the two PF net treatments, intermediate in the Gen. 2 and partial treatments, and highest in the unnetted plots. Despite the high overwintering CM population in the block, and a slightly delayed PF deployment, nets almost eliminated fruit damage.

Methods

Plot setup. A replicated study was set up in an entomology research block in WSU Sunrise Orchard in Rock Island, WA in May of 2025. The block consisted of alternating rows of four apple cultivars (‘Fuji’, ‘Golden Delicious’, ‘Gala’, ‘Jonagold’) at a 3 x 10 ft spacing on a 3-wire trellis system; the Fuji rows were chosen for having the sturdiest framework for supporting the nets, and the most consistent bloom. Each plot (replicate) consisted of 12 trees in single row, separated by pass-thru spaces for easy access to the block. The block was untreated for insect pests in 2024, and had a history of both woolly apple aphid and codling moth infestations. The treatments were randomized on the basis of the bloom assessment. Six treatments comprised of various combinations of net timing and coverage, and predator releases (Table 1) were replicated four times in a randomized complete block design.


Fully netted plot, zip-tied bottom edges


Partially netted plot

Table 1. Net trial treatments (white net, ‘Fuji’), SRO 2D, 2025

Trt. #	Trt. Name	Deployment timing	Deployment Level	Predator Releases
1	Net PF Full	Petal fall[1]	Full[3]	No
2	Net G2 Full	2nd flight[2]	Full	No
3	Net PF Partial	Petal fall	Partial[4]	No
4	Net PF Full + preds	Petal fall	Full	Yes[5]
5	No Net, no preds	---	---	No
6	No Net, + preds	---	---	Yes

[1] Intended for PF, but delayed ca. 2 weeks due to installation of bullhorns and extenders.

[2] Deployed 26 June, immediately after the evaluations of 1st gen. CM damage.

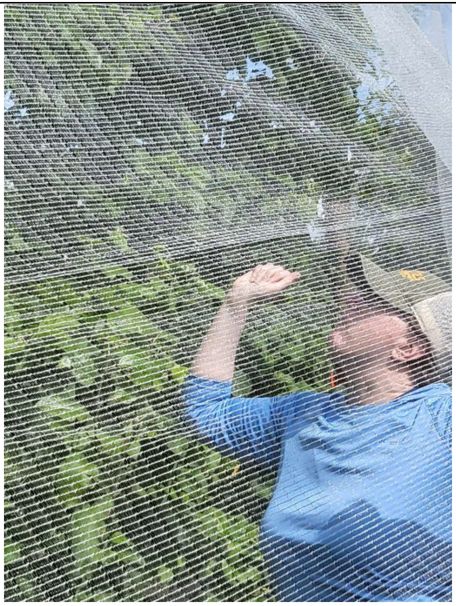
[3] Nets zip-tied between the trunks about 6-8 inches off the ground.

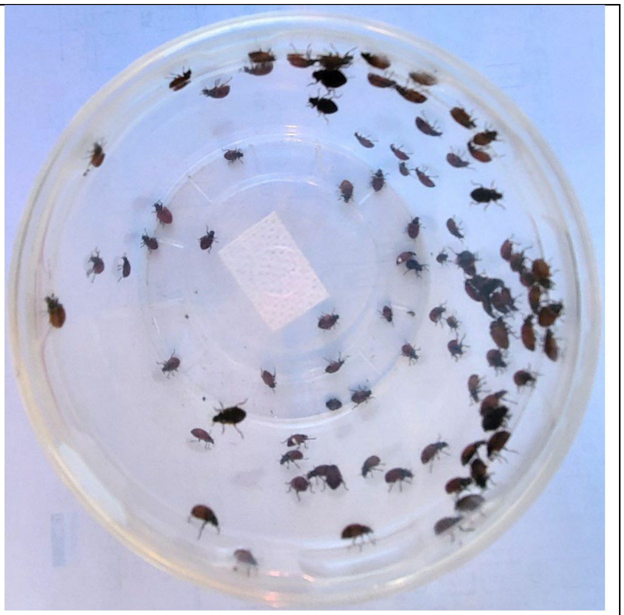
[4] Nets rolled up and zip-tied about 3 feet off the ground

[5] Two releases of lacewings (early instar larvae) on 22 May and 5 June, and one release of adult convergent lady beetles on 10 June; both species at an equivalent rate of 20,000/acre.

To provide optimum support for the drape nets, bullhorns and extenders were screwed to the trellis posts, and 3 additional wires stretched down the row provided extra space for the branches a the tops of the trees. The nets were draped over these wires on May 8-12, rolling up the nets in trt. 2 along the top wire for later deployment. Nets were continuous down the 12-tree plots, and fastened vertically at each end. The net bottoms in the fully netted plots zip-tied about 6-8 inches off the ground between every 3rd tree trunk, rolling up any excess. The partially netted plots had the bottoms rolled up to about 3 ft off the ground, with the rolls secured with zip ties.

Predator Releases. Insectary-reared predators were released on three dates during the early part of the season. Lacewing larvae (early instar) were released on 22 May and 5 June by throwing small portions of the buckwheat medium containing larvae on the foliage, distributing the portions along the length of the plot. Adult lady beetles were released on 10 June by shaking small portions in the canopy the length of the plot. Both predators were released at an equivalent rate of 20,000 per acre in the 10 × 40 ft plots.


Lacewing release.

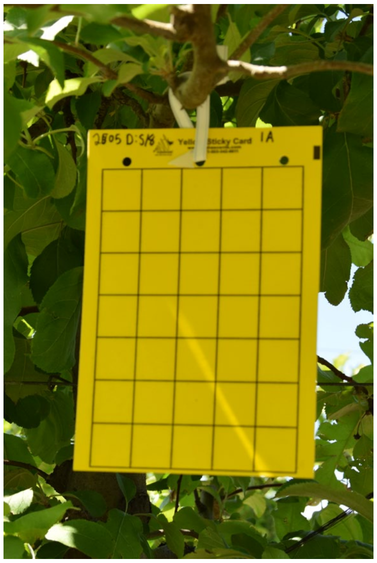

Adult lady beetles for release, 10 June

Sampling Pests and Natural Enemies. Plots were sampled prior to net deployment on 8 May, and every 2 weeks through the remainder of the season. The bottom zip ties of the fully netted plots were cut, and observers ducked inside the net wall to make counts or change traps, and new zip ties installed after sampling was complete. The exception was trt. 3, which did not need to be untied for observers to work inside the net. Three aphid species (woolly apple aphid, WAA; rosy apple aphid, RAA) and green apple Aphid, GAA) were evaluated in a 2-min timed count per plot. One person stood on each side of the row and walked slowly along it for 1 min, using a 4-bank hand talley to record aphid colonies. At the first date, the high numbers of lady beetles in the plots were noted, and added to the timed count. A separate sample for natural enemies of woolly apple aphids was conducted when colonies became sufficiently numerous; up to 10 colonies/plot were examined *in situ*, recording the numbers of parasitized aphids and predators found in association with the colony. Tap counts (2/plot) were done with a 24 inch diameter (452 in^2) beating tray, recording natural enemies. On May 8, a single yellow sticky trap (YSC) and a single earwig shelter per plot were deployed, and checked at the subsequent sample date and biweekly thereafter throughout the season. The YSC was hung on a tree


Woolly apple aphid colony


Tap counts beneath the net

branch or trellis wire between 5-6 ft from the ground in a space cleared of foliage. The traps were changed and their location in the plot moved on each sample date, and the natural enemies found on them recorded. The earwig shelter consisted of a 4 x 10-inch strip of cardboard rolled up to about 1 inch in diameter, and tied to the lower trunk with flagging tape. The same roll was used throughout the season, but its location in the plot was changed on each sample date. The shelters were unrolled and examined in situ, recording earwigs, lacewing cocoons, spiders, and codling moth larvae/pupae. A single CM pheromone trap per plot was suspended from a trellis wire about 7-8 ft from the ground, and liner was changed on each sample date. The trap body was an orange delta type tied to a 6 ft bamboo pole, baited with a CM+DA+AA lure. CM adults were recorded by sex and mating status, and lures changed every 6 wk. Mite evaluations were performed three times during the season by collecting 25 leaves randomly throughout the plot, brushing the leaves on a revolving glass plate, and recording pest and predatory mites.

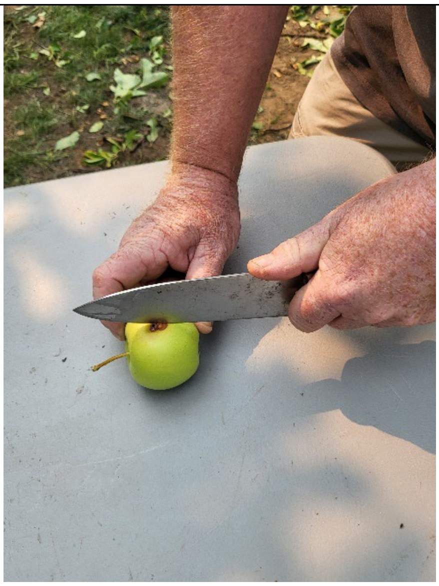
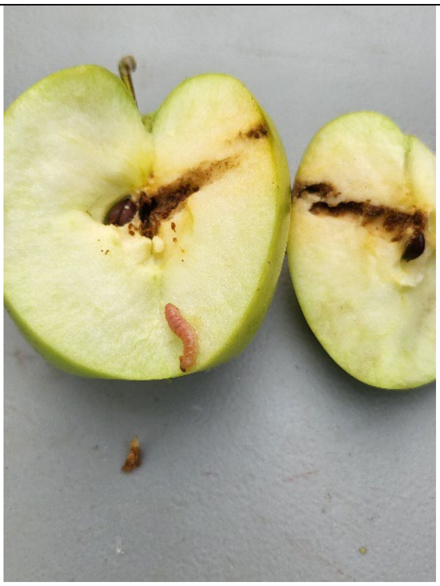


Yellow sticky card (YSC) in canopy

Codling moth delta trap

Codling moth damage was assessed three times during the season: at the end of the first flight (26 June), the second flight (20 August), and just prior to commercial harvest (September 30). Assessments were made using an untimed visual examination, with two observers on opposite sides of the trees walking slowly down the plot. Stings, entries, and intact fruit were recorded with hand talleys. No attempt was made to examine 100% of the fruit; the canopy was scanned top to bottom as the observer walked slowly but without stopping. The examinations took about 1.5-2 min/observer, or 3-4 minutes/plot depending on crop load.

A destructive, whole-plot sample was taken on a subsample of 10 plots to validate the visual damage observations. Fruit were harvested and examined on tables set out for that purpose, cutting the damaged fruit to determine if it was a sting or an entry. Damage was scored as a sting if there was no evidence of penetration or feeding at the fruit core, and as an entry if such evidence existed.

 Cutting fruit for destructive damage sample	 Successful CM entry

Spray Penetration. Spray deposition was measured in one netted (1A) and unnetted (5B) plot to visualize if the nets intercepted the sprays to any extent. Water sensitive cards (WSC; 2 x 3 inches) were stapled on 3.4 m (11 ft) wooden pole on cross-arms at 3 heights: 110 cm (3.6 ft), 210 cm (6.9 ft), and 310 cm (10.2 ft). Four cards were placed at each height, at the north and south ends of the cross-arm, and 2 facing east and west. The cards were labeled with their treatment and position with waterproof laminated tape. Two replicate poles were placed in each of the plots. The plots were sprayed with water on 22 August with an airblast sprayer calibrated to deliver 100 gpa. Both sides of the row were sprayed. The WSC were photographed *in situ* immediately after the sprayer passed including the label for later identification.

 Pole with cross-arms to hold WSC.	 Spraying with water for spray penetraion test.	 WSC (bottom level) in the netted plot.

Data analysis. Data were analyzed using a generalized linear mixed model (SAS Ver. 9.4, PROC MIXED) and means were separated using the Tukey adjustment to correct for multiple comparisons (HSD, $P \leq 0.05$). Season-long pest and natural enemy samples were analyzed as a repeated measures model (10-11 dates). Fruit damage in the 10 destructively sampled plots at harvest was regressed against the visual inspections using PROC REG, with the ‘noint’ option forcing the line through the zero on the x-axis. Spray penetration as evidenced by the WSC was analyzed using DepositScan.

Results and Discussion

Woolly apple aphids: There was a brief increase in WAA densities in late May-early June, but populations remained at low levels until late July. These rose steeply in the next four weeks peaking in mid-September (Fig. 1) in the affected treatments. The addition of predators did not reduce WAA seasonal densities (Fig. 2); it seems as though early season release of predators is likely not effective against late-season outbreaks. Similarly, keeping the nets off until late June produced slightly lower WAA densities, but they were still higher than expected. The partial (full season) netting approach was more successful in keeping WAA densities at a level similar to an unnetted plot; the latter treatments (unnetted with and without predators) were the lowest WAA levels.

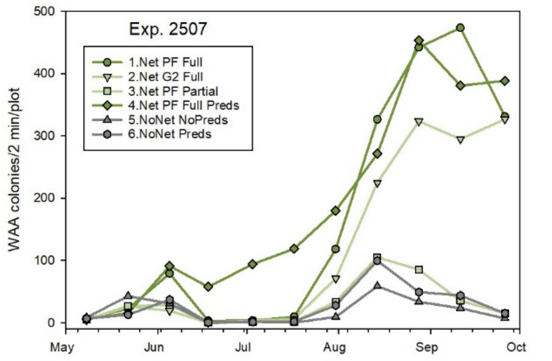


Fig. 1. WAA densities over time.

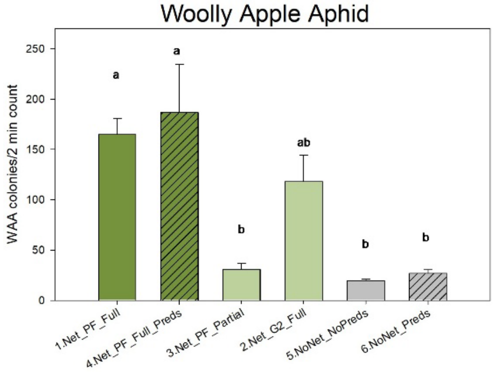


Fig. 2. Mean seasonal WAA densities.

Natural enemies: Earwigs (as sampled in earwig shelters) were abundant in this plot, but no treatment differences were detected, despite the difference in WAA populations (data not shown). Similarly, the densities of generalist predators (spiders, syrphids, coccinellids, *Deraeocoris*, and lacewings) in the tap samples were low overall, with no treatment differences. Tap samples were a relatively ineffective method to sample for the specialist parasitoid, *A. mali*; the YSC were much more effective, and demonstrated that *A. mali* was highest where WAA was highest, in the fully netted plots (Table 2). Despite high populations, *A. mali* failed to control WAA, but merely tracked their numbers. Coccinellid numbers were highest seasonally in the unnetted plots without predators, which may be an artefact of their attractiveness from a distance. Interestingly, coccinellid densities were higher in the late season in the YSC, but were mainly composed of mite predators (*Stethorus* spp.; data not shown); but peaked much earlier in the visual observations. It was also apparent that the visual observations detected coccinellids in much higher numbers than the YSC (Fig. 3). Lacewings were highest in the partial net and unnetted plots (Table 2); again, the attractiveness of the traps to draw from a distance may compromise their ability to characterize an open vs unnetted plot. The fact that lacewings were caught at all in the fully

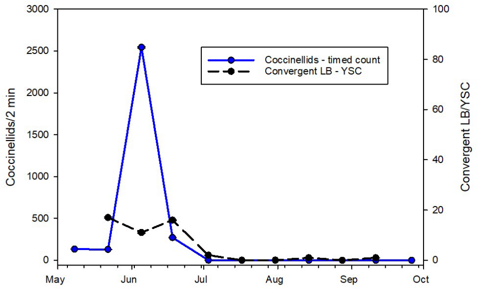


Fig. 3. Coccinellid densities in two different sampling methods. Note Y-axis scales.

netted plot may be an indicator that the nets are more permeable to alate predators than previously thought.

WAA colony evaluations were not possible until later in the season after aphids had built up. Virtually all the natural enemies observed in association with the WAA colonies were *A. mali*, with only a few generalist predators found. Differences in *A. mali* densities varied with WAA densities (data not shown).

Table 2. Natural enemies in yellow sticky card counts, SRO 2D, 2025

Trt	Lacewings	Coccinellids	*H. convergens*	Syrphids	*A. mali*
1.Net PF Full	0.38bc	4.35ab	0.11a	0.25a	269.90a
2.Net G2 Full	0.65bc	2.48b	0.47a	0.20a	32.13b
3.Net PF Partial	2.33a	1.35b	0.14a	0.53a	27.50b
4.Net PF Full Preds	0.18c	1.48b	0.17a	0.13a	127.03ab
5.NoNet NoPreds	1.20abc	9.38a	0.33a	0.50a	32.33b
6.NoNet Preds	1.35ab	5.38ab	0.11a	0.60a	27.23b

RAA densities were extremely high in the early season, but their populations were similar across all treatments. GAA was almost non-existent in this block, disappearing early in the season. Coccinellids were very numerous in visual counts, apparently attracted by the early RAA population; however, the net treatments, surprisingly, had no effect on their densities. Mite densities were low throughout the season, never exceeding 0.5 mites/leaf, with no differences among treatments.

Codling moth: CM trap captures were relatively high initially, as trap deployment occurred likely near the peak of the first flight. Captures dropped in all treatments in the next 4 weeks (Fig. 4), but went to near zero in the G1 fully and partially netted treatments by the second sample date. These remained near zero for the rest of the season. The second flight peaked in mid-July in the unnetted plots, but dropped to zero in early July in the Gen. 2 plots. It seemed clear that the nets inhibited orientation to traps; by proxy, the same may be true of orientation to females under the nets. Fruit damage tells a similar story, but with a few subtle differences (Fig. 5). The unnetted plots had the highest levels of damage overall, exceeding 60% at harvest. The Gen. 2 nets and the partial nets had intermediate levels of damage (ca. 40%), with a steady increase in the partial net plots as the season progressed. The fully netted plots (with and without predators) had the lowest damage levels, remaining <7% despite the high carryover of CM and late net deployment.

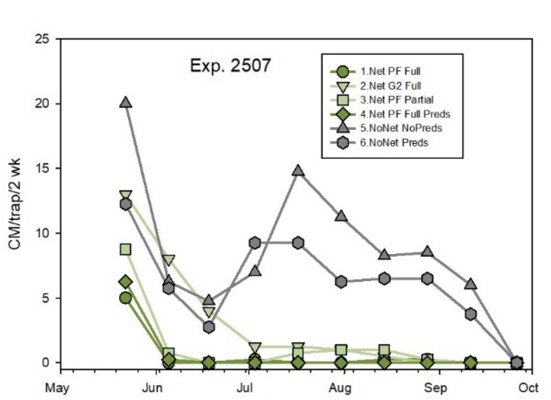


Fig. 4. Codling trap counts, 2025.

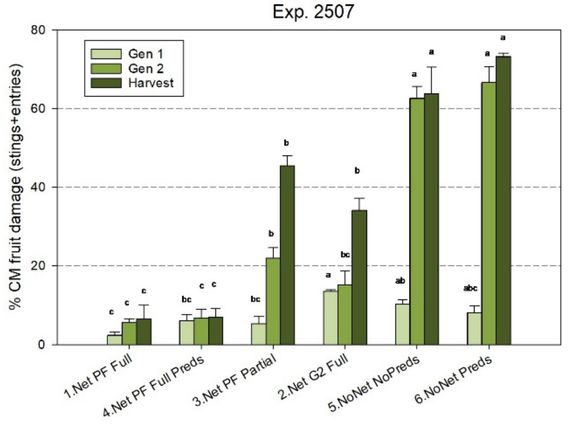


Fig. 5. Codling moth fruit damage, 2025.

Fruit for destructive sample, unnetted (check) plot

Fruit for destructive sample, netted plot

Validation of visual CM damage counts. The visual counts consistently underestimated the total number of fruit and the total number of CM-damaged fruit. However, they did so in a very consistent manner, such that the relationship between treatments was maintained. The R^2 for both of these metrics was >95% (Fig. 6A, B). Estimating the true damage levels may be accomplished by use of the regression equations specific to each.

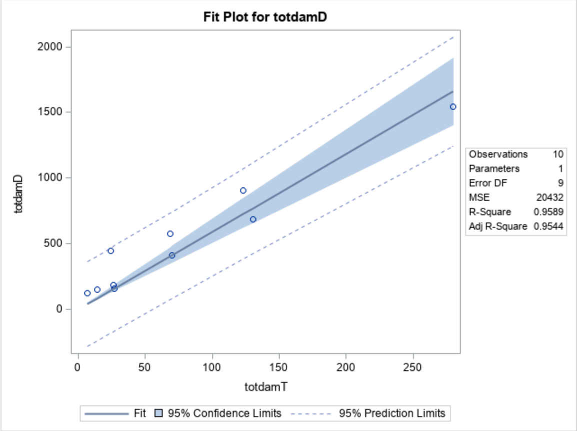


Fig. 6A. Regression for CM-damaged fruit

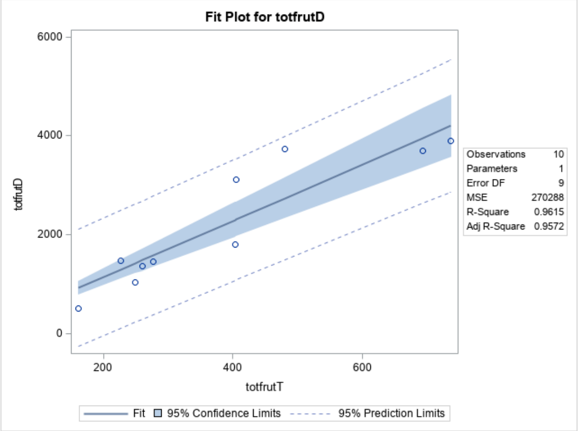


Fig. 6B. Regression for total fruit

Colored nets. While this sub-experiment was incompletely replicated, some interesting trends emerged in the woolly apple aphid counts (Fig. 7). Woolly apple aphids were significantly higher under the black nets than in the grey net or open plots. The two net colors had only two replicates each, while the open 'Golden Delicious' plots had four replicates, the same number as the 'Fuji' white net experiment. While the WAA numbers varied substantially between the two net colors, CM damage was controlled at roughly the same level (≤ 5% at harvest) (Fig. 8) as the white nets. All of the nets used have the same weave and aperture size, so the similarity in damage is expected.

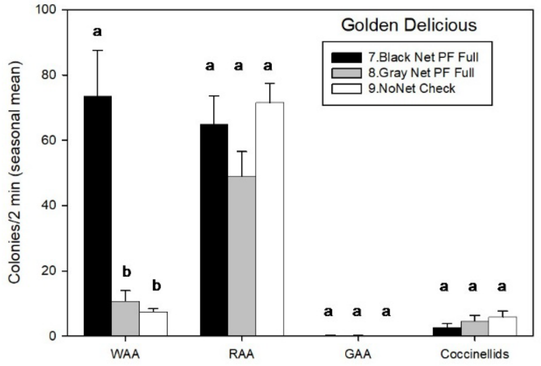


Fig. 7. WAA seasonal densities, Golden Delicious, 2025

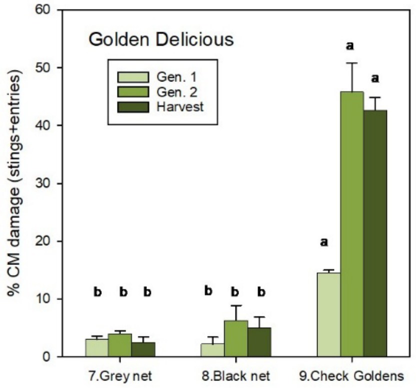


Fig. 8. CM fruit damage, Golden Del., 2025

Spray Penetration. The DepositScan software is unreliable if the deposit exceeds 30% of the area of the WSC, which it did in most cases. A rough visual estimate can be gained by visual examination of the photos (Fig. 9); however, it appeared that the total deposit was more related to the obstruction of a branch or a leaf than obstruction by the net. For instance, the East-Low card in the netted plot appeared to have a greater deposit than that of the open plot. More sophisticated measurements are needed to determine spray penetration, and more specifically, spray efficacy.

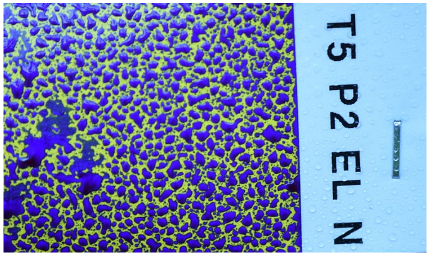
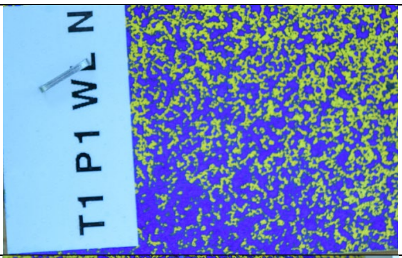
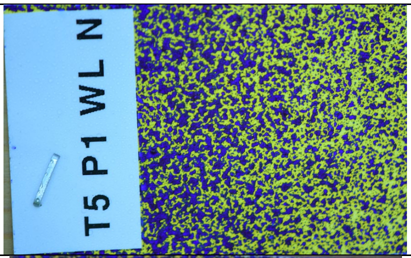
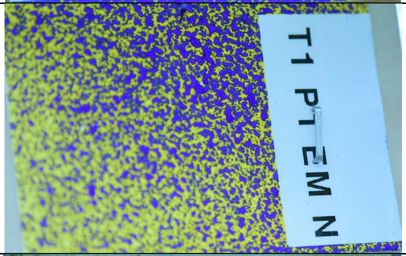
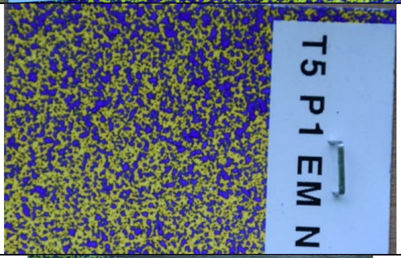
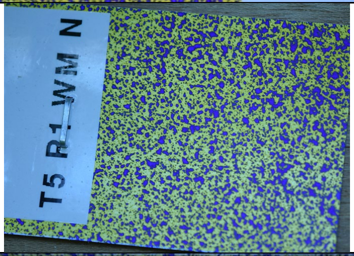
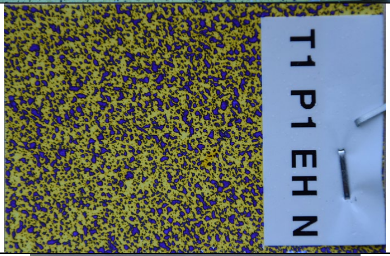
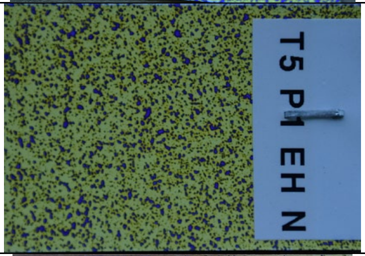
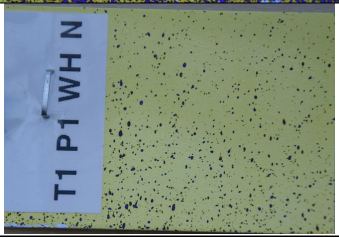
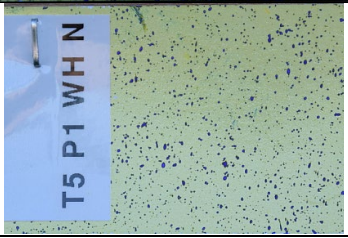
P1 position	Netted – T1	Open – T5
East Low		
West Low		
East Mid		
West Mid		
East High		
West High		

Fig. 9. Water Sensitive cards in netted and open plots, 22 August, 2025. Cards were placed at 3 heights in the canopy, with one set facing the east row middle, and one set facing west.

Title: NEW CODLING MOTH PATHOGENS
Report Type: CONTINUING

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Cooperators: Teah Smith (Zirkle Fruit Company)

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $104,000
Total Project Request for Year 2 Funding: $121,000
Total Project Request for Year 3 Funding: $124,000

Other related/associated funding sources: Applying for several sources of funding in addition to negotiating with industry partners that may sponsor this research.
Funding Duration: 2025 - 2027
Amount: $313,332 total in pending requests
Agency Name: Washington Commission on Integrated Pest Management ($23,498 requested for 2025 – not funded), WSU BioAg program ($39,920 requested for 2025 - funded), WA Specialty Crop Block Grant ($249,914 to be requested for 2025-2027 - funded)**.**
Notes: We will also apply for additional funds to expand the scope of this project. We are also seeking industry partners to sponsor part of this research through licensing agreements with WSU.

WTFRC Collaborative Costs: none

Budget 1
Primary PI: RT Curtiss
Organization Name: Washington State University
Contract Administrator: Gabriel Iacoboni
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Station Manager/Supervisor: Lee Kalcsits
Station manager/supervisor email address: lee.kalcsits@wsu.edu

Item	**2025**	**2026**	**2027**
Salaries	$59,630.00	$74,254.00	$77,224.00
Benefits	$20,995.00	$26,141.00	$27,186.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$19,375.00	$16,605.00	$15,590.00
Travel	$4,000.00	$4,000.00	$4,000.00
Plot Fees			
Miscellaneous			
Total	$104,000.00	$121,000.00	$124,000.00

Footnotes: Salaries for project PI (1@ 0.2 FTE), postdoc (1 @ 0.375 FTE in year 1, 1 @ 0.722 FTE in years 2 and 3) and project technician (1 @ 0.05 FTE); Benefits @ 35% for PI and Postdoc, 37.7% for technician; Supplies: Lab consumables, Mycology supplies, Laminar flow hood, Gene Sequencing; Travel to plots, motor pool rental, fuel, per diem, other related travel.

ORIGINAL PROJECT OBJECTIVES:

1) Explore - Locate diseased codling moth larvae and adults from Washington apple orchards throughout the state
2) Isolate - Establish cultures and sequence prospective pathogens that are isolated from those codling moths
3) Infect - Test the infection potential of pathogens on healthy codling moth caterpillars in lab assays and in field studies
4) Select - By repeatedly selecting for infection, pathogenicity and virulence may increase with each generation
5) Share - Provide outreach and education to describe project findings, and find an industry partner to commercialize

This project addresses the WTFRC high priority for codling moth management by developing new crop protection products with an organic emphasis. In addition, it addresses a need for resistance management tools by developing entomopathogenic fungi strains that have not previously been used to manage codling moth.

2025 SIGNIFICANT FINDINGS

- 3,000 out of 17,000 codling moth final instar larvae that were collected from Washington apple farms in 2025 were found dead.
- These larvae are all candidates for discovery of new pathogen strains.
- We discovered a likely second *Beauvaria* strain in 2025 from WA codling moths
- Processing 2025 samples and characterizing pathogens is ongoing
- We developed a rapid screening tool that will be thoroughly tested in 2026

ORIGINAL METHODS:

All objectives and activities will occur throughout the project and will be ongoing beyond the end of this study. Objectives 1 and 2 will begin in early 2025 using bands that were collected in 2024. In spring 2025, when overwintering codling moth begin to emerge from diapause, we will deploy bands to capture caterpillars of the first generation. Objectives 2 – 4 will continue starting in January 2025 with the three strains of entomopathogenic fungi we isolated in 2024. The following objectives will occur simultaneously:

Objective 1: Explore (June – November each year)

Capitalizing on codling moth pupation behavior, final instar larvae will be captured in cardboard bands affixed to tree trunks in commercial apple orchards in Washington State. The method for using cardboard banding material is used by farmers as a control tactic to physically remove moths from orchards, and as a research tactic to collect moths for use in experiments. We will work with farmers and consultants to locate farms in which we will deploy bands. We will also identify locations where farmers deploy bands that we may collect. Using population modeling, bands will be placed in commercial organic and conventional apple orchards prior to codling moth pupation. Bands will be collected from orchards when modeling indicates the caterpillars have pupated but not emerged as adults. Bands with larvae will be returned to the laboratory where the codling moth final instar larvae will be visually screened for potential presence of pathogens. Likely infected caterpillars will be used in Obj. 2, while those that appear uninfected will be incorporated into the codling moth research colony housed at WSU-TFREC in Wenatchee. Establishment of this colony was funded in

2023 by the Washington Tree Fruit Research Commission. Colony-reared moths will be used in Obj. 3-4 and in other studies.

Secondary to exploring for infected codling moth larvae, we will record and analyze data on the infection rate of codling moth larvae in each generation as currently found in Washington. We will also collect site information and correlate site characteristics with current levels of infection. These data will be useful in understanding how much natural control is currently being achieved, as a comparison with future sprays of the entomopathogenic fungi, and if there are any differences in susceptibility of each generation at each site. These findings will also be used to establish tactics for when and where to spray these fungi if they are developed into biopesticides.

<u>Objective 2: Isolate – (Starting June 2025)</u>

Prospective infected codling moths identified in Obj. 1 will be surface sterilized to reduce contamination before being placed on fungal growth media plates. Information on the provenance of the potentially infected larva will be recorded and follow the caterpillar from the farm until sporulation and beyond. Surface sterilization of each possibly infected codling moth larva will follow this protocol:

Step	Washes	Time
1	70% Ethanol	5 sec
2	Sterile Deionized Water	5 sec
3	0.5% to 1% Bleach	1 min
4	Sterile Deionized Water	5 sec
5	Sterile Deionized Water	5 sec
6	Drying (under sterile flow hood)	Until dry

Following surface sterilization, each infected larva will be transferred individually to sterile SDAY Semi-Selective Media plates that will be labeled with the provenance of the larva. SDAY plates contain Sabouraud Dextrose Agar: 32.5 g, Yeast Extract: 0.5 g, Deionized Water (DI H_2O): 500 mL, and a Penicillin-Streptomycin solution to reduce microbial contamination risk in this fungal growth media. Infected larvae will remain on SDAY plates until sporulation occurs. Upon adequate sporulation, cultures will be split by the "Agar Punch Method" for mycelium culturing. Under sterile conditions, punched-out agar discs containing mycelium will be placed upside down onto the surface (center) of a new media plate to ensure direct contact between the mycelium and the new medium. Isolated cultures will be identified using genetic sequencing.

Using the "Agar Punch Method," pure cultures will be further isolated and propagated. Confirmation of culture purity will periodically be performed by genetic sequencing to ensure the pathogen in culture has not been contaminated.

<u>Objective 3: Infect – (Starting January 2025)</u>

Once pure and identified cultures have been isolated in Obj. 2, they will be used to expose healthy codling moth caterpillars to the prospective pathogens. Caterpillars will be sourced from the WSU-TFREC codling moth colony. Caterpillars will be exposed to a pathogen spore solution and immediately transferred to SDAY plates. Exposed caterpillars will remain on agar plates until they die, and sporulation occurs. Sporulation will be an indication of successful reinfection. Caterpillars that do not exhibit sporulation will be considered unsuccessful infection events and discarded. Spores collected from lab-infected caterpillars will be used to re-isolate pure strains of the pathogens. Pathogens will again be periodically confirmed by DNA sequencing.

As part of this objective, we will record data on infection success to measure changes in the rate of infection over time. These data will be used to further understand the native infection rate and to compare with the findings of the following objective.

Objective 4: Select – (Starting January 2025)

By repeatedly infecting and isolating pure pathogen strains from codling moth caterpillars, fungal pathogenicity may be increased. The methods used in Obj. 1-3 will be repeated, and selected strains will be tested for increased pathogenicity. Pathogenicity of each infection generation will be recorded to document changes in the virulence of the pathogens. In addition, strains will be selected for other key characteristics, including the ability to be mass-produced, persistence in the field, and field efficacy.

Again, pathogenicity data will be recorded to demonstrate changes in the ability of selected strains to infect codling moth caterpillars.

Objective 5: Share (Annually, or as information becomes available)

Information on project activities and achievements will be shared at extension events in English and Spanish (translated and delivered by co-PI Reyes Corral). We will develop a factsheet that may be shared on the WSU Tree Fruit website and/or presented verbally at grower meetings, such as Apple Day, in both English and Spanish. Last, we will publish the results in peer reviewed journals.

In addition to directly sharing findings with the industry, during this project, we will seek out 1-3 industry partners that will sponsor some of the ongoing costs of the research. Those industry partners will be insecticide/biopesticide manufacturing companies that will be offered the right of first refusal in exchange for financial contributions to the research. If this project successfully develops these entomopathogenic fungi into highly virulent strains, these industry partners will license the strains from Washington State University and make them available to growers to use as a control tactic.

2025 RESULTS AND DISCUSSION

Objective 1: Explore (June – November each year)

In 2025, we collected ca. 17,000 codling moths from several apple orchards throughout Washington State. We found 3,000 that were dead of unknown causes in the collection. Exploration for possibly pathogen-infected codling moths will continue in 2026-27.

Objective 2: Isolate – (Starting June 2025)

Fungal specimens

1. Three entomopathogenic fungi were isolated in pure cultures from a study in 2024 (CM-1, CM-2, CM-3).

DNA extraction

2. DNA from two of each of the isolated entomopathogens was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle 1987, Reyes Corral 2025). DNA quality and quantity were confirmed after extraction by using a NanoDrop™ 2000 spectrophotometer (ThermoFisher, Waltham, MA).

ITS gene amplification/sequencing

3. The internal transcribed spacer (ITS1, ITS2) region of ribosomal DNA was amplified using universal fungal primers ITS1F (5’-CTT GGT CAT TTA GAG GAA GTA A-3’) and ITS4 (5’-TCC TCC GCT TAT TGA TAT GC-3’) (Martin and Rygiewicz 2005, Bellemain et al. 2010, Yu et al. 2022). ITS1F and ITS4 were selected to ensure amplification of the entirety of the ITS region.
4. Amplified products were Sanger sequenced (Sanger and Coulson 1975) by Molecular Cloning Laboratories (MCLAB, San Francisco, CA).
5. Sequences were cleaned, aligned, and analyzed in Geneious Prime 2025.0.3 (Dotmatics, Auckland, New Zealand) using BLAST searches against reference databases to identify matches. Returned sequences from the entomopathogenic isolates were identified and deposited in GenBank:

GenBank Batch Submission	Sample ID	GenBank Accession No.	Organism	Top Matching GenBank ID
SUB15683559	CM 1-1	*PX444714*	*Beauveria bassiana*	*OW986954*
SUB15683559	CM 1-2	*PX444715*	*Beauveria bassiana*	*OW986954*
SUB15683559	CM 2-1	*PX444716*	*Hirsutella/Ophiocordyceps*	*KM652176*
SUB15683559	CM 2-2	*PX444717*	*Hirsutella/Ophiocordyceps*	*KM652176*
SUB15683559	CM 3-1	*PX444718*	*Metarhizium robertsii*	*KF624795*
SUB15683559	CM 3-2	*PX444719*	*Metarhizium robertsii*	*LR792768*

Primer design

Given the likelihood that multiple species of entomopathogenic fungi from each of the isolated genera are present in Washington orchards and recognizing that fungal strains can rapidly diverge among and even within orchards, we developed genus-specific primers and probes instead of targeting individual species.

To guide primer/probe development, we combined sequence data from Washington-native entomopathogenic fungi with the top 100+ GenBank records for each genus to generate consensus sequences. By identifying conserved regions within either the ITS1 or ITS2 loci/gene regions, we designed genus-specific primers that enable amplification of multiple species within each group of entomopathogenic fungi.

Designed primers/probes for each of the isolated entomopathogenic fungi were as follows:

Genus	Loci	No. of Sequences used for Consensus	Primer/Probe	Sequence	Length
Beauveria	ITS 2	117	Bea 362 F	5’-CAT TTC AAC CCT CGA CCT CCC-3’	159 bp
			Bea 467 P	5’-GTA ATA CAG CTC GCA CCG GA-3’	
			Bea 520 R	5’-AGT TGG GTG TTT TAC GGC GT-3’	
Hirsutella / Ophiocordyceps	ITS 1	137	Hir 76 F	5’-CCT GCG AAC CCT ATA GCG TT-3’	152 bp
			Hir 159 P	5’-AAG ACT CGA ACT GTC AGC GG-3’	
			Hir 200 R	5’-GTT TTG CTT GTA CGC CGA GA-3’	
Metarhizium	ITS 2	143	Met 416 F	5’-CAG CAC AGC CGT CCC TTA AA-3’	109 bp
			Met 477 P	5’-AAA ACA CTC GCA ACA GGA GC-3’	
			Met 524 R	5’-GGG GTT TTA CGG CAG TGG A-3’	

Real-time PCR multiplex assay development

Sequencing each codling moth larva would require substantial time and resources, making it an impractical approach for large-scale screening. To address this limitation, we designed and optimized a real-time PCR multiplex assay incorporating distinct, non-overlapping dyes across the light spectrum, allowing clear and rapid identification of entomopathogenic fungi from the genera originally isolated in Washington State.

Real-time PCR conditions consisted of an initial denaturation step of 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and a plate read step at the end of each cycle. Each 10 µl reaction contained 1X PerfeCTa Multiplex qPCR ToughMix (Quantabio, Beverly, MA), 300 nM of each primer set, 100 nM of each target probe, nuclease-free H_2O, and DNA template (between 200–300 ng/µl) from each sample. qPCR reactions were conducted using a CFX Opus 96 Real-Time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA).

Objective 3: Infect – (Starting January 2025)

In 2025, we conducted several assays with the isolated fungi. Laboratory assays that exposed these fungi to final instar *C. pomonella* confirmed Koch's postulates and demonstrated that they are indeed infective to *C. pomonella*. These fungi may have applicability for *C. pomonella* management in commercial orchards. Reinfection assays revealed distinct mortality and development patterns across fungal treatments; *B. bassiana* had the highest mortality in the first assays. Visible signs of mycosis and sporulation were evident in all fungal treatment groups within 10–15 d post-exposure. Overall, *B. bassiana* treatments caused the highest mortality and the greatest reduction in successful adult emergence, ca. 47% died before pupating. *Metarhizium robertsii* caused the second highest mortality in the first assays, ca. 17% died following exposure.

Objective 4: Select – (Starting January 2025)

Selection began immediately after the first assays. After each exposure generation we have selected the five strains that killed codling moth larvae most rapidly. After several infection generations, *B. bassiana* now causes >93% mortality in lab assays, while *M. robertsii* causes >96% mortality in lab assays. Selection will continue with these original strains to increase pathogenicity, and other desirable characteristics.

Objective 5: Share (Annually, or as information becomes available)

Findings from this project were shared seven times in 2025 by PI Curtiss and coPIs Northfield and Reyes Corral. We have reached at least 300 people.

CONCLUSION

This project is progressing as intended. We were surprised to find as many dead codling moths in our survey as we did, but to aid in processing sampling, we developed a rapid screening tool, a multiplex assay that will allow us to test specimens for pathogens without needing to first grow the pathogens. This tool will also allow us to rapidly identify new pathogenic strains and develop a library of strains for future use as insecticides. Although this work is continuing, progress is very encouraging.

WTFRC INTERNAL PROJECT – BUDGET SHARED FOR INFORMATIONAL PURPOSES ONLY

FINAL 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
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Cooperators: Gerardo Garcia (WTFRC), Northwest Horticultural Council, Pacific Agricultural Labs (Sherwood, OR), OMIC USA Laboratory (Portland, OR), Cameron Burt & Kelly O'Neill, 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

2025 WTFRC APPLE PESTICIDE RESIDUE STUDY

Since 2011, the Washington Tree Fruit Research Commission (WTFRC) has conducted annual trials to evaluate pesticide residues on 'Gala' apples. This year, we applied fifteen insecticides/acaricides, five fungicides, and three plant growth regulators 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 11 and delivered the next day to Pacific Agricultural Labs (Sherwood, OR) and OMIC USA Laboratory (Portland, OR) for chemical residue analysis.

TRIAL DETAILS

- 18th 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 0.54 inches of rain fell on the trial block on between pink and harvest with almost no rain occurring after petal fall

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 2025.

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*
prohexadione-Ca	Kudos 27.5WDG	12	Pink & petal fall	<0.01	3	0.01*	0.01 (Tha)
Inpyrfluxam	Excalia	4	Petal fall	<0.01	0.01	0.01	0.01 (Can, Mex)
metamitron	Brevis SC	40	Petal fall & 10 mm	<0.01	0.01	0.01	0.01 (many)
ethephon (summer)	Ethephon 2SL	32	45 DAFB	<0.1	5	0.01*	0.1 (Can)
flutianil	Gatten	8	35	<0.01	0.15	0.01*	0.15 (many)
tolfenpyrad	Bexar	27	35 & 21	0.55	1	0.01*	0.01 (Twn,Tha)
indoxacarb	Avaunt	6	35 & 21	0.22	1	0.01*	0.1 (Can)
flupyradifurone	Sivanto prime	14	35 & 21	0.18	0.7	0.01*	0.5 (Twn)
fenbutatin	Vendex 50WP	32	35 & 21	1.5	15	0.01*	2 (Twn)
zeta-cypermethrin	Mustang Maxx	4	35 & 21	0.23	2	0.01*	0.7 (many)
acequinocyl	Kanemite	31	28	<0.05	0.4	0.01*	0.01 (Chn,Tha)
lambda-cyhalothrin	Warrior II	2.56	28	0.031	0.3	0.01*	0.2 (many)
flonicamid	Beleaf 50SG	2.8	28	0.051	0.2	0.01*	0.2 (many)
sulfoxaflor	Transform	2.75	28 & 14	0.067	0.5	0.01*	0.3 (many)
chlorantraniliprole	Altacor eVo	2.2	28 & 14	0.19	1.2	0.01*	0.4 (many)
spinosad	Entrust SC	10	28 & 14	0.040	0.2	0.01*	0.1 (many)
buprofezin	Centaur WDG	34.5	21	1.2	3	0.01*	1 (Twn)
Ipflufenoquin	Axios 20SC	3	21 & 14	0.037	0.15	0.01*	0.01 (Tha)
phosmet**	Imidan 70-W**	92	14	2.7	10	0.01*	2 (Twn)
cyfluthrin	Baythroid XL	2.8	14	<0.05	0.5	0.01*	0.1 (many)
fenazaquin	Magister	36	14	0.39	0.6	0.2	0.3 (many)

[1] Top markets for WA apples with established MRLs; 2 December 2025. https://mrldb.nwhort.org/#top_markets

*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 2025.

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*
Inpyrfluxam	Excalia	4	Pink & petal fall	<0.01	0.01	0.01	0.01 (Can, Mex)
metamitron	Brevis SC	40	80 & 72	0.02	0.01	0.01	0.01 (many)
prohexadione-Ca	Kudos 27.5WDG	12	51 & 45	0.04	3	0.01*	0.01 (Tha)
acequinocyl	Kanemite	31	35 & 21	<0.05	0.4	0.01*	0.01 (Chn,Tha)
lambda-cyhalothrin	Warrior II	2.56	28 & 21	0.048	0.3	0.01*	0.2 (many)
flonicamid	Beleaf 50SG	2.8	28 & 21	0.079	0.2	0.01*	0.2 (many)
tolfenpyrad	Bexar	27	28 & 14	0.28	1	0.01*	0.01 (Twn,Tha)
flupyradifurone	Sivanto prime	14	28 & 14	0.14	0.7	0.01*	0.5 (Twn)
fenbutatin	Vendex 50WP	32	28 & 14	0.84	15	0.01*	2 (Twn)
indoxacarb	Avaunt	6	21 & 14	0.17	1	0.01*	0.1 (Can)
flutianil	Gatten	8	21 & 14	0.013	0.15	0.01*	0.15 (many)
zeta-cypermethrin	Mustang Maxx	4	21 & 14	0.12	2	0.01*	0.7 (many)
chlorantranliprole	Altacor eVo	2.2	21 & 7	0.15	1.2	0.01*	0.4 (many)
spinosad	Entrust SC	10	21 & 7	0.035	0.2	0.01*	0.1 (many)
phosmet**	Imidan 70-W**	92	21 & 7	3.4	10	0.01*	2 (Twn)
buprofezin	Centaur WDG	34.5	14	0.76	3	0.01*	1 (Twn)
sulfoxaflor	Transform	2.75	14 & 7	0.086	0.5	0.01*	0.3 (many)
ipflufenoquin	Axios 20SC	3	14 & 7	0.026	0.15	0.01*	0.01 (Tha)
ethephon	Ethephon 2SL	48	14 & 7	1.0	5	0.01*	0.1 (Can)
fenazaquin	Magister	36	7	0.24	0.6	0.2	0.3 (many)
cyfluthrin	Baythroid XL	2.8	7	<0.05	0.5	0.01*	0.1 (many)

[1] Top markets for WA apples with established MRLs; 2 December 2025. https://mrldb.nwhort.org/#top_markets

*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

DISCUSSION

Our 2025 trials included three plant growth regulators applied in a typical use pattern (standard) and also at the latest timings allowed by the respective product labels (aggressive); residues of metamitron, prohexadione-calcium, and ethephon were not detected in the industry standard protocol, but all three produced residues above key MRLs when sprayed at their minimum preharvest intervals. Residues were generally higher for most products when sprayed in the aggressive protocol, but that trend was not consistent. Apple producers should be mindful of the inherent variability in measuring pesticide residues and incorporate appropriate safety margins in their spray programs to allow for possible aberrant readings.

Products which produced residues in excess Maximum Residue Levels (MRLs) of key export markets in 2025 include: **Bexar, Avaunt, Centaur WDG, Axios 20SC, Imidan 70-W, Magister, Brevis SC, Kudos 27.5WDG, and Ethephon 2SL**. 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.

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

WTFRC APPLE PESTICIDE RESIDUE STUDIES 2011-2024

Since 2011, the Washington Tree Fruit Research Commission has conducted annual field studies to evaluate the harvest residues of numerous insecticides, acaricides, fungicides, and bioregulators commonly used in commercial apple production in WA. To provide a comprehensive overview of all measured residues, the table below summarizes all results regardless of application rates and timings or supplemental treatments such as overhead cooling, application of sunburn protectants, or simulated packing line washing, scrubbing, and waxing of fruit; values in **bold red font** highlight those residue levels which **exceed current maximum residue levels** (MRLs) for apples in some key export markets. Please note that the table does not include MRLs for India, which are currently set at 0.01 ppm for most chemicals. For more details regarding application protocols or results from specific years, please review annual reports of these studies at www.treefruitresearch.org. For more information on MRLs or other regulatory issues, please consult the Northwest Horticultural Council at www.nwhort.org.

STUDY DETAILS

- All trials conducted on 'Pacific' Gala / M.9 Nic.29 trained to central leader/spindle on 3' x 10' spacing
- Applications made with 2 x 25 gal Rears Pak-Blast sprayer calibrated to 100 gal water + 8 oz Regulaid / acre
- Spray protocols included both standard (applications at typical commercial rates and timings) and aggressive (applications at maximum rates and minimum retreatment and pre-harvest intervals) programs

MAJOR FINDINGS

- Many residues reported as potentially problematic in earlier annual reports would now be considered acceptable due to the relaxation of some MRLs in some markets
- Higher residue levels were consistently measured with higher application rates and shorter pre-harvest intervals
- Residues of some pesticides decreased on fruit which received a simulated packing treatment, but results were too inconsistent and unpredictable to consider it a reliable method for reducing residue levels
- Sunburn protection programs with Raynox or Eclipse did not significantly affect measured pesticide residues
- Routine application of overhead cooling did not significantly impact pesticide residue levels
- Carrier volume (100 gal water/acre vs. 200 gal water/acre) effects on residue levels were inconsistent and inconclusive

Minimum, maximum, and median residues vs. MRLs of common pesticides applied to 'Gala'/M.9 Nic. 29 apples near Rock Island, WA. WTFRC 2011-2024.

Chemical name	Trade name	# years evaluated	# samples analyzed	Minimum residue	Maximum residue	Median residue	US MRL[1]	Lowest export MRL[1]
				ppm	*ppm*	*ppm*	*ppm*	*ppm*
Abamectin	AgriMek SC	4	20	0	0	0.000	0.02	0.01 (many)
Acequinocyl	Kanemite	4	20	0	**0.032**	0.000	0.4	0.01 (CHN, THA)
Acetamiprid	Assail 70WP	6	48	0	0.31	0.068	1	0.8 (many)
Afidopyropen	Versys	4	20	0	0	0.000	0.02	0.02 (many)
Benzovindiflupyr	Aprovia	4	16	0	0.043	0.023	0.2	0.2 (many)
Bifenazate	Acramite	8	79	0	**0.43**	0.029	0.7	0.2 (CHN)
Boscalid	Pristine	4	32	0.049	0.86	0.130	3	2 (many)
Buprofezin	Tourismo/Centaur	9	66	0	**1.9**	0.034	3	1 (TWN)
Captan	Captec 4L	2	8	0.15	1.1	0.555	25	5 (CAN)
Carbaryl (summer)	Carbaryl 4L	1	4	**0.62**	**3.1**	**1.355**	12	0.01 (THA)
Carbaryl (thinning)	Carbaryl 4L	2	16	0	0	0.000	12	0.01 (THA)
Chlorantraniliprole	Altacor/Altacor eVo	7	44	0	0.34	0.035	1.2	0.4 (many)
Cyantraniliprole	Exirel	6	60	0.021	**0.6**	0.105	1.5	0.5 (TWN)
Cyclaniliprole	Verdepryn	4	16	0	0.16	0.057	0.3	0.2 (many)
Cyflufenamid	Torino	4	20	0	**0.043**	**0.017**	0.06	0.01 (THA)
Cyflumetofen	Nealta	6	48	0	0.25	0.035	0.3	0.3 (CAN, MEX)
Cyfluthrin	Baythroid XL	3	12	0	0	0.000	0.5	0.1 (many)
Cyprodinil	Inspire Super	11	96	0	**0.19**	0.041	1.7	0.05 (IDN)

Chemical name	Trade name	# years evaluated	# samples analyzed	Minimum residue	Maximum residue	Median residue	US MRL[1]	Lowest export MRL[1]
				ppm	ppm	ppm	ppm	ppm
Diazinon	Diazinon 50W	7	52	0	0.12	0.019	0.5	0.1 (CAN)
Difenoconazole	Inspire Super	11	92	0	0.11	0.021	5	0.5 (CHN)
Emamectin benzoate	Proclaim	3	40	0	0	0.000	0.02	0.02 (many)
~~Endosulfan*~~	~~Thionex 50W~~	~~4~~	~~32~~	~~0~~	~~0.99~~	~~0.000~~	~~na~~	~~na~~
Ethephon (fall)	Ethephon 2SL	1	2	0.72	0.9	0.810	5	0.1 (CAN)
Ethephon (summer)	Ethephon 2SL	3	12	0	0.57	0.260	5	0.1 (CAN)
Ethephon (spring)	Ethephon 2SL	2	6	0	0.14	0.000	5	0.1 (CAN)
Etoxazole	Zeal	7	72	0	0.13	0.017	0.2	0.07 (many)
Fenazaquin	Magister	2	8	0.30	0.52	0.385	0.6	0.3 (many)
Fenbutatin	Vendex 50WP	1	4	0.83	1.1	0.970	15	2 (TWN)
Fenpropathrin	Danitol	11	94	0	0.65	0.175	5	0.01 (THA)
Flonicamid	Beleaf 50SG	3	12	0.024	0.37	0.043	0.2	0.2 (many)
Flubendiamide	Tourismo	4	42	0	0.31	0.040	1.5	0.8 (many)
Fluopyram	Luna Sensation	3	38	0	0.083	0.000	0.8	0.5 (many)
Flupyradifurone	Sivanto prime	3	12	0.089	0.39	0.170	0.7	0.5 (TWN)
Flutianil	Gatten	6	32	0	0.031	0.000	0.15	0.15 (many)
Flutriafol	Topguard	6	64	0	0.13	0.028	0.4	0.3 (many)
Fluxapyroxad	Merivon	5	52	0	0.51	0.048	0.8	0.8 (CAN, MEX)
~~Formetanate~~	~~Carzol SP~~	~~1~~	~~4~~	~~0~~	~~0~~	~~0.000~~	~~na~~	~~na~~
Hexythiazox	Onager	3	40	0.012	0.089	0.022	0.4	0.4 (many)
Imidacloprid	Nuprid 2SC	4	32	0	0.053	0.000	0.5	0.5 (many)
Indoxacarb	Avaunt	3	12	0.066	0.29	0.110	1	0.1 (CAN)
Ipflufenoquin	AXIOS	2	8	0.024	0.062	0.042	0.15	0.01 (THA)
Isofetamid	Kenja 400SC	4	24	0	0.16	0.018	0.6	0.6 (many)
Lambda-cyhalothrin	Warrior II	7	54	0	0.053	0.000	0.3	0.2 (many)
Mancozeb	Penncozeb 75DF	1	4	0	1.8	0.750	0.6	0.6 (MEX)
Mefentrifluconazole	Cevya	4	16	0.057	0.37	0.140	1.5	0.9 (TWN)
Methoxyfenozide	Intrepid	4	32	0	0.21	0.030	2	1.5 (CAN, TWN)
Metrafenone	Vivando	2	28	0	0	0.000	1.5	1 (many)
Myclobutanil	Rally 40WSP	7	68	0	0.73	0.099	0.5	0.5 many)
Novaluron	Rimon	4	34	0.09	0.63	0.325	3	2 (CAN, TWN)
Penthiopyrad	Fontelis	4	42	0	0.034	0.017	0.5	0.4 (many)
Phosmet	Imidan 70-W	6	32	1.1	6.1	2.700	10	2 (TWN)
Pydiflumetofen	Miravis	4	16	0.011	0.071	0.030	0.2	0.2 (many)
Pyraclostrobin	Pristine/Merivon	9	84	0	0.47	0.045	1.5	0.5 (many)
Pyridaben	Nexter	3	40	0	0.044	0.029	0.75	0.01 (THA)
Spinetoram	Delegate WG	9	74	0	0.084	0.011	0.2	0.05 (many)
Spinosad	Entrust	7	64	0	0.11	0.024	0.2	0.1 (many)
Spiridoclofen	Envidor 2SC	4	52	0	0.35	0.042	0.8	0.5 (China)
Spirotetramat	Ultor	4	52	0	0.19	0.020	0.7	0.7 (many)
Sulfoxaflor	Transform	3	12	0.051	0.17	0.097	0.5	0.3 (many)
Thiacloprid	Calypso	1	8	0.081	0.15	0.091	0.3	0.3 (CAN, THA)
Thiophanate-methyl**	Topsin 4.5FL	7	62	0	0.83	0.086	2	2 (MEX)
Tolfenpyrad	Bexar	7	40	0.096	1.1	0.345	1	0.01 (TWN, THA)
Trifloxylstrobin	Luna Sensation	5	46	0	0.033	0.000	0.7	0.5 (CAN)
Triflumizole	Procure 480SC	5	46	0	0.049	0.000	0.5	0.01 (THA)
Zeta-cypermethrin	Mustang Maxx	1	4	0.061	0.11	0.087	2	0.7 (many)
Ziram***	Ziram 76DF	7	68	0	2.8	0.510	7	0.1 (CAN)

[1] Top markets for WA apples excluding India; 11 Oct 2024. https://nwhort.org/export-manual/comparisonmrls/apple-mrls/, https://bcglobal.bryantchristie.com/

* Endosulfan values reported are sum totals of Endosulfan I, Endosulfan II, and Endosulfan sulfate residues

** Thiophanate-methyl values reported are sum totals of thiophanate-methyl and carbenzadim residues

*** Dithiocarbamate residues cannot be directly measured; total Ziram values are estimates based on analysis of the degradation product CS_2

Results of these unreplicated trials 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 university extension staff, crop advisors, and warehouse representatives to develop responsible pest control programs.

EXECUTIVE SUMMARY

Project Title: Pesticide Residues of WA Apples

Keywords: MRL, pesticide, residue, apple

Abstract: The Washington Tree Fruit Research Commission (WTFRC) has conducted annual pesticide residue studies in apple and cherry since 2011 to provide basic data to the tree fruit industry regarding residue levels of commonly used pesticides. This information is used by growers, consultants, and fruit sales desks to help guide management decisions regarding spray programs for fruit being exported to foreign markets which may have more stringent Maximum Residue Level (MRL) tolerances than those allowed for fruit sold domestically.

For these studies, various fungicides, insecticides, acaricides, and plant growth regulators were sprayed at typical rates and timings used by the WA apple industry, as well as in a "worst case scenario" in which products were applied at maximum rates and minimum preharvest and retreatment intervals in an effort to generate the highest possible residues while still following product label guidelines. Applications were made to a high density 'Gala' orchard with an airblast sprayer calibrated to 100 gallons water/acre. Fruit were then sampled at standard harvest timing and submitted to commercial labs for residue analysis.

Results have consistently found that residue levels of nearly all pesticides applied were safely below tolerances set for the United States market by the Environmental Protection Agency (EPA), but many residues have exceeded MRLs for some important apple export markets. Most of these potentially problematic cases have been for countries that have either set their MRLs at the minimum limit of detection for those particular products, or have not posted MRLs for those pesticides, such as India; in those cases, imported fruit in those markets cannot have residues which exceed that nation's default level for MRLs, which is frequently a very stringent limit such as 0.1 or 0.01 ppm.

In response to apple industry interest, our 2025 trial protocols included the plant growth regulators ethephon, prohexadione calcium, and the newly registered chemical thinner, metamitron. No residues of these products were detected when applied at typical industry rates and timings, but residues exceeding MRLs for key export markets were found when those products were applied in our "aggressive" protocol.

Reports of all WTFRC pesticide studies on apple and cherry since 2011 are available at www.treefruitresearch.org.

Proposal Title: Evaluation and Optimization of Robotics Plus and VariMas 3 Sprayers
Report Type: Continuing Report (No cost extension of proposal).

Primary PI: Gwen Hoheisel
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Cooperators: Steve Saunders, Robotics Plus, NZ; Han Smits, Munckhof Mfg, Canada; Keith Veselka, North West Farm Management LLC., Yakima WA

Project Duration: 1-Year

Total Project Request for Year 1 Funding: $53,443 WSU & WTFRC

Other related/associated funding sources: Cost-share by each manufacturer
Funding Duration: 2024 - 2025
Amount: approximately $70,000
Agency Name: Munckhof Mfg. Canada and Robotics Plus, NZ
Notes: Sprayer being donated; at least 3 weeks of 2 FTE for training, optimization, and data collection; expected 7 trips to PNW.

WTFRC Collaborative Costs:

Item	**2024**
Salaries	$1,200.00
Benefits	
Wages	
Benefits	$480.00
RCA Room Rental	
Shipping	
Supplies	
Travel	$425.00
Plot Fees	
Miscellaneous	
Total	$2,105.00

Footnotes:
Salaries: 54 hours of help for collecting data 4 times.

Travel: Wenatchee to Mattawa or Othello (160 miles * 4 trips * 0.665) = $425

Budget 1
Primary PI: Gwen-Alyn Hoheisel
Organization Name: Washington State University
Contract Administrator: Anastasia (Stacy) Mondy
Telephone: 916-897-1960
Contract administrator email address: anastasia.mondy@wsu.edu

Item	2024
Salaries	$22,813.00
Benefits	$8,009.00
Wages	$5,350.00
Benefits	$535.00
RCA Room Rental	
Shipping	
Supplies	$7,192.00
Travel	$7,439.00
Plot Fees	
Miscellaneous	
Total	$51,338.00

Footnotes:

Salaries and Benefits: 1 month of field prep and collection + 1 month of lab analysis time for technician; 2.5 weeks time Hoheisel for lab, field, optimization, tracer collection, and Extension; 2.5 weeks time Khot for field, lab, optimization, and tracer collection. Benefits range from 25.6-44.4% per person.

Wages and Benefits include 9 weeks at 0.5 FTE summer salary for graduate student to help with field prep, optimization, and laboratory analysis. Benefits at 10%.

Supplies include: Swath gobbler $4142 includes tax; misc field supplies (cash register tape, WSP, cut plastic cards, bags, labels, gloves, zip ties, clips, general field supplies, spray nozzles, nutrasol, etc.) $1100; Blue Dye and pyranine tracer $1500; misc lab supplies (vials, ethanol, chem-wipes and general lab supplies) $450

Travel includes: Gwen/Maia (technician) share 20 trips to field * 150 miles RT * $0.655/m.

Lav/Datta (Grad student) 15 trips to field * 150 miles RT * $0.655/m; Trips to the field are for field prep, sprayer set-up, data collection, field clean up. Exact location is yet to be determined as we have to work with manufacturers and cooperators. Two companies in the Mattawa and Othello area have expressed interest.

$2000 for both Robotics Plus and Munckhof to come special for the field trial and optimization and all other travel is included in the cost share for the companies

Objectives

Objective 1. With manufacturers and cooperators, optimize sprayers and conduct deposition studies;
Objective 2. Document best management practices (BMPs) and limitations/incentives to technology adoptions; and
Objective 3. Summarize results for outreach.

Significant Findings

Overview and additional work

- There were significant delays in the fully operational sprayers for 2024. However, in 2024 researchers, orchard managers, and manufacturers worked collaboratively to conduct field trials for Robotics Plus on V-trellis apples. In winter of 2025, laboratory trials were done
- The 3-row Varimas Munckhoff sprayer was transported out of WA by the manufacturer with the intent of replacing it with a single-row intelligent sprayer. We will work with the manufacturer for one more year to get a machine imported, but will then end the grant.

Robotics Plus Prospr

FIELD TRIAL

- There were 2 speeds (5 and 3mph) and 2 rates (100 & 80 GPA) tested.
- Similar and higher deposition was seen in T1 and T3 with the higher rate (100 GPA) of application regardless of speed. Similarly, the treatments with lower rate (T2 and T4, 80GPA) showed similar deposition regardless of speed. Indicating that rate, not speed had more effect on canopy deposition. Increasing speed has the advantage of more timely applications that coincide with critical pest and disease stages as well as labor savings for time in the orchard.
- Of critical importance is to recognize that with the speed change, the fan settings increased at the higher speed. Optimizing the fan speed to canopy shape will be critical for good coverage.
- Tree row volume calculations indicated the smaller canopy could be appropriate for the lower rate of 80GPA instead of the grower standard of 100GPA. The lower deposition at lower rates is expected, and while significant, it is unknown if it is biologically important, and further bioassays should be done.
- Both aerial and ground drift was very low in all treatments with the highest depositions being one row from the sprayed row and canopy deposition was 24 to 64 times higher than any off-target drift that occurred one row over. This stresses that smaller fans with less air volume are well suited for high-density orchards with smaller canopies.
- Deposition was less in many of the middle areas of the canopy regardless of rate and speed. It was thought that the fans were too close and interfered with each other, but the manufacturer discovered that wires were switched on one set of fans. We tested airflow and deposition in the lab with the corrected configuration.

LAB TESTING

- The crossed wires on one set of fans, led to improper application rates in the field.
- When corrected the air patterns and deposition were highly symmetrical on both sides of the canopy. The deposition was more even from top to bottom.

Methods

For both machines, there is a laboratory study to optimize air and field deposition trials to assess coverage. Protocols will follow ISO methods for evaluation of sprayers.

Obj. 1. With manufacturers and cooperators, optimize sprayers and conduct deposition studies

Deposition Trials for either sprayer:

In each replicate run, plastic cards (2×2 inch) will be placed in the appropriate zone and sprayed with a biodegradable fluorescent tracer dye (Pyranine 10G, Keystone Inc., Chicago, IL). Metrological data such as wind velocity, wind gust, air temperature and relative humidity will also be collected at 1 Hz sampling rate using all–in–one weather station (Model: ATMOS 41, METER Inc., Pullman, WA). Rules outlined in ISO 22522 standards (ISO, 2007) will be followed to collect such data. After spray application, the plastic cards will be left for 10-15 min to dry. Each of the plastic card will then be collected in a labelled and color–coded plastic bag (165×149 mm) to identify the location of cards. The deposit samples will be then kept in a cooler in the field, before moving the samples to a cooling chamber (around 35.1°F [1.7 °C]) in the lab.

The plastic cards will be analyzed for deposition data by fluorometry analysis. A known volume of deionized water will be added to the plastic bags containing the deposit sample. The sample bags will then be shaken for 1-min using a mechanical shaker, to thoroughly mix the tracer into deionized water in the sample bags. The rinsate will be transferred into two 10–ml cuvettes (Fisher Scientific, Hampton, NH). Each cuvette will be analyzed twice for fluorescence intensity using the fluorometer (Model: 10AU, Turner Designs, San Jose, CA). The details of the fluorometry analysis has been previously reported (Salyani, 2000; Khot et al., 2012). The data will be reported as deposition in µg/cm^2.

Robotics Plus:

The Prospr sprayer manufactured by Robotics Plus is a fully autonomous, hybrid electric and diesel platform with 2 real-time kinematics (RTK) corrected GPS sensors and 3D LiDAR sensors for mobility. Designs can vary, but for tested machine, there are eight fans total with four fans facing each side of the sprayer (Figures 1A,B) and the distance from the canopy varies because of the V-trellis and offset placement from the top to bottom (Figure 1C). The top and bottom fans can have speed adjusted independently with each fan operating four Albuz ATR80 brown and four ATR80 yellow nozzles. The sprayer is controlled remotely and set-up initially with run parameters of rate, speed, and pressure. The sprayer can maintain the rate as speed alters slightly, but the variable rate application within the block is not yet a feature on this tested model.

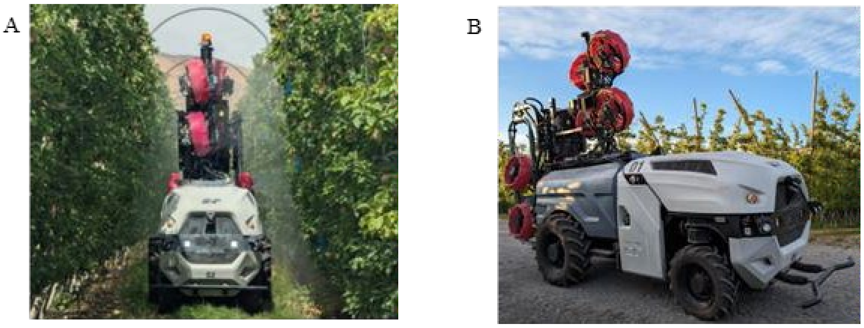

Figure 1. Front (A) and side (B) view of the commercial autonomous Robotics Plus Prospr tested in a V-trellised apple orchard

To quantify and optimize air volume passing through the canopy zones for a set fan speed, a mast with three sonic anemometers mounted 1.5, 2.3, and 3.8 m AGL was secured on an electric all-terrain vehicle. This was driven in tandem with the sprayer in the adjacent row to quantify the air speed passing through the sprayed canopy row. The target was to maintain air volume coming through the canopy at 1 to 3 mph and no gusts greater than 5 mph. The speed (rpm) of upper and lower fans on the sprayer unit were tested and optimized for four spray treatments which consisted of combinations of two application rates and two sprayer travel speeds (Table 1).

Treatment	Application rate (GPA)	Travel speed (mph)	Top fan speed (rpm)	Bottom fan speed (rpm)
T1	100	5	2300	2000
T2	80	5	2300	2000
T3	100	3	2100	1900
T4	80	3	2100	1900

Deposition trials for canopy and in-field drift were performed in late summer with a full canopy on a V-trellis (Fig 2).

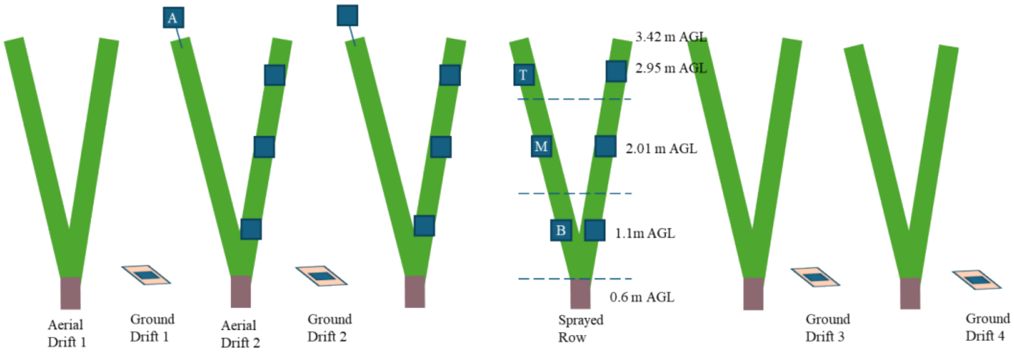

Figure 2. Schematic representation of deposition, and drift sampler locations (~3, 6, 9 feet above ground in the canopy and 1-foot above the canopy for drift rows). in the experimental v-trellised apple orchard plot.

VariMas 3: All pre-trials and deposition trials will be centered around following tests.
<u>Test for optimal settings:</u> According to the manufacturer, the VariMas 3 sprayer was operated in WA orchards in 2023 trials with settings common in the Netherlands that show optimal coverage and 99% drift reduction. Because orchard conditions in Northern Europe are often different than Washington with factors like humidity, sun intensity, and crop protection material can impact deposition. Additionally, we will be going from a 3-row to a single row sprayer. Therefore, it is important to measure the sprayer control (i.e., standard Dutch settings) with different nozzles, PTO speeds, and rates. The VariMas 3 control uses air induction nozzles, 5.5 mph, 300 rpm of PTO, and 70 GPA. Our treatments will consist of stepwise assessments of one variable at a time to see improvements in spray coverage (%). First, air speed will be tested with handheld anemometers at different canopy zones to determine if PTO should be increased for higher speeds. Second, the orchard canopy will be assessed for LWA and then rates will be estimated using Orchard Max application. Third, the speed will be adjusted. After each step, we will operate the sprayer with the WSP for rapid assessments. Once optimized, we will do a replicated deposition trial with spray collector cards in the 2 spray rows (one full spray row and one half sprayed row) and 2 rows adjacent to assess in-field drift. Pertinent methods are detailed in previous section.

Laboratory trials:
At WSU-CPAAS, we measured the vertical air (mph) and liquid spray (gpm) across the spray boom. The WSU Smart Patternator equipped with anemometers and liquid measuring devices (Bahol et al. 2020) was used for this evaluation. Air and liquid were be measured vertically every 12” inches. These patternator tests will be performed in a single location equivalent to canopy distance.

Obj. 2. Document BMPs and limitations/incentives to technology adoptions

Research shows that there are generally five aspects that influence technology adoption (Rogers et al. 2014). During Obj. 1 activities, we will document about the machine and operations from manufacturer, operator driving the sprayer, block manager, and orchard manager. Comments, responses to pointed questions, and observations on operations will be classified into five categories (italicized below) as in Diffusions of Innovation (Rogers et al. 2014). This qualitative analysis will be shared with manufacturers and industry through Obj. 3.

Specifically for sprayers, there has to be a *relative advantage* in either economics, regulations, or worker safety. Technology has to be *compatible* with other farm operations and layers of management. There has to be minimal *complexity* such that it doesn't fail in the ruggedness of operations and operator understanding. Technologies have to be *observed* and trialed. While this is the role of Extension and manufacturers, *trialability* is critical for successful adoption and requires physical use of the equipment.

Obj. 3. Summarize results for outreach

Outreach to growers: Results from this project will be synthesized into peer-reviewed university Extension publications, newsletter articles (e.g., Fruit Matters), and technology know-how videos to be ported on the WSU website. A field day will be planned with these and other technologies, (e.g., in tandem with Smart Orchard Field Day), to educate sprayer optimization and utilization of new technologies. Seven similar field days were offered for grape, blueberry and hops growers in 2023. Therefore, the agenda and manufacturer cooperators are aligned and in agreement with this continued education.

Outreach to regulatory bodies: There is much interest in both the federal and WA State government on ways to improve pesticide application. Hoheisel is a member of the WA state SB5550 committee which is supposed to suggest relevant resolutions to application technology problems. Both Khot and Hoheisel, have participated with the Center for Regulatory Science (CERSA) which is pulling together interested parties to discuss how to improve regulations and communications among manufacturers, registrants, and the EPA. These results in written or presentation form will be shared with all above groups.

Results and Discussion

Both aerial and ground drift was very low in all treatments with the highest depositions being one row from the sprayed row (Fig 3). Canopy deposition was 24 to 64 times higher than any off-target drift that occurred one row over, indicating that the sprayer was adjusted well with the majority of the spray hitting the intended target. This stresses that smaller fans with less air volume are well suited for high-density orchards with smaller canopies.

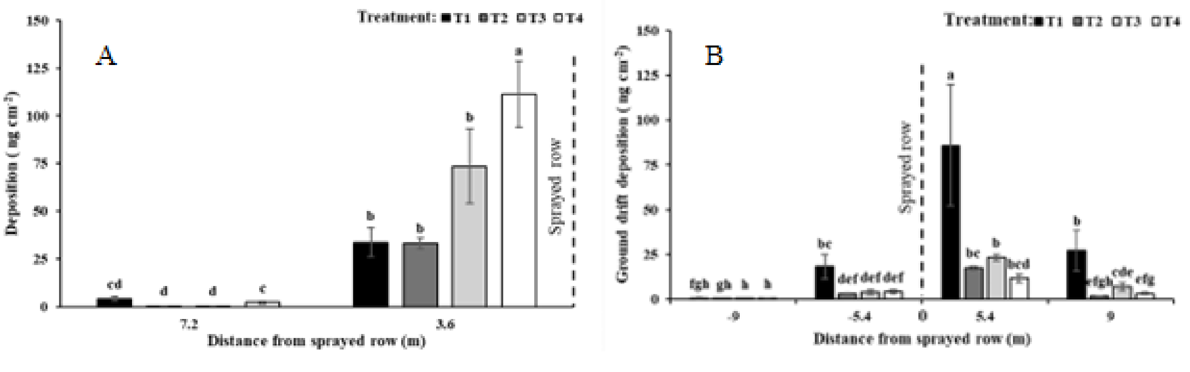

Figure 3. Aerial drift quantified for different sprayer treatments (A). Ground drift quantified at 17 and 30 ft (5.4 and 9 m) on either side of the sprayed row (B).

Testing combinations of speed and rate is of high importance to growers. Tested autonomous sprayers have the advantage of driving at higher speeds (5mph) than a typical tractor-pulled sprayer. But adjusting and calibrating for that increased speed is critical. Similar and higher deposition was seen in

T1 and T3 with the higher rate (100 GPA) of application regardless of speed. Similarly, the treatments with lower rate (T2 and T4, 80GPA) showed similar deposition regardless of speed. Indicating that rate, not speed had more effect on canopy deposition. Increasing speed has the advantage of more timely applications that coincide with critical pest and disease stages as well as labor savings for time in the orchard.

Of critical importance is to recognize that with the speed change, the fan settings increased at the higher speed. Also of importance for more uniform deposition is the distance from the canopy and the ability to adjust the top and bottom fans independently. Our trial changed the fan settings significantly from grower operation to achieve moderate air flow on the opposite side of the canopy. If fans were all identical, it does not account for the offset placement on the machine and increased distance to the canopy. Additionally, changing speed without changing fan speed, could lead to poor coverage. These results show the need for growers to calibrate their air per canopy architecture at least once by either using a low technology flagging on the opposite side of the canopy or using a hand-held anemometer on the opposite side of the canopy so that fan settings can be optimized and programmed into the sprayer controls.

Tree row volume calculations indicated that the smaller canopy architecture could be appropriate for the lower rate of 80GPA instead of the grower standard of 100GPA. The lower deposition at lower rates is expected, and while significant, it is unknown if it is biologically important and further bioassays should be done. Deposition was less in many of the middle zones (Fig 4) regardless of rate and speed. The fans may be interfering with each other, but the manufacturer discovered that one set of fans had the wires crossed and improper rates were coming from the fan.

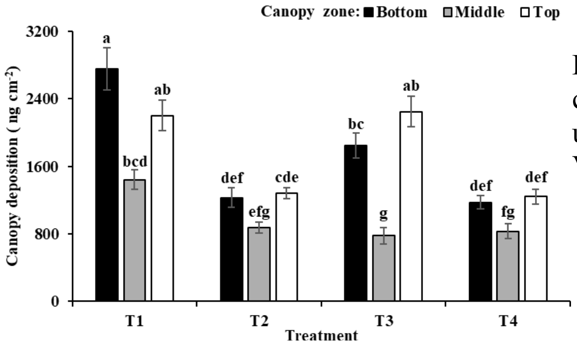

Figure 4. Spray deposition quantified in different canopy zones during spray treatments conducted using Robotics Plus Prospr autonomous sprayer in V-trellised orchard.

Laboratory trials performed after wires were corrected showed a much more symmetrical pattern both on the left to right side of the sprayer as well as vertically from lowest to highest heights (fig 5).

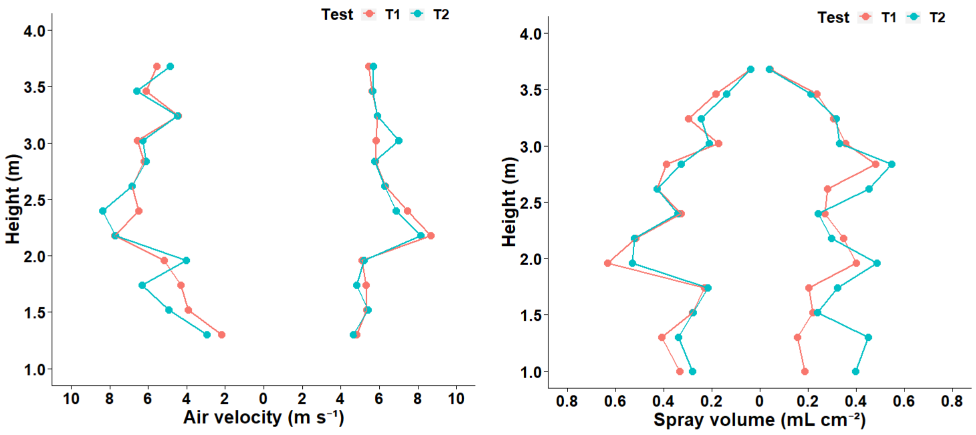

Figure 5. Plots depicting air (left) and spray (right) pattern for two tests cumulative air pattern on the left and right side of the sprayer

Proposal Title: Best practices for releasing lacewings in apples
Report Type: New Proposal

Primary PI: Rebecca Schmidt-Jeffris
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Cooperators: Tianna DuPont (WSU); Steve Arthurs (BioBee); Growers: Teah Smith (Zirkle), Mike Brown (Gebbers), Chris Peters (Gilbert); Chuck Weaver and Aaron Avila (GS Long)

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $26,174
Total Project Request for Year 2 Funding: $27,046
Total Project Request for Year 3 Funding: $27,919

Other related/associated funding sources:

Awarded	
Funding Duration:	in-kind match for this specific project, 2024-2026
Amount:	>$15,000
Agency Name:	BioBee
Notes:	In-kind match of commercial insectary lacewings as needed for Obj. 1a, Obj. 2, Obj. 3
Awarded	
Funding Duration:	in-kind match for this specific project, 2024-2026
Amount:	~$7,500
Agency Name:	Zirkle Fruit
Notes:	In-kind match of commercial insectary lacewings as needed for Obj. 1b
Requested	
Funding Duration:	April 2024 – March 2027
Amount:	$350,000
Agency Name:	Western Sustainable Agriculture Research and Education (WSARE)
Notes:	The project outlined below is project for which we submitted a funding request to WSARE. The WSARE proposal 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.

WTFRC Collaborative Costs: None

Budget 1
Primary PI: Rebecca Schmidt-Jeffris
Organization Name: USDA-ARS
Contract Administrator: Mara Guttman
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Supervisor: Rodney Cooper
Supervisor email address: rodney.cooper@usda.gov

Item	**2024**	**2025**	**2026**
Salaries[1]	$19,829.00	$20,490.00	$21,151.00
Benefits[2]	$6,345.00	$6,557.00	$6,768.00
Wages	$0.00	$0.00	$0.00
Benefits	$0.00	$0.00	$0.00
RCA Room Rental	$0.00	$0.00	$0.00
Shipping	$0.00	$0.00	$0.00
Supplies	$0.00	$0.00	$0.00
Travel	$0.00	$0.00	$0.00
Plot Fees	$0.00	$0.00	$0.00
Miscellaneous	$0.00	$0.00	$0.00
Total	$26,174.00	$27,047.00	$27,919.00

Footnotes:

[1] Salary request is for a GS-5 Step 1 biological science technician to conduct field work, process samples in the lab, and assist the lead technician (funded by WSARE) with the molecular work (6 months/year at 100% FTE, annual salary $39,657). Each following year includes a 4% cost of living adjustment and a within-grade step increase.

[2] The benefits rate for the technician is 32%.

Objectives

1. **Determine which method of releasing lacewings results in the greatest establishment and pest control**
   a. Compare species, life stages, and cards versus loose eggs
   b. Compare drone to ground releases at orchard-scale
2. **Determine which lacewing release ~~rate~~ timing is most effective for aphid control**
   We have determined that release timing is likely the most critical factor for success and are altering this objective accordingly.
3. **Determine the effects of organic pesticides on insectary-reared lacewings**
   a. Determine acute toxicity of organic pesticides to lacewings
   b. Determine the duration that field-aged residues remain harmful

Significant Findings

- Our work now focuses on releasing ***C. rufilabris***, which is currently the only lacewing produced in enough quantity to meet industry demand.
- Results from prior work and this project in 2024 indicates that lacewings applied as **larvae are the most effective**. Successful releases resulted in a 40-50% decrease in aphids and an 80% decrease in mealybugs.
- Lacewings released as **egg cards or larvae have the highest recovery rates**, possibly indicating higher survival.
- However, **egg cards are not recommended**. Lacewing eggs on cards are often heavily predated on by orchard predators and can result in cannibalism from hatching lacewings. The cards were also associated with mealybug pest increases in a 2024 trial, possibly due to recruitment of ants to the area; ants attack other orchard predators.
- Commonalities in trials where releases resulted in pest reduction are: (1) **<0.1 aphid colonies/infested leaves per shoot**, (2) **evening temperatures ≥35°F**, (3) at least one lacewing larvae found post-release, and (4) no applications of harmful pesticides post-release.
- Based on laboratory work and field trials where pesticides were used before and during sampling, most organic pesticides we tested can be applied one day before release. More data is needed on Sonata, Instill-O, OxiDate, Cyd-X, Previsto, Serenade, and Problad Verde. **Lime sulfur causes high levels of mortality even when applied 7 days before release**.
- **Entrust, lime sulfur, and OSO** should not be applied for 14-21 days following a release.
- When lacewing larvae or hatching eggs were released by drone using a flight path perpendicular to the orchard rows, larvae recovery did not differ from ground releases. This indicates that **drone releases are an equally effective delivery method**.
- We attempted to use hatching eggs in the drone trial by holding eggs for ~2.5 days at 80°F in buckwheat hulls. Larvae recovery post-release was much lower than in 2024 (when larvae were used). Additional research is needed to determine the best conditions for hatching eggs into larvae.
- In 2025, we determined that the commonly used COI Fulmer primers with Takara TAQ regularly yield unusable DNA sequences for confirming lacewing identification and are developing a new protocol to improve sequencing results. We are also using this project to develop new primers for the detection of all apple aphid pests in molecular gut content work.

Methods

Obj 1a. The study was conducted at three organic, commercial orchards in 2024. Because the results from 2024 (and prior years) were fairly conclusive regarding life stage and method, this trial was only conducted in Year 1. The experimental design was a randomized complete block design, with location in the orchard the blocking factor (pest pre-counts were too low to use). Treatments were: (1) *C. rufilabris* loose eggs, (2) *C. rufilabris* egg cards, (3) *C. rufilabris* larvae, (4) *C. plorabunda* loose eggs, (5) *C. plorabunda* egg cards and (6) no-release control. Treatments were applied once to five ~0.15 or 0.25-acre replicates per treatment, with ≥50 ft between plots. Releases occurred on 10 April (Rock Island), 19 April (Mattawa), and 26 April (Pateros). We noticed that the egg cards at the Pateros location appeared to have undergone a freeze event (dead eggs) and that initial recovery of larvae was lower than expected, so we conducted a second release at this location on 31 May. For the second release, we only re-released in the most promising treatments (#4 and #5). Treatments were monitored once weekly for six weeks, with Week 5 monitoring skipped at some locations. In Pateros, we monitored Treatments 4-6 for an extended period to account for the second release. Eggs were released at a rate of 100,000/acre and larvae at 20,000/acre. We attempted to hold eggs at room temperature prior to release to speed their rate of hatch, but this was logistically challenging as due to different release dates coordinated with the growers and different stages of maturity of the eggs when they arrived.

Sampling occurred on 12 trees (4 trees×3 rows) in plot centers. We counted the number of infested leaves (green apple aphid, rosy apple aphid) or distinct colonies (woolly apple aphid) on three shoots per tree for each of the 12 trees (Markó et al. 2013, Orpet et al. 2019). Mealybugs on beat trays were counted at all locations, and were also sampled with burlap traps (Grasswitz & Burts 1995) and 1-foot shoot samples (5/plot) at the Pateros orchard, which had high mealybug pressure. We used beat tray samples (12/plot) to count released lacewings and ants and sticky cards (2/plot) for adult lacewings. Lacewings collected from beat trays were immediately placed in molecular grade ethanol and frozen. We are also testing different methods for counting aphids: at the Pateros location, we also counted the number of aphids per beat tray sample.

While it is known that lacewing egg cards cannot be used in orchards with high ant populations, whether predation by other insects or cannibalism is a significant issue with the cards has not been investigated. In 2025, we monitored *C. rufilabris* egg cards in the field (for predation events and cannibalism) and the lab (cannibalism only) using time lapse photography. For the field study, we monitored two sets of ten egg cards attached to trees for one week. In the lab, egg cards placed on pea plants with and without available prey (5 cards each) were monitored for one week. Analysis of the videos are ongoing.

Molecular methods. All collected lacewings (taps, sticky cards) will be sequenced using COI primers, which we have determined can distinguish released from resident lacewings. We will also collect lacewing larvae directly from each shipping container prior to release. This provides us with a point of comparison for lacewings collected in field – the genetics of captured lacewings are compared to the genetics of the "from the bottle" lacewings. In the analysis, individuals clustering with the "from the bottle" lacewings are related and therefore from the release treatment. We are currently testing alternative primer and TAQ combinations to determine which protocol most consistently yields quality DNA sequences.

Lacewing larvae collected from the field will also undergo molecular gut content analysis to determine what pests are consumed by released lacewings and whether they consume resident natural enemies. We have determined that our three aphid pest species do not amplify well with our usual COI barcoded molecular gut content protocol and that they are detected at rarely low rates with a "generic aphid" primer (Chapman et al. 2010). We are in the process of developing new primers that are capable of detecting low levels of DNA of all apple aphid pests.

Obj. 1b. This trial (2024-2025) was conducted in five 'Granny Smith' blocks, which were divided into thirds, resulting in three ~4-acre plots per block. Each block third received a treatment: (1) drone release, (2) ground release, or (3) no-release control. The first 2024 release occurred on 10 April, *C. rufilabris* eggs were released at 20,000/acre. We noticed that the eggs were still very immature (green) when released and subsequently, there was no recovery of released lacewings. Therefore, we conducted a second release on 10 May 2024, this time of 20,000 larvae/acre. The drone pilot (Weaver) also indicated that we should try a different flight pattern; our first release was directly over the orchard rows, the second release was perpendicular. In 2025, due to the high cost of larvae, we released lacewing eggs after holding them at 80°F for 2.5 days in buckwheat hulls. Unfortunately, this resulted in a low larvae recovery and no pest control (see Results). For both trials, we conducted sampling at six fixed "stops" even distributed across the block. At each stop, we collected four tap samples (mealybugs, aphids, lacewings, ants), counted aphid colonies on six shoots, and replaced one sticky card.

Obj. 2. This objective was modified to test release timing, which we have concluded is likely the most important factor for release success. This trial was conducted at the organic Pateros and Rock Island orchards used in 2024 and in a conventional orchard in Moxee. Initially, the conventional orchard was to be minimally managed, but the grower did apply conventional insecticides during the trial. Plots in all trials were 0.15 acres, replicated five times per treatment. The treatments consisted of two sequential releases of 20,000 *C. rufilabris* larvae per acre, spaced two weeks apart (Table 1).

Each of four timing treatments were initiated one week after the previous and compared to a no-release control. In Pateros, we also tested an additional treatment of mealybug destroyer larvae (Koppert) released at a rate of ~5,000/acre (1 capful/tree) on 23 May and 20 June. Sampling was as described in Obj.1. We attempted to block treatments within the Pateros orchard based on pest pressure in prior years, but this resulted in plots of the same treatment being clustered close together within the orchard block; it appears that this affected the trial results. We determined that there is a strong directional effect on pest pressure in that block, but that it differs between

Table. 1. *C. rufilabris* larvae/tray, post-release monitoring period.

Release		Release Dates	Eggs	Cards	Larvae
2025	Pateros #1	16 May + 20 May			0
	Pateros #2	23 May + 6 Jun			0.006
	Pateros #3	30 May + 13 Jun			0
	Pateros #4	6 Jun + 20 Jun			0.003
	RI #1	30 Apr + 14 May			0.019
	RI #2	7 May + 21 May			0.014
	RI #3	14 May + 28 May			0.008
	RI #4	21 May + 4 Jun			0.003
	Moxee #1	8 May + 22 May			0.004
	Moxee #2	15 May + 29 May			0
	Moxee #3	22 May + 5 Jun			0
	Moxee #4	29 May + 12 Jun			0
	Drone	12 May	0.004		
	Ground	12 May	0.015		
2024	Pateros #2	31 May		0.008	
	Pateros #1	26 Apr	0	0	0.004
	Mattawa	19 Apr	0.008	0.017	0.008
	RI	10 Apr	0	0.004	0.017
	Drone	10 Apr	0		
	Ground	10 Apr	0		
	Drone	10 May			0.035
	Ground	10 May			0.035
2023	Desert Aire	1 May	0	0.008	0.013
2022	Desert Aire	4 May	0.004	0.05	0.029
2021	Pateros	5 May	0.008		0.029

species. Therefore, in future work, we will only block treatments based on location within the orchard, both N-S and E-W. In 2026, this trial will be repeated in the Pateros and Rock Island orchards.

Obj. 3a. The effects of 17 organic pesticides were compared to a control (water). We tested eggs of *C. plorabunda* and *C. rufilabris* via two exposure methods: direct application and on residues. In the direct application, treatments were applied as 2 mL of solution (highest labelled rate) using a Potter spray tower, then eggs were individually transferred to single wells in a 96-well plate. In the residue test, treatments were pipetted into the wells of each plate, allowed to sit briefly, then pipetted back up. Plates were then placed in a fume hood until residues dried. This resulted in ~96 replicates per treatment (occasionally, wells were unintentionally not loaded). We monitored egg hatch daily for five days.

We also tested the combined exposure of contact+residue on *C. rufilabris* larvae (*n*=30 replicates per treatment). Larvae were treated and held in 1-oz plastic portion cups, using the Potter tower. A small piece of water-soaked wick and *Ephestia* eggs were provided and replenished every 2 days for moisture and food. Lacewings were monitored daily for mortality until they reached adulthood. We observed that some lacewings did not form a cocoon around their pupae (naked pupa) and recorded this information. In a separate assay, we monitored the effects of 48 h exposure on movement (Ethovision XT14). In 2026, we will assess effects on predation.

Obj. 3b. Because OSO, Entrust, and lime sulfur caused the highest rates of mortality in the lab assay (see Results), we focused on these pesticides for testing field-aged residues. We dipped limbs of individual trees in mixed pesticide solution on 14, 18, and 20 August so that 7, 3, and 1-day old residues were available simultaneously. Leaves from these limbs were used on 21 August to make leaf disk arenas in 1-oz portion cups; one leaf disk was embedded in agar inside the cup and one *C. rufilabris* larvae was placed on the disk. Lacewing larvae were provided with food and moisture as previously described and monitored daily until reaching adulthood. Because only the leaf disk contained pesticide residue, we assessed repellency by recording the location of the larvae at each observation point, and the location where the cocoon was formed.

Cost Comparison. At the conclusion of the study, we w ill also perform a cost comparison for any treatment found to decrease pest abundance relative to the control. Cost estimates will include lacewing purchase, shipping, and labor/drone pilot fees (estimate provided by the grower). Cost will be scaled relative to pest control (percent reduction/dollar spent).

Outreach. Intensive outreach efforts are entirely funded by WSARE:

- Training flip chart, dual language handout, and pocket flip book (2026-2027)
- Website updates (ongoing): https://treefruit.wsu.edu/crop-protection/opm/lacewings/
- Field Days (2026)
- Intensive session to be held on Apple Day (20 Jan 2026) that will include project research updates, a grower experiences panel, an overview from the insectary industry on maximizing release success, and the effects of apple netting on natural enemies
- Fruit Matters Newsletters: https://treefruit.wsu.edu/article/current-guidelines-for-releasing-natural-enemies/
- Instructional videos: how to scout completed (https://treefruit.wsu.edu/videos/lacewing-and-aphid-scouting/); checking order quality filmed, currently in final stages of editing; how to conduct releases (2027)
- Presentations on trial results were presented at five grower meetings in 2025 and scheduled for two meetings in 2026

Results and Discussion

Obj. 1a. We are currently conducting the molecular work to determine which of the *C. plorabunda*-group larvae collected from our trials are released versus resident individuals. As a preliminary measure of recovery, we used the number of *C. plorabunda*-group larvae in treatments where they were not released as a "correction factor", subtracting this value from the average number captured in the plots where they were released. Estimated recovery (per tray, for four weeks post-release) of *C. plorabunda* larvae in loose eggs treatments was: 0.008 in Pateros, 0.007 in Rock Island, and 0.027 in Mattawa. Estimated recovery of *C. plorabunda* larvae in egg card treatments was: 0.013 in Pateros, 0.007 in Rock Island, and 0.019 in Mattawa.

Because the egg/egg card treatments were held for variable amounts of time after shipment, each trial differed in the relative recovery rate of these treatments relative to the *C. rufilabris* larvae treatment (Table 1). When the egg treatments could be held until substantial hatching was observed (Mattawa 2024), recovery of loose eggs was the same as recovery of larvae. However, when releases needed to be conducted soon after receiving the shipment (Rock Island 2024), more larvae were recovered from the larvae release treatment compared to the loose egg and egg card treatments. Across all of our prior projects, larvae and egg card releases have had the highest recovery rates (Table 1), potentially indicating better survival and delivery to the target.

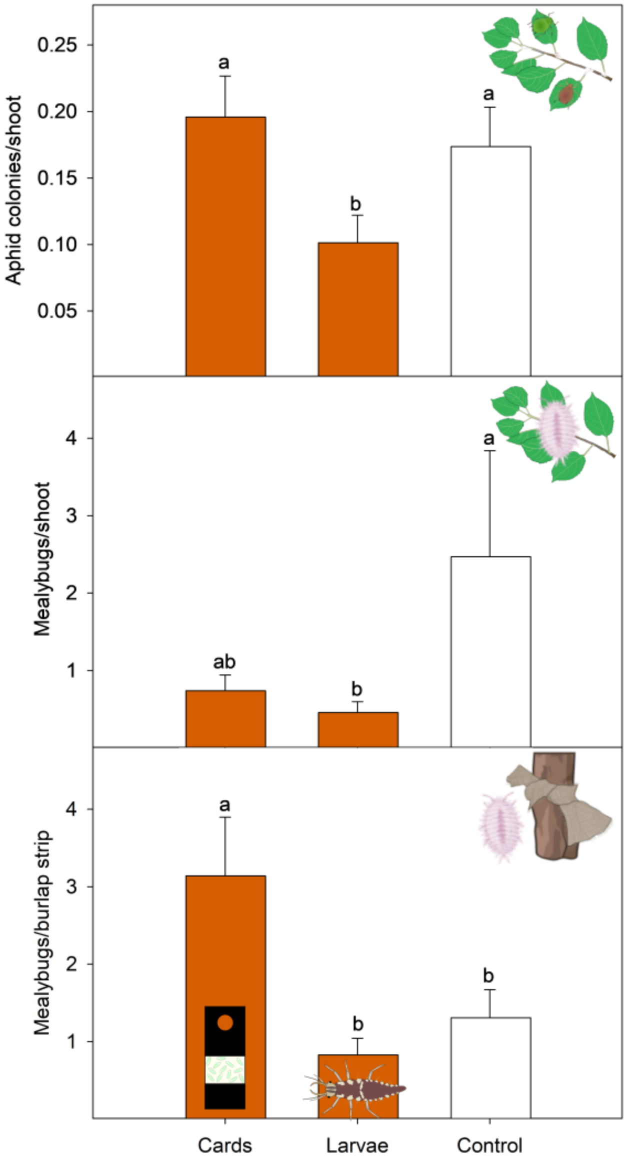

Fig 1. Aphid and mealybug seasonal sums in Pateros 2024 trial (1-8 weeks after second *C. rufilabris* release).

We did not observe any differences in pest counts between treatments in the 2024 Rock Island or Mattawa trials, likely due to releasing too early. Additionally, at the Rock Island location, a spinosad application for thrips was associated with reduced counts of both resident and released lacewings in the following weeks.

In 2024 Pateros, the lacewing larvae release reduced aphids and mealybugs captured in traps (Fig. 1). However, the egg card treatments did not significantly reduce aphids and appeared to increase mealybugs (Fig. 1). Of the three trials, this location had by far the most ants. We speculate that the egg cards may have attracted ants into the release plots and they may have attacked other natural enemies, causing the mealybug populations to increase; the week following the second release, ants were over twice as abundant in the egg card plots compared to the control. Based on this trial, it appears that releases of lacewing larvae can decrease both aphid and mealybug populations in orchards, but that in some situations, egg cards may increase pest populations.

In our egg card monitoring study, many different natural enemies found and consumed the lacewing eggs on the cards, including ants (Fig. 2). Cannibalism by hatching lacewings was also observed.

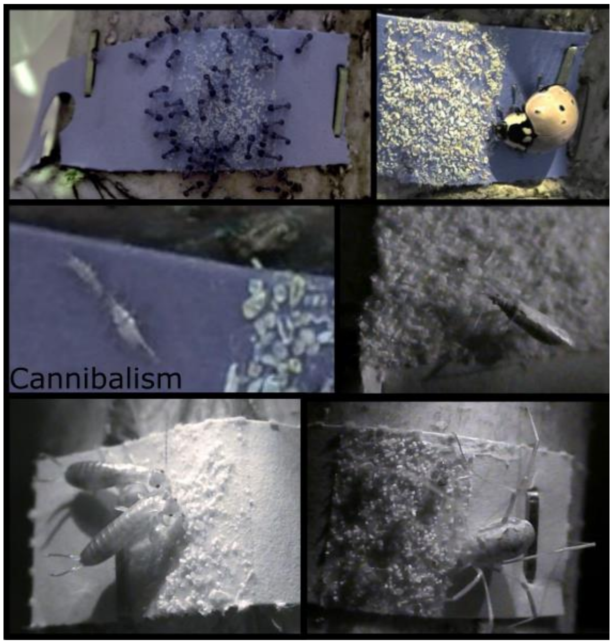

Fig 2. In-field predation of lacewing eggs on cards and cannibalism.

Obj. 1b. In both years of the study, flying a drone perpendicular to orchard rows resulted in lacewing recovery rates that did not statistically differ between the ground and control treatment (Fig. 3). In 2025, using eggs to reduce trial costs (unable to obtain material donation this year) seemed to cause poor recovery (Fig. 3, Table 1). Ideal conditions for hatching eggs and turning them into larvae need to be determined to reduce release cost. No differences in mealybug or aphid abundance were observed between treatments in either trial. For 2026, we will reduce plot sizes to 0.75 acres, which will allow for "typical" drone flight patterns, reduce the cost of larvae, and should also decrease inter-plot variability (which makes it difficult to detect the effects of releases on pests).

Obj. 2. Despite releasing twice (effectively doubling the release rate), recovery rates of larvae were lower than they have been in previous years (Table 1). In 2026, we will test storage conditions of larvae to determine the ideal temperature for overnight holding; we suspect that switching from using a large walk-in cold room in previous to a precision control chamber in 2025 may have inadvertently resulted in holding the lacewings at a cooler temperature (both were set to 50°F). The 2024 drone trial lacewings were held at 55°F (grower's cold storage) and this resulted in an excellent recovery rate (Fig. 3, Table 1), so this will be the first temperature we test. We also did not recover any mealybug destroyers from plots where they were released. In the Rock Island trial, more lacewings were recovered in earlier versus later releases; this may be because as the temperatures increased, the lacewings became more mobile and less likely to be found in the parts of the tree that are sampled by beat tray. The

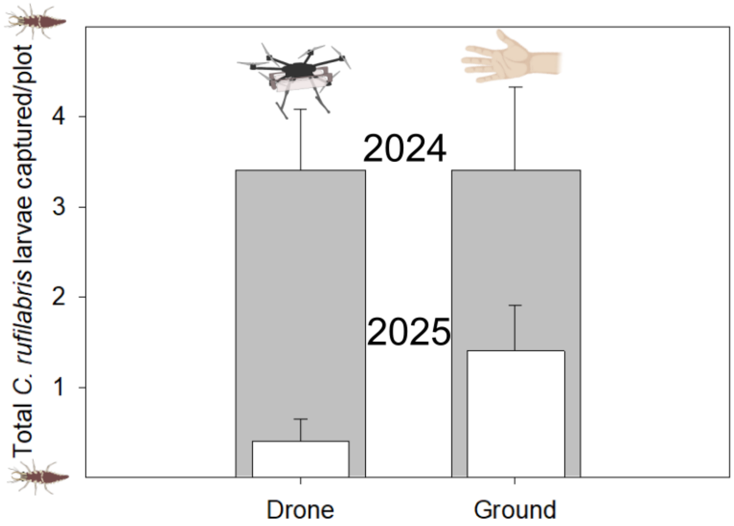

Fig 3. Total number of *C. rufilabris* larvae recaptured per plot in the 2024-2025 drone trials.

amount of larvae recovered did not correspond to whether a release resulted in pest control (see below).

No differences between treatments were observed in pest counts in the Pateros or Moxee 2025 trials. At Pateros, this was attributed to the way that plots were assigned within the orchard (see Methods) and will be corrected by blocking on both a N-S and E-W gradient in 2026. In the Moxee orchard, woolly apple aphid was the primary pest. Populations started extremely high (0.84 colonies/shoot on 8 May) and peaked at 1.0 colonies/shoot (29 May); this is well above where previous releases have been successful (<0.1 colonies/shoot). Colonies were large, with the largest colony in a plot averaging 13.8 cm^2. Woolly populations rapidly decreased between 5 June (0.67/shoot) and 12 June (0.01/shoot) and stayed low for the remainder of the trial (0.002/shoot). We have not yet obtained spray records from the grower to determine if this was due to a pesticide application; this is a conventional orchard.

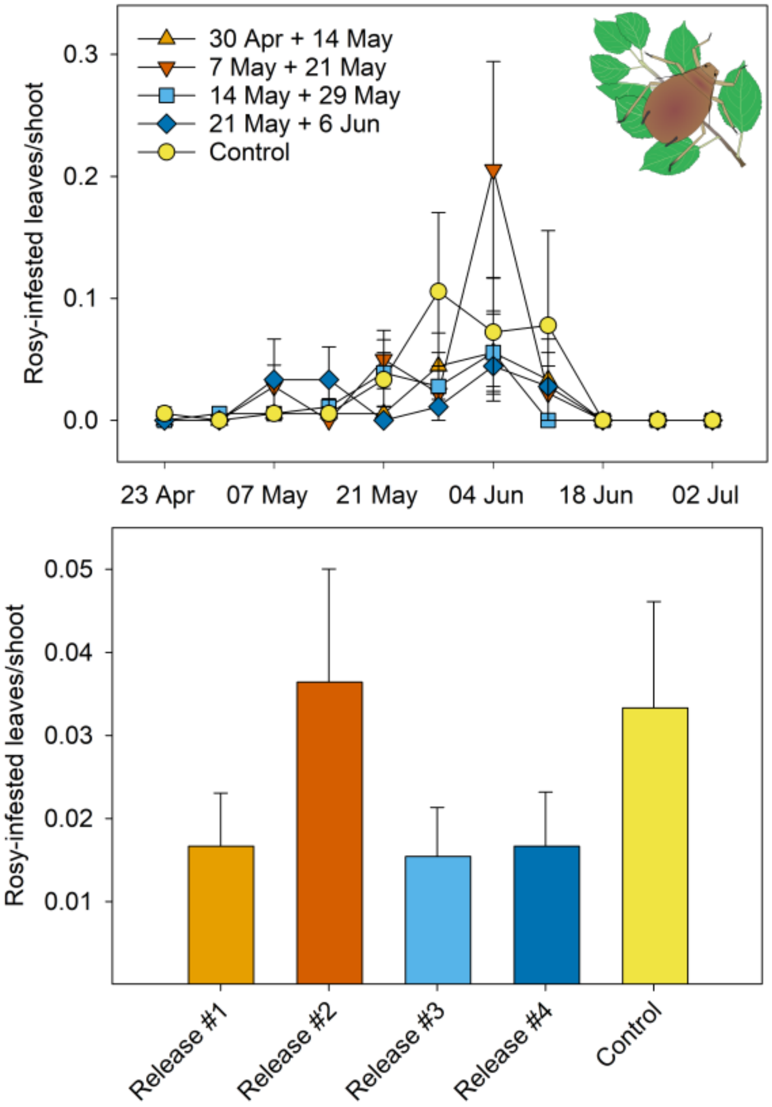

Fig. 4. Rosy apple aphid-infested leaves/shoot during weekly samples and seasonal means from 7 May – 2 July 2025, Rock Island.

Lacewing releases decreased rosy apple aphid by ~50% in all timings in Rock Island, except for the 7 May + 21 May release (Timing #2) (Fig. 4). Larvae recovery was higher in this treatment (Table 1) than Timing #3 and #4, although this may have been due to reduced movement at cooler temperatures and is not necessarily an indicator that more survived. There are no obvious pesticides used immediately before or after Timing #2 dates that were not also used around a release date in another treatment. The number of larvae in a lacewing order can vary dramatically; it is possible that one of the shipments used in Timing #2 was poor.

Obj. 3a. Previous studies have shown that lacewing eggs are much less sensitive to pesticides than larvae. Our results provide an initial indication of this; there were very few pesticides that differed from the control, and of these, the effects were typically moderate (Table 2). Egg hatch was slightly higher for *C. plorabunda* (93%) compared to *C. rufilabris* (87%) (averaging treatments that did not differ from the control). Oil residues (but not direct spray) resulted in near complete prevention of egg hatch in both species. The oil residue never dried, so eggs were essentially

Table 2. Percent of unhatched lacewing eggs (corrected relative to the control) 5d after initial exposure to pesticides as direct sprays or residues. Treatments significantly different from the control are indicated with an asterisk (*).

	C. plorabunda		*C. rufilabris*	
	Direct	Residue	Direct	Residue
Dipel	0	0	0	0
Cyd-X	0	0	0	8
Entrust	0	0	0	2
Serenade	0	0	0	0
Sonata	0	0	10	0
Blossom Protect	0	0	0	10
Previsto	0	0	0	0
Cueva	0	0	0	0
Instill-O	0	15*	2	21*
Lime sulfur	0	3	17*	7
Problad Verde	0	3	5	5
Double Nickel	0	0	0	7
OSO	0	3	7	29*
Regalia	0	0	0	1
Kaligreen	0	10	2	0
OxiDate	1	0	1	25*
Oil	0	100*	0	97*

smothered. This effect would likely be much less extreme in the field and warrants further testing with larvae and residues on leaves. Instill-O residue caused a 15% and 21% reduction in egg hatch compared to the control in *C. plorabunda* and *C. rufilabris*, respectively. Lime sulfur was the only product to reduce egg hatch as a direct spray, and only in *C. plorabunda*. Additionally, OSO and OxiDate residues reduced egg hatch in *C. plorabunda*.

Contact+residue exposure to lime sulfur, OSO, or Entrust increased *C. rufilabris* larval mortality (Table 3). Instill-O, Regalia, and OxiDate exposure appeared to inhibit cocoon formation (Table 3). Many of the pesticides tested slowed *C. rufilabris* development (Table 3); pupal duration increased the most in OSO and lime sulfur (+3 days), followed by Entrust, OxiDate, Instill-O, Cyd-X, and Previsto (+2 days), then Regalia, Cueva, and Serenade (+1 day). Movement was reduced in Instill-O, Blossom Protect, Double Nickel, Problad Verde, Previsto, Cyd-X, and Regalia treatments. In the field, more time spent in susceptible stages and lethargy both increase predation risk; lethargy also inhibits prey-finding, risking starvation.

Obj. 3b. Exposure to 1, 3, and 7-day old lime sulfur residues increased *C. rufilabris* larvae mortality relative to the control, while Entrust and OSO residues did not (Table 4). None of the residues increased development time. Larvae were more frequently observed off pesticide residues in the OSO 3- and 7-day treatments and the lime sulfur 3-day treatment (Table 4). A higher proportion of cocoons were formed on the lid (away from the pesticide residues) in all 7-day and 3-day residue treatments. It is possible that fresher residues (1-day) caused enough lethargy to mask repellency effects. Additionally, lime sulfur may lose repellency to larvae at 7-days post release, resulting in more time spent on the residues; however, at this point the residues are still toxic; this may explain why larval mortality was higher on the 7-day lime sulfur residues compared to the 3-day residues. To avoid loss of released larvae, growers may need to wait more than two weeks following a lime sulfur application.

Citations

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Markó, V. et al. 2013. Biocontrol Sci. Technol. 23: 126-145.
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Table 3. Effects of organic pesticide contact+residue exposure as larvae on *C. rufilabris* mortality, development, and movement.

	% mortality					% forming	Development time (days)		Ethovision	
Pesticide	*n*	Larvae	*n*	Pupae	Total	cocoons	to pupation	Total	*n* recorded	Distance moved (cm)
DiPel	30	10	27	0	10	93	10.3 ± 0.3	19.7 ± 0.2	12	1.81 ± 0.99
Cyd-X	30	10	27	26	33	67	11.8 ± 0.6*	20 ± 0.4*	12	0.72 ± 0.34*
Entrust	30	33*	20	5	37	75	11.9 ± 0.4*	20.5 ± 0.3*	10	3.84 ± 3.39
Serenade	29	17	24	17	31	83	10.8 ± 0.4*	20.6 ± 0.3*	12	1.11 ± 0.45
Sonata	30	13	26	4	17	88	10.6 ± 0.3	20.4 ± 0.2*	11	2.08 ± 1.13
Blossom Protect	28	7	26	4	11	88	9.7 ± 0.3	19.6 ± 0.3	12	0.23 ± 0.16*
Previsto	30	10	27	11	20	67	11.3 ± 0.5*	19.7 ± 0.3	12	0.49 ± 0.26*
Cueva	30	20	24	13	30	84	11 ± 0.6*	20 ± 0.2*	12	0.95 ± 0.77
Instill-O	30	13	26	19	30	58*	11.8 ± 0.5*	20.1 ± 0.2*	12	0.16 ± 0.13*
Lime sulfur	30	90*	3	0	90*	67	12.7 ± 0.9*	21.3 ± 0.9*	11	4.59 ± 2.12
Problad Verde	30	17	25	4	20	88	10.7 ± 0.3	20.1 ± 0.3*	11	0.37 ± 0.16**
Double Nickel	30	10	27	7	17	85	10.3 ± 0.4	19.4 ± 0.2	11	0.28 ± 0.14**
OSO	29	59*	12	25	69*	50*	12.9 ± 0.8*	19.8 ± 0.3	11	2.71 ± 1.39
Regalia	30	10	27	22	30	70	11 ± 0.5*	19.6 ± 0.2	12	0.72 ± 0.60*
Kaligreen	30	7	28	11	17	75	10.5 ± 0.3	19.7 ± 0.1	10	2.95 ± 1.59
OxiDate	30	13	26	15	27	54*	11.8 ± 0.4*	20.2 ± 0.3*	12	2.26 ± 1.20
Oil	30	10	27	7	17	78	10.7 ± 0.4	19.9 ± 0.2*	11	0.82 ± 0.41
Control	30	10	27	7	17	89	9.8 ± 0.3	19.3 ± 0.2	12	3.03 ± 1.84

Table 4. Effects of organic pesticide field-aged residue exposure (in days) as larvae on *C. rufilabris* mortality, development, and repellency.

	% Mortality									Duration (d)			% Repelled	
Pesticide, age	*n*	Larvae	*n*	Cocoons	*n*	Pharates	*n*	All	*n* survivors	Larvae	Cocoon	Total	Larvae	Cocoons
Lime sulfur, 7	30	50*	15	0	15	13	30	57*	12	6.8 ± 0.4	8.2 ± 0.2	15.2 ± 0.4	35	17
Lime sulfur, 3	30	30*	21	5	20	0	30	33	20	7.5 ± 0.3	8.6 ± 0.1	16.0 ± 0.3	51*	30*
Lime sulfur, 1	30	43*	17	6	16	6	30	50*	15	6.4 ± 0.3	8.5 ± 0.1	14.9 ± 0.3	39	47*
Entrust, 7	30	13	26	12	23	9	30	30	21	7.0 ± 0.3	8.4 ± 0.1	15.3 ± 0.3	25	14
Entrust, 3	30	7	28	14	24	0	30	20	24	7.0 ± 0.2	8.1 ± 0.1	15.1 ± 0.2	36	42*
Entrust, 1	30	7	28	11	25	8	30	23	23	7.0 ± 0.3	8.1 ± 0.1	15.1 ± 0.4	36	48*
OSO, 7	30	7	28	4	27	11	30	20	24	6.8 ± 0.2	8.2 ± 0.1	15.0 ± 0.3	25	17
OSO, 3	30	3	29	10	26	4	30	17	25	7.5 ± 0.3	8.4 ± 0.1	15.9 ± 0.3	47*	40*
OSO, 1	30	7	28	11	25	4	30	20	24	6.9 ± 0.3	8.3 ± 0.1	15.2 ± 0.3	51*	58*
Control	30	7	28	7	26	12	30	23	23	6.8 ± 0.3	8.3 ± 0.1	15.2 ± 0.2	29	4

Project Title: Whirligig mite: A new biocontrol agent for apples and pears

Report Type: Continuing Project Report

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Cooperators: Adam Spencer (Applied Bionomics)

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $53,143
Total Project Request for Year 2 Funding: $56,438
Total Project Request for Year 3 Funding: $0

Other related/associated funding sources: Awarded
Funding Duration: in-kind match for this project, as needed

Amount:	~$1,000 to date, continuing to supply as needed
Agency Name:	Applied Bio-nomics
Notes:	In-kind match of commercial insectary whirligigs (Crazee mite), as needed for the project. Crazee mites are priced at $0.28 per mite.

WTFRC Collaborative Costs: None

Budget 1
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Item	**2024**	**2025**	**2026***
Salaries[1]	$33,442.00	$35,938.00	$0.00
Benefits[2]	$10,701.00	$11,500.00	$0.00
Wages	$0.00	$0.00	$0.00
Benefits	$0.00	$0.00	$0.00
RCA Room Rental	$0.00	$0.00	$0.00
Shipping	$0.00	$0.00	$0.00
Supplies[3]	$9,000.00	$9,000.00	$0.00
Travel	$0.00	$0.00	$0.00
Plot Fees	$0.00	$0.00	$0.00
Miscellaneous	$0.00	$0.00	$0.00
Total[4]	$53,143.00	$56,438.00	$0.00

Footnotes:

*No funding is requested for Year 3. This year will be used to complete any remaining molecular work, analyze sequence results, and conduct outreach activities.

[1] Salary request is for a GS-6 Step 1 biological science technician to conduct field work (5 months/year at 100% FTE, annual salary $44,207) and a GS-9 Step 1 biological science technician to conduct molecular work (3 months/year at 100% FTE, annual salary $60,088). The Year 2 (2025) budget includes a 4% cost of living adjustment and a within-grade step increase for both positions.

[2] The benefits rate for both technicians is 32%.

[3] The supplies request is for the molecular gut content work, including pipette tips, reagents, and sequencing services (estimated $8,500/year). An additional $500/year is requested for field work and bioassay supplies, including Petri dishes, flagging tape, and sample vials.

[4] This is the total budget for the entire project, to be split 50% between Apple Crop Protection and Fresh and Processed Pear Research. **Our total request from ACP is $54,790.50** ($26,571.50 in Year 1 and $28,219 in Year 2).

Objectives

1. **Evaluate effects of whirligig releases on codling moth fruit damage and other pest populations in apples.**
   Release timing will target codling moth, but this timing will allow us to monitor the effects on other apple pests that are also consistently present in our research orchard, including woolly apple aphid, rosy apple aphid, green apple aphid, and thrips.
2. **Verify that whirligigs eat target pests in orchards.**
   Our prior work has only evaluated the diet of whirligigs collected near potato fields and examined lab predation of orchard pests. This objective will confirm that they consume pests in orchards after release.
3. **Screen non-target effects of conventional and organic pesticides on whirligigs in laboratory assays.**

Project Goals. The primary goal of this project is to determine if augmentation or conservation of whirligigs shows promise for improving control of key orchard pests. We will do this by determining (1) if high release rates result in decreases of monitored pests, (2) if released whirligigs establish, (3) the diet breadth of released whirligigs, and (4) which orchard pesticides are whirligig-compatible. If so, results from the project will lay the groundwork for a larger effort to determine ideal release rates, timing, and best practices for establishment. As this project is nearing its conclusion (2026) we will apply for other funding sources (such as a Specialty Crop Block Grant), using what we have learned from this project to determine exact research objectives and as preliminary data for the granting agency. Additional goals of the project are improving grower knowledge of whirligig identification, basic biology, and pesticide compatibility, which can be accomplished in the short time frame of this project.

Significant Findings

2025

- Field work on this project was paused due to the 2025 federal hiring freeze preventing the grant team from hiring necessary seasonal technical staff for this and other projects, in addition to other federal disruptions
- As of late 2025, whirligigs can now be ordered from multiple distributors in the US and shipped to Washington
- Although previously published research indicated that whirligigs can be held for a 48-h assay without food or water, we determined that dry laboratory air can be a substantial source of mortality. Whirligigs were provided *Ephestia* eggs as food during all new assays. We also ran small humidifiers over the workspace when loading assays and immediately sealed Petri dishes once whirligigs were loaded to prevent drying.
- Mortality in the 2024 assay was likely due to how long various treatments were left in unsealed Petri dishes before Parafilm was added. None of the treatments significantly differed from the control at 48 h in the new assay. During the mortality evaluations, Magister and Aza-Direct residues were observed to cause lethargy and erratic movement at 48 h.
- Assail, Agri-mek, Magister, and Exirel exposure reduced the number of prey killed.
- Assail, Agri-mek, Magister, Nealta, and Exirel reduced movement in subjects monitored by Ethovision.
- 16S primers detected potential prey more frequently than COI primers, detecting thrips and aphids, unlike COI. However, 16S could not detect the DNA of mites. Results from the molecular gut content work combining both primer types indicate that whirligig mites frequently consume thrips and rust mites. Prior work with species-specific primers indicates that they also are regular woolly apple aphid primers.

2024

- We were successful in obtaining an APHIS permit to have insectary-reared “Crazee mite” shipped to the Wapato laboratory and released at the USDA research farm in Moxee, nearly a year ahead of the anticipated schedule.
- Despite precautions to exclude ants, we concluded that a series of three trials (two apple, one pear) at Moxee farm were disrupted due to harassment of the released whirligigs by ants.
- On short notice, a fourth trial using a “local” whirligig population was initiated in a commercial orchard. The single-tree design used at Moxee farm was unsuitable for this location because the trees were trellised, but initial results still indicate that whirligigs hold some promise.
- In the commercial orchard, the release treatment reduced rosy apple aphid populations in the first week post-release, but not afterward, likely due to rapid dispersal out of plots.
- Because we will not need to rely on our small laboratory colony, our future field trials will expand plot sizes (0.10-0.25 acres/plot).
- Very few whirligigs were recaptured from field trials for gut content analysis. In order to better understand the feeding ecology of whirligigs and improve molecular methods, we are using samples from previous projects. 39% of whirligigs tested positive for woolly apple aphid and 30% tested positive using a generic aphid primer that detects green and rosy apple aphid.
- We determined that 16S barcoded primers, unlike COI, can amplify aphid and western flower thrips DNA and optimized a laboratory procedure for using this primer set.

Methods

Objective 1: Field Releases

Trial location and preparation. All trials consisted of single-tree replicates. There were five replicates per treatment in the research orchard trials and nine replicates per treatment in the commercial orchard trial. The USDA-ARS research farm and the commercial orchard used are both located in Moxee, WA. The research farm is unsprayed and the commercial orchard is organic.
In the research orchard trials, we painted a thick layer of Tanglefoot on the base of each focal tree and its adjacent trees prior to releases. This was done to prevent ants from harassing released whirligigs; the ant population at the research orchard is extraordinarily high.

Sampling – Research Orchard, Apples. Sampling was done on the release (or control) tree and the trees immediately adjacent to it both down and across the row (Fig. 1). For each sample type, we collected an “inner” sample from the focal tree (release or control) and an “outer” sample from the four adjacent trees. Each adjacent tree made up ¼ of the “outer” sample to keep the sampling intensity consistent between the “inner” and “outer” samples.

For each sample, we counted the number of infested leaves (GAA, RAA) and number of colonies (WAA) per four 1-foot shoots on each sample. One beat tray sample was collected from four limbs to count ants, whirligigs, and other natural enemies. We collected a 20-leaf sample for brushing to count predatory mites (phytoseiids, *Zetzellia mali*) and pest mites (rust mites, all spider mite species). One sticky card per sample was used to sample adult thrips (cut into quarters for the “outer” sample).

Sampling – Research Orchard, Pears. Leaf samples were collected from the pear orchard as described for apples, but pear psylla [eggs, small nymphs (I-III), large nymphs (IV-V)] were also counted. In addition to the

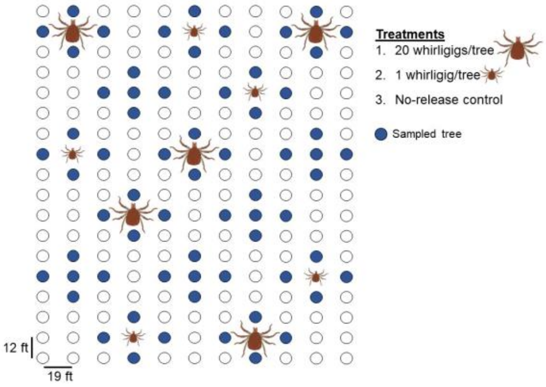

Fig. 1. Plot layout

previously mentioned arthropods, beat trays were also used to count adult pear psylla. Sticky cards were used to count adult thrips, pear psylla, and *Trechnites* parasitoid wasps.

Sampling – Commercial Orchard, Apples. This orchard was trellised, so we modified our sampling protocol. We sampled each focal tree and one adjacent tree on either side, going down the row (three trees). Due to the tighter spacing, we did not collected separate "inner" and "outer" samples per plot. For each three-tree plot, we collected the following samples per tree: two beat tray samples, two aphid shoot samples, and ten leaves, in addition to one sticky card.

Pre-count sampling of shoots, leaves, and beat trays was conducted 0-1 days before release and post-release sampling was performed weekly for two weeks in the research orchard trials and weekly for four weeks in the commercial orchard. In the research orchard, sticky cards were only used to sample for one week (cards left out from 13-20 May). In the commercial orchard, sticky cards were collected and replaced weekly for the duration of the trial.

Releases. Insectary whirligigs were obtained from Applied Bio-nomics through their distributor Evergreen Growers Supply and arrived via overnight shipping on 3 May. They were stored at 15°C until use, per the recommendations of the insectary. A proprietary blend of food is provided for the whirligigs in their shipping containers. Colony whirligigs are maintained on potato and tomato plants infested with potato psyllids. Preliminary morphological examination and molecular work indicate that they are very likely the same species. Whirligigs were collected by aspirating single individuals into modified pipette tips from either the insectary container or the colony. The appropriate number of whirligigs was then added to each release tree (Table 1) by unblocking the pipette tip opening. We used the laboratory colony population of whirligigs for the commercial orchard trial because our existing APHIS permit only allowed releases at the research farm address.

Table 1. Whirligig 2024 release trial details.

Trial	Orchard	Variety	Rates tested (/tree)	Whirligig source	Release date
1	Research	Fuji	20, 2, or 0	Insectary	6 May
2	Research	Red Delicious	20 or 0	Insectary	8 May
3	Research	Pear	20 or 0	Insectary	8 May
4	Commercial	Gala	20 or 0	Lab colony	6 June

Objective 2: Gut content analysis

Our original plan was to perform gut content analysis on any whirligigs captured on the beat trays from these trials. However, no whirligigs were captured in the research orchard and only a small number were collected from the commercial orchard (see Results and Discussion). Instead, we used specimens collected from other studies to better understand the feeding ecology of whirligigs in orchards. Data are presented for specimens collected from apple and pear orchards; the pear specimens were processed as a component of other projects, using those funds.

Whirligigs were collected in molecular grade ethanol and frozen for use in gut content analysis. Methods for gut content analysis were similar to other gut content studies conducted at the USDA-ARS lab (Cooper et al., 2022; Krey et al., 2020). The COI gene was PCR-amplified from each whirligig using universal primers (LCO1490/HCO2198) (Folmer et al., 1994). Barcoded primers were used to identify sequences from each specific sample. The barcoded COI amplicons were sequenced (PacBio sequencer, WSU CORE facility). Sequences were grouped into Operational Taxonomic Units (OTU) and each OTU was analyzed using the Basic Local Alignment Search Tool (BLAST) feature of the National Center for Biotechnology Information (NCBI) website. In preliminary work, we determined that while this method works for most key natural enemies and pests, DNA of pear psylla and the three aphid pests (GAA, RAA, WAA) does not amplify well using these universal primers. Instead, we developed a pear psylla primer (Ohler) and adapted a general aphid primer to use in conjunction with the universal primers for detecting these pests (Chapman et al., 2010). We have also worked to optimize a procedure for using an alternative barcoded primer, 16S. This primer is much better at amplifying the DNA of many key pests (aphids, western flower thrips, psyllids) than COI. However, COI is useful for detecting lepidopterans (e.g., codling moth, leafrollers), and some mites (phytoseiids, *Zetzellia mali*, rust mites).

Objective 3: Pesticide non-target effects

Pesticides were tested at the highest labelled field rate (100 GPA equivalent) and compared to a water control. 2 mL pesticide or water (control) was applied to each side of plastic Petri dishes (60 mm diam) using a Potter tower and allowed to air dry. Then, a single adult whirligig was added to each arena. No food or water was provided. There were 27 replicates (arenas) per treatment, except for the control (*n*=53). Whirligig mortality was recorded at 24 and 48 h. After the 48-h evaluation, up to 10 surviving individuals per treatment from the mortality assays (including the water control) were monitored using motion tracking software (Ethovision) for 25 min. Then, we added 10 IV-V instar potato psyllids from colony to 10 arenas per treatment (except *n*=19 for the control) and counted the number of psyllids consumed in 24 h. Whirligigs that died during the feeding assay were excluded from the analysis.

Planned work for 2026 includes a separate assay to test pesticide repellency, by treating half of a Petri dish with pesticide and comparing the amount of time spent by a whirligig on the treated and untreated side of the dish (30 min recordings in Ethovision, 10 replicates/pesticide). We are also seeking suggestions for additional pesticides to test.

Planned Outreach. We will summarize project results each year in the early spring in an article for *Fruit Matters* newsletter. Additionally, in early 2026, we will write a new page for the Orchard Pest Management section (“Beneficials”) of the WSU Tree Fruit site that summarizes what is currently known about whirligig mites in orchards and then update the page at least once per year with information learned from the project. Results will also be presented at a minimum of one grower meeting each year and at the Orchard Pest Management and Disease Conference. In Ireland, growers shown whirligig mites in the field indicated that they had mistaken it for European red mite and some growers had even applied unnecessary miticides (Cuthbertson, 2000; Cuthbertson and Murchie, 2006; Cuthbertson and Murchie, 2010). The Irish researchers provided growers with identification cards showing differences between whirligigs and European red mite, which led to several growers reducing their pesticide applications (Cuthbertson and Murchie, 2010). We will create similar identification cards for distribution at grower meetings. We will also add videos of both magnified and unmagnified whirligigs to our Orchard Pest Management page; their movement patterns are very distinctive and unmagnified videos of whirligigs moving in the field will help growers identify this natural enemy.

Results and Discussion

Obj. 1. Moxee farm was an ideal location for testing releases of whirligigs for their ability to control codling moth because of the high pressure (unsprayed, no MD) and because the wide tree spacing allows for single-tree release treatments. Our 2024 trials were planned with the assumption that we would be using our small laboratory colony for releases, because of the time necessary to obtain an APHIS permit to ship and release the insectary whirligigs from the Oregon facility. Therefore, our trials were all designed as single-tree releases to minimize the number of whirligigs needed. We relied on the Tanglefoot trunk barrier to exclude ants, which are unusually abundant at the USDA research farm in Moxee. Ants are known to harass natural enemies to prevent them from consuming aphids and this tending behavior is observed throughout the Moxee orchard, with resident ants primarily tending the large population of rosy apple aphids. Unfortunately, the sticky bands immediately became coated with dust and (due to a neighboring dairy) filth flies, rendering them ineffective. This may have caused released whirligigs to be attacked by ants and drop from the trees. The trees were also heavily pruned at a late timing, resulting in little foliage on the trees during our releases, which likely decreased shelter and prey for the whirligigs. Whirligigs may have also been more mobile than we anticipated. As a consequence of one or more of these factors, we recovered no whirligigs in any of our three research orchard trials. Because conditions in the orchard and the experimental design were not suited for the trial work, we limited the research orchard trials to one release and did not monitor codling moth damage.

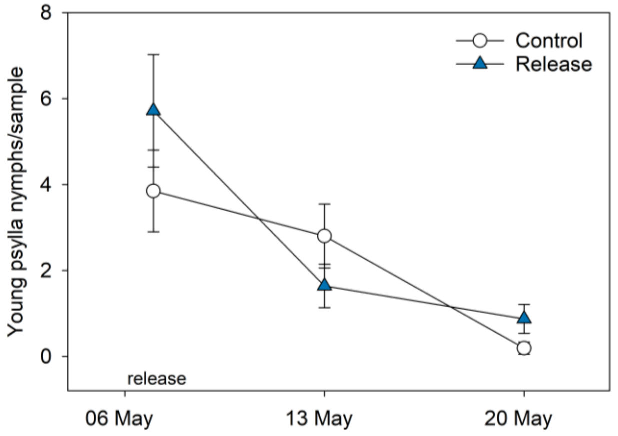

Fig. 2. Young pear psylla nymphs per leaf brush sample comparing whirligig release treatments in a research pear orchard.

Because of the logistical issues at the research orchard, one month later, we set up an additional trial at a commercial orchard using a similar experimental design. Because this orchard was trellised, a single-tree release design was not ideal, but releasing across larger plots was not possible because our APHIS permit to release the insectary whirligigs did not include this location. Therefore, were only able to release smaller numbers from our laboratory colony on single trees. However, this design combined with a trellised orchard likely allowed released whirligigs to quickly disperse from release trees. No whirligigs were found in release trees one week post-release, four were found at two weeks, and then one at three weeks. One whirligig was found in a no-release tree at three weeks post release. This location also already had whirligigs present during the trial, although in relatively low abundance. These whirligigs would likely have not yet been active in the orchard during our planned release timing and our released whirligigs may have then represented a larger increase in predators relative to the resident natural enemy population. At this time, we are unable to distinguish between released and resident whirligigs.

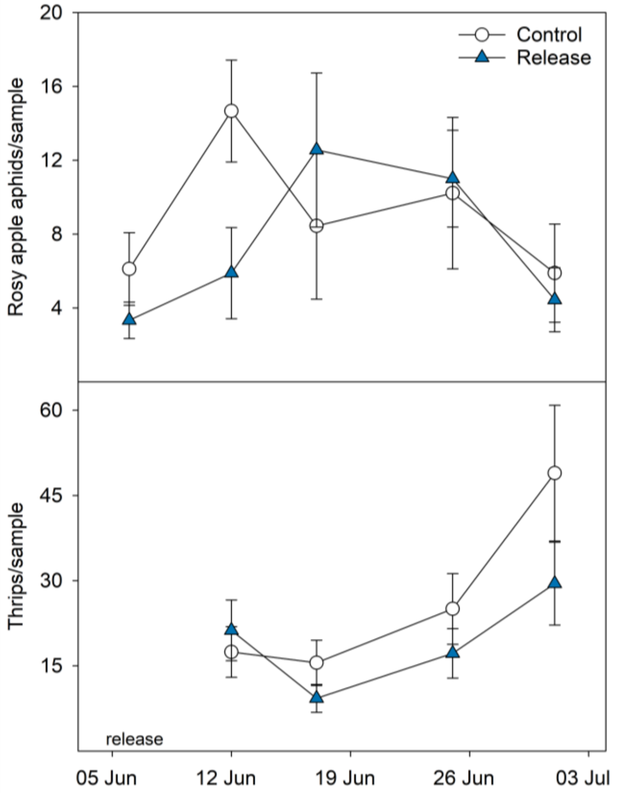

Fig. 3. (a) Rosy apple aphids and (b) thrips per sample collected

No differences were observed between treatments in the most abundant pests (RAA, GAA) in either of the research apple trials. Despite the early timing, ants were abundant, especially in the Fuji block (~0.4/tray). In the pear trial, there were fewer pear psylla young nymphs (I-III instar) in the release treatment at two weeks post-release (Fig. 2); this difference was marginally significant in statistical testing. In the commercial apple orchard, there were fewer rosy apple aphids in release plots at one week post-release (Fig. 3a). This short-lived effect could be due to the rapid dispersal of whirligigs out of the monitored release trees, but demonstrates that they do have the potential to decrease pest populations in the field. There was also a trend for fewer thrips captured on sticky cards in release trees compared to the no-release control (Fig. 3b).

Table. 3. Percent of whirligig samples collected in 2023 from two apple orchards testing positive using a woolly apple aphid (WAA) specific primer and a generic aphid primer.

Orchard Location	*n*	% WAA+	% Gen. Aphid+
Moxee (Research)	12	42	25
Benton City	34	38	32

To mitigate the logistical issues encountered in 2024, in 2026, we have planned two trials. Both will consist of releases across larger plots (0.10-0.25 acres/plot). We will test the insectary suggested rate of 1,000/acre as a starting point for proof-of-concept of whirligig releases at scale. One trial will occur across multiple blocks of apples at the Moxee research farm and one at a commercial apple orchard. In 2024-2025, we reduced the ant population through bait applications. We have communicated with the USDA farm crew to minimize pruning to allow for adequate bud production in 2026, to increase the foliage available for whirligigs and the availability of fruit for pest damage assessments.

Obj. 2. Our results to date indicate that whirligigs may be effective predators for pest aphids in apples. Despite relatively low populations of woolly apple aphids at both of the sampling locations, 39% of whirligigs tested positive for woolly apple aphid DNA (Table 3). The generic aphid primer is less sensitive and does not amplify woolly apple aphid DNA, but does detect green and rosy apple aphid DNA; 30% of specimens tested positive using this primer. COI barcoded primers detected DNA from the common orchard predatory mites *Galendromus occidentalis* and *Zetzellia mali* in whirligig mites, as well as less common predatory mites such as Bdellidae, Tydeidae, and Ameroseiidae, which they may have encountered in the ground cover. Apple rust mite (*Aculus schlectendali*) was commonly detected. There were also COI detections of other eriophyoid mites: *Aceria tosichella* and *Brevulacus reticulatus*, and *Caraboacarus stammeri*, a mite that is phoretic on beetles. Two COI samples also contained DNA of crab spiders (*Xysticus*). Many more species were detected with 16S barcoded primers, although 16S did not detect the DNA of any mites. 16S primers detected six species of thrips (including western flower thrips), five species of leafhoppers, six species of aphids (including woolly apple aphid, but not green apple or rosy apple aphid), three species of springtails, three species of flies, and *Xysticus*. Both COI and 16S primers detected ladybeetle DNA in several samples; we are working to determine if this was contamination from ladybeetle samples processed at the same time as the whirligig samples. Although whirligig mites clearly act as predators of several orchard natural enemies, their potential as predators of pests appears to surpass this risk. At both sites, many whirligigs tested positive for western flower thrips (21-41%). In the Moxee orchard, where apple rust mite was abundant, 53% of whirligig mites tested positive for this pest. This is promising for their potential to control pear rust mites in pear orchards.

None of the samples from past studies that tested positive for WAA DNA the WAA-specific primer (*n*=12) tested positive for WAA DNA using the 16S barcoded primers. Similarly, of none of the samples testing positive with the Chapman aphid primer (*n*=16) tested positive for DNA of any aphid with the 16S barcoded primers. In additional preliminary work with various other orchard predators, we found that none of 27 samples that tested positive with the WAA primer tested positive for WAA DNA with the 16S primers. Of the 26 samples testing positive with the Chapman primer, only four tested positive for any aphid DNA with the 16S primers (two tested positive for *Aphis* spp.). Although 16S primers can detect aphid DNA, the detection rate appears to be much lower than for more specific primers. Therefore, it appears that 16S may not be suitable for detection of aphid consumption by predators in apple orchards. Instead, we have now dedicated our molecular efforts to creating and optimizing highly sensitive species-specific primers for WAA, GAA, and RAA.

Table. 4. Percent of whirligig samples collected from two sites in 2022 and 2023 (one commercial, one research) testing positive for various arthropod groups using either COI or 16S barcoded primers. *n*=101 for Benton County, *n*=52 for Moxee.

	Positive for:	Benton County	Moxee
COI	*Galendromus occidentalis*	3	0
	Zetzellia mali	0	2
	Other predatory mite	6	0
	Aculus schlechtendali	3	52
	Other eriophyoid mite	3	0
	Xysticus	2	0
	Thrips tabaci	0	2
	Cicadellidae (leafhoppers)	0	2
16S	*Frankliniella occidentalis*	41	21
	Thrips tabaci	0	2
	Other plant-feeding thrips	9	2
	Predatory thrips	1	0
	Cicadellidae (leafhoppers)	3	6
	Delphacidae	1	0
	Wooly apple aphid	1	0
	Other aphid	7	6
	Springtails	12	10
	Xysticus	6	2
	Misc. flies	2	2

Molecular work from two previous studies in pear (funded by other projects) was recently completed and is presented here. Both primers used (pear psylla, twospotted spider mite) are species specific and therefore relatively sensitive for small amounts of DNA. The majority of specimens (54%) tested positive for pear psylla DNA, while very few (5%) tested positive for twospotted spider mite DNA (Table 5). Variation in number of specimens collected is due both to the differences in abundance at a given site, and differences in sampling intensity, which varied by the goal of each project. It is also important to note that pear psylla and twospotted spider mite abundance varied between these sites.

Obj. 3. Our 2024 non-target effects study was designed to replicate the methods of previously published studies, which noted that they did not include food or a moisture source (Laurin and Bostanian, 2007a; Laurin and Bostanian, 2007b). We had moderate mortality in the control (24%), especially compared to some of the other "low mortality" treatments (7-13%). We attribute this partially to the control whirligigs being exposed to more airflow than the other treatments; we loaded this treatment first and parafilm was added to seal all of the Petri dishes at the end of loading at roughly the same time. We have noted that whirligigs are susceptible to drying out in laboratory environments with rapid air circulation (outside of the colony).

In our 2025 assay, none of the treatments significantly differed from the control in mortality at 48 h and mortality in the control was 2%. This indicates that the addition of food, use of humidifiers, and sealing dishes immediately after adding the mite reduced mortality to appropriate levels to compare treatments. During the mortality evaluations, we noticed that at 48 h, all individuals in the Magister and Aza-Direct treatments were moving slowly, with irregular twitching leg movements. Whirligigs exposed to Assail, Agri-mek, Magister, Nealta, and Exirel residues exhibited reduced movement in the Ethovision monitoring assay; most individuals in the Magister and Exirel treatments did not move at all during the monitoring period. In other studies with aphids, we observed whirligigs killing, but not consuming prey items. We monitored Petri dishes containing only potato psyllid nymphs for 24 h (*n*=9 dishes, 10 nymphs per dish) and determined that 0-1 psyllids per dish die without a predator present (4.5%). Therefore, all death in the consumption assays was attributed to a combination of actual and non-consumptive predation (with any "other" mortality assumed to be accounting for by comparing treatments to the control). Whirligig mites consumed fewer prey in the Magister and Exirel treatments and fewer prey were killed (eaten +dead) in the Assail, Agri-mek, Magister, and Exirel treatments. There were no differences between treatments in the proportion of prey that died without being consumed. There was also some mortality in the whirligigs during the prey consumption assay: two individuals died in the Assail and Aza-Direct treatments and one individual died in the Agri-mek, Centaur, Delegate, Esteem, Magister, and Movento treatments. The other treatments (and the control) had no deaths. The sample size during the prey consumption assay (*n*=10 in treatments, *n*=19 in the control) was too small to allow for statistical comparison of mortality; however, it is possible that some of these treatments may cause a "slower kill" of whirligigs that is not apparent until more time passes. Whirligigs appear to be fairly tolerant of pesticide applications. Orchards where they are present (or where they are released) should be avoid applications of Assail, Agri-mek, Magister, Exirel, and Aza-direct if possible. Growers should maximize the time between application of these pesticides and releasing whirligigs.

Table. 5. Percent of whirligig samples collected from pear orchards testing positive using species-specific primers for pear psylla (PP) and twospotted spider mite (TSM).

Year	Orchard Location	*n*	% PP+	% TSM+
2023	Cashmere	1	100	100
2022	Leavenworth	93	46	-
2022	Tieton #1	3	0	0
2023	Tieton #1	9	22	22
2023	Tieton #2	1	100	100
2023	Tieton #3	1	100	100
2023	Wapato #1	6	67	33
2023	Wapato #2	26	92	-
	Total	140	54	2

Citations

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Table 5. Effects of exposure to freshly dried pesticide residues on adult whirligig mites (*Anystis baccarum*).

Pesticide			% Mortality			% Prey consumption				Ethovision assay	
Product	AI	MOA Group	*n*	24 h	48 h	*n*	% eaten	% dead	% eaten + dead	*n*	Distance moved (cm)
Assail	acetamiprid	4A	27	0	0	8	62.8 ± 4.7	8.7 ± 3.5	71.5 ± 6.0*	10	80.8 ± 65.3*
Delegate	spinetoram	5	27	7	7	9	74.4 ± 6.5	6.7 ± 2.4	81.0 ± 6.4	10	465.3 ± 201.4
Agri-mek	abamectin	6	27	7	19	9	63.7 ± 9.1	2.1 ± 1.4	65.9 ± 8.9*	9	169.7 ± 94.1*
Esteem	pyriproxyfen	7C	27	0	7	9	68.1 ± 4.6	12.2 ± 4.6	80.4 ± 4.2	9	470 ± 195.8
Centaur	buprofezin	16	27	4	4	9	66.6 ± 7.6	10.2 ± 2.9	76.8 ± 6.3	10	457.6 ± 241.3
Magister	fenazaquin	21A	27	7	7	9	43.1 ± 3.4*	8.1 ± 2.3	51.2 ± 4.6*	11	3.5 ± 1.8*
Movento	spirotetramat	23	27	4	4	9	77.4 ± 5.8	10.2 ± 3	87.7 ± 3.2	10	298 ± 154.8
Nealta	cyflumetofen	25A	27	0	4	10	70.0 ± 5.2	7 ± 2.1	77.0 ± 4.2	10	74.7 ± 47.5*
Altacor	chlorantraniliprole	28	27	11	15	10	77.3 ± 4.8	10.6 ± 2.7	87.9 ± 4.2	9	492.8 ± 240.7
Exirel	cyantraniliprole	28	27	0	0	10	53.3 ± 7.5*	15 ± 5.4	68.3 ± 5.5*	10	2.6 ± 2.5*
Aza-Direct	azadirachtin	UN	27	0	11	9	68.0 ± 4.3	14.4 ± 3.8	82.4 ± 4.3	9	235.4 ± 141.5
Control	-		53	0	2	19	74.2 ± 4.9	14.7 ± 3.8	88.9 ± 2.4	10	467.7 ± 289.2

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

Report Type: Final Project Report

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Project Duration: 3 Year (NCE approved on 8/19/2024)

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
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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

Overall leveraged funding throughout the life of the project: $173,692.00

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.* Substantially accomplished – see results below. Although we have generally accomplished the goal, in-depth characterization of the sequence data to further explain the results is ongoing).
2. *To identify benefits of specific apple rootstock-AMF associations including protection against pathogenic root fungi, nitrogen uptake, and tolerance to water stress.* Fully accomplished – see results below.

SIGNIFICANT FINDINGS (over the life of the project):

- Plant-AMF relationships are complex and may need to be tailored accordingly. Matching host genetics with compatible AMF species has the potential to enhance agricultural practices in nursery and orchard systems.
- When grown in the presence of replant pathogens (i.e., live orchard soil), G.11 rootstocks pre-colonized with the AMF *R. irregularis* had significantly higher stomatal conductance compared to non-inoculated control plants, regardless of watering regime. This result highlights the role of AMF in maintaining water supply to plants experiencing a combination of water-stress and replant pressure, especially less vigorous apple rootstocks (with relatively small root systems).
- When grown in *live* orchard soil and *well-watered*, G.11 rootstocks pre-colonized by *R. irregularis* recovered significantly more nitrogen (from ^{15}N-enriched fertilizer) than non-inoculated plants growing under the same conditions.
- When grown in *live* orchard soil and *water-stressed* (i.e., combined water-stress and replant pressure), inoculation with *R. irregularis* benefited the ability of G.11 rootstocks to retain/accumulate nitrogen in root tissue. Under these conditions, a plant's need to maintain/prioritize root growth over shoot growth would be expected to be relatively high.
- Results suggest that AMF community composition can be successfully manipulated at the nursery prior or in the field prior to planting. In our experiment, incorporating *R. irregularis* (RTI-Ag) into live soil (at the time of planting) provided several benefits to G.11 rootstocks experiencing replant pressure.
- Spore/propagule count is not directly correlated with the ability of commercially available AMF products to colonize apple roots.
- In general, *Rhizophagus* and *Claroideoglomus* spp. appear to colonize apple root tissue rapidly and maintain strong associations over time. However, product consistency and reliability can be highly variable depending on the manufacturer.

- In G.11, pre-established (nursery-derived) associations with *Rhizophagus* spp. tended to be maintained after planting into both live and pasteurized soil treatments.

- G.11 rootstocks established new relationships with commercially available AMF (previously identified as "promising"), regardless of whether they were planted into pasteurized or live soil (i.e. native microorganisms present in live soil did not prevent these interactions). Rootstocks also established new relationships with a variety of native AMF present in live soil, but associations varied in the presence of commercial inoculum.

- Native microorganisms present in "live" soil can restrict development of and/or alter pre-existing (nursery-derived) AMF communities. Ultimately, however, outcomes between pre-existing AMF communities and biotic components present in soil will depend strongly on the specific groups of microorganisms that are present.

METHODS:

As previously described in the Continuing Report (CR) for 2024, an AMF product assessment was conducted to identify "promising" commercially available products for use in subsequent experiments. **One of these experiments was designed to assess the ability of introduced AMF products to compete with native AMF in live orchard soil and in nursery-derived apple roots (Obj. 1).** Based on product test panel results (CR 2024), *F. mosseae* (RTI-Ag) and *C. claroideum* (RTI-Ag) represented the most "promising" commercially available products in terms of their ability to colonize apple; both products were selected for use in a subsequent greenhouse experiment.

Pasteurization, a proxy for soil fumigation, may provide insight into the ability of commercial AMF products to colonize roots post-fumigation. Therefore, the experiment included both live and pasteurized orchard soil treatments (soil collected from WSU-Sunrise Research Orchard, Rock Island, WA). As described in the original proposal, treatments were: 1) live orchard soil, 2) live orchard soil + *F. mosseae*, 3) live orchard soil + *C. claroideum*, 4) pasteurized orchard soil, 5) pasteurized orchard soil + *F. mosseae*, and 6) pasteurized orchard soil + *C. claroideum*. In addition, G.11 rootstocks from two different nurseries were used: TRECO (Woodburn, OR) and Cameron (Eltopia, WA). Altogether, the experiment included 84 trees (7 replicate trees x 12 treatment/nursery combinations). Prior to planting, a small amount of fine root tissue was collected from various locations on the root system to obtain representative samples of nursery-derived (or pre-existing) AMF communities. This root tissue was stored at −80°C (-112°F) until processing. Trees were planted into 2.7L pots containing live or pasteurized potting mix with or without AMF inoculum (12g per pot). Rootstocks were then grown for a period of 8 weeks under supplemental lighting (16h photoperiod) and watered as needed; plants did not receive supplemental nutrients. Upon harvest, plant growth characteristics including trunk diameter (cm), total plant biomass (g), root volume (mL), and leader shoot length (cm) were measured. At the same time, new (white) root tissue was collected from each plant for DNA isolation/assessment of AMF colonization. DNA extraction (root surface + endosphere) was conducted using the DNeasy Plant Pro Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. DNA samples were sequenced using Glomeromycota-specific primers (AML1/2) in Fall of 2025. Changes to nursery-derived AMF community composition following cultivation in orchard replant soil (live vs. pasteurized) were assessed using a Glomeromycota-specific phylogenetic tree, a tool which was previously constructed in collaboration with Dr. Loren Honaas and Dr. Huiting Zhang (as described in CR 2023). This analysis was recently completed (Winter of 2025) and results are reported here.

Assessing benefits of specific apple rootstock-AMF associations (Obj. 2). Experiments designed to explore *functional* benefits of "compatible" apple rootstock/AMF associations were previously conducted as described/reported in CR 2024. One of these experiments, however, could not be completed until Fall/Winter of 2025; the methods and results for this particular experiment are reported here. The primary aim of the experiment was to test for AMF-mediated tolerance to water stress in apple. An additional aim of this study was to assess whether AMF 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. Briefly (and as previously described in CR 2024), this experiment was performed using the dwarfing rootstock G.11 because 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 (Casagrande-Biasuz & Kalcsits, 2021). Experimental treatments included both live and pasteurized orchard soil (WSU-Sunrise Research Orchard, Rock Island, WA) with and without the AMF *Rhizophagus irregularis* (formerly *Glomus intraradices*; Mycointech; 12g per 2.7L pot). After 5 weeks, colonization by *R. irregularis* was confirmed via microscopy. Trees were then maintained at 2 different soil moisture contents: well-watered (WW; 80-90% field capacity) and water-stressed (WS; ~40% of field capacity) for an additional 4 weeks. Altogether, there were 8 treatments: Live x No AMF x WW, Live x AMF x WW, Live x No AMF x WS, Live x AMF x WS, Past x No AMF x WW, Past x AMF x WW, Past x No AMF x WS, Past x AMF x WS. These 8 treatments were each replicated 6 times. Volumetric water content sensors were inserted into the root zone (2 per treatment) to ensure target volumetric soil water content was maintained over the duration of the experiment. Stomatal conductance and stem midday water potential (plant physiological responses related to water usage) were measured as described in the original proposal. At the start of the water stress experiment (5-weeks post planting), labelled nitrogen (ammonium-^{15}N nitrate; $^{15}NH_4NO_3$) was spiked into a subset of pots via watering (100 mg per pot). This nitrogen source is physically accessible to both the plants and the AMF. The same amount of unlabeled NH_4NO_3 was added to a different set of pots as a control. Aluminum dishes were placed under each pot to avoid the loss of water containing labelled N and work surfaces were covered with a sheet when using labelled N to avoid cross contamination. After 4 weeks under the different soil moisture regimes, all trees were destructively harvested. Sample processing of leaf, roots, and wood for assessment of labeled nitrogen (^{15}N) uptake were conducted in Fall of 2025 by the UC Davis Stable Isotope Facility (Davis, CA, USA).

RESULTS AND DISCUSSION:

Testing the ability of commercial AMF to compete with those in native orchard soil and those pre-existing in apple roots (i.e., nursery-derived) (Obj. 1): The experiment was harvested in Fall of 2024, microbial DNA present in apple root tissue was extracted, and DNA samples were sequenced. The amount of root tissue colonized by AMF was also assessed at harvest (2 months post-planting). Across treatments, average percent root length colonization ranged between 40-70% in G.11 plants from TRECO and 25-60% in those from Cameron Nursery (as previously reported in CR 2024). No significant differences in percent AMF root length colonization were observed between uninoculated treatments and those containing commercial inoculum (*C. claroideum* or *F. mosseae*), regardless of soil status (live or pasteurized) or nursery origin (TRECO vs. Cameron). However, in the uninoculated TRECO control treatment, pasteurization had a positive effect on the degree of AMF colonization. AMF colonization was significantly higher in TRECO rootstocks grown in pasteurized soil compared to those grown in the presence of microorganisms contained in live soil (Mann-Whitney test; p=0.04). In comparison, soil pasteurization had no effect on AMF colonization levels in the Cameron rootstocks. This may be partly explained by the fact that initial AMF community composition (prior to planting) was significantly different in G.11 rootstocks from TRECO vs. Cameron nurseries (NMDS; Bray-Curtis dissimilarity index). Taken together, these results suggest that native microorganisms present in live soil can significantly restrict the degree of mycorrhization by pre-established/nursery-derived AMF. However, pre-existing community structure can influence subsequent outcomes between root-associated AMF and soil microbes.

Sequence based analysis of AMF community composition provided more detailed information. Ordination analysis indicated that in the absence of a native soil microbial community (pasteurized soil), inoculation with *C. claroideum* significantly altered pre-established AMF community composition in G.11 rootstocks from both TRECO and Cameron nurseries (1-way ANOSIM; p=0.01 and 0.04, respectively) (Figure 1A and 1B). This result indicates that, in the absence of a functional soil microbial community, *C. claroideum* was competitive and/or antagonistic towards pre-established AMF communities, regardless of differences in starting community structure related to nursery origin. In comparison, inoculation with *F. mosseae*, had no significant effect on the structure of the existing AMF community, regardless of nursery location.

In live soil, AMF community structure varied considerably among treatments. In G.11 rootstocks from TRECO, AMF communities that developed after planting into uninoculated control and *C. claroideum* treatments were significantly different from those of the pre-plant community (1-way ANOSIM; p=0.02 and 0.005, respectively) (Fig. 1C). In G.11 rootstocks from Cameron Nursery, inoculation with *F. mosseae* was the only treatment in which initial AMF community composition was significantly altered (1-way ANOSIM; p=0.004) (Fig. 1D). These results suggest that outcomes between biotic components in the live/bulk soil and pre-existing AMF communities will depend on the specific groups of microorganisms that are present in each compartment.

Phylogenetic analysis was conducted to further assess whether the apple rootstocks served as a significant source of inoculum from the nursery where they were produced**.** In the TRECO rootstocks, the nursery-derived AMF community was represented only by *Rhizophagus* spp. which included a diversity of ASVs (Fig 2, Clade 1). Most of these associations were maintained at relatively high abundance across both pasteurized and live treatments, except when rootstocks were grown in live soil + *C. claroideum* (a treatment which was characterized by the loss of several *Rhizophagus* ASVs).

In comparison, the AMF community pre-established in rootstocks from Cameron nursery was much more diverse, representing ASVs from all 4 main clades (*Rhizophagus*, *Glomus, Funneliformis*, and *Claroideoglomus* spp.) as well as a handful of "unknown" AMF, whose sequences could not be assigned to any known taxonomic group (Fig 2, Clade 6). Unlike TRECO rootstocks, AMF communities pre-established at Cameron nursery included only a single ASV from the *Rhizophagus* group (ASV 3 – also dominant in TRECO rootstocks). However, this ASV was maintained in all pasteurized and live treatments, except when rootstocks were grown in live soil + *F. mosseae.* Regardless of nursery location, associations with *Rhizophagus* that were established prior to planting tended to be maintained across both live and pasteurized treatments.

As mentioned above, no *Glomus* spp. ASVs were detected in TRECO rootstocks prior to planting or in pasteurized soil treatments. A handful of *Glomus* spp. ASVs were, however, identified when plants were grown in live soil (uninoculated control, live + *F.mosseae* and, Live + *C. claroideum*). This suggests that TRECO rootstocks were able to establish new relationships with *Glomus* spp. present in live soil, regardless of the presence of either inoculum. In the rootstocks from Cameron nursery, a variety of *Glomus* spp. existed prior to planting and two of these (ASV 5 and 9) were maintained at relatively high levels across both live and pasteurized treatments (except in the Live + *F. mosseae* treatment).

Despite representing one of the most promising commercially available products (in terms of the ability to colonize apple seedlings in pasteurized soil; CR 2024), we could find no evidence of successful establishment of *F. mosseae* in pasteurized or live soil, regardless of nursery origin. The *F. mosseae* commercial inoculant did, however, contain a *Claroideoglomus* sp. (ASV 34, Clade 4) which colonized rootstocks in both pasteurized and live soil containing the inoculum. This ASV was identified in both TRECO and Cameron rootstocks and only in the following treatments: Pasteurized + *F. mosseae* and Live + *F. mosseae*. As previously discussed in CR 2022, this represents another example in which what the manufacturer advertises is not always what is actually present.

In addition, ASV 47 (Clade 4) represented a *Claroideoglomus* species that was present in the *C. claroideum* inoculum which successfully formed relationships with G.11 rootstocks from both nurseries in pasteurized and in live soil. This ASV was identified in both TRECO and Cameron rootstocks and only in the following treatments: Pasteurized + *C. claroideum* and Live + *C. claroideum*. Finally, it is worth noting that a handful of "unknown" AMF ASVs closely related to *Paraglomus* species (ASV 84, 86 and 149; Fig 2) were detected in live soil treatments from both TRECO and Cameron nurseries. This finding is in alignment with other sequence-based studies in which *Paraglomus* spp. were detected in apple roots and orchard soil systems of Central Washington (Van Horn, et al., 2021).

Assessing benefits of specific apple rootstock-AMF associations (Obj. 2): **Plant tolerance to water stress.** AMF colonization of G.11 rootstocks was assessed microscopically after 5 weeks. The root systems of plants that had been inoculated with *Rhizophagus irregularis* spores were colonized; 25% and 33% root length colonization in live and pasteurized orchard soil, respectively. At this time, no AMF were detected in uninoculated control plants grown in live or pasteurized orchard soil. During the 4-week water-stress experiment which followed, plant physiological response data, including stomatal conductance and stem water potential, were collected. Stomatal conductance refers to the diffusion of gases (e.g., water vapor) through plant stomata. Dry soil reduces the transpiration of water through a plant. Therefore, stomatal conductance will be higher when plants are *less* water stressed. As expected, G.11 rootstocks cultivated in "live" orchard replant soil with a 30-40% water deficit, had reduced stomatal conductance relative to those cultivated in well-watered soil (~80% field capacity) (Fig. 3). In the presence of replant pathogens (i.e., live orchard soil), *R. irregularis* had a highly significant, positive effect on plant water uptake (i.e., stomatal conductance) under both water-stressed and well-watered conditions ($p = 0.0001$ and $p = 0.0009$, respectively). Therefore, in these treatments (AMF x WW and AMF x WS), water acquisition likely occurred through a combination of direct root uptake and *R. irregularis* hyphal uptake/transfer to plants. This result provides clear evidence of *R. irregularis* directly functioning in a beneficial role in "live" orchard soil and represents a specific AMF-rootstock relationship that could be harnessed to improve drought tolerance.

When cultivated in pasteurized soil, however, colonization by *R. irregularis* had a significant inhibitory effect on stomatal conductance ($p=0.012$) when plants were well-watered and no effect on stomatal conductance when plants were water-stressed. The reason for this result is not clear.
To date, microbe-microbe interactions in the apple endosphere/rhizosphere are still poorly understood, and functional outcomes of AMF mycorrhization are likely to depend on complex interactions between environmental conditions and other soilborne microorganisms.

Plant nitrogen acquisition. In the current study, we also assessed whether *R. irregularis* benefited plants in terms of nitrogen uptake. When cultivated in live soil and *well-watered*, plants pre-colonized by *R. irregularis* recovered significantly more ^{15}N (from the ^{15}N-enriched fertilizer; ammonium-^{15}N nitrate; $^{15}NH_4NO_3$) than non-inoculated plants growing under the same conditions (Fig. 4; p=0.01). Under these conditions (live x AMF x WW), there was almost a doubling in the amount of ^{15}N that the mycorrhizal plants acquired from soil (relative to uninoculated plants; live x No AMF x WW). This amount (6 mg) was similar to the level of ^{15}N acquired when plants were well-watered and grown in the absence of replant pathogens (pasteurized x AMF x WW). When well-watered plants were grown in the absence of replant pathogens (i.e. pasteurized soil), ^{15}N recovery between AMF and non-AMF treatments was not significantly different (Fig. 4). This result indicates that, in live/well-watered soil, colonization by *R. irregularis* can benefit plant absorption of water and nitrogen by helping to compensate for and/or reduce root tissue damage caused by soilborne pathogens.

In *water-stressed* treatments, however, *R. irregularis* did not significantly affect ^{15}N recovery, regardless of presence/absence of soil microbes (Fig. 4). This was surprising considering the significant increase in stomatal conductance when inoculated plants grown in live soil were water-stressed (Fig 3). That said, plant uptake of nitrogen is partly regulated/balanced by the amount of water available in the soil. Therefore, there may have been less of a need to acquire N from soil in water-stressed treatments. Crops can become simultaneously water and nitrogen limited during drought episodes (Plett, et al., 2020); however, in this experiment, water stress did not appear to negatively affect plants' ability to access inorganic nitrogen.

Plant nitrogen partitioning. Compared to well-watered plants, watered-stressed plants generally contained *proportionally* more ^{15}N in roots than in shoots, regardless of soil replant status (Fig. 5). When water limits growth, plants may prioritize/retain nitrogen in roots to maintain vital functions. When experiencing a combination of replant pressure and water-stress, a plant's ability to prioritize root growth over shoot growth may become even more important. In live soil, plants that were pre-colonized by *R. irregularis* partitioned a significantly higher proportion of recovered ^{15}N into roots when water-stressed (AMF x WS) than when well-watered (AMF x WW) (Kruskal-Wallis test, p=0.02; Dunn's multiple comparison tests, p=0.01). In pasteurized soil, a similar trend appeared, but no significant differences were identified between these two treatments. Taken together, results suggest that *R. irregularis* can significantly benefit the ability of G.11 to retain/accumulate nitrogen in root tissue under combined water-stress and replant pressure.

FIGURES:

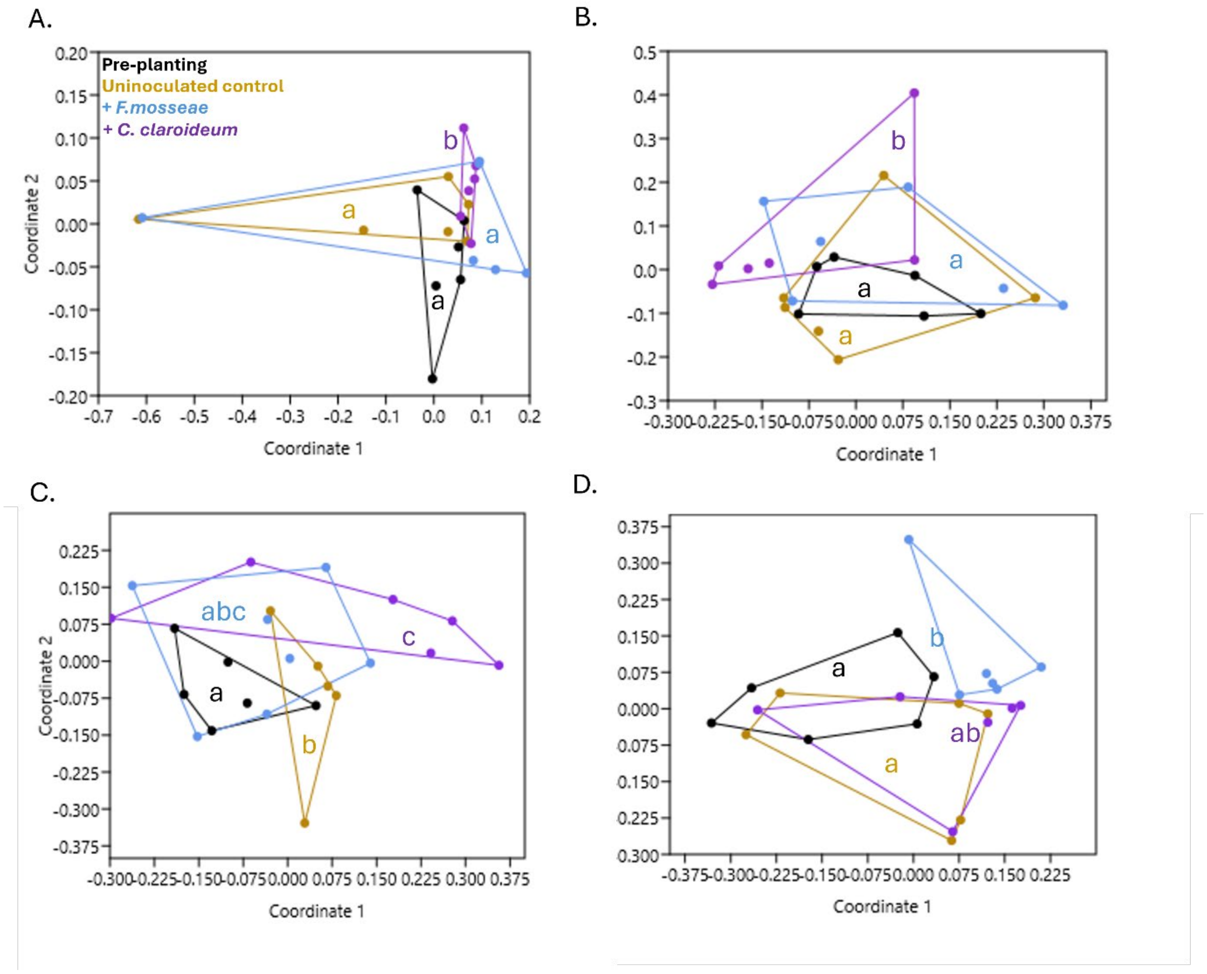

Figure 1. Effect of different treatments on pre-established (nursery-derived) AMF community composition in pasteurized (A and B) vs. live (C and D) orchard soil. Root tissue was collected prior to planting G.11 rootstocks into SRO orchard soil (Pre-planting; Black) and 2 months post-planting into either: uninoculated control soil (Brown), soil inoculated with the AMF *F. mosseae* (Blue), or soil inoculated with the AMF *C. claroideum* (Purple). Convex hulls enclose all samples derived from the same soil treatments. In panels A and C, rootstocks were obtained from TRECO Nursery; in panels B and D, rootstocks came from Cameron Nursery. Ordination of Glomeromycota (AMF) communities was conducted by NMDS analysis of amplicon sequence variant (ASV) data using the Bray-Curtis dissimilarity coefficient. Letter groups indicate significant differences ($p < 0.05$) between treatments as indicated by 1-way ANOSIM conducted using Bray-Curtis dissimilarity coefficient.

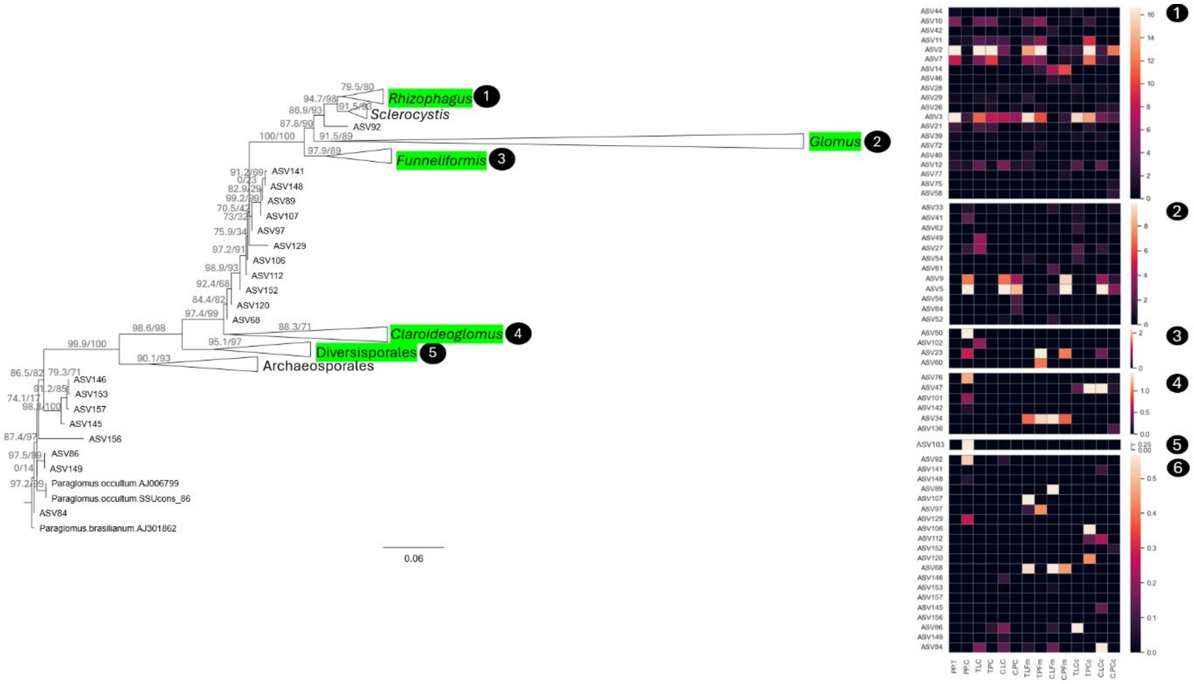

Figure 2. Figure 2. (left) A simplified version of the full phylogenetic tree for Phylum Glomeromycota used to track changes to nursery-derived AMF communities (Obj. 1). Triangles represent clades (i.e., groups of related sequences) that have been "collapsed" in order to visualize tree structure. Triangle 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 green contain amplicon sequence variants (ASVs) recovered from our sequencing analysis along with reference sequences. **(right)** Heatmaps showing relative abundance of ASVs within the major clades shown on the left (labeled 1-5). Group 6 contains ASVs from our sequencing analysis that are not taxonomically annotated (i.e., no reference sequences). Experimental treatments are listed along the x-axis of the heat map: Experimental treatments are listed along the x-axis of the heat map: PP.T and PP.C = TRECO (T) and Cameron (C) pre-planting (PP); TLC and TPC = TRECO live and pasteurized controls; CLC and CPC = Cameron live and pasteurized controls, Fm = +*F. mosseae*, Cc = +*C. claroideum*. 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 6 biological replicates for each treatment.

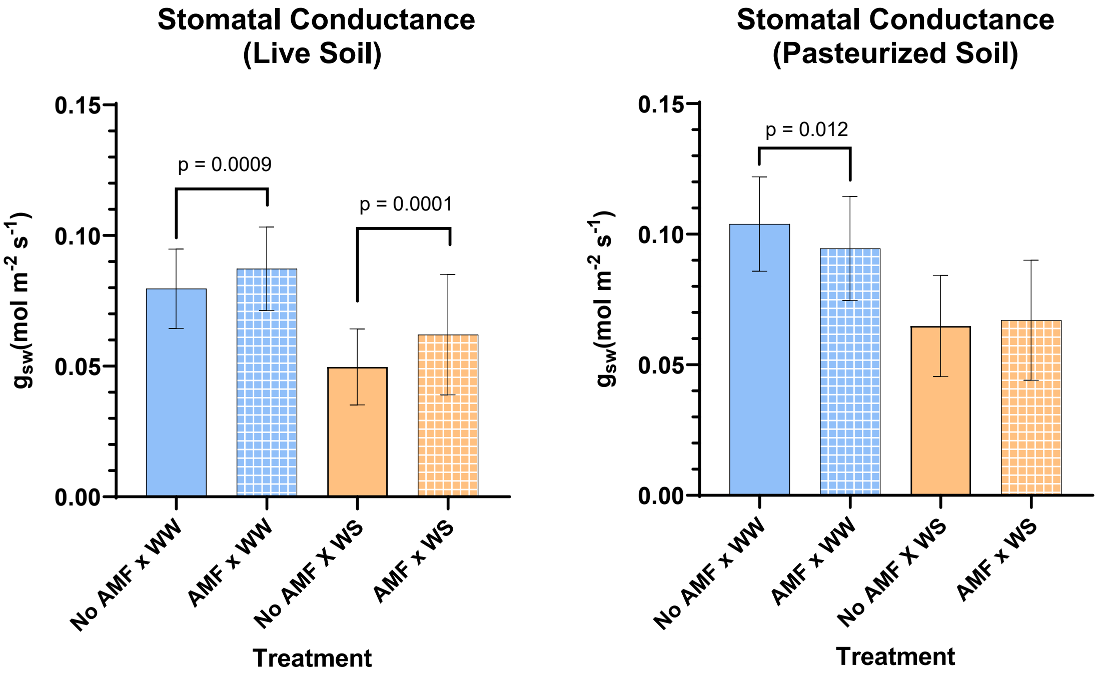

Figure 3: Bar graphs showing mean stomatal conductance by G.11 rootstocks cultivated in live (left) or pasteurized (right) orchard soil with (WS; orange bars;) or without (WW; blue bars) a water deficit (30-40% field capacity). Hatched bars represent treatments in which plants were colonized by the AMF *R. irregularis* prior to experiencing the different watering regimes. For all treatments, stomatal conductance measurements were taken from 6 different plants on the same set of 20 sampling dates (over the course of 1 month). Paired t-tests were used to control for inherent variability between days; treatments experiencing the same watering regime with or without AMF were “paired”.

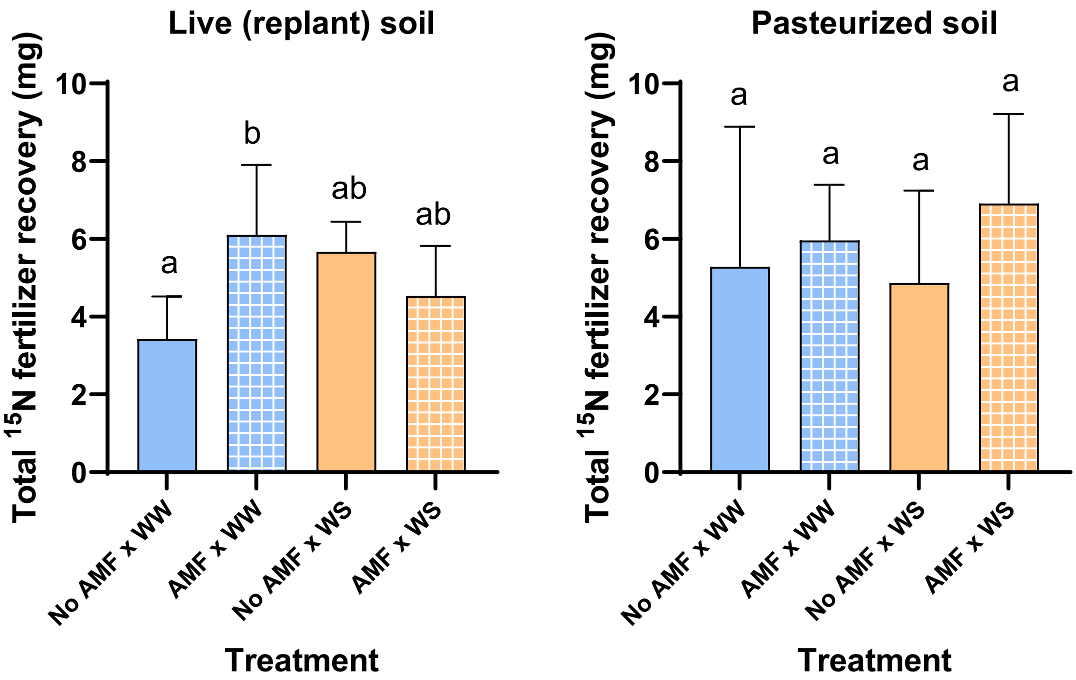

Figure 4. Bar graphs showing total ^{15}N fertilizer recovery into plants cultivated in either live "replant" soil (left) or pasteurized soil (right) after 1 month. Well-watered (WW) treatments are shown in blue, while water-stressed (WS) treatments are colored orange. Hatched bars represent treatments in which plants were colonized by the AMF *R. irregularis* prior to experiencing the different watering regimes; n=6 for all treatment combinations, except Live x AMF x WS where n=5. Within each soil type, statistically significant differences between treatments are represented by different letter groups (Kruskal-Wallis test, Dunn's multiple comparison tests, $p<0.05$).

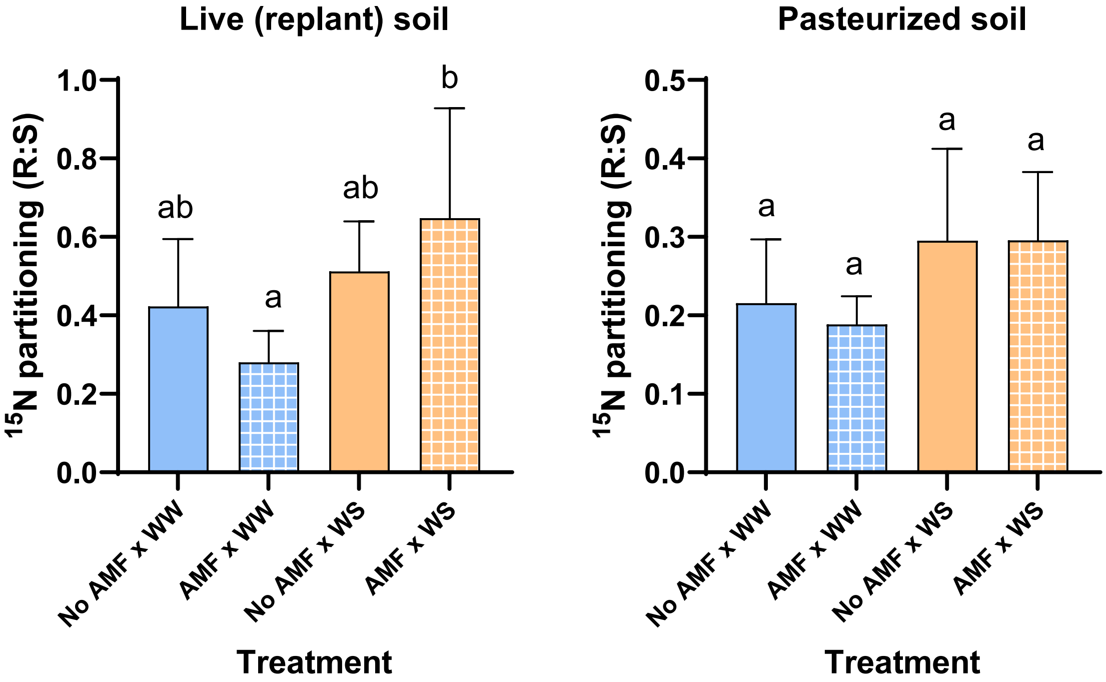

Figure 5. Bar graphs showing partitioning of ^{15}N recovered from ^{15}N-enriched fertilizer into roots vs. shoots (R:S) in G.11 rootstocks cultivated in live (replant) and pasteurized soil under different watering regimes. Well-watered (WW) treatments are shown in blue, while water-stressed (WS) treatments are colored orange. Hatched bars represent treatments in which plants were colonized by the AMF *R. irregularis* prior to experiencing the different watering regimes (n=6 for all treatment combinations, except Live x AMF x WS where n=5). Within each soil type, statistically significant differences between treatments are represented by different letter groups (Kruskal-Wallis test, Dunn's multiple comparison tests, p<0.05).

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Executive Summary

Project title: Assessing Barriers to and Benefits of AMF Colonization in Apple

Key words: AMF, apple rootstocks, drought tolerance, nitrogen uptake, replant disease

Abstract: Microorganisms living inside plant tissues (endophytes) often play very specific and crucial roles in promoting the health and growth of their host plant. Arbuscular mycorrhizal fungi (AMF) are root-colonizing endophytes present in most terrestrial ecosystems, including agricultural soils. AMF have been shown to provide a spectrum of benefits to their plant hosts including improved tolerance to water stress, access to nutrients, and disease resistance. Although many AMF species are generalists, functional benefits may be fungus- and plant-species dependent. A primary aim of this research was to assess a range of benefits of specific apple rootstock/AMF associations. Previously identified "compatible" rootstock/AMF combinations leading to rapid establishment of a relationship were selected for testing. Specifically, we tested the ability of two different AMF species (*Claroideoglomus etunicatum* and *Claroideoglomus claroideum*) to enhance plant defense against infection by the fungal replant pathogen *Rhizoctonia solani* in G.41 and G.890 tissue-cultured plantlets. A separate experiment was conducted to test for *R. irregularis-mediated* tolerance to water stress and nitrogen uptake ($^{15}NH_4NO_3$) in G.11 rootstock. Experiments provided clear evidence of AMF species directly functioning in beneficial roles with commercially available apple rootstock genotypes. Colonization of G.11 rootstocks by *R. irregularis* led to significant increases in stomatal conductance in live orchard soil in both water-stressed (30-40% water deficit) and well-watered (~80% field capacity) treatments. This result highlights the role of a specific apple rootstock-AMF associations in maintaining water supply to plants experiencing a combination of water-stress and replant pressure, especially in less vigorous apple rootstocks with relatively small root systems. When grown in *live* orchard soil and *well-watered*, G.11 rootstocks pre-colonized by *R. irregularis* recovered significantly more nitrogen (from ^{15}N-enriched fertilizer) than non-inoculated plants growing under the same conditions. In addition, under combined water-stress and replant pressure (conditions in which a plant's need to prioritize root growth over shoot growth would be expected to be relatively high), inoculation with *R. irregularis* benefited the ability of G.11 rootstocks to retain/accumulate nitrogen in root tissue. Finally, the AMF *C. etunicatum* (but not *C. claroideum*) significantly enhanced plant defense against subsequent infection by *R. solani* in G.41 (but not G.890), relative to uninoculated controls. Results of this study provide insight into specific AMF-rootstock relationships which could be harnessed to improve disease control, drought tolerance and/or sustainability, and represent a first step towards assessing the utility of and improving upon current practices to promote the establishment of mycorrhizal associations in orchard systems.

Project Title: Assessing refugia plantings for biocontrol services

Report Type: Final Project Report

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Cooperators: Teah Smith (Zirkle), Dianna Sanchez (Stemilt)

Project Duration: 2 Years

Total Project Request for Year 1 Funding: $48,401
Total Project Request for Year 2 Funding: $50,235

Other related/associated funding sources: Awarded
Funding Duration: 2022–2025
Amount: $249,560
Agency Name: Washington State Department of Agriculture Specialty Crop Block Grant
Notes: Ensuring reliable pollination for Washington apples with cultural practices and conservation.

Other related/associated funding sources: Applied, not funded
Funding Duration: 2025
Amount: $24,000
Agency Name: Washington Commission in Integrated Pest Management
Notes: Assessing beneficial insects in planted refugia

WTFRC Collaborative Costs: none

Budget 1
Primary PI: Dr. RT Curtiss
Organization Name: Washington State University
Contract Administrator: Office of Research Support and Administration
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Station Manager/Supervisor: Kimi Lucas (interim)
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Item	**2024**	**2025**
Salaries	$32,619.00	$33,924.00
Benefits	$13,232.00	$13,761.00
Wages		
Benefits		
RCA Room Rental		
Shipping		
Supplies	$550.00	$550.00
Travel	$2,000.00	$2,000.00
Plot Fees		
Miscellaneous		
Total	$48,401.00	$50,235.00

Footnotes:

[1] Salary for PI Orpet = pay rate of $7,260.42/month X 12 months X 10% FTE (salary originally budgeted for Orpet in 2025 will be used instead for an additional 5% FTE to Curtiss and 8.78% FTE to technical assistant); Salary for Co-PI Curtiss = pay rate of $7,083.33/month X 12 months X 5% FTE; Salary for technical assistant = pay rate of $3,900/month X 12 months X 42% FTE. All personnel have a 4% COLA increase for year 2.

[2] Benefits rates of 32.8% (Orpet), 32.2% (Curtiss), and 42% (technical assistant)

[3] Natural enemy sampling supplies (sticky cards, plastic bags)

[6] Travel to field sites, approximately 100 miles a week for 25 weeks/year

Completed Objectives

1. Quantify natural enemy and plant communities in refugia plantings with weekly sampling at sites in the Columbia Basin across three growing seasons.
2. Assess spillover of natural enemies and effects on pests from the wildflower plantings by sampling in transects starting in the apple orchard edge row and extending up to 1,000 ft into the orchard.
3. Create a codling moth parasitoids reference collection housed at WSU-TFREC, to document and assist with identifications in Objective 2.
4. Share findings, including practical advice on economics of plantings relative to biocontrol benefits.

Significant Findings

- Refugia supported high numbers of beneficial insects, including syrphid flies (whose larvae are aphid predators) and bigeyed bugs (*Geocoris;* generalist predators). More of these taxa were found in refugia than in apple orchards. In the first year or study, abundance of these beneficials within apple orchards was not correlated with proximity to refugia.
- Refugia supported lower numbers of the omnivorous apple pest *Campylomma* than apple orchards, suggesting that the refugia will not induce *Campylomma* problems.
- Green lacewings and mite-eating ladybugs were scarcely found in refugia, and similar numbers of aphid-eating ladybugs were found in orchards as refugia. However, in-field observations suggest that the sampling method used (yellow sticky cards with no lures) may be ineffective for monitoring aphid-eating ladybugs. Inferring sources and spillover of highly mobile insects like green lacewing adults is difficult with the study design.
- Abundance of woolly apple aphids and leaves rolled by leafrollers within apple orchards was not correlated with proximity to refugia plantings.
- Codling moth parasitoids were extremely rare in collections from several Washington locations.

Methods

Objective 1. – Quantify natural enemy and plant communities in refugia

Monitoring for beneficial insects was conducted in flower plantings and at varying distances into adjacent apple orchards. Flowers were previously planted by orchardists and have been studied for pollinator diversity since 2022 in association with the WSDA Specialty Crop Block Grant-funded project entitled "Ensuring reliable pollination for Washington apples with cultural practices and conservation" led by the original PI Orpet; this project was subsequently led by PI Curtiss for the 2024 and 2025 seasons. This project, funded by Washington Tree Fruit Research Commission, supported staff to examine stored sticky cards from 2023 and quantify biocontrol agents and deploy new sampling in 2024 and 2025.

Field procedures varied between years. In 2023, six sites were studied. In 2024 and 2025, five of those were studied; one was excluded because the orchard was cut down. In all years, insects were monitored at each site in a flower planting plot and in three plots in the adjacent apple orchard (Figure 1). In 2023, the three orchard plots were: three rows in from the flowers, 20 meters in from the flowers (about 6 rows), and 20 meters from the opposite end of the orchard (Figure 1A). The plots were modified in 2024/5, replacing the far edge plot with an orchard-central plot that was 50–100 meters from the flowers, depending on the size of the orchard (Figure 1B). In all years, all plots were 20 m long. The flower plots were 0.3–1.0 m wide, and orchard plots were the width of one drive row.

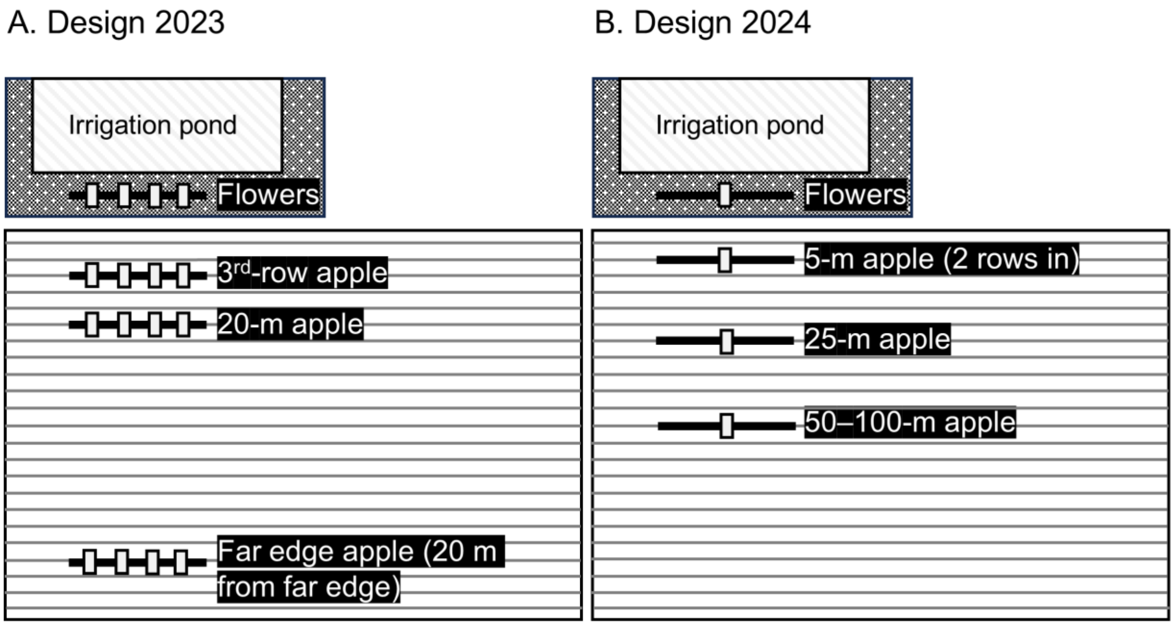

Figure 1. Diagram of sampling design used in 2023 (A) and 2024/2025 (B). An example is given where flowers were planted around an irrigation pond. Other plantings could be strips by roadsides or fields next to orchards. Orchard dimensions and row spacing varied between sites.

In 2023, four sticky cards were placed in each plot, and in 2024/2025 only one card was placed in each plot. Cards were replaced once every two weeks after bloom until fall. The card dimensions were 8 × 5 inches or 4 × 6 inches.

In all years, two blue vane traps, which are effective for collecting Hymenoptera (including parasitoids), were deployed 3 m from the ground in each plot for one 24-h interval every two weeks.

In all years, a survey of flowering plants was made on each visit by walking through each plot and recording all species in bloom.

Objective 2. – Quantify spillover of beneficials and monitor pests in orchard transects

Additional pest sampling was done in the orchard plots beyond the sticky card and blue vane trap sampling described in Objective 1 in refugia and orchards. Ten first-year shoots in each orchard plot were inspected on each visit. For green apple aphids, apple grain aphids, and rosy apple aphids, the number of infested leaves were counted. For woolly apple aphids, the number of infested leaf axils was recorded. For leafrollers, the number of rolled leaves was recorded.

Objective 3. – Create a codling moth parasitoid reference collection

Investigator RT Curtiss collected a small number of Hymenopterans that emerged from field-collected laboratory codling moth colonies. It was planned to request reference insects from the Washington State University insect museum to build a collection of known codling moth parasitoids to compare new specimens with. However, so few were collected from WA codling moths that they are currently an insignificant part of the biodiversity. Likewise, none were collected by other methods.

Objective 4. – Share findings.

Outputs of this project included lists of beneficial insect and plant species found in refugia plantings, quantification of aphid and leafroller pests correlated with beneficial insect spillover into orchards, and a better understanding of codling moth parasitoid fauna. Findings were shared on a project website all about refugia plantings, at least one extension presentation each year of the project, and a field day showcasing an example planting in the second year.

Results and Discussion

Objective 1. – Quantify natural enemy and plant communities in refugia

Sticky card data showed beneficial insects were found in variable numbers in flower refugia. The woolly apple aphid parasitoid *Aphelinus mali* was scarcely found in refugia plots relative to orchard plots in any year (Figure 2A, B, C). Syrphid flies appeared to make great use the refugia (Figure 2D, E, F), but green lacewings did not (Figure 2G, H, I). Bigeye bugs (*Geocoris)* were consistently most abundant in refugia, but scarcely found in orchards (Figure 3A, B, C). The opposite was true of the small black ladybugs that eat mites (Figure 3D, E, F), whereas similar numbers of aphid-eating ladybugs (i.e., two-spotted ladybugs, transverse ladybugs, convergent ladybugs) were found in refugia as orchards (Figure 2G, H, I). Distribution of mite and aphid lady bugs between plots varied greatly between years, with aphid lady bugs seeming to prefer refugia to orchards in 2024 and 2025.

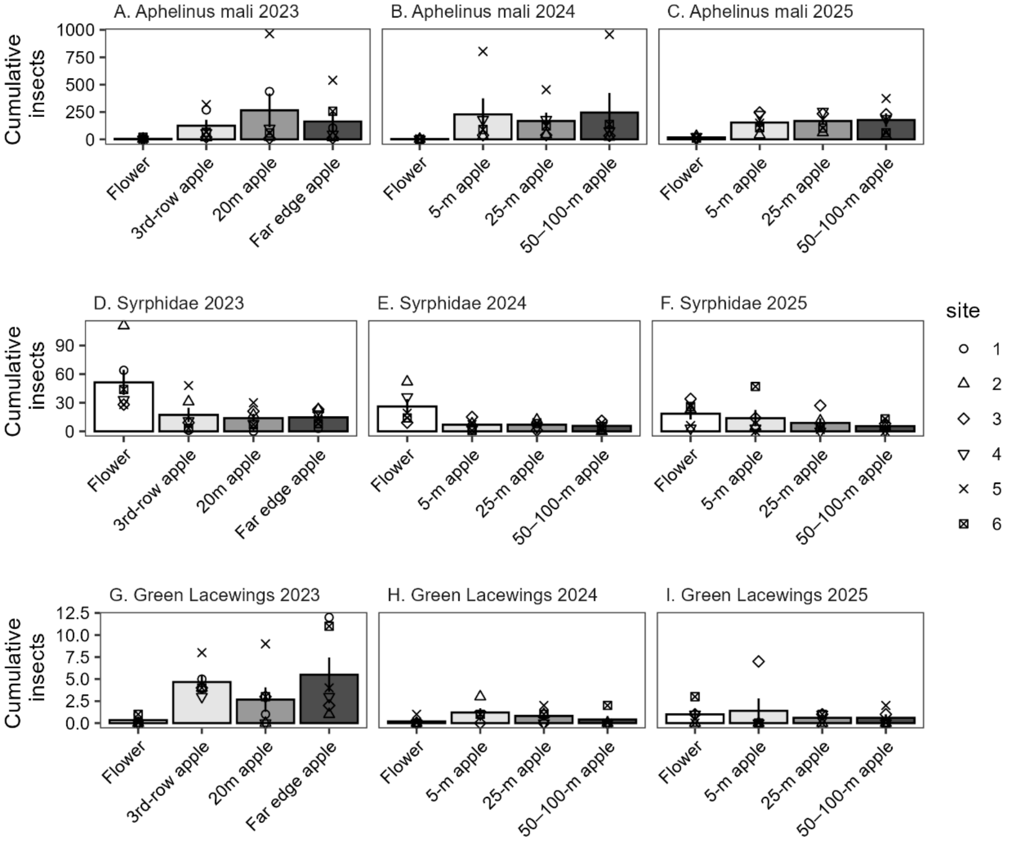

Figure 2. Cumulative number of beneficial insects found on sticky cards during 2023 at six sites, and 2024 and 2025 at five. Individual points show data for one site, and bars show the mean of all sites visited with standard error bars.

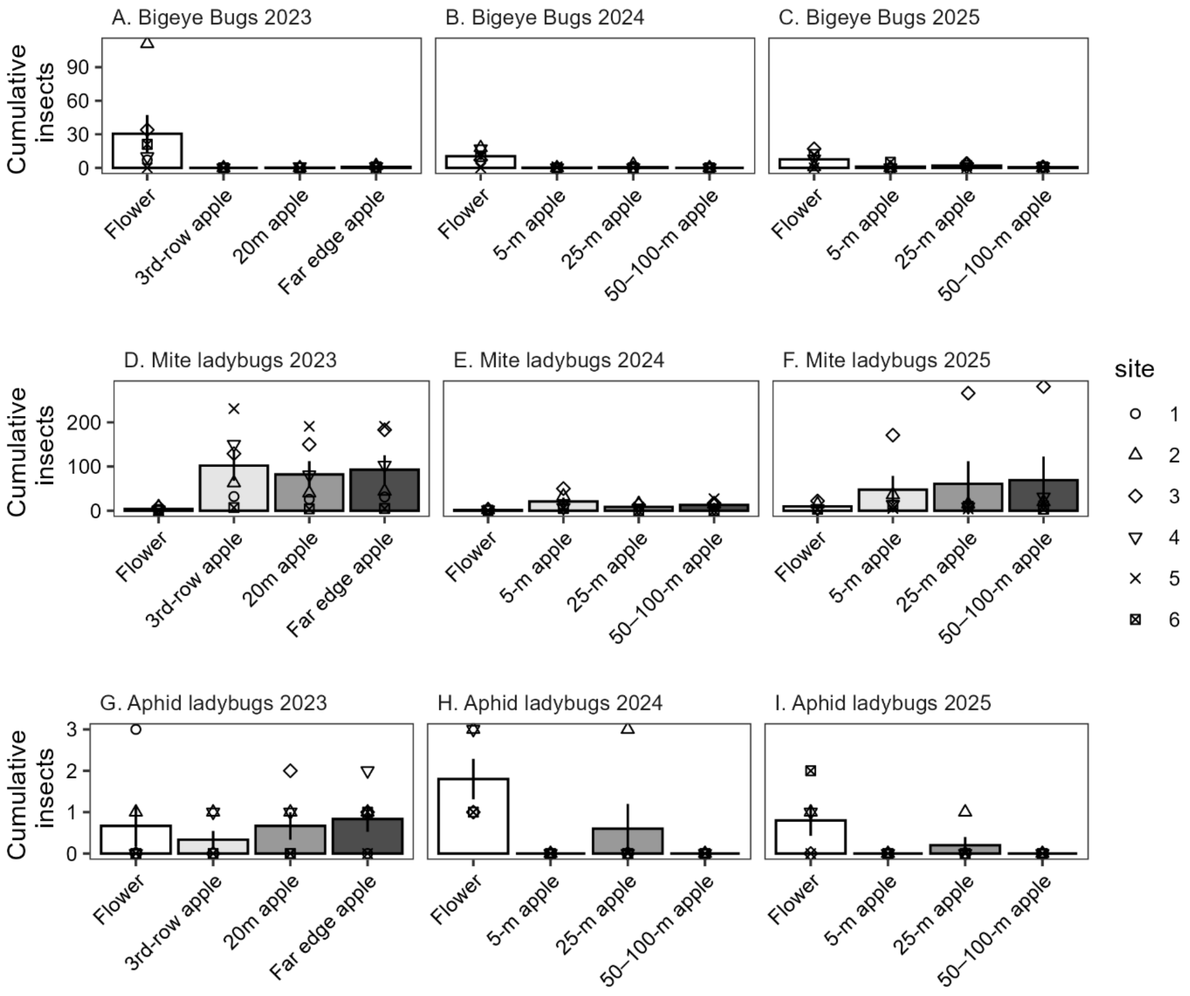

Figure 3. Cumulative number of beneficial insects found on sticky cards during 2023 at six sites, and 2024 and 2025 at five. Individual points show data for one site, and bars show the mean of all sites visited with standard error bars.

Monitoring showed that refugia likely improves the stability of syrphid and predatory bug populations. Adult syrphids eat nectar from flowers. Orchards had few flowers and low flower diversity (Figure 4), so they supported a smaller syrphid community than would be possible if season-long flowers were available, like in refugia plantings. Bigeye bugs were surprisingly more abundant in refugia than orchards. These predatory bugs are known to attack aphids in orchards, indicating importance of refugia for their conservation and use in integrated pest management.

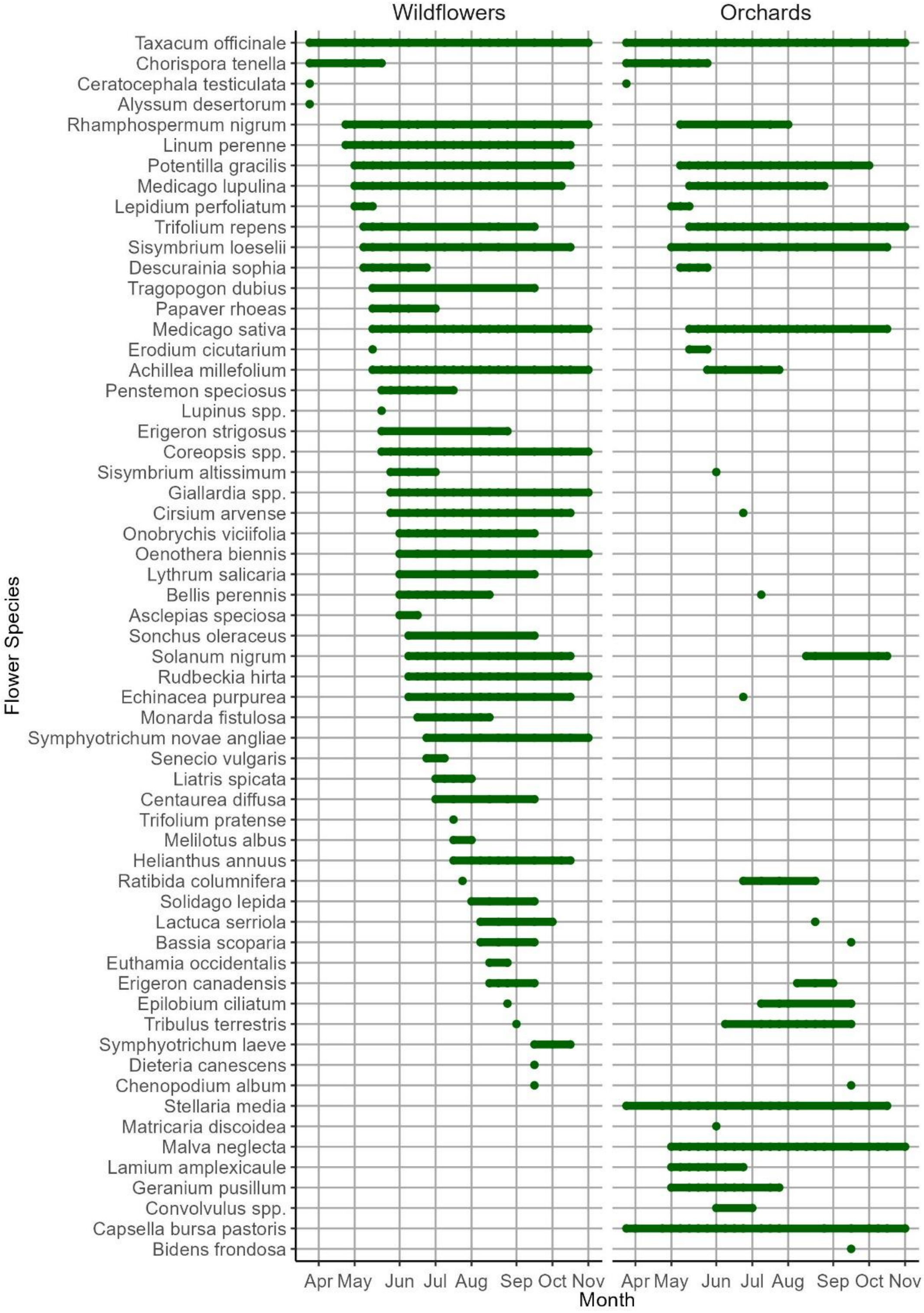

Figure 4. Flowering period of each species observed across sites and years in wildflower refugia and all orchard plots combined, arranged by earliest detection in wildflower refugia.

Some beneficial insects seemed indifferent to the planted refugia. For example, *Aphelinus mali* are specialists of woolly apple aphid, so it is not surprising that few were found in refugia. The same may be true of the small black ladybugs that eat mites; perhaps few mites occur in the refugia. On the other hand, green lacewings are highly mobile generalists and were expected to occur in refugia, but they were rarely observed compared with orchards. Similarly, there were about as many aphid-eating ladybugs in refugia as orchards. Sampling methods for lacewings and ladybugs may have been inadequate to capture their presence in the refugia. Likewise, the sticky card method may be ineffective for monitoring some taxa like aphid-eating ladybugs. In past studies, many ladybugs and ladybeetle larvae were observed in some refugia in some years, particularly on yarrow plants, which may host alternative prey for the ladybugs.

The blue vane traps that were deployed are highly effective at collecting pollinators and other Hymenoptera.

Objective 2. – Quantify spillover of beneficials and monitor pests in orchard transects

Woolly apple aphids (Figures 5A, B, C) and rolled leaves (Figures 5D, E, F) in apple orchards were found in similar abundance regardless of distance from refugia in all years of this study. *Campylomma* bugs from sticky cards were also found in similar abundance regardless of distance from refugia (Figure 5G, H, I). Many fewer *Campylomma* were found on sticky cards in the refugia than in orchards, suggesting that refugia are probably not sources of this apple pest.

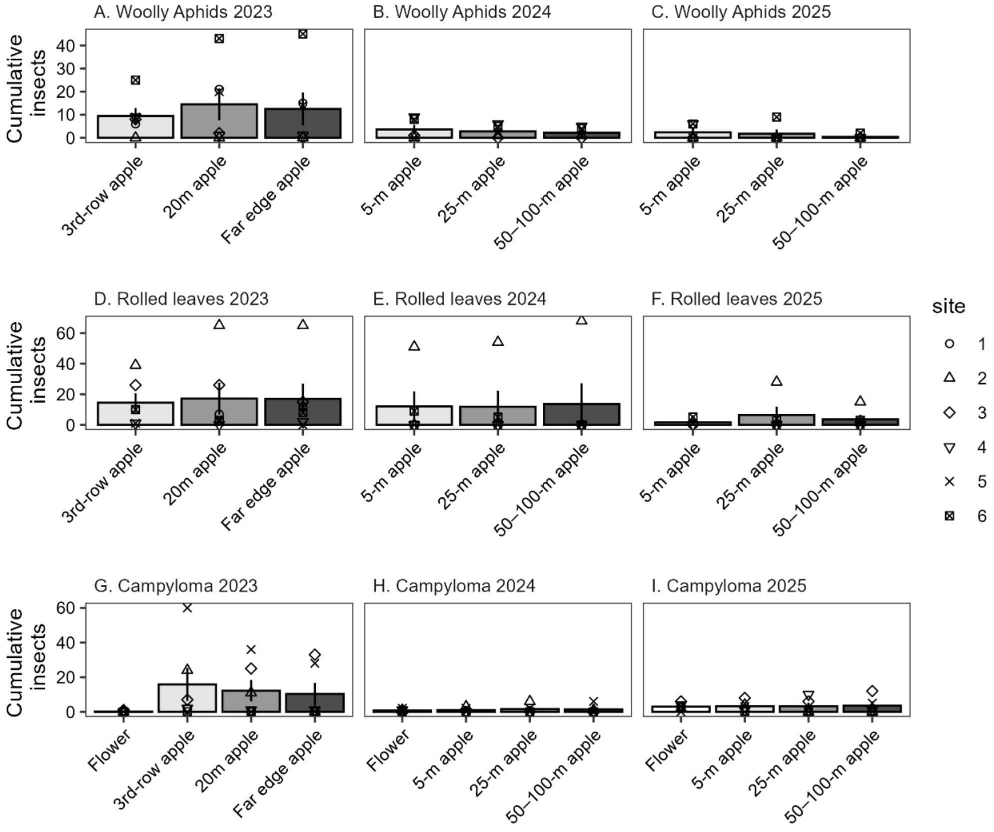

Figure 5. Cumulative number of pest insects found with apple tree sampling (panels A, BC, D, E, F) or sticky cards (panels G-I) during 2023 at six sites and 2024 and 2025 at five. Individual points show data for one site, and bars show the mean of all sites visited with standard error bars.

Likewise, two of the leafhopper vectors of cherry X-disease, *Colladonus montanus reductus and Colladonus geminatus,* were uncommon in refugia relative to orchards (Figure 6). A third vector species, *Euscelidius variegatus*, was more abundant in refugia. However, the abundance of *E. variegatus* was so low in orchards, including edges close to refugia, that it seems that the refugia probably were not a major source of X-disease to neighboring cherry orchards; evidence from movement studies in Oregon suggest *E. variegatus* is not very dispersive, which is consistent with our observations here.

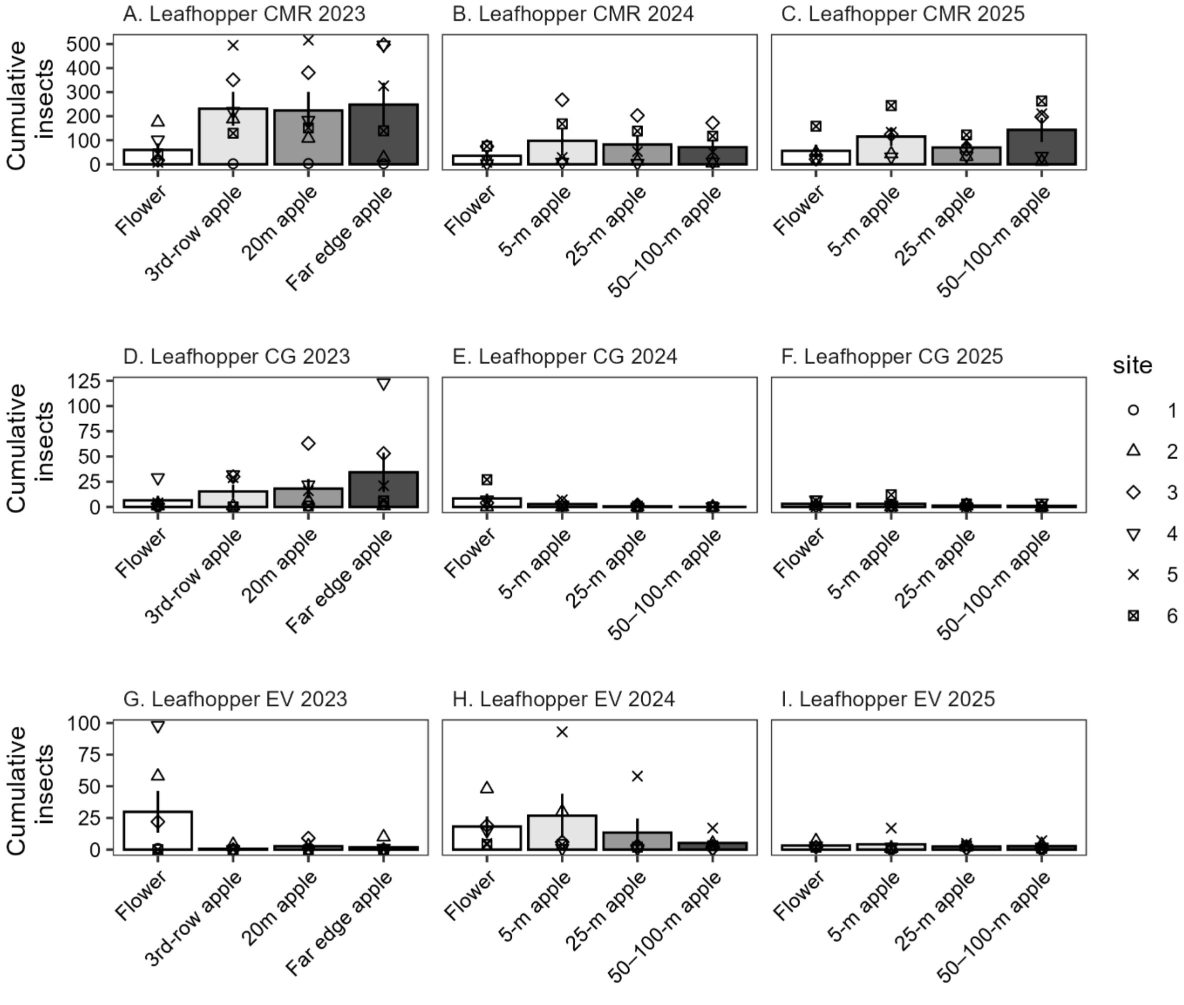

Figure 6. Cumulative number of leafhoppers Colladonus montanus reductus (CMR), Colladonus geminatus (CG), and Euscelidius variegatus (EV) found on sticky cards during 2023 at six sites and 2024 and 2025 at five. Individual points show data for one site, and bars show the mean of all sites visited with standard error bars.

Measuring the effect of refugia on crop areas has proven difficult. There was generally no clear pattern of insects spilling over from refugia into orchards. However, spillover is difficult to infer with the study design. We expected to find more of some insects in orchard plots closer to refugia, with clear declines in abundance as distance from refugia increased. However, if insect dispersal is great and is highly influenced by orchard-specific factors like prey density, patterns may not be clear. A relatively small number of refugia-generated insects may colonize orchards each spring and cause a subsequent numerical response to high prey density. This may result in more beneficial insects observed in the orchard (where there is food, e.g., woolly apple aphids) than in the refugia despite the early importance of refugia as an initial source population of predators.

Despite the limitations of the study, it seems reasonable to suggest that the refugia studied are less important for specialized insects that feed on apple pests because their prey will always be higher in the orchard. This is the case for the woolly apple aphid specialist *A. mali.* However, if refugia contained firethorn (none of the studied refugia had this plant), which is a plant that woolly apple aphids can feed on, then refugia may have been a source of *A. mali.* Generalists that require flowers during the adult stage and are highly mobile, e.g., syrphid flies, likely benefit from refugia.

Likewise, the direct effect of refugia on pests is also difficult to infer. Rosy apple aphids were less common than other pests (Figure 7). Similarly, very few green apple aphids (27 insects total), and apple grain aphids (12 insects total) were found in the orchards we monitored.

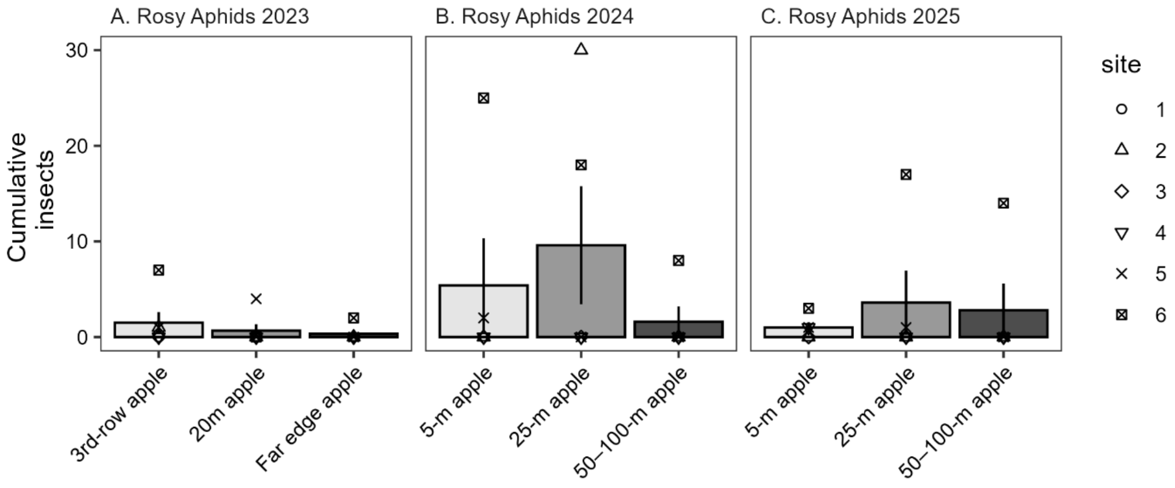

Figure 7. Cumulative number of rosy apple aphids found with apple tree sampling during 2023 at six sites and 2024 and 2025 at five. Individual points show data for one site, and bars show the mean of all sites visited with standard error bars.

Objective 3. – Create a codling moth parasitoid reference collection

Parasitoids in field-collected codling moth colonies were collected each year in extremely small numbers. Identification and creation of a reference collection is ongoing. However, their rareness in collections suggests that they are an insignificant component of codling moth management. In contrast, fungal pathogens have been found in codling moths far more frequently than parasitoids, suggesting that they may eventually be useful as management tools.

Objective 4. – Share findings.

Development of educational materials to share is ongoing. Investigator M Luppino established a project website (https://cahnrs.wsu.edu/tfrec-orpet/insect-habitat-survey/) that included photos of flower plots and insects found. Luppino spoke about the project at ten events, including at NCW Apple Day in 2025, the 2025 WA Hort EXPO, reaching more than 600 individuals directly across the three years of the project. Field days with orchard manager cooperators were held at one of the flower plantings each year.

Conclusion

The results indicate that refugia are heavily used by syrphid flies, which are important aphid predators. There was not clear evidence that other beneficial taxa were utilizing refugia en masse or spilling over into neighboring orchards. The same is true of pests; there was no evidence that these refugia are pest sources or contribute to pest outbreaks. However, pollinators greatly benefitted from these refugia, and there were significant positive effects to their populations.

All objectives were completed by the end of 2025. Three years of insect sampling were conducted, and samples were quantified. Technical staff are now trained for sampling procedures and for identification of beneficial insects on sticky cards for future projects. Findings are in the process of being published in peer reviewed journals as of the writing of this report. The previous lead investigator, Orpet, left Washington State University to Oregon State University January 1, 2025, so the lead role was passed to RT Curtiss for the final year of the project. Curtiss oversaw project administration. Sampling and insect quantification was managed by Luppino, who filled this role in previous years. Orpet stayed as a co-PI to assist with administration and data analysis.

Proposal Title: Patulin risk assessment and improved detection

Report Type: Continuing Proposal

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Cooperators: Fruit Processors for sampling fruit, Dale Whittington (University of Washington) will assist with PAT analyses

Project Duration: 3 Years

Total Project Request for Year 1 Funding: $45,649
Total Project Request for Year 2 Funding: $49,539
Total Project Request for Year 3 Funding: $08,100

Other related/associated funding sources: None

WTFRC Collaborative Costs: None

Budget 1
Primary PI: Achour Amiri
Organization Name: Washington State University
Contract Administrator: Kevin Rimes
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Item	6/1/2025	6/1/2026	6/1/2027
Salaries[1]	$26,730.00	$27,799.00	$0.00
Benefits[1]	$8,019.00	$8,340.00	$0.00
Wages	$0.00	$0.00	$0.00
Benefits	$0.00	$0.00	$0.00
RCA Room Rental	$0.00	$0.00	$0.00
Shipping	$0.00	$0.00	$0.00
Supplies[2]	$10,100.00	$12,600.00	$7,300.00
Travel[3]	$800.00	$800.00	$800.00
Plot Fees	$0.00	$0.00	
Miscellaneous	$0.00	$0.00	
Total	$45,649.00	$49,539.00	$8,100.00

Footnotes:

[1] Salaries and benefits of a Postdoctoral Scientist for nine months at 0.6 FTE and 30% benefit rate to lead the research outlined in the project. A 4% annual increase is accounted for in Year 2.

[2] Supplies include media and petri plates to grow, test, and maintain fungi; sequencing of fungi in years 1 & 2; patulin analysis in Years 2 & 3; DNA extraction kits and reagents; quantitative PCR and dye for patulin detection and fungi associated with; as well as sampling bags, pipette tips, crates.

[3] Travel to processing facilities and extension meetings every year.

OBJECTIVES

We propose this three-year project to address the following objectives:

1- Characterize the ability of "non-expansum" *Penicillium* species to produce patulin in vitro and on apples.

2- Investigate the role of asymptomatic fruits as a source of patulin (PAT) producing fungi and develop detection assays for patulin-producing fungi in asymptomatic fruits.

3- Update and improve existing SOP to better mitigate patulin and conduct outreach activities for growers, packers, and processors.

These objectives address the postharvest research priority #4 delineated in Figure 1. The goal is to establish a comprehensive approach to understand the sources of patulin contamination, improve detection, and mitigation of PAT contamination which aligns with the 2025 ACP priority #2 for Enhanced Postharvest Decay management.

SIGNIFICANT FINDINGS

- In 2024, 24 isolates from 5 *Penicillium* species were inoculated to Gala apples.
- Patulin (PAT) was quantified at 2 and 5 months of cold storage in regular atmosphere.
- Two species did not produce PAT at either period.
- PAT production was significantly greater at 5 months compared to 2 months
- As expected, most *Penicillium expansum* isolates produced PAT at 2 and 5 months
- Among the *Penicillium* species found to be virulent on apples, many isolates of *P. solium* and *P. crustosum* did not produce patulin, while a few isolates of these same species produced smaller amounts but were still above the 50 µg/kg limit.
- In 2025, 35 isolates from 13 *Penicillium* species were inoculated to Gala and Honeycrisp apples. Apples are currently in storage and will be used to quantify PAT and a larger number of isolates and species.
- Preliminary results indicate ability of some non-expansum species to produce PAT although at lower quantities compared to the main species *P. expansum*.

METHODS

Objective 1. Characterize the ability of "non-expansum" *Penicillium* species to produce patulin (PAT) in vitro and on apples. [Years 1-2]

Activity 1.1. Evaluation of Penicillium species for their toxigenic potential in vitro [Year 1].

Ten *Penicillium* species, including *P. expansum*, *P. solitum*, *P. roqueforti*, *P. commune*, *P. paneum*, *P. psychrosexuale*, *P. crustosum*, *P. carneum*, *P. palitans*, and *P. citrinum,* isolated from decayed apples throughout the ending project CP-21-102A, will be investigated for PAT production. Two to five isolates from each species, depending on their frequency, will be selected from the culture collection of the Pathology lab and tested in vitro on three liquid growth media: malt extract broth (MEB), 5% glucose/yeast extract/peptone (5-GYEP), and yeast extract medium (YES) to account for medium effect on PAT production as previously demonstrated (Dombrink-Kurtzman and Blackburn, 2004). Spore suspensions of each isolate will be prepared and diluted to 10^5 spores/milliliter (mL) and 100 microliters (µl) of the suspension will be used to inoculate 50 mL the three media in glass flask in triplicates. The flasks will be incubated at 72°F (22°C) at 140 rpm on a rotary shaker for 7 to 15 days. Afterward, the medium in each flask will be aseptically filtered and stored at -112°F (80°C) and used for PAT extraction as described below. Non-inoculated media and non-inoculated media supplemented with PAT at a known concentration, will serve as negative and positive controls, respectively.

Activity 1.2. Evaluation of Penicillium species for their toxigenic potential on detached apples [Years 1&2].

The isolates examined in Activity 1.1. will be assessed for PAT production on apples from two cultivars: Honeycrisp and Grany Smith, to account for potential cultivar effects (Snini et al. 2016). Fruit will be harvested at commercial maturity from the WSU-Sunrise orchard in East Wenatchee and will be surface disinfected for 2 min in 0.6% sodium hypochlorite, rinsed three times with sterile water, and air-dried in a laminar flow hood. The apples will be punctured (3 mm diameter x 3 mm depth) at two equidistant points around the stem-bowl area using a sterile syringe. Each wound will be inoculated with 20 µL of spore suspension (10^5 pores/mL) from each isolate or with sterile water as a negative control. Six replicate apples will be used for each isolate and cultivar combination in duplicate. Inoculated apples will be stored in clamshells at 35°F (1.5°C) for five months. The decayed tissue surrounding the wounded area will be collected and frozen at -112°F until it is used for PAT extraction. Apple samples inoculated with sterile water or spiked with known concentrations of pure PAT will serve as negative and positive controls, respectively.

Patulin Extraction and quantification: Patulin will be extracted from the liquid medium (Activity 1.1) and inoculated apples (Activity 1.2) using the QuEChERS (quick, easy, cheap, effective, rugged, and safe) method adapted from a previously published procedure. A total of 10 mL of the medium (Activity 1.1) or 10 g of the decayed apple tissue (Activity 1.2) will be extracted with 10 mL of 1% HOAC in acetonitrile, using a pellet of ceramic homogenizer and one pouch of QuEChERS extraction salt (4g MgSO4, 1NaCL). A volume of 5 mL of the organic phase will be transferred to a dispersive SPE tube (0.9 g MgSO4, 0.1850 g PSA and 0.045g GCB). A volume of 1 mL of supernatant will be filtered through a 0.2 µm filter then evaporated to dryness in a fume hood. Dried samples are then dissolved in 1 mL distilled water containing 0.1% acetic acid. Patulin will be quantified by liquid chromatography-mass spectroscopy (LC–MS) method previously validated (project #: CP-21-102A) and analyses will be conducted at the Mass Spectrometry Center, University of Washington.

Activity 1.3. Molecular investigation of "non-expansum" Penicillium species for presence of genetic markers linked with patulin production and genes expression levels [Years 1&2].

While testing a sub-sample of *Penicillium* species in vitro and on detached fruit is valuable for assessing their ability to produce PAT, it is also essential to determine whether these species possess the genetic capacity for PAT production. PAT biosynthesis is regulated by a genetic cluster comprising 10 genes, and research has shown that not all strains contain the complete set of these genes. Consequently, species that may possess only 4 or 7 genes per cluster may exhibit reduced PAT production relative to species that contain all 10 genes. To investigate this hypothesis, it is imperative to assess the presence of the patulin gene cluster responsible for PAT biosynthesis (Tanous et al., 2014;

Artigot et al., 2009). DNA will be extracted from the *Penicillium* species described in Activity 1.1 using standard laboratory protocols. Primers developed by Tanous et al. (2014) for *P. expansum*, along with additional primers to be developed in this project for other species identified in Washington, will be utilized to screen for the presence of PAT-related gene clusters or fragments of the clusters.

Based on the outcomes derived from the identification of the PAT biosynthesis genes in the target 10 *Penicillium* species, the expression of the PAT cluster genes will be analyzed using Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) to verify their potential for PAT production. This analysis is crucial for assessing whether the absence of certain genes within the PAT cluster affects the species' capacity to produce PAT. A correlation will be established between PAT production in vitro (Activity 1.1), in vivo (Activity 1.2), and the expression of PAT-related genes (this activity). Total RNA will be extracted from the apples utilized in Activity 1.2, following standard laboratory protocols. Specific primer pairs targeting genes from the PAT biosynthesis cluster will be designed to facilitate the investigation of their expression. Reverse transcription will be conducted on the purified RNA to generate complementary DNA (cDNA) for subsequent quantitative PCR analysis.

Expected benefits to the industry and potential pitfalls: Accurately assessing the ability of numerous *Penicillium* species, beyond the well-documented *P. expansum*, to produce PAT and contribute to food contamination is essential, particularly in light of our recent findings that indicate elevated levels of resistance to postharvest fungicides among non-*P. expansum* species (Pandey and Amiri, 2024). The knowledge gained through this investigation will facilitate a more effective risk assessment associated with these species and will be critical in developing mitigation strategies.

Objective 2. Investigate the role of asymptomatic fruits as a source of patulin (PAT) producing fungi and develop detection assays for patulin-producing fungi in asymptomatic fruits. [Years 1-3]

Activity 2.1. Sampling asymptomatic apples from commercial processing facilities [Year 1].

To validate the findings previously reported from only 4 lots with asymptomatic fruit surveyed in the concluding project #: CP-21-102A regarding the presence of fungi in asymptomatic fruits, an additional survey of asymptomatic fruit is necessary. This study will investigate the presence of fungal organisms within the core of the fruit, as well as the levels of PAT contamination. Coordination with fruit producers, packers, and processors will be essential. We will randomly collect approximately 50 asymptomatic fruits from various bins, adhering to the protocol established in project #: CP-21-102A. Five lots will be surveyed in Year 1. The fruits will be placed in clamshell containers to prevent bruising and contamination and will subsequently be transferred to the Plant Pathology Laboratory at WSU-TFREC in Wenatchee for further analysis. Each fruit will be examined for any symptoms, lesions, or infections, and only those identified as asymptomatic will be retained for further study. Additionally, fruits that have been previously inoculated with a virulent strain of *P. expansum*, recognized for its capacity to produce significant quantities of patulin, will serve as a positive control. The collected samples will be appropriately labeled, and each apple will be aseptically split into two halves: one half will be used to isolate potential fungi from the core, while the other half will be frozen at -112°F to be used for PAT detection using the method described in Objective 1. Fungal isolation from one half of the fruit will be achieved by plating a portion of the core of each apple on Petri plates containing an agar medium and incubating the plates for 7 to 10 days at 70°F. The colonies will be identified using key morphological and microscopic characteristics, and unknown fungi of interest will be characterized using a DNA-based technique.

Activity 2.2. Development and validation of a quantitative molecular detection assay for patulin-producing Penicillium species in asymptomatic apples. [Years 2-3].

Quantitative PCR (qPCR) is a highly sensitive method used to detect and quantify pathogens as well as for assessing the expression of genes that are crucial in microorganisms. Herein, we aim to exploit the qPCR technique to develop a precise straightforward easy assay to detect and quantify *Penicillium* species in asymptomatic apples, allowing for timely decisions regarding the removal of contaminated fruits prior to processing. This method will focus on the detection and quantification of the expression of the patF gene, which is part of the PAT gene cluster and encodes an essential enzyme in PAT biosynthesis (Tannous et al., 2014; 2015). The presence of patF in an organism signals a substantial potential for patulin production. Preliminary results from Activity 1.3 will ascertain whether patF is the ideal target gene for such an assay in *Penicillium* species. Additional genes within the PAT cluster will be considered if necessary.

Five *Penicillium* species will be selected from Activity 1.1 based on their ability to produce PAT and on the expression of genes detected in Activity 1.3. The *Penicillium* isolates will be grown on potato dextrose agar for 7 days at 70°F and spore suspension will be prepared in distilled water and diluted to 10^2, 10^4, 10^6, 10^8 spores/mL. Fuji apples will be washed and surface sterilized as described in Activity 1.2. Afterward, the cores of the apples will be injected with 30 µL of the spore suspension of the different *Penicillium* isolates at the specified concentrations using a syringe. Control apples will be injected with 30 µL of sterile water. Six replicate apples will be used for each isolate and spore concentration combination in duplicate. The apples will be incubated in clean clamshells for 30 days at 34°F. Afterward, total RNA will be extracted from the fruits following the procedure described in Activity 1.3. After conducting a quality check and performing retro-transcription, the resulting cDNA will be used for qPCR analysis with patF specific primers. The ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and β-tubulin genes will be used as housekeeping genes and positive control for qPCR. We will compare the ability of the qPCR assay to detect different loads (spore concentrations of the *Penicillium* species) and the expression of the patF gene will quantify patulin levels.

Activity 2.3. Develop and validate a dye-based assay for visual patulin detection with the naked eye.

The purpose is to provide an efficient, quick, easy and cost-effective method for fruit processors to detect PAT-producing fungi and PAT presence in asymptomatic fruits. The RPA-SG method combines Recombinase Polymerase Amplification (RPA), a highly sensitive and rapid DNA amplification technique, with the use of SYBR Green, a fluorescent dye that enables visual detection of amplified DNA for naked-eye detection of patulin-producing fungi in fruit samples.

New primers targeting the patF gene of the patulin metabolic pathway will be designed using available sequences of multiple PAT-producing fungal species, and their efficiency will be compared to the one developed recently by Roumani et al. (2023). Different PAT-producing *Penicillium* species used in Activity 2.2. will be used at different concentrations to determine the specificity and sensitivity of the RPA-SG assay. Fuji apples will be inoculated and incubated as described in Activity 2.2. Genomic DNA will be extracted using standard lab protocols. Recombinase Polymerase Amplification (RPA) followed by detection of amplification with the use of SYBR will be achieved as described by Roumani et al (2023). The presence of green-fluorescent color int the sample tubes will indicate PAT contamination in the sample.

To validate the qPCR and PRA-GS assay, their reliability, accuracy, and reproducibility must be confirmed by comparing their detection with current biochemical methods such as high-performance liquid chromatography coupled with a Mass spectrometer (LCMS). Patulin will be extracted from 10 g of asymptomatic and symptomatic apples inoculated by *Penicillium* species using QuEChERS extraction as in Activity 1.2. The limit of detection (LOD), relative specificity (SP), relative sensitivity

(SE), accuracy (AC), positive and negative predictive values (PPV/NPV) of the qPCR and PRA-GS methods will be compared to PAT quantification with LCMS.

Expected benefits to industry and potential pitfalls: Stringent quality control measures, including the detection of latent fungal infections, are essential to minimize patulin contamination and ensure consumer safety. Combining the detection (qPCR & PRA-GS) and quantitative (qPCR) assays will ease not only detection but also the quantification of patulin contaminations for timely and informed decisions. While the qPCR assay may require some expertise, we anticipate the PRA-GS assay to be cost-effective, easily accessible, and instrumental to stakeholders. Once the assays are validated, a cost assessment will be conducted to recommend their implementation for PAT and associated fungi detection.

Objective 3. Conduct outreach activities for growers, packers, and processors. [Years 2-3]

The knowledge anticipated from this project, in conjunction with that of the completed project CP-21-102A, will be invaluable in providing science-based information and recommendations to stakeholders in Washington. To disseminate findings to stakeholders, we will undertake outreach activities, including:

Talks:

Amiri and Leannec will present data at the end of Year 1 to 3 at local and regional commodity meetings such as Northwest Field Days, Hortshow and Apple Review days. We will provide yearly updates to the state fruit processors.

Publications:

Leannec and Amiri will publish their findings in peer-reviewed journals in Year 3. Leannec will summarize data and publish a newsletter in the WSU Fruit Matter to update the industry on the major findings from this project.

Updated SOP and factsheet:

We expect at the completion of this project to have a better understanding of the patulin contamination issues in WA. This will allow us to provide accurate recommendations to improve current SOPs and to develop a factsheet summarizing the patulin issue and mitigation strategies.

Results and Discussion

Objective 1. Characterize the ability of “non-expansum” *Penicillium* species to produce patulin (PAT) in vitro and on apples

In 2024 and 2025, our efforts focused on assessing patulin (PAT) production on apples. Fifty, isolates from 8 *Penicillium* species were assessed on inoculated Gala apples after 2 and 5 months of cold storage.

In 2024, the 15 *P. expansum* isolates all produced PAT at levels between 0.4 and 275 mg/kg after two months of storage and between 170 and 950 mg/kg after 5 months of storage (Figure 1). The single isolate of *P. commune* (virulent) and three isolates of *P. solitum* (virulent) did not produce PAT at both periods, whereas one *P. solitum* isolate PDC3 produced 452 and 53.4 mg/kg after 2 and 5 months, respectively (Figure 1). Isolate *P. roqueforti* (LOP5-1-1) and two isolates of *P. sajarovii* produced between 5 and 276 mg/kg PAT.

These preliminary results confirm the high mycotoxigenic ability of P. expansum isolates, especially as the fruit decay longer. Although the number of "non-expansum" isolates tested was low, there is some evidence that these species can produce PAT as well, although some variability between the isolates was observed, indicating that not all of them may produce PAT.

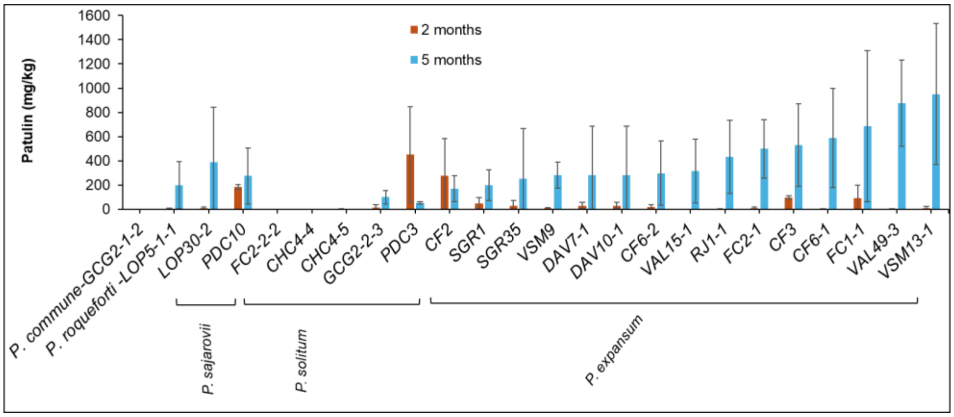

Figure 1. Quantification of patulin produced by different *Penicillium* isolates inoculated to Gala apples after 2 and 5 months of storage.

In 2025, 35 isolates from 13 species were inoculated to two cultivars (Gala and Honeycrisp) and are stored in cold storage for PAT quantification in 2026.

Ongoing and Future Activities

Activity 1.1. Evaluation of Penicillium species for their toxigenic potential in vitro. The ability of 35 isolates from 13 Penicillium species to produce PAT in vitro is being assessed.

Activity 1.2. Evaluation of Penicillium species for their toxigenic potential on detached apples. The ability of 35 isolates from 13 *Penicillium* species to produce PAT on Gala and Honeycrisp is being assessed.

Activity 1.3. Molecular investigation of "non-expansum" Penicillium species for presence of genetic markers linked with patulin production and genes expression levels. The presence of PAT cluster genes conferring the ability to produce patulin will be assessed in multiple non-expansum species to correlate with data from activities 1.1 and 1.2.

Activity 2.1. Sampling asymptomatic apples from commercial processing facilities. Asymptomatic apples will be collected in 2026 from processing facilities to investigate latent infections that may be related to PAT contamination.

Activity 2.2. Development and validation of a quantitative molecular detection assay for patulin-producing Penicillium species in asymptomatic apples. qPCR assays will be developed to detect and quantify different Penicillium species producer of PAT.

Activity 2.3. Develop and validate a dye-based assay for visual patulin detection with the naked eye [2027]

Project Title: New materials and delivery system for fire blight control

Report Type: Continuing Project Report

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Cooperators: Garrett Bishop (G. S. Long), Virna Stillwaugh (Northwest Horticultural Council)

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $64,308
Total Project Request for Year 2 Funding: $66,480
Total Project Request for Year 3 Funding: $68,739

Other related/associated funding sources: Awarded
Funding Duration: 2024 - 2028
Amount: $999,591
Agency Name: USDA-AFRI
Notes: The funded USDA project aims to develop biobased delivery systems to enhance the stability and biocidal efficacy of commercially available biopesticides under field conditions, including essential oils, bacteriophages, and biocontrol agents.

WTFRC Collaborative Costs: None

Budget 1
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Item	**2025**	**2026**	**2027**
Salaries	$40,176.00	$41,783.00	$43,454.00
Benefits	$14,132.00	$14,697.00	$15,285.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$10,000.00	$10,000.00	$10,000.00
Travel			
Plot Fees			
Miscellaneous			
Total	$64,308.00	$66,480.00	$68,739.00

Footnotes: The salary includes one postdoc in the Huang lab (42% FTE) and one postdoc in the Zhao lab (30% FTE), each paid $4,650 per month with a benefit rate of 35.2%. Supplies include the associated costs of consumables, materials, and chemicals required for this project.

OBJECTIVES

1. To determine the effectiveness of PAMAM dendrimers and AgNPs in inhibiting *E. amylovora in vitro* and *in vivo*.

2. To develop a versatile cell-based microcarrier for encapsulating PAMAM dendrimers and enabling *in situ* generation of AgNPs, aimed at enhancing stability, delivery, and biocidal efficacy.

3. To assess the biocidal efficacy of encapsulated PAMAM dendrimers and AgNPs in controlling fire blight in greenhouse and field trials and to deliver results to growers.

SIGNIFICANT FINDINGS

- Laboratory in vitro assays demonstrated that 20 nm AgNPs exhibited substantially stronger antimicrobial activity against *E. amylovora* than 100 nm particles, achieving rapid bacterial inactivation at elemental silver concentrations as low as 0.004-0.04 µg/mL.
- PAMAM dendrimers showed strong dose-dependent antimicrobial activity, with both G0 and G1 achieved ~5-log reductions within 30 minutes at 10 mM.
- A strong synergistic interaction was observed when AgNPs (20 nm) were combined with PAMAM G0, achieving 5-log reduction within 30 minutes, substantially exceeding the efficacy of either antimicrobial alone.
- Neither PAMAM dendrimers G0 at 10 mM nor AgNPs at 0.2% exhibited any phytotoxicity on Gala/Pink Lady leaves and Pink Lady flowers.
- Detached-flower assays revealed that Gala flowers showed 2-3 log reductions (best with PAMAM G0), whereas Pink Lady flowers showed approximately 2-log reductions.

METHODS AND PROCEDURES

1. To determine the effectiveness of PAMAM dendrimers and AgNPs in inhibiting *Erwinia amylovora in vitro* and *in vivo*.

In this objective, we will assess the antimicrobial efficacy of PAMAM dendrimers and AgNPs against *E. amylovora*. *In vitro* assays will be performed to determine the effective concentrations and optimal treatment times for both materials, while *in vivo* assays will evaluate their performance on blossoms under controlled environment in the lab. In addition, the phytotoxicity of these two materials on flowers will be assessed in greenhouse setting.

2. To develop a versatile cell-based microcarrier for encapsulating PAMAM dendrimers and enabling *in situ* generation of AgNPs, aimed at enhancing stability, delivery, and biocidal efficacy.

In this objective, yeast cells (*Aureobasidium pullulans*) will be utilized as biobased microcarriers to enhance the delivery of two novel antimicrobial materials. For PAMAM dendrimers, a rapid vacuum infusion technique will be employed to encapsulate them into yeast cells with high loading efficiency. For AgNPs, an *in-situ* synthesis approach will be used to directly generate AgNPs in yeast cells by leveraging their bio-catalytic properties. We will evaluate the encapsulation efficiency and yield and assess the role of microcarriers in enhancing the stability, controlled release, and biocidal efficacy of the encapsulated materials under simulated field conditions.

3. To assess the biocidal efficacy of encapsulated PAMAM dendrimers and AgNPs in controlling fire blight in greenhouse and field trials and to deliver results to growers.

In this objective, we will conduct greenhouse trials to evaluate the efficacy of PAMAM dendrimers, AgNPs, and their encapsulated formulations against fire blight under controlled conditions. The top-performing formulations will then undergo field testing in Dr. Zhao's apple orchards to monitor fire blight symptoms and potential fruit marking under real-world conditions. Preliminary findings will be shared with growers through direct communication and extension activities, with results published in scientific journals and industry magazines.

Types and timing of anticipated results: In the first year, we expect to determine the effective concentrations of PAMAM dendrimers or AgNPs against *E. amylovora*. The phytotoxicity profiles of these two materials will be also understood.

Any potential problems or limitations that may be encountered: In the first year, we do not expect any difficulties in conducting these experiments as the experiment designs are straightforward. The team has extensive experience in evaluating the antimicrobial activity of various materials.

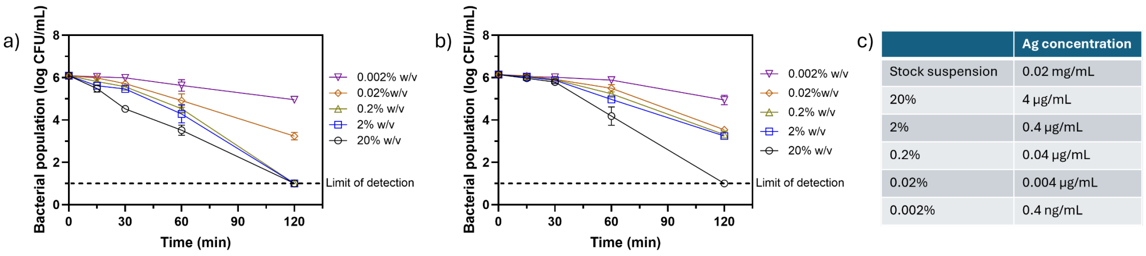
RESULTS AND DISCUSSION

1. Antimicrobial activity of AgNPs against *E. amylovora*

To evaluate the *in vitro* antimicrobial efficacy of AgNPs, bacterial suspensions of *E. amylovora* (~6 log CFU/mL) were treated with two sizes of AgNPs (20 nm and 100 nm) at varying concentrations and incubated for 15-120 min before plate counting.

AgNPs demonstrated strong antimicrobial activity against *E. amylovora* in vitro, with nanoparticle size playing a critical role in determining potency. As shown in **Fig. 1**, 20 nm AgNPs achieved significantly higher bacterial reduction than 100 nm AgNPs, consistent with the known size-dependent antimicrobial behavior of silver nanoparticles. Smaller AgNPs possess a larger surface-area-to-volume ratio and greater cellular interaction, contributing to improved oxidative stress generation and membrane disruption.

For 20 nm AgNPs, the minimal effective concentration ranged from 0.02–0.2%, corresponding to 0.004-0.04 µg/mL of elemental Ag. These levels are substantially lower than typical silver-based bactericidal thresholds, highlighting the strong intrinsic activity of the selected formulations. The rapid inactivation (within 15-120 minutes) indicates that AgNPs are highly effective candidates for blossom protection when applied at appropriate doses.



	Ag concentration
Stock suspension	0.02 mg/mL
20%	4 µg/mL
2%	0.4 µg/mL
0.2%	0.04 µg/mL
0.02%	0.004 µg/mL
0.002%	0.4 ng/mL

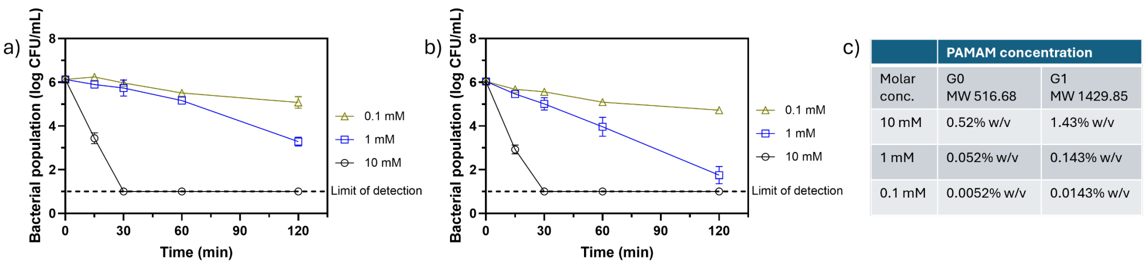
Figure 1 *In vitro* antimicrobial efficacy of silver nanoparticles (AgNPs) against *E. amylovora* as a function of elemental silver concentrations and treatment time. Bacterial suspensions (~6 log CFU/mL) were treated with 20 nm (a) and 100 nm (b) AgNPs at concentrations ranging from 20% to 0.002% of the stock suspension and incubated for up to 120 minutes. (c) The accompanying table summarizes the corresponding elemental silver concentrations for each treatment level.

2. Antimicrobial activity of PAMAM G0 and G1 dendrimers against *E. amylovora*

To evaluate the *in vitro* antimicrobial efficacy of PAMAM dendrimers, PAMAM dendrimers G0 and G1 were added to *E. amylovora* suspensions (~6 log CFU/mL) at 1-10 mM, incubated for 15-120 min, and surviving bacteria were quantified to determine effective doses.

As shown in **Fig. 2**, PAMAM dendrimers also exhibited strong antimicrobial effects, with PAMAM G1 consistently outperforming PAMAM G0 across tested concentrations. At 10 mM, both G0 and G1 achieved approximately a 5-log reduction of *E. amylovora* within 30 minutes, but G1 required lower mass concentration due to its structural characteristics and higher charge density.

The minimal effective concentration for both dendrimers was approximately 1 mM, though G1 required a lower dilution factor (14×) to achieve 10 mM compared to G0 (38.7×), highlighting improved efficiency and practical advantages for formulation. These findings confirm PAMAM dendrimers as promising antimicrobial candidates for fire blight management due to their rapid membrane-disruptive mechanisms. Given the substantially higher cost of PAMAM G1, PAMAM G0 was selected as the primary candidate for subsequent experiments.



	PAMAM concentration	
Molar conc.	G0 MW 516.68	G1 MW 1429.85
10 mM	0.52% w/v	1.43% w/v
1 mM	0.052% w/v	0.143% w/v
0.1 mM	0.0052% w/v	0.0143% w/v

Figure 2 *In vitro* antimicrobial activity of PAMAM dendrimers G0 and G1 against *E. amylovora* at varying molar concentrations. Bacterial suspensions (~6 log CFU/mL) were treated with PAMAM G0 (a) or PAMAM G1 (b) at 0.1, 1, and 10 mM and incubated for up to 120 minutes. (c) The accompanying table summarizes the corresponding w/v concentrations for each molar level, reflecting differences in molecular weight between G0 and G1.

3. Phytotoxicity test for AgNPs and PAMAM G0

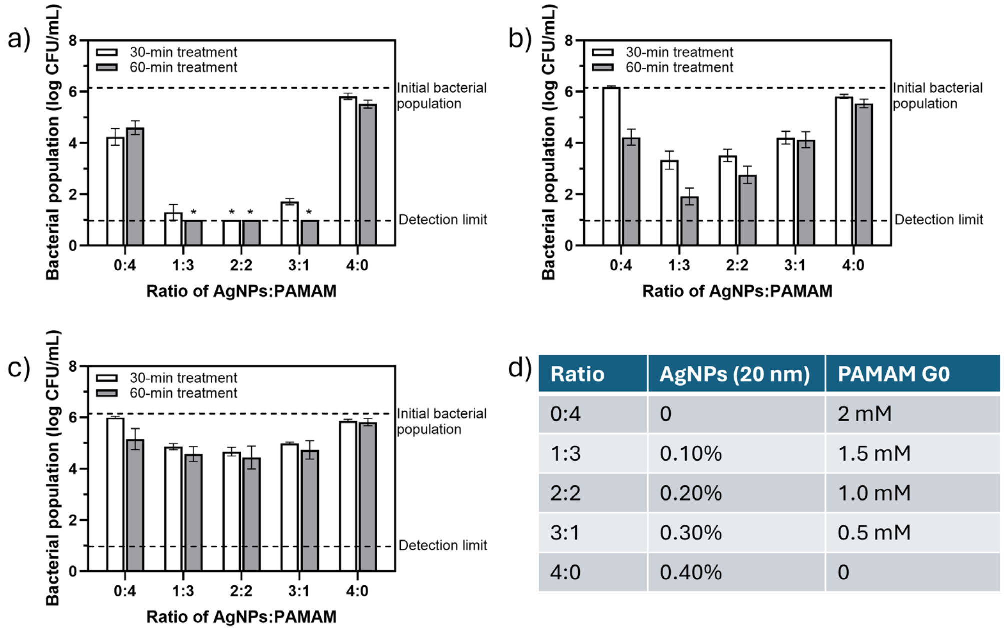
We did not observe any phytotoxicity on leaves of Gala and Pink Lady as well as flowers of Pink Lady after spraying PAMAM dendrimer G0 (10 mM) or AgNPs (0.2%) in the greenhouse.

4. Synergistic antimicrobial effect of AgNPs + PAMAM

To assess synergistic interactions, *E. amylovora* suspensions were treated with combined AgNPs (20 nm) and PAMAM G0 at 100%, 75%, and 50% of the target dosage, and log reductions were compared with individual treatments.

A strong synergistic antimicrobial effect was observed when 20 nm AgNPs were combined with PAMAM G0 (as shown in **Fig. 3**). At the full (100%) dosage, mixtures containing both agents achieved rapid and extensive bacterial inactivation, reaching a 5-log reduction within 30 minutes. This performance substantially exceeded that of either AgNPs or PAMAM G0 used alone at equivalent concentrations, demonstrating that the two materials act cooperatively to enhance membrane disruption and bactericidal activity. At reduced dosages (75% and 50%), the synergistic trend remained detectable, though less pronounced, highlighting dosage sensitivity of these combined treatments.

The observed synergistic antimicrobial effect likely results from complementary mechanisms: a) PAMAM dendrimers increase membrane permeability, facilitating silver ion or nanoparticle entry; and b) AgNPs generate ROS and interact with intracellular components once internalized. This strong complementary effect supports combining AgNPs and dendrimers in future microcarrier-based delivery systems to lower the required antimicrobial load while maintaining efficacy.



Ratio	AgNPs (20 nm)	PAMAM G0
0:4	0	2 mM
1:3	0.10%	1.5 mM
2:2	0.20%	1.0 mM
3:1	0.30%	0.5 mM
4:0	0.40%	0

Figure 3 Synergistic antimicrobial activity of AgNPs (20 nm) and PAMAM G0 against *E. amylovora* across different dosage levels. (a), (b), and (c) show bacterial log reductions for formulations applied at 100%, 75%, and 50% of the target dosage, respectively, using mixing ratios ranging from 0:4 to 4:0. (d) The table summarizes the corresponding AgNPs and PAMAM G0 concentrations for each mixing ratio at 100% dosage.

5. Antimicrobial efficacy on detached apple flowers (Gala and Pink Lady)

Detached Gala and Pink Lady flowers were inoculated with *E. amylovora* (100 µL at ~7 log CFU/mL) and incubated at room temperature for 2 hours to allow bacterial attachment. Following incubation, flowers were treated with AgNPs, PAMAM G0 (10 mM), or their combination under two humidity conditions (60% RH and 100% RH) to simulate field-relevant blossom environments. Treated flowers were then held at room temperature for 2-6 hours prior to bacterial enumeration. Spraying the flowers with sterile DI water served as the control treatment.

Detached Gala flowers exposed to 60% and 100% RH showed clear treatment-dependent reductions in *E. amylovora* (**Fig. 4 a&b**). At 60% RH, PAMAM G0 (10 mM) achieved approximately a ~2.4 log reduction within 2 hours, while AgNPs (0.2% w/v) alone produced <1- log reduction. The combined AgNP + PAMAM G0 treatment yielded modest reductions at ~1.2 log reduction within 2 hours and ~1.5 log reduction within 6 hours. Under 100% RH, efficacy decreased across all treatments, though PAMAM G0 (10 mM) still performed best (~2.3 log reduction), followed by AgNPs at the concentration of 0.2% w/v (~1.0 log). Importantly, bacterial reductions measured after 2 hours were comparable to those observed after 6 hours, indicating that the antimicrobial effects plateau early and that a 2-hour treatment window is sufficient for assessing efficacy on floral tissues.

For Pink Lady flowers (**Fig. 4 c&d**), similar bacterial population reductions were observed which were generally limited to <1-2 logs across both humidity conditions. Among the treatments, PAMAM G0 performed best, achieving approximately 1.8-log reduction within the 6-hour treatment period.

It is noted that although synergy was evident in the *in vitro* suspension tests, the synergistic effect on flowers was modest, likely due to: flower surface complexity and microstructure, reduced antimicrobial diffusion on hydrophobic petal tissues, and biological shielding provided by floral exudates. In addition, higher humidity (100% RH) tended to reduce treatment efficacy overall for both apple flowers, possibly by supporting bacterial survival or diluting antimicrobial residues on the flower surface. However, daytime relative humidity in Washington State orchards is typically low (30-50% RH), suggesting that the antimicrobial performance observed under lower-humidity conditions in this study is highly relevant to field applications and that treatment efficacy may be further enhanced under realistic orchard environments.

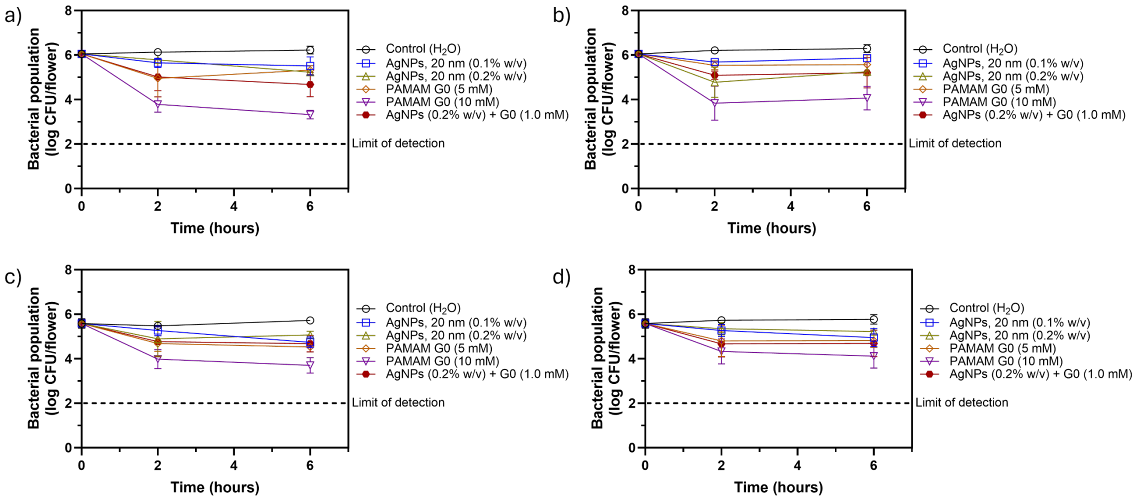


Figure 4 Antimicrobial efficacy of AgNPs (20 nm), PAMAM G0, and their combination on detached apple flowers under varying relative humidity conditions. (a) and (b) show results for Gala flowers at 60% RH and 100% RH, respectively, while (c) and (d) present corresponding results for Pink Lady flowers at 60% RH and 100% RH.

In addition, these observations also highlight the limitations of whole-flower spraying assays. To better represent natural infection processes and improve treatment deposition, future experiments will focus on direct inoculation and treatment of the flower stigma, the primary infection court for fire blight. Targeting stigma will enable more realistic and biologically meaningful assessments of antimicrobial performance under bloom-relevant conditions.

SIGNIFICANCE TO THE INDUSTRY AND POTENTIAL ECONOMIC BENEFITS

Fire blight, caused by *Erwinia amylovora*, is one of the most destructive diseases affecting apple production, resulting in more than $100 million in annual losses in the United States. Infections typically occur during bloom and the early post-bloom period, when flowers are highly susceptible, and historical data indicate that 5-10% of Washington apple orchards experienced significant fire blight outbreaks between 1993 and 2018.

Current management strategies rely heavily on repeated applications of antibiotics, which has raised increasing concern over antibiotic resistance, regulatory restrictions, and long-term sustainability. The emergence of resistant *E. amylovora* strains threatens the continued effectiveness of existing control programs and increases production risks for growers. Although biological control agents offer an alternative, their inconsistent performance and limited stability under field conditions have constrained widespread adoption.

This project directly addresses these challenges by developing non-antibiotic, high-efficacy antimicrobial formulations that provide rapid and robust suppression of *E. amylovora* during the critical bloom period. By reducing reliance on antibiotics and improving disease control consistency, the proposed approach has the potential to lower crop losses, reduce production costs associated with repeated sprays, and mitigate resistance risks. Successful implementation of this technology would deliver a sustainable, scalable, and economically beneficial solution for the apple industry, strengthening long-term orchard productivity while minimizing environmental impact.

FUTURE PLAN

In the second year, we will evaluate the antimicrobial efficacy of the proposed two antimicrobial materials (AgNPs and PAMAM dendrimer G0) through field trials. In addition, we will use biobased microcarriers (i.e., yeast cells) to encapsulate the two proposed antimicrobial materials to enhance their delivery and antimicrobial efficacy against *E. amylovora*. Encapsulation efficiency and yield will be quantified, and the role of the microcarriers in improving stability, controlled release, and biocidal efficacy will be evaluated under both laboratory and greenhouse conditions.