2025 Apple Crop Protection Research Review

Ladybug and parasitoid nose to nose, WAA and WAA mummies with p-toid exit holes.
Photo Source: Teah Smith

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Hybrid Format
Wenatchee, WA

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

Report Type: Final Project Report

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

Project Duration: 3 Year

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

Other related/associated funding sources: Awarded
Funding Duration: 2024 - 2024
Amount: $20,000
Agency Name: Washington Commission on Integrated Pest Management
Notes: Application funded further exploratory research on viral control of codling moth, with specific objectives related to but not covered in this project. Project title: “Efficacy testing of novel viral pesticide CrpeNPV against codling moth and other agricultural pest insects”; there were no other related or associated funding sources for this project

WTFRC Collaborative Costs: None

Budget 1
Primary PI: William Walker
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Item	2021	2022	2023
Salaries	$40,089.00	$41,425.00	$42,762.00
Benefits	$14,031.00	$14,499.00	$14,967.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$4,076.00	$4,076.00	$4,075.00
Travel			
Plot Fees			
Miscellaneous			
Total	$58,196.00	$60,000.00	$61,804.00

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

OBJECTIVES

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

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

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

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

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

The identification of a novel virus, CrpeNPV, that can infect codling moth provides new opportunities to explore enhanced formulations of viral control of codling moth utilizing both CpGV and CrpeNPV concurrently or in sequence during different seasonal generations. Fundamental research on coinfection of codling moth with CpGV and CrpeNPV is required. Cultivars of CpGV and CrpeNPV would be combined and exposed to codling moth larvae and then screened for efficacy. In addition to registration for organic use as described above in objective number one, appropriate measures will be taken as necessary for registration of use of CrpeNPV in USA for codling moth control.

SIGNIFICANT FINDINGS

- Dose-response and survival time assays with CrpeNPV against our colony codling moth insects, which was obtained from local orchards, demonstrated that the CrpeNPV virus was effective in killing local codling moth larvae.
- Compared to CpGV applied alone, CrpeNPV applied alone was less lethal than CpGV against our codling moth colony insects at low doses used to establish efficacy of the virus via dose-response analysis. However, at higher doses of CrpeNPV that approximate field application rates of CpGV, CrpeNPV was observed to be as equally efficacious as CpGV
- When CrpeNPV was combined with CpGV at a ratio of one-to-one, enhanced lethality was not observed compared to when either virus was applied individually; neither were inhibitory effects observed.

RESULTS AND DISCUSSION

Administrative delays in hiring a dedicated technician persisted until the end of summer of the second year of the project (2022) resulting in substantial delays in launching this project. The process to initiate hiring on our side began during the first year of the project directly after the funding became available, and was finally resolved with the onboarding of the technician in August of 2022. Competency training

was provided to the technician during the autumn of 2022, and experimentation according to the objectives of the project have been ongoing continuously since then. Because of this, a No Cost Extension was sought and granted. However, due to the delay in the initiation of this project until autumn of 2022, more than a full year after the start date, current activities have been performed across a span of only two and a half years at time of filing these reports. Some of the experiments described below are yet incomplete, and currently ongoing. These experiments will be completed through the formal end date of the experiment at the end of April 2025, and if desired, an amended final report may be filed.

Objective 1. For Objective 1, the initial goal was to assess if combining different strains of the virus would result in greater efficacy in killing the codling moth larvae, as a result of recombination of genetic material between the different strains resulting in hybrid strains. If higher or faster larval mortality would be observed in assays where different viral strains were combined, then viral particles could be purified from larval hosts and genetically screened to determine if hybrid viral particles had formed. An alternative hypothesis would be that presence of different strains of virus co-infecting the same larvae could result in higher or lower rates or larvae mortality, simply due to the presence of the different strains in the same larval organisms even without viral hybridization events occurring.

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

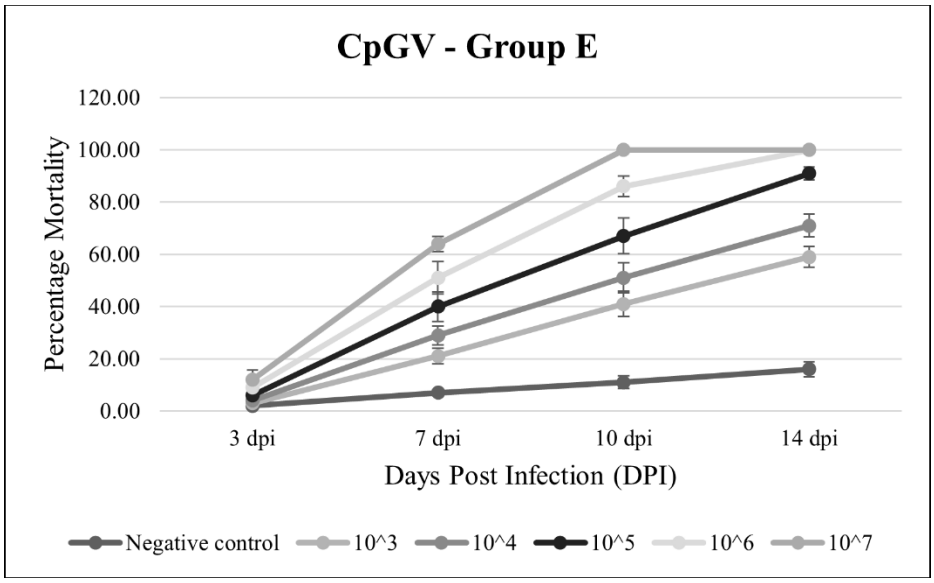

Figure 1. Dose-response mortality assay for codling moth neonate larvae exposed to CpGV - Group E. Twenty larvae tested per replicate (n=20), with five replicates for each treatment. 10 microliters of virus solution or distilled water control were applied at specified concentrations for all treatment groups at the specified dosage, ranging from 1 x 10^7 CpGV occlusion bodies (OB)/mL down to 1 x 10^3 OB/mL. Dilutions were made in autoclaved distilled water, which was also used as the negative control. Treatments were applied evenly to the surface of artificial diet in assay vials and allowed to dry for two hours, after which a single newly hatched neonate larvae was transferred to each assay vial. Larvae were scored for mortality on days 3, 7, 10 and 14 after initiation of experiments (dpi). Error bars indicate standard error values.

After establishing dose response baselines for CpGV, we decided it would be optimal to conduct further assays at concentrations comparable to field application rates based on input from stakeholders. Thus, samples of commercially available Cyd-X and Virosoft were obtained. Ten-fold dilution series were generated from these samples and five dosage steps were tested, such that doses were tested at Dose 1 - 10%, Dose 2 - 1%, Dose 3 - 0.1%, Dose 4 - 0.01%, Dose 5 - 0.001% of undiluted formulation. This dilution series was chosen considering that a standard field application rate is 3 oz of virus per 100 gallons of water per acre is equivalent to a dilution of approximately 0.023% dilution, which falls between Dose 3 and Dose 4.

At the higher dosage levels, substantially higher and faster mortality rates were observed compared to the lower dose-response dilution series (Figure 2). With the Group E, Virosoft product, even at the lowest dosages (0.001% of undiluted formulation) 45% mortality was apparent by day 3 post infection, and by 7 days post infection, nearly 100% mortality was observed across all dosages (Figure 2A). A different trend was observed when Group E (Virosoft) was combined with Group A (Cyd-X). 18-25% mortality was observed across all dosages after 3 days post-infection and 100% mortality was not observed for any and all dosages until by 10 days post-infection Figure 2B). Currently lacking in our datasets is Group A (Cyd-X) alone, which will be critical to the interpretation of the combined strains bioassay (Figure 2B). Bioassays with the higher dosages of Group A are currently ongoing and will be included in the final presentation.

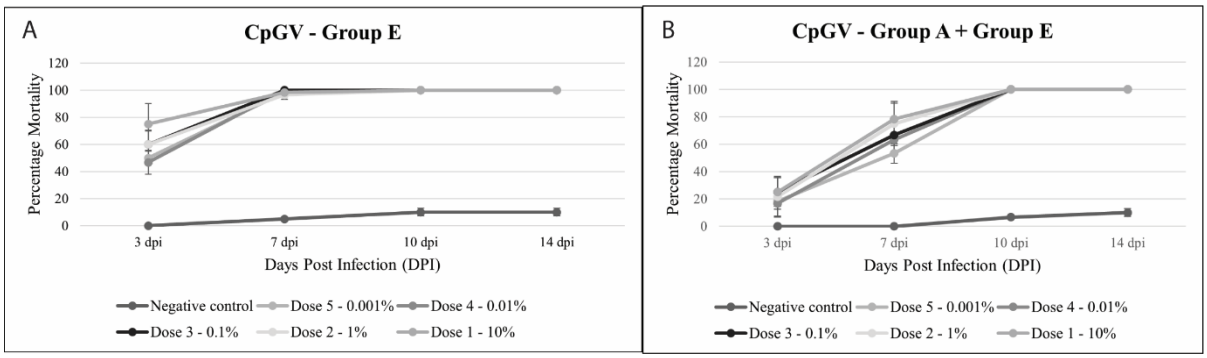

Figure 2. Dose-response mortality assay for codling moth neonate larvae exposed to CpGV – A) Group E. B) Group A+E. For each, twenty larvae tested per replicate (n=20), with three replicates for each treatment. 10 microliters of virus solution or distilled water control were applied at specified concentrations for all treatment groups at the specified dosage, ranging from Dose 1 - 10% of undiluted formulation to Dose 5 - 0.001% undiluted formulation, with 10-fold steps in between. Dilutions were made in autoclaved distilled water, which was also used as the negative control. For B) each dilution series was first generated, and then 1 mL of each strain was combined and applied to the larval diet, resulting in each strain being applied at half the dosage compared to when the strain was applied alone. Treatments were applied evenly to the surface of artificial diet in assay vials and allowed to dry for two hours, after which a single newly hatched neonate larvae was transferred to each assay vial. Larvae were scored for mortality on days 3, 7, 10 and 14 after initiation of experiments (dpi). Error bars indicate standard error values.

It must be noted that in Figure 2B, each virus was combined and presented to the codling moth larvae each at half the dosage compared to presentation of Group E alone (Figure 2A). For these experiments, dilution series for each strain were generated separately, and then at each dosage, 1 mL of each were combined and then applied to the larval diet. However, this 50% reduction in dosage of each strain by itself should not be expected to result in the differences observed for treatment with Group E alone versus the combination of Group A and Group E, considering that in Group E alone the 100% larval mortality was observed by day 3 of the experiment and the dilution series across all five dosages spans more than a 50% difference. It must be mentioned that the experiments conducted in Figure 2A and 2B were conducted at the same time of the year with the same dilution series aliquots for Group E, so differences may not be attributed to sampling differences. Previously published findings that Group E CpGV (R5 Strain) is more potent at killing susceptible codling moth larvae than a

50%/50% mixture of Group A and E strains (Graillot et al., 2016) mirror results presented here. However, this study reported only LC50 values and scored mortality at 7 days post infection and did not examine speed of kill effects.

Pending analysis of data for bioassays conducted with Group A alone, the delayed mortality observed when Groups A and E were combined may be attributable to a form of interference that is known to occur in instances of co-infection (Du et al., 2022) whereby different virus types compete for primacy of infection and suppress the infection of other virus types; this phenomenon has been observed to occur even between different strains of the same species of virus. Co-infection of codling moth by different strains of CpGV has been examined elsewhere. Alternatively, it was reported that a CpGV mutant displayed deficits in viral replication, and these deficits resulted in competitive disadvantages during co-infection of the mutant and wild-type versions of the virus (Elmenofy et al., 2015). Whether interference or differences in replication efficacy or something else entirely accounts for the differences in viral efficacy in codling moth larvae subjected to Group E virus versus the combination of Groups A and E, increased mortality rates were not observed when combining the different viral strain types, thus the possibility of hybridization to form more lethal virus types was excluded.

Objective 2. For Objective 2, the general goal was to genetically engineer a version of the CpGV Group A genome that has been modified in the laboratory to be amenable to genetic engineering, including the insertion of foreign genes (Hilton et al., 2008). The specific goal was to incorporate an insect-specific spider toxin, known as *Hvt*, into the CpGV Group A genome to introduce a secondary mode of action during viral infections, which could result in enhanced lethality and also serve as a safeguard against development of resistance. For this line of experiment, an artificial bacmid construct was designed that would ultimately enable expression of the *Hvt* spider-toxin in codling moth larvae that have been infected with the genetically engineered CpGV virus (Figure 3). Production of this construct was commercially outsourced, and was generated and provided to us during the final year of the project. At which time, we undertook efforts to transfer the *Hvt* transgene construct from the standard cloning bacmid into to the CpGV Bacmid so that codling moth larval infection experiments could be conducted to produce the genetically modified CpGV-*Hvt* virus and assess effects of the *Hvt* toxin. These transfer experiments were conducted using a commercially available bacmid transformation kit that allows transfer of genetic material from cloning bacmids to end-use bacmids, such as the CpGV Bacmid. Protocols were followed as described in previous reports on genetic engineering of the CpGV Bacmid (Hilton et al., 2008, Gebhardt et al., 2014). Despite our efforts, at present time of writing, we have not been able to recover genetically engineered CpGV bacmid.

Figure 3. Expression construct for the Hvt toxin gene. Relevant features include the Hvt spider-toxin gene open reading frame (ORF) positioned downstream of the *Drosophila melanogaster* (Dmel) heat shock protein 70 (hsp70) gene expression driver, which includes the regulatory region and promoter sequences. The Dmel hsp70 promoter has previously been shown to drive constitutive gene expression across different insects including moths (Uhlírová et al., 2002).

Objective 3. For Objective 3, the goal was to investigate effects of co-infections of CpGV and CrpeNPV on codling moth larvae. After establishing baseline parameters for CpGV (Genome Group E) and CrpeNPV, co-infection experiments have been conducted with these two specimen. Initial assays were first conducted with CrpeNPV alone, to generate a dose-response curve, as with CpGV (Group E) using the same five dosages on a ten-fold dilution series, compared to distilled water as a negative control. For CrpeNPV infections alone, maximum larval mortality was observed to be 81% for the highest dose (1 x 10^7 OBs/mL) on day 14 (Figure 4A). Compared to infections with CpGV (Group E) for which 100% larval mortality was observed with the highest dose as early as day 10 (Figure 1), overall larval mortality effects caused by CrpeNPV were lower than larval mortality effects caused by CpGV (Group E) at all dosages and time-points examined. These observations are consistent with results obtained in the initial report on efficacy of CrpeNPV against codling moth (Wennmann et al., 2019), which compared efficacy of CrpeNPV against codling moth to CpGV strains representative of genome groups A and B, but not E. When both viruses were combined at equal ratios, mortality rates were lower early in the period of infection, 3 dpi and 7 dpi, more similar to when CrpeNPV was presented alone; later in the infection, 10 dpi and 14 dpi, larval mortality rates were higher, more similar to when CpGV was presented alone (Figure 4B, Table 1). It must be noted that for these co-infections, since dilution series formulations for each virus were combined at each dosage, the effective dosage of each virus was 50% of the dosage when either virus was presented alone. These results are similar to findings of a previous report, in which it was observed that co-infection of cutworm larvae (*Agrotis segetum*) with both an NPV (AgseNPV-B) and a GV (AgseGV) did not result in changes in mortality rates when either virus was presented alone (Wennmann et al., 2015). These findings may be attributable to the principle of super-infection exclusion, by which infection of individual cells by one virus precludes simultaneous infection by other viruses (Beperet et al., 2014).

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

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

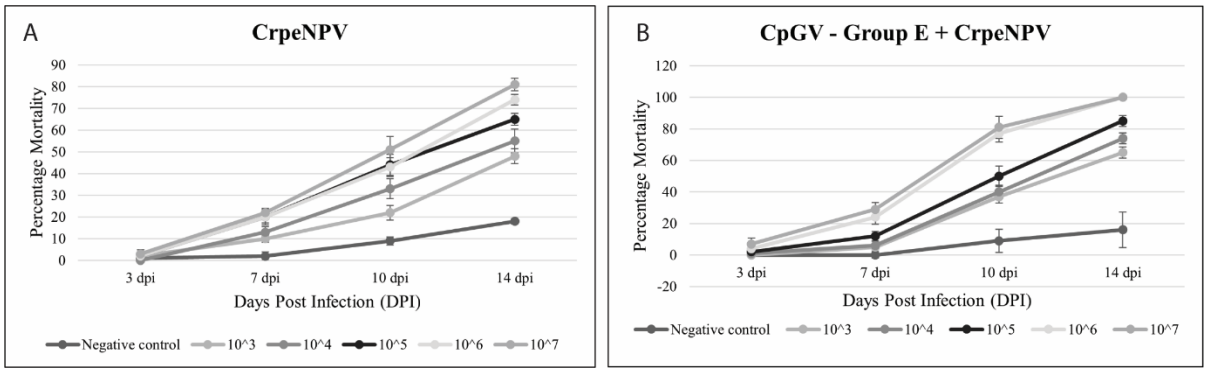

Figure 4. Dose-response mortality assay for codling moth neonate larvae exposed to A) CrpeNPV or B) CpGV – Group E and CrpeNPV. In either condition, twenty larvae tested per replicate (n=20), with five replicates for each treatment. 10 microliters of virus solution or distilled water control were applied at specified concentrations for all treatment groups, ranging from 1 x 10^7 CpGV occlusion bodies (OB)/mL down to 1 x 10^3 OB/mL. Dilutions were made in autoclaved distilled water, which was also used as the negative control. For B, 1 mL of each virus at same concentration was combined. Treatments were applied evenly to the surface of artificial diet in assay vials and allowed to dry for two hours, after which a single newly hatched neonate larvae was transferred to each assay vial. Larvae were scored for mortality on days 3, 7, 10 and 14 after initiation of experiments (dpi). Error bars indicate standard error values.

Next, we examined higher dosages of CrpeNPV and CrpeNPV combined with CpGV – Group E that reflected field application rates of CpGV, which are typically applied at 3 oz per 100 gallons of water per acre. The CrpeNPV virus was supplied to us at a reported concentration of 1 x 10^{11} OBs per mL. This is comparable to OB concentrations for commercial formulations of CpGV, which may range from 1 x 10^{10} to 1 x 10^{14} OBs per mL, or even higher. Regardless, a 10-fold dilution series was established, and five dosage steps were tested, such that doses were tested at Dose 1 – 1 x 10^{10}, Dose 2 - 1 x 10^{9}, Dose 3 - 1 x 10^{8}, Dose 4 - 1 x 10^{7}, Dose 5 - 1 x 10^{6} of undiluted sample. At these higher dosages, larval mortality rate averaged ranged from 70% to 85% at three days post infection, and by seven days post infection, 100% larval mortality was observed across all dosage applications (Figure 5A). These findings are comparable to results when CpGV – Group E was applied across a dilution series spanning the field application rate equivalents (Figure 2A). Similarly, when these higher dilution series dosages of CrpeNPV and CpGV – Group E were combined at a one to one ratio, similar larval mortality rates were observed, with average mortality spanning 51.67% to 85% across all dosages at three days post infection, and by seven days post infection 100% larval mortality was observed for all dosages. These findings suggest that when CrpeNPV is applied at similarly high viral loads as commercial formulations of CpGV, similar levels of codling moth larval mortality may be achieved.
In a recent publication, it was shown that CpGV (either Group A or Group E) and CrpeNPV may both co-infect single codling moth larvae individuals. However, CrpeNPV was not able to facilitate replication of CpGV – Group A in codling moth larvae displaying Type I resistance that were resistant to CpGV – Group A (Hinsberger et al., 2021). This stands in contrast to the ability of CpGV – Group E to facilitate replication of CpGV - Group A in Type I resistant larvae. In another report, CrpeNPV was shown to effectively kill codling moth larvae that were resistant to CpGV (Wennmann et al., 2019), for Type I, Type II and Type III resistant populations. Thus, at least for Type I resistant codling moth larvae, CrpeNPV may effectively kill the larvae, but would not facilitate co-infection with the CpGV – Group A virus. However, presentation of both CrpeNPV and CpGV, either at the same time or in rotation may serve to preclude development of resistance to either virus in susceptible codling moth populations due to the fact that these viruses represent different genera of baculoviruses.

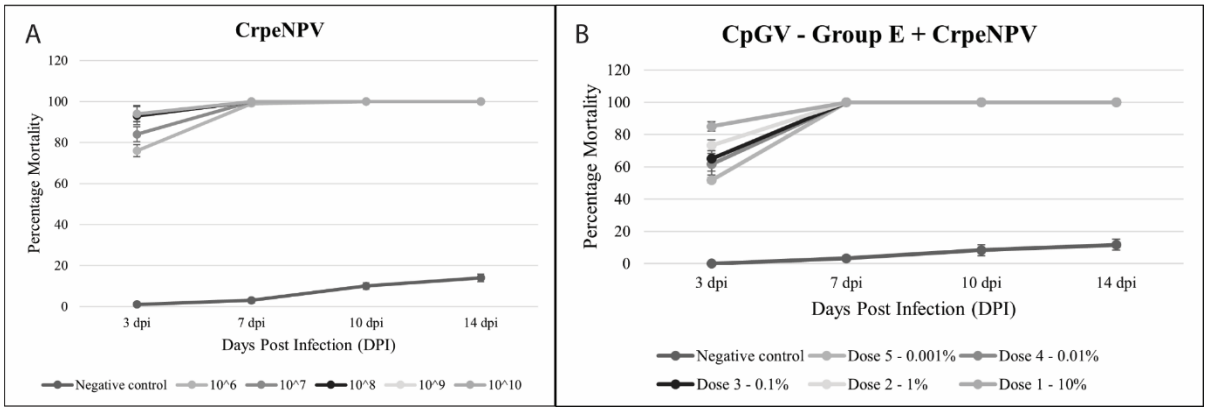

Figure 5. Dose-response mortality assay for codling moth neonate larvae exposed to field application equivalent dosages of A) CrpeNPV or B) CpGV – Group E and CrpeNPV. In either condition, twenty larvae tested per replicate (n=20), with five replicates for each treatment. 10 microliters of virus solution or distilled water control were applied at specified concentrations for all treatment groups, For A) ranging from 1 x 10^{10} CpGV occlusion bodies (OB)/mL down to 1 x 10^{6} OB/mL. Dilutions were made in autoclaved distilled water, which was also used as the negative control. For B) 1 mL of each virus at same concentration was combined. Treatments were applied evenly to the surface of artificial diet in assay vials and allowed to dry for two hours, after which a single newly hatched neonate larvae was transferred to each assay vial. Larvae were scored for mortality on days 3, 7, 10 and 14 after initiation of experiments (dpi). Error bars indicate standard error values.

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

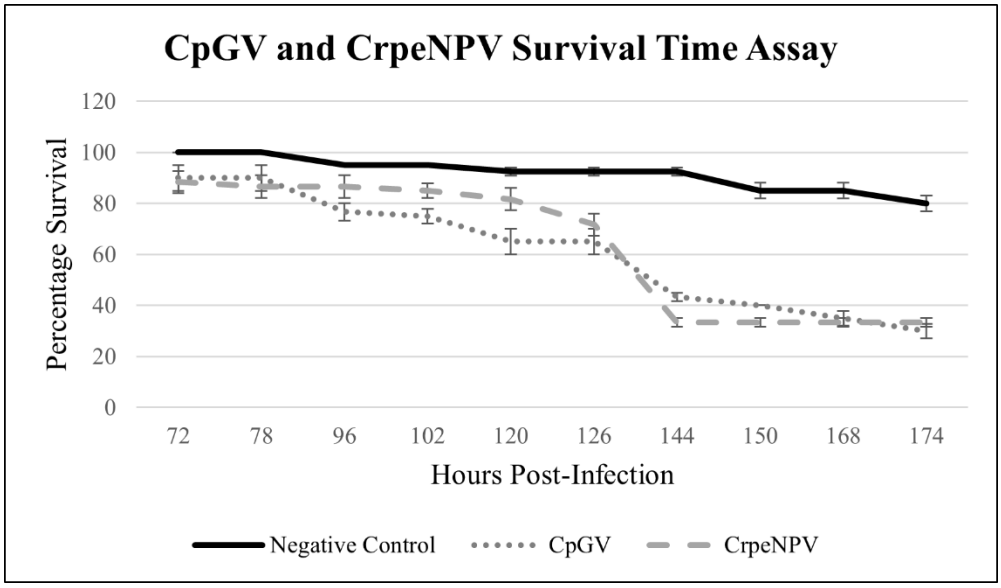

Figure 6. Survival time assays for codling moth neonate larvae exposed to CpGV (Group E) or CrpeNPV. Twenty larvae tested per replicate (n=20), with three replicates for each treatment. 10 microliters of virus mixture or distilled water control were applied at specified concentrations for all treatment groups, at a single concentration of 3.7 x 10^2 occlusion bodies (OB)/mL. The dilutions for each virus was made in autoclaved distilled water, which was also used as the negative control. Treatments were applied evenly to the surface of artificial diet in assay vials and allowed to dry for two hours, after which a single newly hatched neonate larvae was transferred to each assay vial. Larvae were scored for mortality twice daily 72 hours through 174 hours after initiation of experiments (hpi). Error bars indicate standard error values.

In closing, it must be mentioned that these findings in this report reflect laboratory assays conducted with virus formulations applied to artificial larval diet. A next step would be to provide CrpeNPV sprayed on apple tree cuttings after which neonate larvae would be permitted access and damage to apples would be assessed. Currently we do not have appropriate permissions to conduct trials of any kind with CrpeNPV outside of our certified quarantine laboratory, which limits our research with this virus. Accordingly, CrpeNPV is not currently approved for usage in the USA. However, that may not be the case at some point in time in the future.

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Executive Summary

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

Keywords: CpGV, CrpeNPV, codling moth, biological control, virus

Abstract: The codling moth granulovirus (CpGV) has been used for decades, primarily in organic apple orchards, to control codling moth by killing larval hatchings before or shortly after they enter the apple. However, in recent years, the first scientific report of codling moth resistant to CpGV in the USA, and indeed right here in Washington State, was published. Since then, concerns and suspicions have been mounting that the extent of CpGV-resistant codling moth in Washington is greater and more widespread than the published record indicates. Novel approaches and solutions for pest management of codling moth, especially in organic tree fruit orchards, are thus needed. To address this issue, we proposed a three-pronged approach to research ways to improve codling moth management based on the use of insect viruses. First, we sought to investigate the hypothesis that novel CpGV genotypes generated through coinfection of codling moth larvae by mixed CpGV genotypes may result in higher or faster rates of larval mortality. We approached this with larval co-infection studies with two commercially available CpGV formulations, each representing a different strain of the virus. In our studies, we did not observe enhanced rates of larval lethality when both strains of CpGV were simultaneously presented to the larvae, compared to presentation of either strain alone. Second, we sought to genetically engineer a modifiable version of the CpGV genome to include an insect-specific spider toxin. This would be done to introduce an additional mode of action directly into the virus as a means to promote enhanced lethality and/or counter-mechanisms to the development of resistance in virus-susceptible codling moth populations. Currently, our efforts to generate genetically engineered clones of the CpGV genome have been unsuccessful. This endeavor remains a work in progress. Third, we sought to explore further the efficacy of a novel baculovirus, known as CrpeNPV, against codling moth larvae. CrpeNPV was discovered in South Africa in the litchi moth, which is closely related to the codling moth, and subsequently reported to be able to kill codling moth larvae, including those that were resistant to CpGV strains. We confirmed dose-response efficacy of CrpeNPV against locally sourced codling moth and demonstrated that CrpeNPV is equally effective as CpGV in killing codling moth larvae at comparable field application rates of the virus. Finally, when CrpeNPV was combined with CpGV we did not observe enhanced larval mortality compared to when either virus was presented alone, though inhibitory effects were not observed either. Currently, to our knowledge, the CrpeNPV is not approved for usage in the USA as a biopesticide. If that status changes, it may be considered in future applications as part of effective codling moth management strategies, including management of resistance to CpGV.

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: $69680

Other related/associated funding sources: Requested, Awarded, None

WTFRC Collaborative Costs: None

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2022	2023	2024
$43,683.00	$44,775.00	$45,894.00
$13,979.00	$14,328.00	$14,686.00
$11,655.00	$11,600.00	$9,100.00
$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 via the piRNA pathway.

Objective 1. Identify candidate target genes for RNAi through transcriptomic analyses and injection trials
Procedures: Whole transcriptome datasets will be generated and analyzed for several life stages of codling moth, including early and late embryo, early and late larval instars, pupae and adults. Lead-PI Walker has extensive experience with this approach in entomology research (Walker et al., 2016; Walker et al., 2019; Walker et al., 2023). For each life stage an appropriate amount of individual

specimen will be collected to ensure that a sufficient quantity of RNA may be extracted to generate high quality transcriptomes. Codling moth specimen will be taken from our in-house codling moth colony. Standard protocols will be used to extract RNA from all sample types and subsequently prepare sequencing libraries that will serve as substrate for next generation RNA sequencing (RNA-Seq). Sequencing will be conducted in-house with our recently acquired Oxford Nanopore Mk1C sequencer, and the output sequence data will be assessed for quality and arranged into who transcriptome data sets containing consensus transcripts for each gene that is expressed at each life stage. Bioinformatic analyses will be conducted on output sequence data to assess which genes are expressed and relative expression abundances compared to all other genes in each sample. Further analyses will be conducted to compare codling moth expressed genes to transcriptomic data sets of other related insects to characterize unique and conserved genes in the codling moth.

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

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

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

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

Procedures: Candidate genes identified in the whole transcriptome datasets will be targeted for disruption by delivery of complementary dsRNA effector molecules via larval feeding. Genes will be targeted that are expressed in larval but also pupal, adult, and embryonic stages of life. For these candidate genes gene-specific dsRNA will be generated in-vitro, using corresponding gene-specific genomic DNA (gDNA) as a template, with standard molecular biology methods (Walker and Allen, 2010, 2011). dsRNA will also be generated from template gDNA corresponding to a plant gene to serve as a negative control to the experimental conditions. Additionally, dsRNA will be generated from template gDNA corresponding to a universal cellular housekeeping gene, inhibitor of apoptosis (IAP), known to be expressed throughout all life stages, and widely across all insects; RNAi against IAP has been shown to induce rapid mortality in a diversity of insects such as mosquitoes (Pridgeon et al., 2008) and plant bugs (Walker and Allen, 2011). Initial RNAi experiments will be conducted targeting disruption of IAP, as a positive control, in order to optimize protocols and methodology (RNAi against

IAP would not be expected to serve as an eventual biopesticide target due to its widespread presence across insects and other domains of life such as fungi).

Initial feeding assays will be conducted via topical application of purified dsRNA solution to standard codling moth artificial diet (Wang et al., 2015). To control for effect of dsRNA feeding on insect mortality, control experiments will be performed through feeding of dsRNA targeting disruption of a selected plant-specific gene that would not be present in the codling moth genome. Initially high concentrations of dsRNA will be applied to the food. For targeted genes that result in successful RNAi outcomes, lower concentrations of dsRNA will be assayed as well in order to assess minimum and optimal concentrations for eventual tree fruit trials. Individual neonate larvae will be placed in feeding chambers and allowed to feed unrestricted, while being monitored for growth, development, and mortality.

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

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

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

Potential Problems and Contingencies:

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

codling moth (Garczynski et al., 2017). Larval, pupal and adult injections would be made into the midgut region as described for codling moth (Wan et al., 2019) and other insects (Walker et al., 2010, 2011).

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

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

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

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

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

and fruit materials to measure the extent to which the various formulations elicit increased feeding behavior by codling moth larvae.

Within our experimental orchards, presence of codling moth will first be assessed with sticky traps baited with codlemone pheromone (Knight et al., 2002). Then, at the onset of codling moth activity, formulation spraying regiments will be implemented with validated mixtures of target-gene dsRNA, "Da Mec" and/or aforementioned feeding stimulants. Initially, dsRNA will be tested at highest dose observed to be effective in artificial diet feeding assays. Randomized block trial replicates will be utilized with respect to different treatment conditions plus no-dsRNA treatment controls. After each flight period, degree of damage to apples will be assessed and compared across each block trial with appropriate statistical measurements employed to assess effectiveness of dsRNA treatments in reducing or preventing codling moth damage to apple fruit.

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

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

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

RESULTS AND DISCUSSION

For Objective 1, whole transcriptome RNA-sequencing has been conducted on neonate and fifth instar whole larvae, 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 the *chitin synthase A* (CHSA) gene, which has recently been demonstrated to be a good RNAi target with a larval mortality phenotype across multiple Lepidoptera Families (Rana et al., 2020), though it has not been examined as an RNAi effector in any Tortricidae.

In addition to whole transcriptome messenger-RNA (mRNA) sequencing, small RNA transcriptome sequencing has been conducted on neonate, third instar and fifth instar larvae to identify "trigger" sequences that would direct effector dsRNA molecules into an alternative RNAi cellular pathway, known as the piRNA pathway (Flynt 2020). This approach has been pursued in collaboration with Dr. Alex Flynt after initial observations of no long dsRNA mediated RNAi phenotype, as described below with regards to Objective 2.

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

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

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

As mentioned above, for Objective 1, an alternative RNA-sequencing approach, aimed at sequencing only small RNAs has been conducted, to identity trigger sequences that activate the piRNA-mediated RNAi pathway. As such, small RNA transcriptomes have been generated across several codling moth larval stages, and several different piRNA trigger sequences have been identified. 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 (Walker et al., 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 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 the juvenile hormone pathway that are known to affect larval growth and 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.

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

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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:

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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

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

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

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

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

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

Progress on Objectives (2024)
(i) Objective 1: Assess dynamics of codling moth populations across orchards with variation in intensity of mating disruption and insecticide use.

From 2022 to 2024, we were able to gather data from our own field work and commercial growers from the state of Washington and the OK-SIR program in British Columbia. The complete dataset is made of over 2 million records and was used to measure the average lag that growers commonly see between predictions from the phenology model and moth captures in pheromone traps. We also built a series of models to attempt to predict future codling moth abundance based on degree days and cumulative trap catch. In our approach, early trap catch data is input into a model and an estimate of the future population dynamics is generated, based on projections of phenology or past capture patterns.

Population dynamics assessments in 2024: In the past year, we brought on Izzy MacDonald, a senior undergraduate student from the data analytics department to work part-time in data management and analysis. We focused on assessing the changes in codling moth population dynamics caused by variations in intensity of mating disruption and insecticide use. We aimed to use our understanding about the effect of commonly used insecticides and mating disruption on codling moth larval populations to project moth capture patterns affected by control treatments. From previous research, we were able to identify the most popular treatment programs used by growers in the state of Washington (Table 1). Most insecticide treatments for the codling moth essentially kill larvae upon hatching as older larvae are protected within fruits. Thus, the residual effect of sprays should be apparent in the hatching pattern of treated pest populations and can be tracked down to adult emergence (Fig. 1).

Table 1. Treatment programs and expected efficacies, as reported by Jones (2021), used to assess the efficacy of control treatments for codling moth management.

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

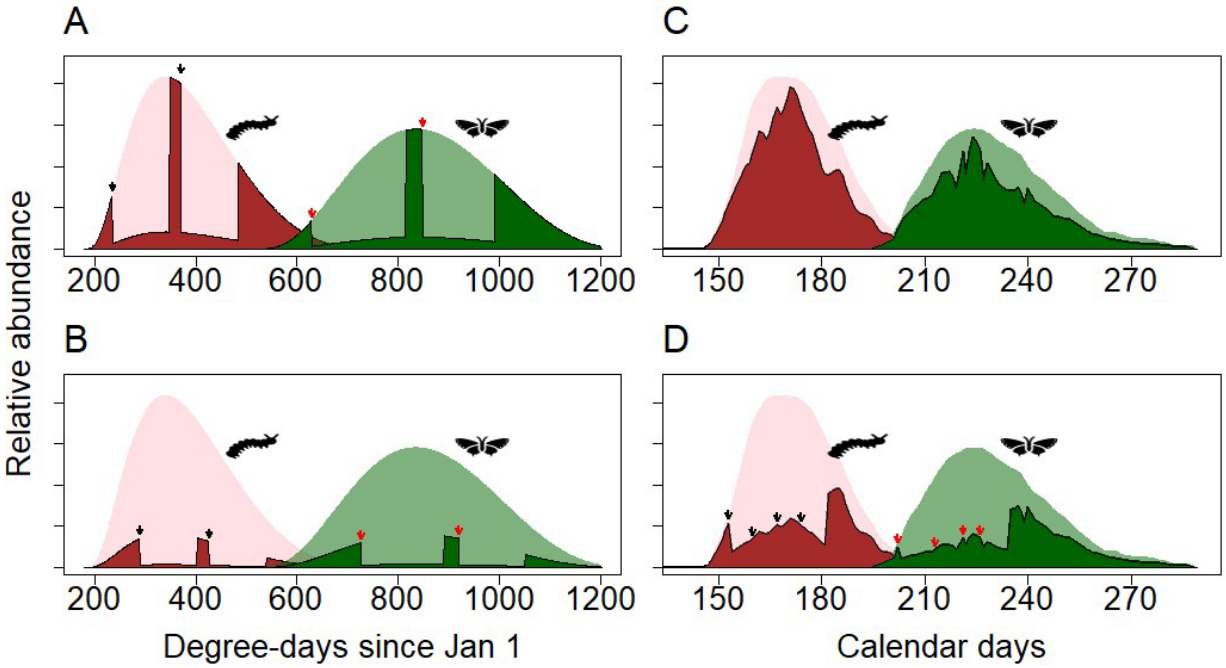

Fig 1. Theoretical effect of four treatment programs on the codling moth larvae (brown for treated vs. pink for untreated) and adults (dark green for treated vs light green for untreated) for the first summer generation: conventional traditional (A), delayed first cover (oil application not shown) (B), mating disruption (C), and mating disruption plus organic traditional (D). Models that involve programs based solely on insecticide treatments (A and B) are showed in degree-days, while those that involve mating disruption are showed in calendar days with temperature records collected in Pullman (WA) between April and October 2023 (C and D). 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 can be used to assess the effect of control treatments on codling moth populations. We used the approach we developed in 2023 to produce projections of codling moth captures in pheromone traps from populations that have been treated with control programs. Our goal is to develop a model that can classify pest populations as with or without an effective control treatment, based on data collected from pheromone trap networks by growers. Preliminary results from computer simulations 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.

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

During 2024, we were able to gather a new dataset from seven orchards in Washington, which contained detailed information about pheromone trap location and density, as well as weekly trap captures from 2018 to 2021 (Fig. 2). We used this new dataset to further test the forecasting model we developed in 2023, and to study the effect of trap density on moth capture efficacy and prediction accuracy.

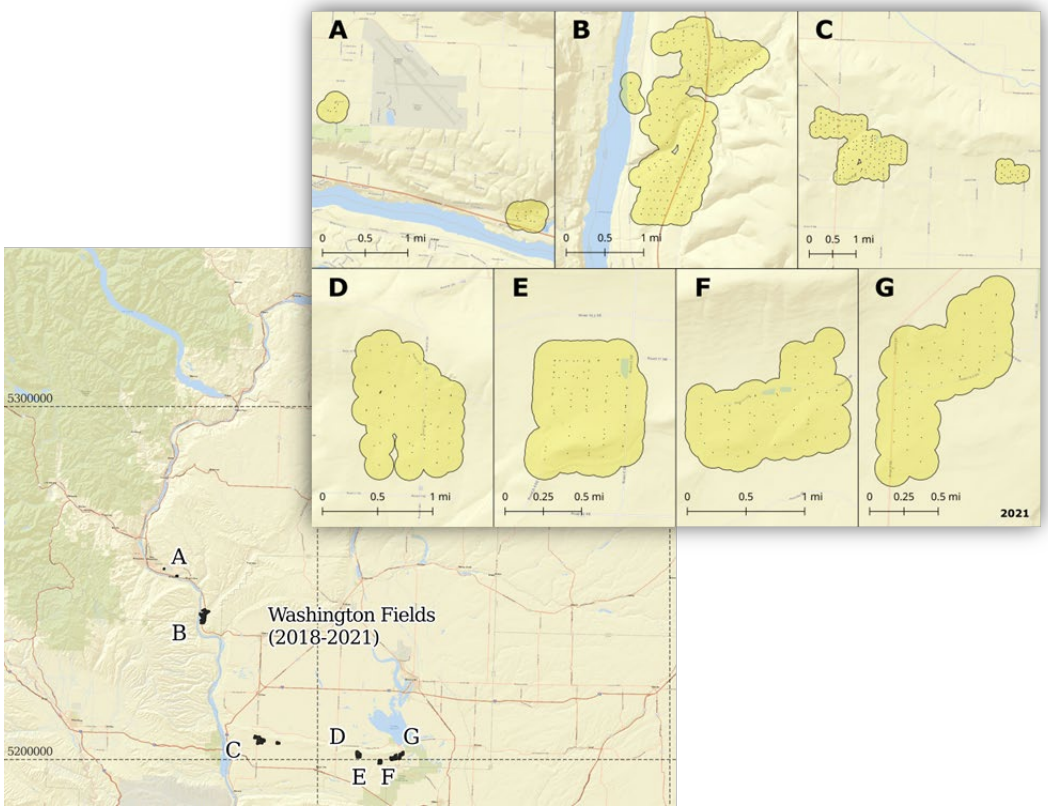

Fig. 2. Map showing the location of seven orchards from which new data was collected to test developed forecasting models and evaluate the effect of trap density on moth capture efficacy and prediction accuracy.

Predictive modeling. We found that the model fit with the OK-SIR dataset closely resemble the data collected in Washington between 2018 and 2021 (Fig. 3). Also, after running a new validation analysis with the new data, we found that our model maintains accuracy close to 80% (19% average error) when predictions are made at 350 degree days but falls to 66% when predictions are made at 300 degree days.

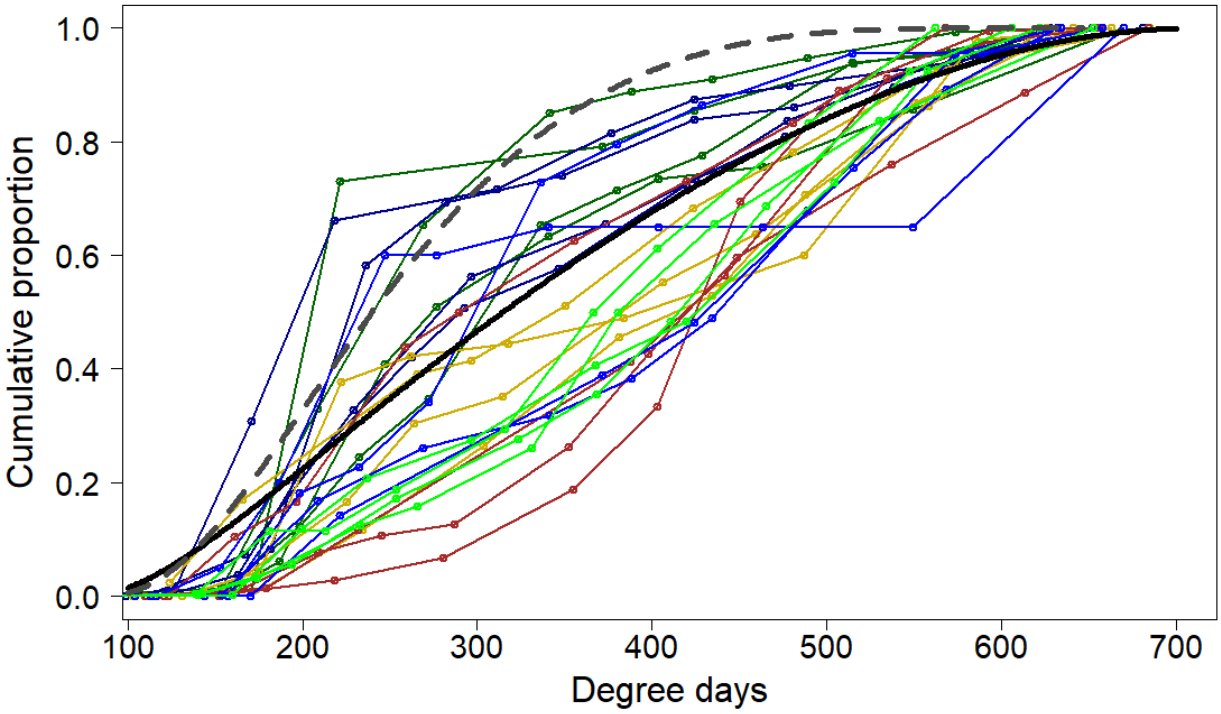

Fig. 3. Cumulative proportion of moth captures in pheromone traps for the first generation of the codling moth in seven WA orchards. Each broken line represents the average of captures in an orchard and year, and orchards are represented with different colors. The black solid curve represents the model fit to the OK-SIR dataset in 2023 and the grey dashed curve is the phenology model used in WSU DAS.

Although our datasets do not have reliable information on insecticide use, or the deployment of mating disruption or sterile insect release programs, they do have detailed information about pheromone trap density. We have noticed that the cumulative proportion of moth captures increases more gradually when capture rates are low and that they become steeper and closer to the phenology model as capture efficacy increases (Fig. 3). For example, one would expect that the cumulative proportion of captures in pheromone traps from a perfect pheromone trap network which captures moths as they emerge, will resemble the phenology model. But for more realistic trap networks the lag between adult emergence and capture should be longer.

We used a probabilistic simulation model to relate the probability of a single moth being captured in a pheromone trap with the inclination of the curve that describes the cumulative proportion of captures across degree days. We then used the inclination of curves for specific orchards to calculate orchard-specific sampling efficacies in terms of moth capture probability. As expected, we found that moth capture probability was low and ranged between 0.5 and 6.5%, depending on trap density. Our analysis shows that the highest capture probability is achieved

with 0.12 traps per hectare (approx. 1 trap every 10 hectares) and decreased for larger trap densities (Fig. 4). This finding highlights the potential interference between traps due to the wide capture range achieved by codling moth pheromone traps, which suggests that it might be unsafe to restrict management actions to areas adjacent to traps registering large numbers of captures.

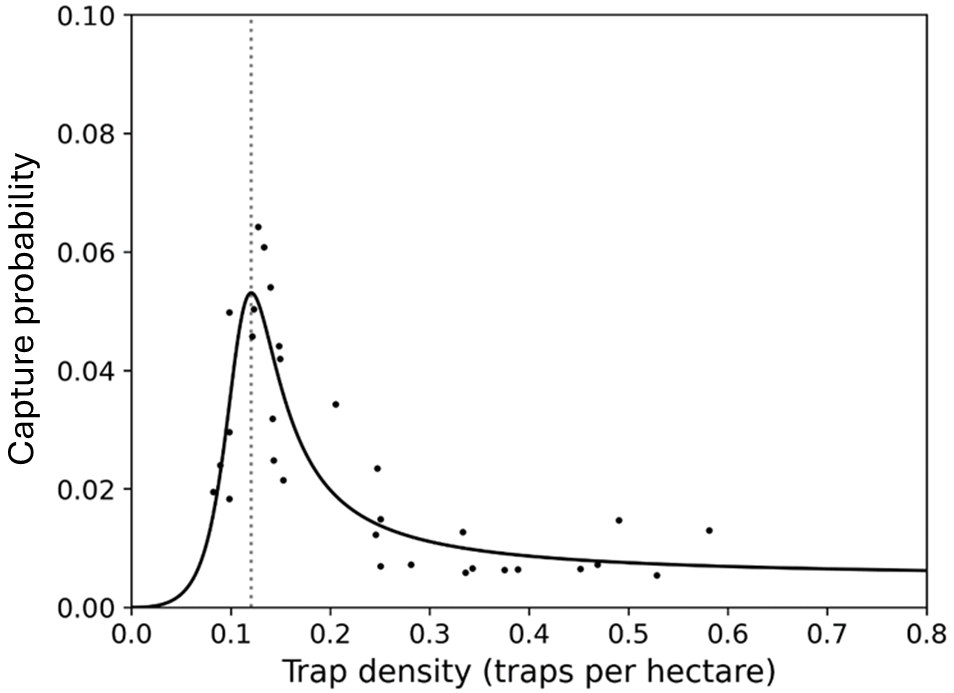

Fig. 4. Capture probability for a single moth estimated for 30 orchards in WA and British Columbia as a function of pheromone trap density. The vertical dashed line represents the maximum moth capture probability, which is achieved at 0.12 traps per hectare.

We also investigated if prediction accuracy of our forecasting model is improved by adjusting predictions for the capture probability of specific orchards. We found that the average error rate of the model went from 19% to 5.8% when predictions were adjusted to orchard-specific capture probabilities. Moreover, we found that while error increased with population size (cumulative number of captures) in the original forecasting model, this trend is reversed with the correction for moth capture efficacy (Fig. 5). We hypothesize that the greater error at low densities when the correction for moth capture efficacy is incorporated is because of insecticide use, or the deployment of long-term control strategies like mating disruption or sterile insect release.

We plan to build our models into the WSU DAS. This will require an appropriate software structure that allows users to input their own data and run the developed models in real-time to produce predictions of future population dynamics. We also plan to build further collaborations with growers in Washington and British Columbia to obtain data of moth captures associated with management actions to test our control treatment assessment tool and make further improvements to our forecasting models.

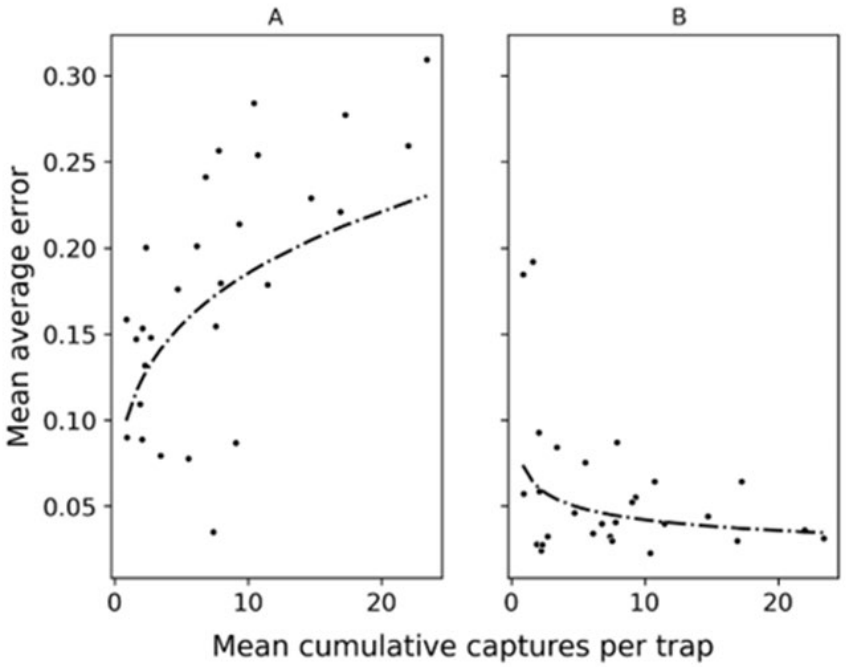

Fig. 5. Forecasting error as a function of cumulative trap captures for the original forecasting model (A) and the model incorporating a correction for the per-orchard moth capture probability (B).

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

In 2024 we ran 4 workshops through the decision aid system in March and April to talk about how codling moth models are run and interpreted. These workshops were attended by approximately 60 individuals in total, who spent over 3 hours with our team discussing codling moth models. We also gave two talks on the method at the meeting of the Entomological Society of America in 2024. We plan to increase our outreach efforts in 2025 now that the models are developed and get more user buy in to using new features on DAS.

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

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

Project Duration: 2-Year

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

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

WTFRC Collaborative Costs: NONE

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

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

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

Budget 2
Co PI 2: Louie Nottingham
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Contract Administrator: Office of Research Support and Administration
Telephone: 509-335-9661
Contract administrator email address: ORSO@wsu.edu
Station Manager/Supervisor: Carol Miles
Station manager/supervisor email address: milesc@wsu.edu

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

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

ORIGINAL PROJECT OBJECTIVES:

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

SIGNIFICANT FINDINGS:

Objective 1

– 2023 Key Findings

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

– 2024 Key Findings

- 14496 larvae were added to the colony from cardboard bands, apples, and eggs laid in mating arenas
- The mating arenas used in 2023 continued to induce moths to lay eggs
- The most important potential finding of this study was unexpected. While extracting larvae from cardboard bands we discovered three new entomopathogenic fungi that may be developed as biological insecticides. Their study is the subject of a pending WTFRC proposal for 2025.

Objective 2

– 2023 Key Findings

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

– 2024 Key Findings

- Although egg laying through a fourth generation was induced, with successive generations we have observed reductions in egg production, thus it is critical to constantly introduce new individuals from the field.
- Several products were tested in 2024 in partnership with insecticide companies
- We tested survival of eggs to potentially ovicidal compounds, adult responses to potentially repellent compounds, and efficacy of final instar toxicants
- In addition, we used larvae from the colony to test the three newly discovered codling moth entomopathogenic fungi.

Objective 3 – 2023/2024 Key Findings

- Now that we have solved the mating and egg laying issues, we are prepared to continue producing enough moths to provide them for other research projects such as the pending entomopathogenic fungi project.

Objective 4 – 2023/2024 Key Findings

- This objective will be addressed following final negotiation with insecticide companies on publication of findings.

METHODS

Objective 1: Establish a codling moth colony

Codling moths, sourced and aggregated from Washington State apple farms, were reared in the laboratory on artificial codling moth diet (Frontier Agricultural Sciences product #F9370B) using well-established protocols to ensure that the colony would establish and grow to sustainable levels within one year. The research colony was housed at WSU TFREC in Wenatchee, WA. Apple trees in commercial and research orchards were banded for final instar codling moth caterpillars to colonize in summer 2022 and 2023. Bands were removed from cold storage in January-February 2023 and 2024. We recovered several hundred codling moth final instar larvae and pupae from these bands to establish and sustain the colony. Upon returning colonized bands to the laboratory, individuals were prepared for a break in diapause and caged for emergence and mating. Eggs (F0 generation) from field-collected mated females were then placed on commercial pre-mixed diet. Larvae were fed upon the diet until they were of sufficient size to pupate. Emerging F0 generation adults laid the eggs of the F1 generation, the first generation potentially available for research use. All generations were reared at constant day length (16L:8D), temperature (24-28 °C (75-82 °F)), and RH (50-70%) to synchronize development.

Through the project we continued banding in other WA locations to maintain the colony's genetic diversity. There was a quarantine process much like initial colony establishment before they were incorporated into the main colony to ensure we did not introduce diseases into the colony. Beyond the end of the project, we will also periodically re-collect moths to maintain representative genetics.

Although past WSU codling moth colonies were maintained, this proposed codling moth colony was not be managed exactly the same way. Previous WSU colonies used a codling moth pinto bean diet from made from scratch, and instead, we used a commercial premixed diet because it requires less space, equipment, and personnel. We followed the rearing protocols for codling moth recommended by the diet company (Frontier Agricultural Sciences).

Potential ongoing problem: Occasionally colonies crash and many of the individuals will die. There are often simple explanations for a colony crash, including poor genetics, and proliferation of disease through the colony. Though there is a possibility that we will continue to experience a colony

crash, we will continue to take steps, such as sterilizing equipment, limiting entry into the growth rooms, and treating egg surfaces with dilute bleach to minimize the risk. We are prepared to field collect new individuals if necessary to replenish the colony.

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

In 2024 the Washington representative codling moth colony grew to a large enough size to accommodate removal of individuals. Thus, we began testing new and current insecticides' efficacies. We employ direct contact assays and choice assays for adults. Against eggs, ovicidal compounds were tested using topical assays. Survival following exposure to insecticides was monitored for up to 14 days.

Topical ovicides were tested by directly spraying eggs laid on egg deposition substrates. Egg hatch was monitored after exposure to insecticides for up to 14 days.

Funds obtained from industry will contribute to the long-term viability of the colony as well as the salaries of personnel tasked with maintaining the colony.

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

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

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

Objective 4: Update Extension resources to include new product information

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

RESULTS AND DISCUSSION

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

2023 Results

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

Figure 1. Checking cardboard bands for CM larvae

Figure 2. CM larva to be collected from infested apple

Figure 3. Some mating arena set-ups tested

Because it was possible that the cardboard band process negatively impacted moth fitness, beginning in July 2023, we changed tactics and began extracting codling moth larvae from infested apples collected on farms in Chelan, Douglas, and Grant Counties, while placing cardboard bands on farms in Chelan, Douglas, Grant, Yakima, and Okanagan Counties (Fig. 2). Larvae extracted from

apples and cardboard bands, 766, were fed on artificial diet, pupated, and emerged as adults. The first several rounds of adult moths were once again placed into the mating arenas, and neither mating nor egg laying occurred. The mating arena design that our colony failed to mate in was based on descriptions found in White and Hutt 1970, 1971; Hathaway et al. 1971, 1972, 1973; Howell and Clift 1972; Bathon et al. 1991; Toba and Howell 1991; Dyck 2010. Again, we changed tactics and tested several new and modified mating arena setups (Fig. 3). By the end of July, one of the tested mating arenas successfully allowed mating, egg deposition, and produced viable larvae (Fig. 4,5). From these mating individuals we obtained 302 F1 generation larvae by the middle to end of September. The majority of these individuals entered diapause and are housed in cold storage and will not be used until late January 2024. Cardboard bands from several on-farm locations will be processed through winter 2023/24.

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

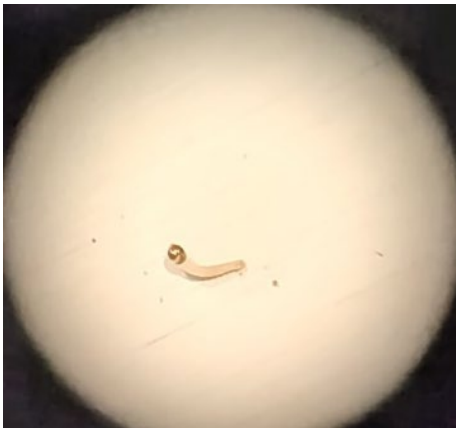

Figure 5. First successful F1 generation CM larva

2024 Results

In the second year of this project, we added 14496 codling moths to the colony. These moths came from several sources, including cardboard bands, apples, and progeny of colony-reared moths. We had very high emergence from live codling moths extracted from bands, ca. 93% of the extracted larvae emerged as adults. From those field-collected moths, we successfully reared four generations from late 2023-late 2024 (Generations F0-F3). Moving forward with the colony beyond the end of the WTFRC funding, we now know that extracting adults from bands is a viable method of quickly building up the colony. In the mating arenas PI Curtiss designed in 2023, eggs were consistently laid by F0-F3 moths, however, the egg to adult production decreased with each successive generation (Table 1).

	F0	F1	F2	F3
% Moths Emerged	81.95	15.36	2.61	0.07

Table 1. The percentage of each lab generation of adult moths that emerged over 2024.

Improvements to our rearing process still need to be made. In the future we plan to test other commercial diets and compare them with the pinto bean diet. In addition, we have recently observed a high proportion of F2 and F3 larvae ceasing to develop when the diet conditions begin to degrade. We recently started transferring them to fresh diet to determine if percent successful pupation and

emergence can be increased by providing new food. That test will be completed several months following the end of the WTFRC project.

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

2023 Results

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

2024 Results

In 2024 we had enough colony-reared moths to begin product testing. We conducted three studies with colony-insects.

1) An egg mortality assay that compared two new products efficacy at causing mortality in topical applications with applications of 440 superior oil. The products were organic, botanical oils that are used against other pests as topical ovicides. For this study we tested all the products in 1% solutions. The 440 oil prevented ca. 95% of the eggs from hatching, test product A prevented ca. 55% of the eggs from hatching, and test product B prevented ca. 40% of the eggs from hatching. At this time, we do not have permission to release additional information about these products, however at the tested concentrations the new products were not more effective than 440 oil. In 2025, we are negotiating with the company to conduct additional tests with the products at higher concentrations.
2) An adult oviposition choice assay that tested the repellent effects of a botanical compound that contained garlic oil. For this cage assay, we compared moths' oviposition on apples treated with the test product or water. The assay allowed moths to choose between only two apples for oviposition. The findings were somewhat inconclusive, but the data suggests that there may be some repellency of the garlic oil. More replication is needed. In 2025, we may conduct more testing if the company wishes to pursue this study again.
3) A final instar larvae mortality/sublethal effect assay using five different test compounds compared to a positive control (Malathion), and negative control (water). One of the test compounds contained a neem product. Our findings suggest that this stage is particularly difficult to affect. It took over 20 days for the last malathion-exposed moth to die, and no other test compounds caused significant mortality or prevented pupation. More studies in 2025 will be conducted with the newly found codling moth entomopathogenic fungi, and compared with other products.

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

2023 Results

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

2024 Results

Although we produced over 14,000 moths in 2025, we only provided a few hundred to other researchers. As this colony continues to develop, we expect to provide more moths to other researchers in the future.

Objective 4: Update extension resources to include new product information

No new updates were produced in 2023 due to the previously described issues. Updates from 2024 testing will be found in 2025 documents such as the crop protection guide, and 2-3 arthropod management tests publications.

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

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

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

EXECUTIVE SUMMARY

Project Title: Crop Protection Product Efficacy Testing for Codling Moth – Laboratory

Keywords: *Cydia pomonella*, management, research colony, new materials

Abstract: Codling moth management in Washington State requires effective mating disruption and application of conventional or organic insecticides. New insecticide products and tactics need to be tested under laboratory conditions, but without access to a colony of codling moths for assays, new product testing is severely hampered. This project established a research codling moth colony at WSU-TFREC in Wenatchee to be used to test new crop protection products and tactics. The first year of the project was constrained by repeated failures to induce oviposition in the laboratory, but by the second year, that issue was resolved and eggs were consistently laid in mating containers. By the project end, we performed assays with new topical ovicides, antifeedants, and potential repellants using the colony. There are still improvements to be made in the rearing process, however, moving forward we have established protocols that will allow for future testing of new products and tactics.

Proposal Title: DEVELOPING THE TOOLKIT FOR CODLING MOTH HOTSPOT MANAGEMENT
Report Type: Continuing Report

Primary PI: **RT Curtiss**
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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: **A**pplied
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 (Pending, $350,000)
WTFRC Collaborative Costs: none

Budget 1
Primary PI: RT Curtiss
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Station Manager/Supervisor: Chad Kruger
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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
- Potential members of the advisory group were engaged, but we have not yet met as a group
- WSU Institutional review board approval of the interview questions was delayed, so this objective will be conducted starting in Spring 2025.

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)

- Year one of mark-release-recapture field experiments were conducted
- Year one of high-density trapping, banding, and fruit damage field studies were conducted
- Insecticide assays will be conducted in spring 2025 as final instar caterpillars are 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 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, 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 2025

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 – ca. 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

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 year 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 will 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:

Year one of mark-release-recapture field experiments and high-density trapping, banding, and fruit damage field studies were conducted. However, as of the writing of this report, samples are still being processed by project technicians. Reporting of an incomplete data set at this time would be premature and potentially misleading. Over 140 traps from the mark-release-recapture study still need to be processed this winter before the year-one data set can be analyzed. We have processed 455 trap samples from the high-density trapping experiment, but still have several hundred to complete. In addition, 351 bands have been evaluated from the banding experiment.

Insecticide assays will be conducted in spring 2025 as final instar caterpillars are extracted from overwintering bands

OBJECTIVE 3:

As reported above in the bullet point summary, in 2024 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 in 2025 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 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 2025. Bands are being stored so the codling moth larvae in them can be used in other experiments included in this project in 2025.

OBJECTIVE 4:

CoPI DuPont and Dani Gray (added to this project in 2024 as a cooperator) hosted Codling Moth Study Circles December 3 and 4, 2024 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.

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 1. Images from WSU Extension hosted Codling Moth Study Circles December 3 and 4, 2024. **121** participants attended in person and online in Yakima and Omak. Orchardists, researchers and consultants shared their knowledge and experience managing codling moth. Participants reported learning 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. **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. These study circles were funded by the Washington Tree Fruit Research Commission through a grant awarded to researchers RT Curtiss, Tobin Northfield, Robert Orpet, and Tianna DuPont for sharing knowledge regarding codling moth management in hotspots.

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

Report Type: Final Project Report

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Project Duration: 3 Year

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

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

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

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

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

ORIGINAL PROJECT OBJECTIVES:

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

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

SIGNIFICANT FINDINGS

Objective 1 – 2022-2024 key findings

- 297 total releases in 2022-2024 resulted in variable capture by lure and mating disruption (MD) type
- Early spring capture was almost always poor with all lures
- Each year, and all years combined, passive mating disruption (hand applied reservoir dispensers) suppressed capture for 4 out of 5 lures
- Each year, and all years combined, the CMDA+AA lure had the most consistent capture across the three MD schemes
- Sufficient replication across all years was achieved to accurately estimate traps/acre and population densities

Objective 2

- PI Curtiss has presented findings at 4 grower meetings in 2022, 3 in 2023, and at 6 in 2024. At two recent extension events where these findings were presented, 98% of attendees reported that they will adopt these findings and alter their management. Extension will continue beyond the end of this project.
- The decision matrix table is presented herein, but it will be modified for publication and readability in print materials in 2025.
- The project webpage, and project-related fact sheets are in development as of the writing of this report but will be completed in early 2025.

METHODS

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

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

Plots: Experiments were conducted in commercial apple orchards in geographically diverse locations across Washington State during the summers of 2022, 2023, and 2024. Orchards contained a variety of apple cultivars, rootstocks, irrigation schemes, and tree training systems on 8-10-acre plots. All orchards were treated with codling moth pheromone mating disruption using: 1) actively dispensing aerosol emitters (i.e., ISOMATE® CM Mist Plus (Vancouver, WA)) at 0.5-1/acre, 2) passively dispensing reservoir dispensers (i.e., ISOMATE® CM Flex, and Scentry NoMate® CM Spiral (Billings, MT)) at recommended rates, or 3) no mating disruption. Conventional chemical controls were applied as needed by farmers.

Experimental design and moth releases: The experiment released externally marked sterile codling moths (75 cups/week for 20 weeks/year) for on-farm evaluation of codling moth lures. The cost for moths increased every year, but we were able to keep other costs down to compensate for the differences. Sterile, mixed-sex codling moth adults were obtained from the Okanagan-Kootenay Sterile Insect Release (OKSIR) facility in Osoyoos, British Columbia, Canada. Upon eclosion, moths at the OKSIR facility were immediately placed in petri dishes at an approximate ratio of 1:1 males:females (ca. 800 moths/petri dish) and treated in a Cobalt-60 irradiator. The dishes of irradiated moths were then packed into battery-powered coolers (2.8 Cu. Ft. Portable Fridge/Freezer: Edgestar co. Austin, Texas) held at approximately 2-5 °C (36-41 °F) and shipped to Washington State. Moths arrived before noon the same day they were packed allowing for immediate release into field plots. Because moths were transported as mixed-sex batches in chill coma directly from the shipper to field sites for immediate release, the sexes could not be separated prior to release.

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

Figure 1. Toriani Kent, Project Technician, releasing pink moths into the orchard canopy (R. Courtney, Good Fruit Grower Magazine)

The experiment employed a cardinal-direction mark-release-recapture design with a single central trap following protocols from Curtiss (2021) (Figure 2). Release locations were marked with flagging tape in the four cardinal directions from the single trap at distances of 20, 40, 60, and 80 m (66, 131, 197, 262 ft) . In each replicate, approximately equal numbers of females and males were released, and the number of moths was increased with increasing distance. Each of the four 20 m (66 ft) release points received ~400 sterile males/~400 sterile females, the four 40 m (131 ft) release points each received ~800 sterile males/~800 sterile females, the four 60 m (197 ft) release sites each received ~1600 sterile

males/~1600 sterile females, and each of the four 80 m (262 ft) release sites received ~3200 sterile males/~3200 sterile females.

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

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

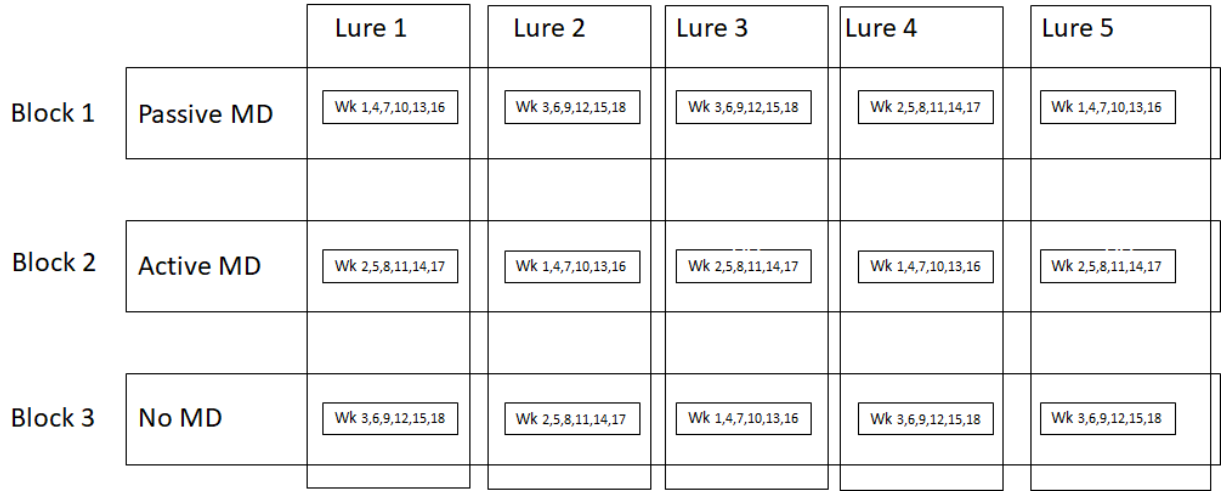

Figure 3. Example experimental layout and timeline.

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

Anticipated results and potential pitfalls: One-third of the total planned replications of each treatment were planned in each year, so major analysis was not planned to occur until the end of the third field season. However, due to some moth supply issues in 2023, eight releases planned for that year did not occur. In 2024, we tried to make up some of the lost releases and conducted five more than originally planned. Over the three years we only missed three planned replications, and they were all due to OKSIR supply issues. We anticipated data would suggest the need for higher trapping densities for orchards under the more efficacious lure types and mating disruption.

Some replications did not have adequate capture for meaningful analysis, and were not included in the analysis.

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

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

Dissemination: Our progress on this project will continue to be shared based on requests from the industry (i.e., distributor and packing house meetings) and at extension events (field days, fruit schools, workshops, etc.) beyond the end of WTFRC funding.

RESULTS AND DISCUSSION

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

Sterile codling moth releases were conducted in 45 commercial orchards each year from 2022–2024. Orchards were divided into three geographically distinct blocks corresponding to latitudes and longitudes 46-47°N and 119–121°W (Royal city region), 47–48°N and 119–121°W (Quincy Region), and 48–49°N and 119–121°W (Okanogan Region). Fifteen orchards were in each geographic block, with five blocks for each treatment: no mating disruption, passive mating disruption, and active mating disruption. All releases were performed when scheduled, unless moth supply issues interfered.

There were 100 total releases performed over 20 weeks of the summer 2022, and due to moth supply issues only 92 releases were performed in 2023, 105 releases were conducted in 2024. Each orchard (lure × mating disruption combination) received at least three releases, resulting in 17-22 acceptable replications of each combination across the three geographic blocks and the three years of releases. There were no statistical differences in combined male+female moth capture due to geography. However, some trends emerged. Capture in the early spring and late fall is poor across all lures, indicating that growers may not be receiving accurate wild moth population data when populations are low and weather conditions are not favorable for flight. Passive mating disruption appears to suppress trap-finding more than active mating disruption, indicating that active mating disruption may be deployed at too low densities to fully suppress mating in our plots. The CMDA+AA lure had the most consistent capture across the three mating disruption schemes and provided the overall highest combined capture.

Preliminary population density estimates based on the 2022-2024 replications also show some trends. All lures can be used in all mating disruption schemes to detect codling moths. However, the CMDA+AA and Megalure 4k lures both appear to detect codling moth at the lowest population levels across management schemes. The CML2, 10x, and CMDA lures had more variable capture, but appear less able to detect codling moths until populations are high when mating disruption is present.

The results presented in this final report are from three seasons and include the combined male+female recapture data, but there are some important considerations arising for farmers. First, the lure used in monitoring programs needs to be carefully matched with the mating disruption program. Second, codling moth capture-based decision making on apple farms is more accurate with the results of this study demonstrating a better understanding of the interactions between the lures and mating

disruption types. Last, spray decision-making based on monitoring traps may be inaccurate in the early spring when accuracy is critical because codling moth responses to traps are poor due to variable and unfavorable weather conditions. A parallel study from the Curtiss Lab found that temperature impacts moth capture significantly, with low temperatures suppressing capture and high temperatures increasing capture more than expected.

Now completed, this project provides accurate treatment guidance for industry decision makers. Accuracy in spray decisions can lead to cost savings by preventing unnecessary sprays, and/or inducing a spray to prevent crop losses. The cost savings, and/or gains will contribute to the long-term sustainability of farming apples in Washington. The continued investment of the WTFRC-ACP in study will provide the industry with more precise codling moth predictions upon which to base spray decisions.

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

PI RT Curtiss has presented preliminary project findings at four grower meetings in 2022, three in 2023, and six in fall 2024. At least 450 growers and decision-makers were present collectively at these meetings. The decision matrix table is presented in table 1. As of the writing of this report (Dec 23, 2024), the project webpage and project-related fact sheets are still in development. Project fact sheets will be completed by early 2025.

In addition to project-specific activities, we applied for a Western SARE grant ($347,287) to expand the research aspects of the project in 2023-2024 and add an extension-focused year (2025) to disseminate our findings. Our preproposal was accepted, and we were invited to write a full proposal that was ultimately rejected. The Western SARE proposed project would have allowed us to expand the scope of this project, cover unanticipated cost increases, and fund additional personnel. Unexpected cost increases are primarily for sterile moths which increased considerably since our original quote in summer 2021 (quoted at $24/unit in 2021, cost $30/unit in 2022, increased to $38/unit in 2023) when this project was in preparation. The increased costs of sterile moths caused us to not have sufficient funds for hiring hourly staff. Despite the lack of staff, we were able to complete all the releases for which we received moths, but we had to postpone work on the project fact sheets and attend fewer grower meetings in 2023 and 2024.

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

		LURE TYPE				
		CML2	**CM 10x**	**CMDA**	**CMDA+AA**	**Megalure 4k**
MATING DISRUPTION TYPE	**Passive**	Recapture: 0.335% n=22 Dispersive Distance: 91m Population Est.: 514/ha Trap area: 2.60 ha # Traps / 4.05 ha: 1.56	Recapture: 0.315% n=19 Dispersive Distance: 85m Population Est.: 863/ha Trap area: 2.27 ha # Traps / 4.05 ha: 1.78	Recapture: 0.287% n=20 Dispersive Distance: 91m Population Est.: 986/ha Trap area: 2.60 ha # Traps / 4.05 ha: 1.56	Recapture: 0.352% n=20 Dispersive Distance: 86m Population Est.: 595/ha Trap area: 2.32 ha # Traps / 4.05 ha: 1.74	Recapture: 0.258% n=18 Dispersive Distance: 88m Population Est.: 1133/ha Trap area: 2.43 ha # Traps / 4.05 ha: 1.66
	Active	Recapture: 0.341% n=22 Dispersive Distance: 90m Population Est.: 599/ha Trap area: 2.54 ha # Traps /4.05 ha: 1.59	Recapture: 0.514% n=21 Dispersive Distance: 87m Population Est.: 273/ha Trap area: 2.38 ha # Traps / 4.05 ha: 1.70	Recapture: 0.365% n=15 Dispersive Distance: 90m Population Est.: 616/ha Trap area: 2.54 ha # Traps / 4.05 ha: 1.59	Recapture: 0.440% n=19 Dispersive Distance: 88m Population Est.: 445/ha Trap area: 2.43 ha # Traps / 4.05 ha: 1.66	Recapture: 0.637% n=19 Dispersive Distance: 85m Population Est.: 217/ha Trap area: 2.27 ha # Traps / 4.05 ha: 1.78
	None	Recapture: 0.446% n=17 Dispersive Distance: 93m Population Est.: 315/ha Trap area: 2.72 ha # Traps / 4.05 ha: 1.49	Recapture: 0.377% n=18 Dispersive Distance: 92m Population Est.: 475/ha Trap area: 2.66 ha # Traps / 4.05 ha: 1.52	Recapture: 0.578% n=18 Dispersive Distance: 90m Population Est.: 238/ha Trap area: 2.54 ha # Traps / 4.05 ha: 1.59	Recapture: 0.359% n=20 Dispersive Distance: 92m Population Est.: 267/ha Trap area: 2.66 ha # Traps / 4.05 ha: 1.52	Recapture: 0.576% n=18 Dispersive Distance: 91m Population Est.: 278/ha Trap area: 2.60 ha # Traps / 4.05 ha: 1.56

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

EXECUTIVE SUMMARY

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

Keywords: *Cydia pomonella*, mating disruption, management, monitoring, population dynamics

Abstract: Codling moth, the key apple, pear, and walnut pest worldwide, is managed with applications of insecticides and mating disruption in Washington State. Their populations are monitored using baited traps, but current population predictions are based on management without mating disruption using a pheromone-only trap lure. Those predictions are not relevant to current management and monitoring practices. This project clarifies population predictions in orchards using no mating disruption, and orchards using both passive and active mating disruption technologies when monitoring traps used one of five currently available lures. The lures tested were the CML2 (pheromone-only), CM10x (pheromone-only), CMDA (pheromone/kairomone), CMDA+AA (pheromone/kairomone), and the Megalure 4K (kairomone-only). There were thus, 15 mating disruption + lure combinations tested in this project. Using previously established analysis methods, and a mark-release-recapture study, we herein provide population density predictions for each of the 15 combinations tested. This study further clarifies capture in monitoring traps in modern orchards that use mating disruption.

WTFRC INTERNAL PROJECT – BUDGET SHARED FOR INFORMATIONAL PURPOSES ONLY

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

Project Title: Pesticide residues of WA apples

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

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

Project Duration: 3 Years

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

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

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

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

Footnotes:
Schmidt estimates 8% of his time is dedicated to this project on an annual basis
Most pesticides tested are donated by their registrants or an ag chemical supply company
1 Wages & benefits primarily for Garcia (spray applications), crew help for Garcia, and Stone (data entry & review)
2 Travel costs include hauling equipment to & from plots and driving samples to analytical lab in OR

2024 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 twelve insecticides, six fungicides, and three miticides 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 12 and delivered the next day to Pacific Agricultural Labs (Sherwood, OR) for chemical residue analysis.

TRIAL DETAILS

- 17th 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 0.18 inches of rain fell on the trial block on August 23-24 (19 days before harvest)

Measured residues vs. maximum residue levels (MRLs) for STANDARD industry apple pesticide programs in 100 water/acre utilizing typical rates, timings, and retreatment intervals. 'Gala'/M.9 Nic.29, Rock Island, WA. WTFRC 2024.

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*
flutianil	Gatten	8	35	<0.01	0.15	0.01*	0.15 (many)
benzovindiflupyr	Aprovia	7	35	0.028	0.2	0.01*	0.2 (many)
pydiflumetofen	Miravis	3.4	35	0.022	0.2	0.01*	0.2 (many)
tolfenpyrad	Bexar	27	35 & 21	0.41	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	0.11	0.7	0.01*	0.5 (Twn)
fenbutatin	Vendex 50WP	32	35 & 21	1.10	15	0.01*	2 (Twn)
zeta-cypermethrin	Mustang Maxx	4	35 & 21	0.089	2	0.01*	0.7 (many)
acequinocyl	Kanemite	31	28	<0.01	0.4	0.01*	0.01 (Chn,Tha)
lambda-cyhalothrin	Warrior II	2.56	28	0.038	0.3	0.01*	0.2 (many)
flonicamid	Beleaf 50SG	2.8	28	0.043	0.2	0.01*	0.2 (many)
cyflumetofen	Nealta	13.7	28 & 14	0.13	0.3	0.01*	0.3 (Can,Mex)
sulfoxaflor	Transform	2.75	28 & 14	0.069	0.5	0.01*	0.3 (many)
chlorantraniliprole	Altacor eVo	2.2	28 & 14	0.26	1.2	0.01*	0.4 (many)
buprofezin	Centaur WDG	34.5	21	1.7	3	0.01*	1 (Twn)
lpflufenoquin	Axios 20SC	3	21 & 14	0.033	0.15	0.01*	0.01 (Tha)
phosmet**	Imidan 70-W**	92	14	2.2	10	0.01*	2 (Twn)
mefentrifluconazole	Cevya	5	14	0.065	1.5	0.01*	0.9 (Twn)
cyclaniliprole	Verdepryn	11	14	0.057	0.3	0.01*	0.2 (many)
cyfluthrin	Baythroid XL	2.8	14	<0.05	0.5	0.01*	0.1 (many)
fenazaquin	Magister	36	14	0.49	0.6	0.2	0.3 (many)

[1] Top markets for WA apples with established MRLs; 1 October 2024. https://nwhort.org/export-manual/, https://bcglobal.bryantchristie.com/

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

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

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

Measured residues vs. maximum residue levels (MRLs) for AGGRESSIVE apple pesticide programs in 100 gal water/acre utilizing maximum labeled rates, and minimum preharvest intervals. 'Gala'/M.9 Nic.29, Rock Island, WA. WTFRC 2024.

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*
benzovindiflupyr	Aprovia	7	35	0.038	0.2	0.01*	0.2 (many)
pydiflumetofen	Miravis	3.4	35	0.038	0.2	0.01*	0.2 (many)
acequinocyl	Kanemite	31	35 & 21	<0.01	0.4	0.01*	0.01 (Chn,Tha)
lambda-cyhalothrin	Warrior II	2.56	28 & 21	0.047	0.3	0.01*	0.2 (many)
flonicamid	Beleaf 50SG	2.8	28 & 21	0.043	0.2	0.01*	0.2 (many)
tolfenpyrad	Bexar	27	28 & 14	0.48	1	0.01*	0.01 (Twn,Tha)
flupyradifurone	Sivanto prime	14	28 & 14	0.20	0.7	0.01*	0.5 (Twn)
fenbutatin	Vendex 50WP	32	28 & 14	1.00	15	0.01*	2 (Twn)
indoxacarb	Avaunt	6	21 & 14	0.29	1	0.01*	0.1 (Can)
flutianil	Gatten	8	21 & 14	0.031	0.15	0.01*	0.15 (many)
zeta-cypermethrin	Mustang Maxx	4	21 & 14	0.11	2	0.01*	0.7 (many)
chlorantranliprole	Altacor eVo	2.2	21	0.34	1.2	0.01*	0.4 (many)
cyclaniliprole	Verdepryn	11	21 & 7	0.16	0.3	0.01*	0.2 (many)
cyflumetofen	Nealta	13.7	21 & 7	0.21	0.3	0.01*	0.3 (Can,Mex)
phosmet**	Imidan 70-W**	92	21 & 7	5.8	10	0.01*	2 (Twn)
buprofezin	Centaur WDG	34.5	14	1.9	3	0.01*	1 (Twn)
sulfoxaflor	Transform	2.75	14 & 7	0.17	0.5	0.01*	0.3 (many)
ipflufenoquin	AXIOS 20SC	3	14 & 7	0.062	0.15	0.01*	0.01 (Tha)
fenazaquin	Magister	36	7	0.52	0.6	0.2	0.3 (many)
cyfluthrin	Baythroid XL	2.8	7	<0.05	0.5	0.01*	0.1 (many)
mefentrifluconazole	Cevya	5	7 & 1	0.30	1.5	0.01*	0.9 (Twn)

[1] Top markets for WA apples with established MRLs; 1 October 2024. https://nwhort.org/export-manual/, https://bcglobal.bryantchristie.com/

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

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

CONCLUSIONS

Laboratory analysis revealed once again that no material tested in our 2024 study produced a residue that exceeded the tolerance level set by the US Environmental Protection Agency. These findings are further evidence that apple growers following directions on product labels should expect their fruit to be in full compliance for domestic sales regarding pesticide residues. Several products we tested did produce **residues which exceed Maximum Residue Levels** (MRLs) set in important export markets for Washington apples in both our standard and aggressive application protocols: **Bexar, Avaunt, Centaur WDG, AXIOS 20SC, Imidan 70-W, and Magister.** 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.

As we have typically observed in previous studies, the aggressive application protocol often produces higher residue levels than our standard protocol. This dynamic is not fully consistent, however, as demonstrated by our current results with fenbutatin. These types of findings highlight the potential variability in pesticide residues from one sample to another. Growers and consultants should always consider that any single sample of their fruit could produce a residue that is an outlier from an expected range of results.

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.

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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 carbendazim residues

*** Dithiocarbamate residues cannot be directly measured; total Ziram values are estimates based on analysis of the degradation product CS2

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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.

Proposal Title: Evaluation and Optimization of Robotics Plus and VariMas 3 Sprayers
Report Type: Continuing Report (No cost extension of 1-year proposal).

Primary PI: Gwen Hoheisel
Organization: Washington State University
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Co-PI 2: Lav Khot
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Telephone: 509-786-9302
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Address: 24106 North Bunn Rd
City/State/Zip: Prosser, WA 99350

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.
- Laboratory tests to examine and even out air flow for Prospr will be conducted in January - February 2025.
- 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 in the spring of 2025. This was a manifest of the WTFRC trip to the manufacturer in Netherlands. A no cost extension of this grant has been awarded to conduct these trials when the sprayer arrives.

Robotics Plus Prospr

- 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. Deposition was also less in many of the middle areas of the canopy regardless of rate and speed. This is likely due to placement and angle of fans, and the air flow interaction among fans. Trying to even out air flow and deposition is warranted but needs to be examined more with laboratory studies quantifying air patterns from each fan.
- 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.

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).

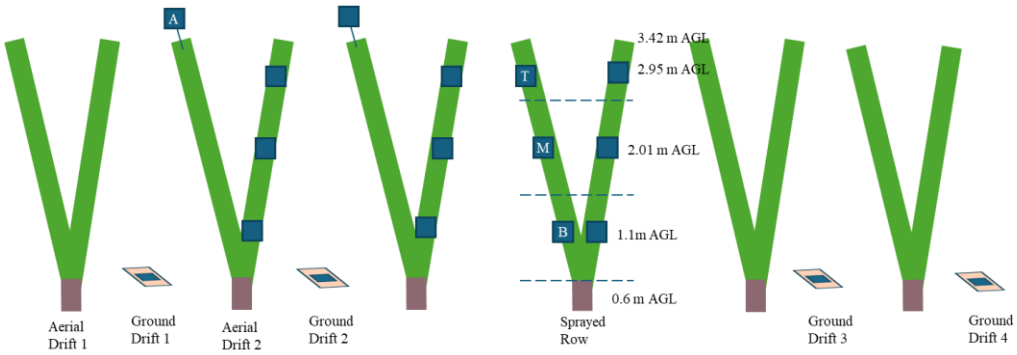

Figure 2. Schematic representation of deposition, and drift sampler locations (~3, 6, 9 feet above ground in the canopy and 1-foot above the canopy for drift rows). in the experimental v-trellised apple orchard plot.

VariMas 3: All pre-trials and deposition trials will be centered around following tests.
Test for optimal settings: 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 will measure 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) will be used for this evaluation. Air and liquid will be measured vertically every 12” inches. These patternator tests will be performed in a single location equivalent to canopy distance. Test results are extremely quick as data gets collected and plotted on a computer. Adjustments based on canopy characteristics can be made. The pulse width modulation (PWM) or other nozzles will be evaluated for droplet size using a commercial droplet sizer (ViziSize P15, Oxford Lasers, UK). AAMS flowmeter will test output on nozzles to ensure they are working before going to the field.

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.

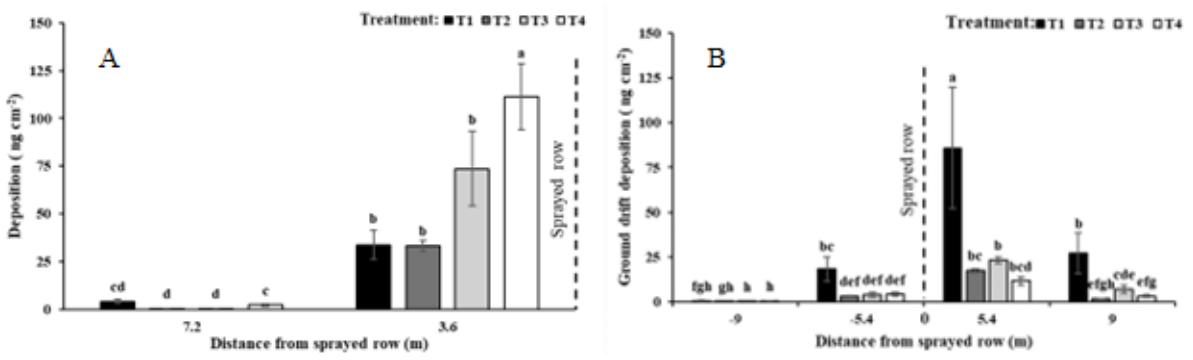

Figure 3. Aerial drift quantified for different sprayer treatments (A). Ground drift quantified at 17 and 30 ft (5.4 and 9 m) on either side of the sprayed row (B).

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 also less in many of the middle zones (Fig 4) regardless of rate and speed. This is likely due to placement and angle of fans, and the air flow interaction among fans. Trying to even out air flow and thus deposition is warranted but needs to be examined more with laboratory studies quantifying air patterns from each fan.

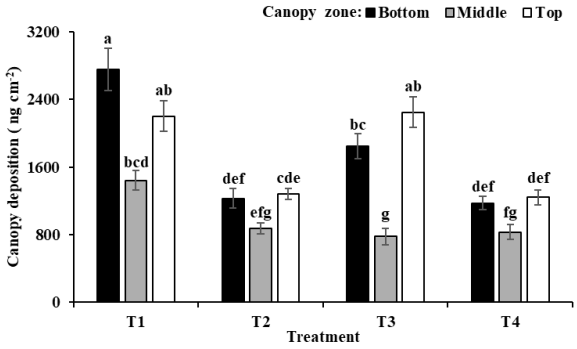

Figure 4. Spray deposition quantified in different canopy zones during spray treatments conducted using Robotics Plus Prospr autonomous sprayer in V-trellised orchard.

Proposal Title: Best practices for releasing lacewings in apples

Report Type: New Proposal

Primary PI: Rebecca Schmidt-Jeffris
Organization: USDA-ARS
Telephone: 509-454-6556
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Address: 5230 Konnowac Pass Road
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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
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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

- Finalized molecular analysis of data collected from previous projects (2021-2023) and this project in 2024 indicates that lacewings applied as **larvae are the most effective**. Lacewings released as egg cards or larvae have the highest recovery rates.
- We found **no evidence that released lacewings consume resident lacewings or other predators** in 2021-2023 samples. Molecular work on 2024 samples is ongoing.
- Lacewing egg products could achieve the same level of larvae recovery as released larvae, but only if eggs were held for the ideal length of time prior to release. This was difficult for us to coordinate while scheduling sampling times with three different growers at four simultaneous trials. Therefore, we will exclusively use larvae releases in future trials.
- Based on conversations with industry stakeholders, only *C. rufilabris* producers can supply enough lacewings to meet the demand of the tree fruit industry, so the majority of our work is now focusing on this species.
- In the Pateros trial, ***C. rufilabris* larvae releases reduced populations of aphids by 42-65%**, depending on the sampling method. In that trial, egg card releases reduced aphids in tap counts by 81%, but did not differ from the control in shoot counts.
- In the Pateros trial, ***C. rufilabris* egg card plots had 65-140% <u>more</u> mealybugs** in beat tray and burlap strip samples than the control, but did not differ from the control in mealybug shoot sample counts. Increased mealybug populations in the egg card treatment may have been due to the attraction of ants, which may have defended the mealybugs from predators. Ant populations were moderate in this orchard and slightly higher in the egg card treatment. In the shoot samples, **plots where *C. rufilabris* larvae were released had 81% fewer mealybugs** than the control.
- There were no significant differences in pest counts at the other trials. It appears that **release timing is critical to success**.
- When lacewing larvae were released by drone using a flight path perpendicular to the orchard rows, larvae recovery was identical to ground releases. This indicates that **drone releases are an effective delivery method**.
- Our initial non-target effects testing confirms that **lacewing eggs are relatively insensitive to pesticide applications**; most products caused little to no reduction in egg hatch. Oil residue prevented egg hatch of both lacewing species, but did not prevent hatch when exposure was through a contact spray. Instill-O residues reduced egg hatch in both species. Lime sulfur via direct contact and OSO and Oxidate residues reduced *C. rufilabris* egg hatch. *C. rufilabris* egg hatch rate and speed were lower than *C. plorabunda*. Our future planned trials with larvae will be more informative regarding product compatibility.

Methods

1a. Compare species, life stages, and cards versus loose eggs (Years 1-2)

Obj. 1a Treatments
1. *C. rufilabris* eggs sprinkled
2. *C. rufilabris* egg cards
3. *C. rufilabris* larvae sprinkled
4. *C. plorabunda* eggs sprinkled
5. *C. plorabunda* egg cards
6. No-release control

The study was conducted at three organic, commercial orchards in 2024. 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 applied once to five ~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 per 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 2024, we used preliminary trials in the lab to fine-tune our use of field cameras and determine the best methods for filming at night. We acquired small, battery-powered infrared lights and five additional field cameras, in addition to upgrading our camera lenses to better visualize small arthropods. In 2025, we will monitor *C. rufilabris* egg cards in orchards for predation events and in the lab for cannibalism events (Brinno TLC300). We will record ten egg cards per trial for one week post-release. We will record any predators visiting the cards and visit length. Ten egg cards per lacewing species will be similarly monitored in the lab to determine if cannibalism is common.

Molecular methods. All collected lacewings (taps, sticky cards) will be sequenced using COI primers (Palomares-Pérez et al. 2019), 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.

Lacewing larvae collected from the field will also undergo molecular gut content analysis (Krey et al. 2020, Cooper et al. 2022) to determine what pests are consumed by released lacewings and whether they consume resident natural enemies. Because we have determined that our three aphid pest species do not amplify well with our usual molecular gut content protocol, we will also screen

each sample using primers that we have determined amplify RAA/GAA (Chapman et al. 2010) and WAA (Orpet et al. 2019). We are also using this project as an opportunity to develop a multiplex PCR for simultaneously identifying prey consumption of multiple apple aphid pests.

Originally, we planned to repeat this trial in only one of the study orchards in 2025. However, we have concluded that efforts are better spent focusing on the question of timing and conducting trials testing this objective at three orchards simultaneously (see Obj. 2). At the Pateros location, we plan to test one additional treatment of a release program using mealybug destroyer larvae, following insectary release recommendations (Koppert).

1b. Compare drone to ground releases at orchard-scale (Years 1-3)

This trial 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. The first 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, 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. 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.

2. Determine which lacewing release ~~rate~~ timing is most effective for aphid control (Years 2-3)

This objective has been modified to test release timing, which we have concluded is likely the most important factor for release success. This trial will occur in all three trial orchards in 2025-2026. Plot set up and sampling will be as described in Obj. 1. Here, the treatments will consist of two sequential releases of 20,000 *C. rufilabris* larvae per acre, spaced two weeks apart. Each of four timing treatments will be initiated one week after the previous and compared to a no-release control. This will determine the ideal timing for treatment of orchards with lacewing larvae, which have been the most reliable method we have tested. We will also begin a USDA in-house study to determine the best methods for purchasing eggs and "converting" them into larvae to be released at the same rates tested here; our goal is develop a program that is more economical for growers (purchasing eggs) while still maximizing efficacy (releasing larvae).

	Week:	0	1	2	3	4	5	6	7	8	9	10
Treatment #	1		release		release							
	2			release		release						
	3				release		release					
	4					release		release				
	Control											

Obj. 2. Revised treatments based on timing. Sampling will occur each week. Week 1 will correspond to ~5 d after the final sulfur thinning spray for each orchard.

3a. Determine acute toxicity of organic pesticides to lacewings (Year 1)

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.

In 2025, we will similarly test the effects of residues and direct application on *C. rufilabris* larvae (*n*=30 lacewings/treatment). Lacewings will be monitored daily for mortality until pupation. We will also monitor pupation, adult eclosion rates, and time to development.

In separate assays, we will also monitor effects of 48 h exposure on movement (Ethovision XT14) and predation (*n*=10 lacewings/treatment).

3b. Determine the duration that field-aged residues remain harmful (Years 2-3)

In the USDA-ARS research orchard, individual apple trees will be sprayed with pesticide solution (highest labelled rate) or water to drip using a backpack sprayer. Trees will be treated at different times so that 3, 7, and 14 day-old residues of each pesticide are simultaneously available as needed. We will make 3.8 cm leaf disk arenas, on which a *C. rufilabris* larvae will be placed (30 individuals per treatment×residue-age combination). Larval mortality and egg hatch will be assessed as described above.

Obj. 3 Treatments
Dipel
Cyd-X HP
Entrust SC
Serenade Opti
Sonata
Blossom Protect (+Buffer)
Previsto
Cueva
Instill-O
Rex lime sulfur
Problad Verde
Double Nickel LC
OSO 5%SC
Regalia
Kaligreen
OxiDate 5.0
440 Superior Spray Oil
Control - water

Cost Comparison. In Obj. 1-2, we will 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 (2026-2027)
- Dual language handout (2026-2027)
- Pocket flip book (2026-2027)
- Website (ongoing)
- Field Days (2026)
- Intensive session at Tree Fruit Days (Jan 2026)
- Fruit Matters Newsletters (March, annually)
- Instructional videos: how to scout completed (https://treefruit.wsu.edu/videos/lacewing-and-aphid-scouting/); checking order quality filmed, currently in editing; how to conduct releases (2026)
- Update were made to the Orchard Pest Management lacewing page: https://treefruit.wsu.edu/crop-protection/opm/lacewings/
- Five grower presentations scheduled for 2025 at winter meetings

Results and Discussion

1a. Compare species, life stages, and cards versus loose eggs

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. Comparing the number of *C. plorabunda*-group larvae in treatments where they were not released as a preliminary method for

separating released versus resident indicates that in Pateros, most of the captured *C. plorabunda*-group are from releases (Fig. x). In Mattawa, we estimate that ~0.5 larvae/plot were resident and that the rest of the *C. plorabunda*-group were from releases. In Rock Island, the same number of *C. plorabunda*-group larvae were found in a treatment where they were not released (*C. rufilabris* eggs) as the two *C. plorabunda* release treatments; it is therefore possible that all of the individuals found in *C. plorabunda* release plots were actually resident lacewings.

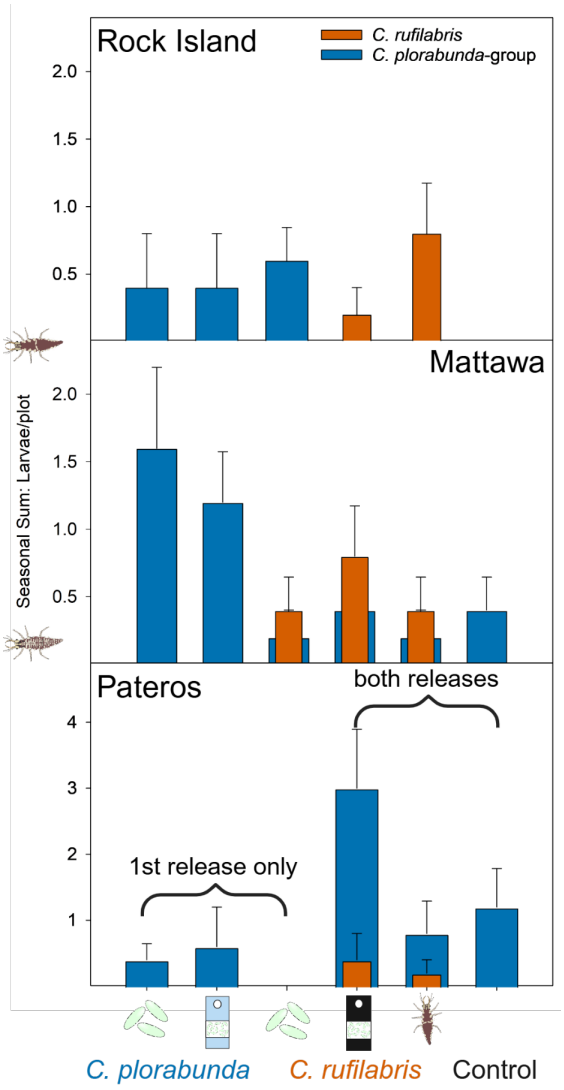

Fig. 1. Total *Chrysoperla* lacewing larvae captured per plot in the four-week post-release period. *C. plorabunda*-group individuals may be released or resident (awaiting completion of molecular work). Seasonal sums are larger for the "both releases" summary of Pateros treatments because this includes both four-week post-release periods (8 total).

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 (Fig. 1). When the egg treatments could be held until substantial hatching was observed (Mattawa trial), 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), 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. This is supported by recently completed gut content work from previous studies (2021-2023), where larvae from egg treatments were less likely to test positive for woolly apple aphid DNA (25%) compared to those collected from egg card (73%) or larvae (55%) treatments. For consistency between trials in future work, we will only use *C. rufilabris* larvae. However, we will also begin to investigate the best methods for growers to order eggs and convert them to equivalent rates of larvae, to reduce cost.

We did not observe any differences in pest counts between treatments in the Rock Island or Mattawa trials. Initially, it appeared that spring temperatures were warming up faster than average, so we conducted mid-April releases at these locations. However, the weather returned to cooler, wetter conditions during and post-release. We speculate that releases were conducted too early. Additionally, at the Rock Island location, a spinosad application for thrips control made on 25 April was associated with reduced counts of both resident and released lacewings in the following weeks (data not shown).

In Pateros, seasonal means of both aphids (Fig. 2) and mealybugs (Fig. 3) differed between treatments in the four-week period after the second release. For this analysis, we only compared the two

treatments that were released twice (*C. rufilabris* egg cards and larvae) and the control. *C. rufilabris* released as larvae had fewer aphid colonies per shoot than the egg card or the control treatments (Fig. 2). There were fewer aphids per beat tray in both the card and larvae treatments, but the difference was only statistically significant for the card treatment.

Table 1. Total number of lacewing larvae recaptured per treatment in this year's and previous trials. *indicates identification work in progress, -indicates a treatment not tested in that trial.

		C. plorabunda		*C. rufilabris*		
	Trial	Eggs	Cards	Eggs	Cards	Larvae
2024	Rock Island	*	*	0	1	4
2024	Mattawa	*	*	2	4	2
2024	Pateros #1	*	*	0	0	1
2024	Pateros #2	*	*	-	2	0
Prior	2021	2	-	2	-	7
Prior	2022	1	-	1	12	7
Prior	2023	2	1	0	2	3
	Average:	1.7	1.0	0.8	3.5	3.4

There were also significantly fewer mealybugs per shoot in the larvae release treatment (Fig. 3); this was also observed as a numerical trend via the other two sampling methods (burlap, beat tray). Although there were numerically fewer mealybugs/shoot in the egg card treatment compared to the control, there were statistically more mealybugs in the beat tray and burlap strip samples. 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 then tended the mealybugs, 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.

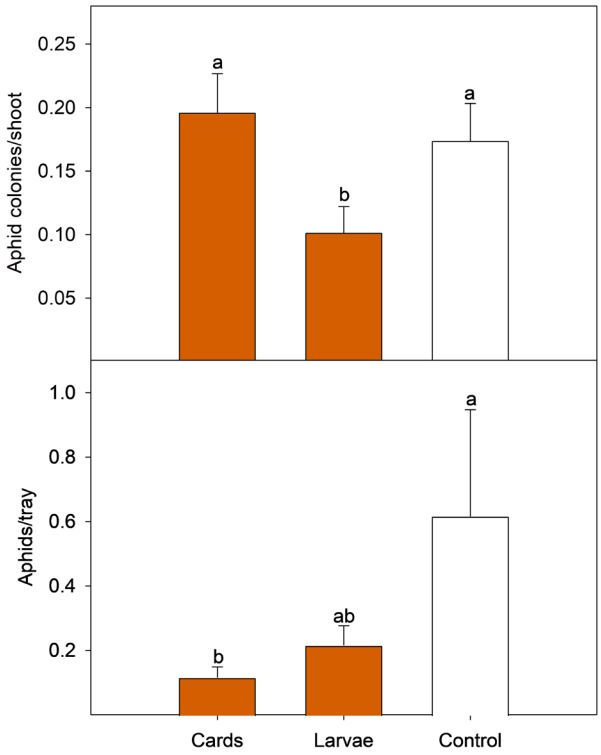

Fig. 2. Mean number of aphid colonies/shoot and aphids/tray in the four-week post-release period after the second set of *C. rufilabris* releases in Pateros.

1b. Compare drone to ground releases at orchard-scale

After the initial (10 April) release of *C. rufilabris* eggs resulted in no recovery of larvae, we conducted a second release on 10 May. Poor recovery after the first release (Fig. 4) was likely due to a combination of releasing very immature eggs and using a relatively low release rate (20,000 eggs/acre). Following the second release, recovery of larvae was substantial and equal between both treatments (Fig. 4). This demonstrates that drone delivery of lacewing larvae, flying perpendicular to the orchard rows, is as effective as hand

releases.

Results of this trial and previous release studies indicate that released lacewings can be found in the orchard for 3-4 weeks post release before they pupate (Fig. 4.). Because of the large plot sizes of this trial, we were also able to analyze the number of adults observed in each treatment (they were unable to move between plots as quickly). Adults were found in the orchard six weeks after the larvae were released (Fig. 5). In the first week *C. rufilabris* adults were observed, there were none in the control plots, but small numbers of them were captured the following two weeks as they moved out of release plots. There were no differences in the total number of adult *C. rufilabris* captured in drone versus ground release treatments, although both of these treatments were greater than the control (Fig. 5). We also found no differences between treatments in counts of resident lacewing larvae or adults (Fig. 5), indicating that our released *C. rufilabris* did not affect resident lacewings.

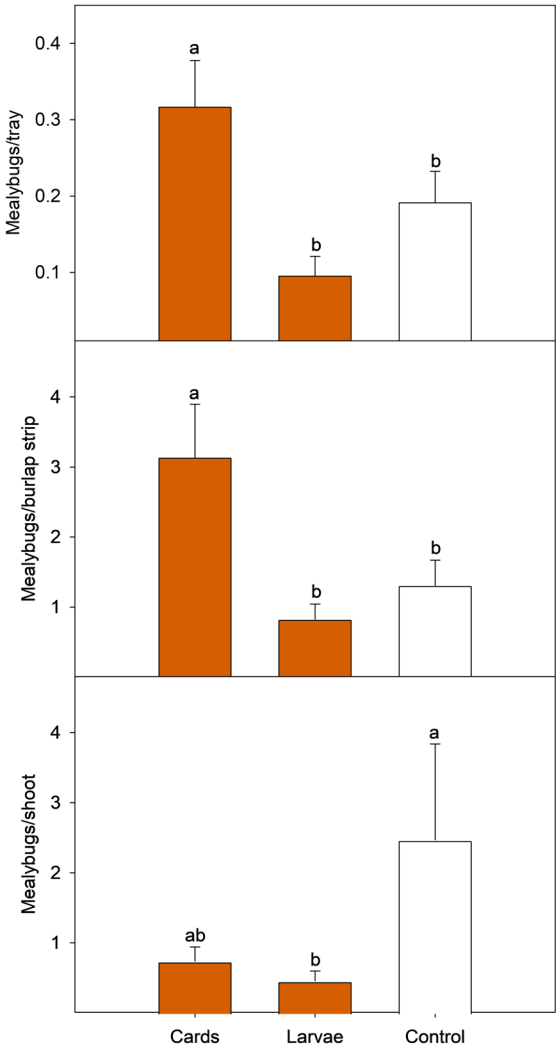

Fig. 3. Mean number of mealybugs per tray, burlap strip, and shoot in the four-week post-release period after the second set of *C. rufilabris* releases in Pateros.

No differences in mealybug or aphid abundance were observed between treatments in this trial. This further emphasizes the importance of determining the proper timing of release for success. One additional challenge with this trial was variation in pest pressure between sections of each orchard block used. Because the treatments were randomly assigned to each section within a block, the control plot was placed in the center in 3 out of 5 replicates; pest pressure was higher closer to the outer edges of the block. In 2025, we will attempt to account for this by using this year's pest counts to better randomize the location of each plot within the orchard.

3a. Determine acute toxicity of organic pesticides to lacewings

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 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*. Our planned work testing effects of these pesticides on larvae will likely be more informative regarding organic pesticide compatibility with releases. However, this provides initial indication that of these two insectary populations, *C. rufilabris* may be more sensitive than *C. plorabunda*. Additionally, Instill-O and oil residues appeared to be consistently harmful to the eggs of both species.

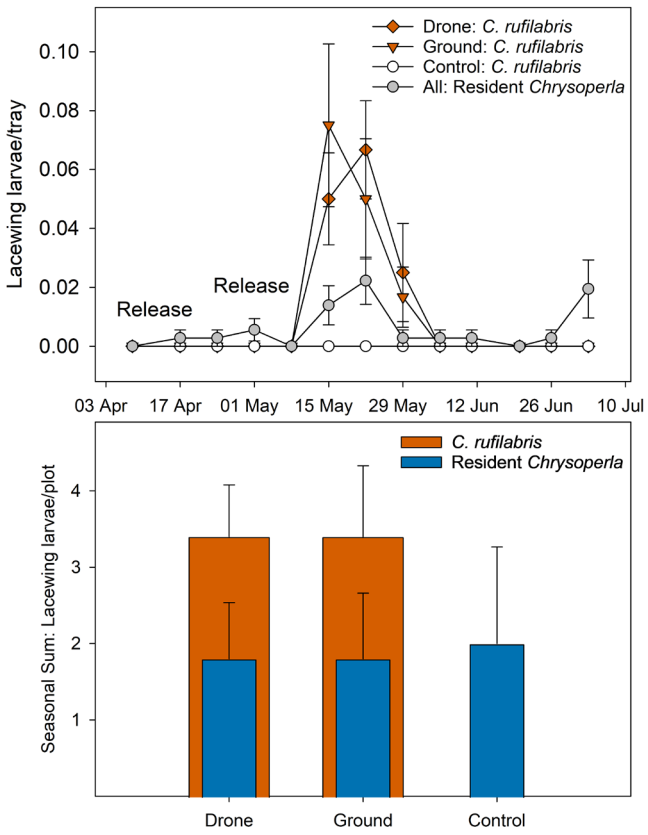

Fig. 4. Mean number of lacewing larvae/tray and seasonal sums of lacewing larvae per plot in the trial comparing drone to ground releases of *C. rufilabris*.

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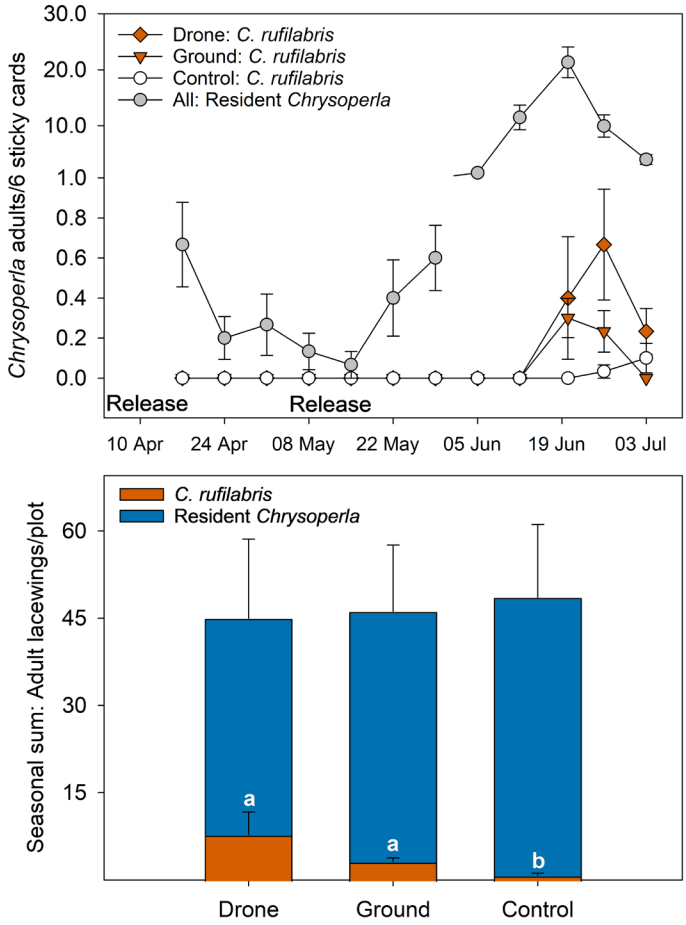

Fig. 5. Lacewing adults per plot on each sampling date and seasonal sums in the trial comparing drone to ground releases of *C. rufilabris*.

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 highlighted.

	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

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:	$592 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

- 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.
- An additional 153 samples were processed using barcoded COI and 16S primers and we are awaiting results from the sequencing service. 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.
- Our non-target effects testing protocol will be modified to include food, due to mortality in the control.

- Our initial non-target effects trial indicates that the insectary whirligigs were the most sensitive to Delegate, Esteem, Centaur, and Aza-Direct residues, with Aza-Direct causing the highest level of mortality. Esteem and Magister caused the greatest reduction in prey consumption.

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.

Table 1. Whirligig 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

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).

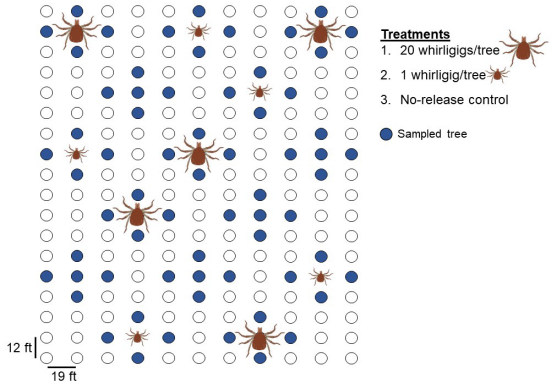

Fig. 1. Plot layout

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 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.

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. In preliminary work, 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 lepidopteran DNA (codling moth, leafrollers).

Objective 3: Pesticide non-target effects

Pesticides (Table 2) 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 30 replicates (arenas) per treatment. Whirligig mortality was recorded at 24 and 48 h. Then, up to 10 surviving individuals per treatment from the mortality assays (including the water control) were monitored using motion tracking software (Ethovision) for 30 min. Then, we added 6 fifth instar potato psyllids from colony to 6 arenas per treatment and counted the number of psyllids consumed in 24 h. To date, we have used this procedure to test the insectary whirligigs, but will also test our colony population in 2025.

We also plan on repeating this assay again with the insectary population due to moderate mortality in the control; we will modify the existing procedure to decrease mortality (see Results and Discussion).

In a separate assay, we will also 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).

Planned Outreach. We will summarize project results each year in the early spring in an article for *Fruit Matters* newsletter. Additionally, in early 2025, 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.

Table. 2. Pesticides tested in assays

Trade Name	MOA	AI
Assail 70WP	4A	acetamiprid
Delegate WG	5	spinetoram
Agri-mek SC	6	abamectin
Esteem 35 WP	7C	pyriproxyfen
Centaur WDG	16	buprofezin
Movento 240 SC	23	spirotetramat
Nealta	25	cyflumetofen
Magister SC	21A	fenazaquin
Exirel	28	cyantraniliprole
Altacor eVo	28	chlorantraniliprole
Aza-Direct	UN	azadirachtin

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.

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.

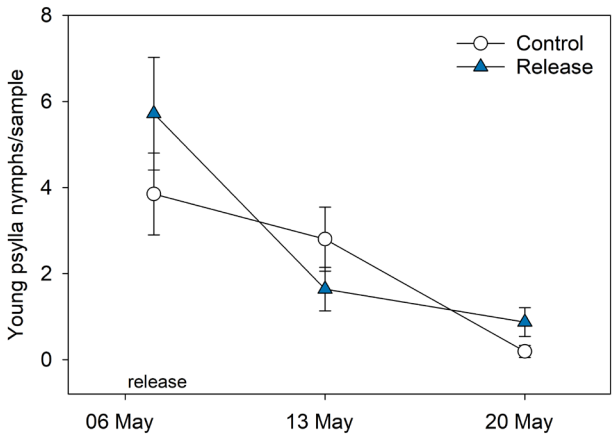

Fig. 2. Young pear psylla nymphs per leaf brush sample comparing whirligig release treatments in a research pear orchard.

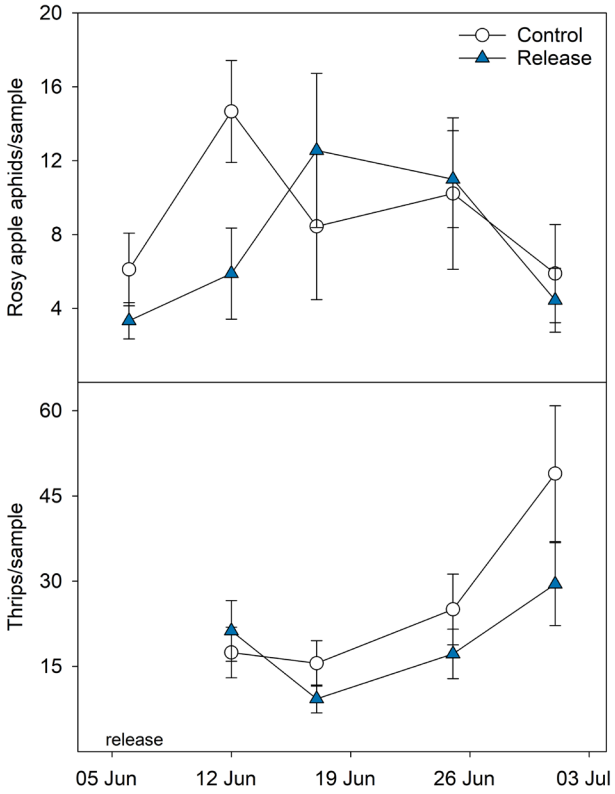

Fig. 3. (a) Rosy apple aphids and (b) thrips per sample collected

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).

To mitigate the logistical issues encountered in 2024, in 2025, 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. We are currently updating our APHIS permit to include a commercial orchard as one of the release sites; the initial permit was only for the research farm. In 2024, we reduced the ant population through bait applications and will continue these efforts in 2025. We have communicated with the USDA farm crew to minimize pruning to allow for adequate bud production in 2025, to increase the foliage available for whirligigs and the availability of fruit for pest damage assessments.

Obj. 2. 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, but does detect green and rosy apple aphid DNA; 30% of specimens tested positive using this primer. These 46 specimens and an additional 107 specimens were processed for generic barcoded primer PCR using COI and 16S regions and we are awaiting the results from the sequencing facility. Preliminary work with four specimens found matches for western flower thrips, rose aphid, and springtail DNA using the 16S primer. Our results to date indicate that whirligigs may be effective predators for pest aphids in apples.

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

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 4). Variation in number of specimens collected is due both to the differences in abundance at a given site, but also due to 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.

Table. 4. 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

Obj. 3. Our 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; although we loaded this treatment first, 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). Therefore, we will repeat this trial to ensure a fair comparison between treatments. We determined that 48h mortality is reduced to 0% in

untreated whirligigs when they are provided with a small amount of frozen *Ephestia* eggs and will provide this food source to mites in our next set of assays.

Because of the high mortality in the control, these results are preliminary and may change when we repeat the trial. For this survival analysis, we compared each treatment to Exirel because it had the lowest mortality. Delegate, Esteem, Centaur, and Aza-Direct significantly decreased survival relative to Exirel; Aza-Direct had the lowest survival. Although we set up six feeding assay arenas per treatment, after 24 h, only two individuals in the Delegate treatment were alive, preventing us from analyzing this treatment. Only Esteem and Magister reduced prey consumption compared to the control.

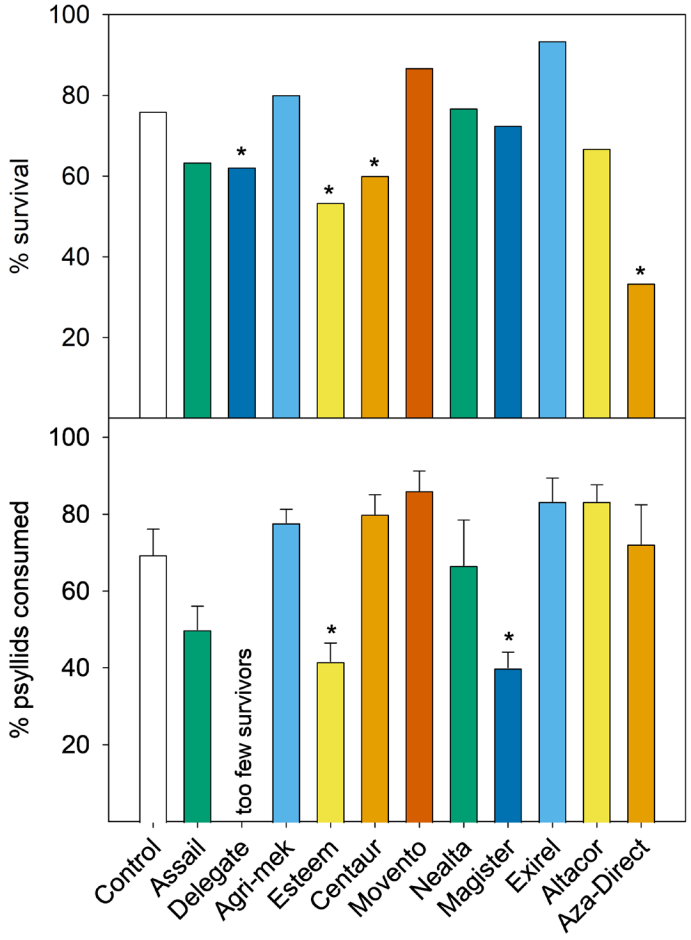

Fig. 4. (a) Percent of whirligigs surviving after 48 h exposure to pesticide residues and (b) % of potato psyllids consumed by pesticide-exposed whirligigs. Asterisks indicate a treatment that was significantly different from either Exirel (% survival) or the control (% psyllids consumed).

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

Report Type: Continuing 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

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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

OBJECTIVES

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

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

SIGNIFICANT FINDINGS:

- Spore/propagule count is not directly correlated with the ability of commercially available AMF products to colonize apple roots.

- Among the commercially available AMF products tested, *F. mosseae* (RTI-Ag) and *C. claroideum* (RTI-Ag) represented the most "promising" products in terms of their ability to colonize apple.

- All 5 AMF products manufactured by RTI-Ag successfully colonized apple in an "agriculturally relevant" timeframe (6 weeks).

- 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 and well-watered treatments. This result represents a specific AMF-rootstock relationship that could be harnessed to improve drought tolerance.*

- *The AMF C. etunicatum enhanced plant defense against infection by the fungal replant pathogen Rhizoctonia solani AG-5 in G.41.*

- Relative to pasteurized orchard soil, "live" orchard soil appeared to restrict development of nursery-derived AMF. In the context of orchard management, however, introducing AMF after fumigation may not be the best approach to harnessing AMF-rootstock relationships.

- *Results suggest that introduced commercial inoculants can negatively impact the development of pre-established AMF communities after planting.*

- *The loss of soil microbes (pasteurization) affected the ability of R. irregularis to benefit water uptake in G.11.*

METHODS:

AMF product panel testing (pre-requisite for Objective 1). Commercially available AMF products designed for soil application and readily available to Washington growers were identified. Seven different, single-species AMF inoculants were selected and tested for their ability to interact successfully with apple in accordance with recommended application rates/procedures (Table 1). AMF species belonging to the genus *Paraglomus* have been repeatedly identified in apple roots cultivated in a variety of orchard soils in Central Washington (Van Horn, et al., 2021; Somera et al., 2021); however, products containing these species are not commercially available. Therefore, *Paraglomus* spp. were obtained from the International Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM) (Table 1). Spore counts of all products were conducted via sucrose density gradient centrifugation. Product viability was initially confirmed in corn as described in the Continuing Report (CR) for 2023. Products were then tested for their ability to successfully colonize 6-week-old apple seedlings (Gala) in sterile growth medium (described in CR 2023). After 6 and 12-weeks, plants were harvested and the extent of mycorrhizal colonization in each treatment was assessed via trypan blue staining (Fig. 1). In addition to providing helpful information about AMF products available to growers in Washinton State, the assessment was aimed at identifying "promising" commercially available products for use in a subsequent experiment (described below).

Assessing the ability of introduced AMF to compete with native AMF in "live" orchard soil (main experiment for Obj. 1). In a recent study conducted by the Somera lab, it was shown that pre-established (nursery-derived) AMF strongly influence AMF community structure after planting and limit effective colonization by mycorrhizal inoculants (CR 2023; Zhang et al., 2024). In other words, apple rootstocks serve as a significant source of inoculum from the nursery where they are produced. This preliminary study was, however, conducted in pasteurized potting mix containing a limited 5-species consortia of AMF. In Summer of 2024, a subsequent experiment was conducted to test the ability of commercial AMF to out-compete those in native orchard soil and in nursery-derived apple roots. Based on product panel test results (Fig. 1), *F. mosseae* (RTI-Ag) and *C. claroideum* (RTI-Ag) were selected for use as commercial inoculum. Pasteurization, a proxy for soil fumigation, may provide insight into the ability of commercial AMF products to colonize roots post-fumigation. Therefore, the experiment was conducted in both "live" and pasteurized orchard 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 a representative sample of nursery-derived (or pre-existing) AMF communities. This root tissue was stored at −80°C (-112°F) until processing. Trees were planted into 2.7-L 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 (16-h photoperiod) and watered as needed; plants did not receive supplemental nutrients. Upon harvest, new (white) root tissue was collected from each plant for DNA isolation and 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. Plant growth characteristics measured at this time included: trunk diameter (cm), total plant biomass (g), root volume (mL), and leader shoot length (cm). Changes to nursery-derived AMF community composition following cultivation in orchard replant soil (live vs. pasteurized) will be 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).

Assessing benefits of specific apple rootstock-AMF associations (Obj. 2). Recent work in the Somera Lab identified compatible AMF-apple rootstock combinations that led to rapid establishment of a relationship (CR 2023; Cook et al, 2024). One caveat of this study was that colonization by AMF could not be interpreted as directly functioning in a plant beneficial role. Separate experiments designed to explore functional benefits of select apple rootstock/AMF associations identified in the compatibility experiments were conducted in Spring of 2024. Specifically, we tested the ability of two different AMF species (*C. etunicatum* and *C. claroideum*; Mycointech, Tarragona Spain*)* to enhance plant defense against infection by the fungal replant pathogen *Rhizoctonia solani* AG-5 in apple. Tissue-cultured plantlets of uniform size (10 replicates each), obtained from North American Plants, Inc. (McMinnville, OR), were used because nursery-derived apple rootstocks come with pre-existing AMF communities. The Geneva rootstocks G.41, G.890 and G.11 were selected for testing based on previous research efforts to identify compatible rootstock/AMF associations (CR 2023; Cook et al., 2024). Briefly, rootstock/AMF associations were pre-established in pasteurized potting soil by placing single species AMF inoculum into the root zone prior to planting. After 5 weeks, mycorrhizal colonization was confirmed. The ability of the specific rootstock/AMF associations to enhance plant defense against *subsequent* pathogen infection was then tested by challenging mycorrhizal and non-mycorrhizal plants with the fungal replant pathogen *Rhizoctonia solani* AG-5. Inoculum of *Rhizoctonia solani* AG-5 was prepared on whole oat grains and inoculum viability was confirmed prior to soil infestation. Infested oat grains were dried, ground and hand-mixed into pasteurized orchard soil (0.25% w/w; 6.25g inoculum in 2.7L soil). It should be noted that the experiment with G.11 was conducted separately from the experiment with G.41 and G.890 due to micropropagated plantlets arriving at different times. In all cases, mycorrhizal (*C. etunicatum* or *C. claroideum*) and uninoculated control plants were transplanted into 2.7L pots with or without the pathogen (7 replicate plants per treatment). G.890 x *C. claroideum* could not be assessed due to insufficient inoculum (Mycointech did not send us the requested amount of material). Thus, there were 8 treatments (instead of 9) (56 plants in total). All treatments were harvested after 4 weeks. DNA was extracted from fine root tissue (as described above) and the amount of *R. solani* AG-5 DNA per gram of root tissue was quantified using a real-time qPCR assay specific for *R. solani* AG-5. We are currently in the process of quantifying "at harvest" AMF colonization levels.

In collaboration with Dr. Lee Kalcsits, a separate experiment was conducted in Summer of 2024 to test for *R. irregularis*-mediated tolerance to water stress in apple. 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 *R. irregularis* (Mycointech; 12g per 2.7L pot). After 5 weeks, AMF colonization was confirmed via microscopy. Trees were then maintained at 2 different soil moisture contents: non-stressed (80-90% field capacity) and water-stressed (~40% of field capacity) for an additional 4 weeks (Fig. 3A). 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 (Fig. 3B). Stomatal conductance and stem midday water potential (plant physiological responses related to water usage) were measured as described in the original proposal (shown in Fig. 3C and D, respectively). An additional aim of this study was to assess whether *R. irregularis* benefits plants in terms of nitrogen uptake. **Isotopic labeling of nitrogen represents a powerful addition to the current toolkit with which to analyze the functional benefits of AMF symbioses.** At the start of the water stress experiment (5-weeks post planting), labelled nitrogen (ammonium-15N nitrate; $^{15}NH_4NO_3$) was spiked into a subset of pots via watering (100 mg per pot). 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 surfaces were covered with a sheet when using labelled N to avoid cross contamination.

RESULTS AND DISCUSSION:

AMF product panel testing (pre-requisite for Obj. 1): High spore/propagule viability is an essential prerequisite for any mycorrhizal product designed for use in agroecosystems. All products tested were *purported* to contain ≥ 50 viable spores/propagules per gram. This was confirmed for all products except *C. etunicatum* (RTI-Ag) (Table 1). It should also be noted that spore/propagule counts for *R. irregularis* (Lallemand) were purported to be 2,000 per gram; the actual spore count was significantly lower (265 per gram). That said, simply counting spores doesn't determine viability, as some spores may be dead or unable to germinate. All products, apart from *Paraglomus* spp., were found to be viable in corn (Table 1). Results were inconclusive for *Paraglomus* spp. because the mycorrhizal structures of this group typically stain weakly (or not at all) in trypan blue (a detail learned over the course of experiment). Next, the ability of the products to successfully colonize *apple* roots in an "ecologically relevant" timeframe was tested (Fig.1). By 6-weeks post-inoculation, all 5 AMF products manufactured by RTI-Ag had successfully colonized apple; colonization levels of RTI-Ag products ranged from 2-50% 6 weeks post-inoculation and 7-60% 12 weeks post-inoculation (Fig. 1). Higher spore counts did not necessarily correspond with a greater ability to colonize apple roots. For example, apple root colonization was relatively low for *D. eburnean* (INVAM) and *R. irregularis* (Lallemand), two products with relatively high spore counts (530 and 265 spores/gram, respectively (Fig. 1). This was surprising, considering that *R. irregularis* was previously shown to be highly compatible with apple, regardless of rootstock genotype (Cook et al., 2024). In the current study, *F. mosseae* (RTI-Ag; 197 spores/gram) and *C. claroideum* (RTI-Ag; 106 spores/gram) represented the most "promising" commercially available products in terms of their ability to colonize apple (Fig. 1). For this reason, both products were selected for use in Experiment 1 (as described in the original proposal).

Assessing the ability of "introduced" AMF to compete with native AMF in "live" orchard soil (primary experiment for Obj. 1). As mentioned above, this experiment was designed to test the ability of commercial AMF to out-compete those in *native* orchard soil and those pre-existing in apple roots (i.e., nursery-derived). The greenhouse experiment was completed in Summer of 2024 and microbial DNA present in apple root tissue has been extracted. DNA samples are expected to be sequenced by mid-February. The amount of root tissue colonized by AMF was also assessed at harvest (Fig. 2). Soil treatment (pasteurized vs. live) was found to be a significant source of variation affecting % AMF colonization (2-way ANOVA; $p=0.008$) in G.11 plants from TRECO Nursery. AMF colonization of root tissue was significantly greater in pasteurized, uninoculated treatments relative to "live", uninoculated treatments (Mann-Whitney test; $p=0.04$; Fig. 2). This result suggests that soil-borne fungi/pathogens may have restricted mycorrhization in "live" (uninoculated) soil. In this treatment, mean AMF levels were reduced by approximately 30% relative to that of pasteurized soil (Fig. 2). In a recent study, we showed that rootstocks serve as a significant source of AMF inoculum from nurseries where they are produced (Zhang et al., 2024). Thus, the AMF communities present in plants cultivated in pasteurized, uninoculated control treatments most likely developed from pre-existing AMF obtained at the TRECO nursery.

When commercial inoculants were present, however, no significant differences in AMF abundance were observed between "live" and pasteurized soil (Fig. 2). This result may be indicative of antagonistic interactions between introduced commercial inoculants and pre-established (nursery-derived) AMF. In fact, when *C. claroideum* was present in pasteurized soil, AMF abundance was significantly reduced relative to uninoculated, pasteurized controls (One-way ANOVA; Dunnett's multiple comparison test; $p=0.03$). In the Zhang et al. study, it was shown that introduced AMF have the potential to alter resident (pre-existing) AMF communities in ways that negatively impact the entire plant-associated microbiome, a finding which may have important implications for manipulating AMF community re-assembly post-fumigation. Regarding G.11 rootstock from

Cameron Nursery, soil treatment was not a significant source of variation affecting AMF colonization and no significant differences in the amount of AMF colonization were identified between pasteurized and "live" treatments. Sequence based analysis of AMF community composition is expected to provide more detailed information.

Assessing benefits of specific apple rootstock-AMF associations (Obj. 2):

Tolerance to water stress. *R. irregularis* colonization of G.11 rootstocks cultivated in both “live” and pasteurized orchard soil was confirmed after 5 weeks. Surprisingly, no AMF were detected in uninoculated control plants (grown in “live” or pasteurized soil) at this time. Upon completion of the water-stress experiment, plant physiological response data was assessed. 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. 4). In addition, colonization by *R. irregularis* (as represented by the hatched bars in Fig. 4) led to a significant increase in stomatal conductance in both of these treatments (water-stressed and well-watered). 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.

Interestingly, in pasteurized soil, colonization by *R. irregularis* led to a significant *decrease* in stomatal conductance in the water-stressed treatment (Fig. 4). This result is difficult to explain, especially as we do not yet have AMF colonization data from harvest (currently in process). If *R. irregularis* was still present in the root tissue at this time, one hypothesis is that soil pasteurization led to loss of microbial diversity/function needed to facilitate water uptake by *R. irregularis*. Although soil-borne pathogens in “live” soil may restrict the degree of mycorrhization, microbe-microbe interactions in the endosphere/rhizosphere are poorly understood, and functional outcomes of AMF mycorrhization are likely to depend on complex interactions between environmental conditions and other soil/rhizosphere microorganisms. For example, Hestrin et al. (2019) showed that synergistic interactions between AMF and soil microbial communities substantially enhanced plant N acquisition from organic matter. In the current study, we will also be able to assess how the loss of soil microbes (pasteurization) affects the ability of *R. irregularis* to benefit nitrogen uptake in apple. Sample processing of leaf, roots, and wood for assessment of labeled nitrogen uptake is currently underway.

Protection against pathogenic root fungi. The purpose of this experiment was to assess the ability of select AMF (*C. etunicatum* and *C. claroideum*) to enhance plant defense against infection by the fungal replant pathogen *Rhizoctonia solani* AG-5. It is well known that the ability of apple to suppress root infection by replant pathogens depends, in part, on rootstock genotype. Therefore, a variety of micropropagated Geneva rootstock genotypes were used (G.11, G.41 and G.890). After 5 weeks, mycorrhizal colonization was confirmed in all three rootstock genotypes (13-19% in G.41, 28% in G.890 and 32-47% in G.11). At this time, the degree of mycorrhization detected in uninoculated control plants was minimal (0-3%). It is interesting to note that mycorrhizal colonization by both *Claroideoglomus* spp. was also higher in G.11 than in G.890 or G.41 after 5-weeks in a previous study (CR 2024; Cook et al., 2024).

It was expected that root infection levels would be lower in AMF plants than in uninoculated controls (i.e., non-AMF plants) and that the specific apple rootstock/AMF combinations would differentially influence plant capacity to harbor *R. solani* AG-5. In G.41, a significant difference in the amount of *R. solani* AG-5 DNA present in root tissue was found between uninoculated control plants and those pre-colonized by *C. etunicatum* (Fig. 5; p=0.02). This result showed that the AMF *C.*

etunicatum enhanced plant defense against infection by the fungal replant pathogen *Rhizoctonia solani* AG-5 in G.41. No other apple rootstock/AMF combinations significantly reduced infection levels relative to the uninoculated controls. Because AMF occupy the same ecological niche (the root cortex) as soil-borne plant pathogens, roots colonized by AMF are unlikely to be simultaneously colonized by non-mycorrhizal fungi (and vice versa). That said, prior to transplantation, higher levels of colonization by *C. etunicatum* were detected in G.11 (32%) and G.890 (28%) than in G.41 (19%). As mentioned above, colonization by AMF can not necessarily be interpreted as directly functioning in a plant beneficial role. We are currently in the process of quantifying "at harvest" AMF colonization levels.

In Fig. 5, it may appear that G.11 did a better job than G.41 or G.890 at defending itself against *R. solani* AG-5, regardless of treatment. However, the experiment with G.11 was conducted separately from the experiment with G.41 and G.890 due to micropropagated plantlets arriving at different times. The level and persistence of *R. solani* AG-5 inoculum in soil may have been different among these two experiments. As bulk soil was not collected upon planting, this cannot be determined. Nevertheless, both water-stress and *R. solani* infection studies mark significant steps toward laying the groundwork for harnessing potential apple rootstock–AMF species preferences for integration into nursery and orchard management systems.

TABLES AND FIGURES:

Table 1. List of commercially available AMF products tested for their ability to successfully colonize apple roots (updated following submission of 2023 Continuing Report).

Manufacturer	Product/AMF Species	Recommended Application Rate	Spore Count (spores/gram)*	Product Viability‡
Symborg (Oxnard, CA)	MycoUp® (*Glomus iranicum*)	0.1 g (diluted in H_2O)	ND◊	Viable
Lallemand (Quebec, Canada)	LALRISE® MAX WP (*Rhizophagus irregularis*)	0.5 g (diluted in H_2O)	265	Viable
RTI-Ag (Gilroy, CA)	*Rhizophagus intraradices*	1 tsp per plant	585	Viable
RTI-Ag	*Funneliformis mosseae*	1 tsp per plant	197	Viable
RTI-Ag	*Claroideoglomus etunicatum*	1 tsp per plant	37	Viable
RTI-Ag	*Claroideoglomus claroideum*	1 tsp per plant	106	Viable
RTI-Ag	*Septoglomus deserticola*	1 tsp per plant	52	Viable
INVAM (University of Kansas)	*Diversispora eburnea*	1 tsp per plant	530	Viable
INVAM	*Paraglomus brasilianum*	1 tsp per plant	242	Undetermined §
INVAM	*Paraglomus occultum*	1 tsp per plant	122	Undetermined §

**All products purported to contain spore concentrations ≥ 100 per gram*
‡ The Most Probable Number (MPN) method was used confirm product viability in corn
INVAM; International Collection of Vesicular Arbuscular Mycorrhizal Fungi; University of Kansas
◊ Clear spores; difficult to count
§ Structures reported to stain weakly (or not at all) in trypan blue and other traditional stains (INVAM)

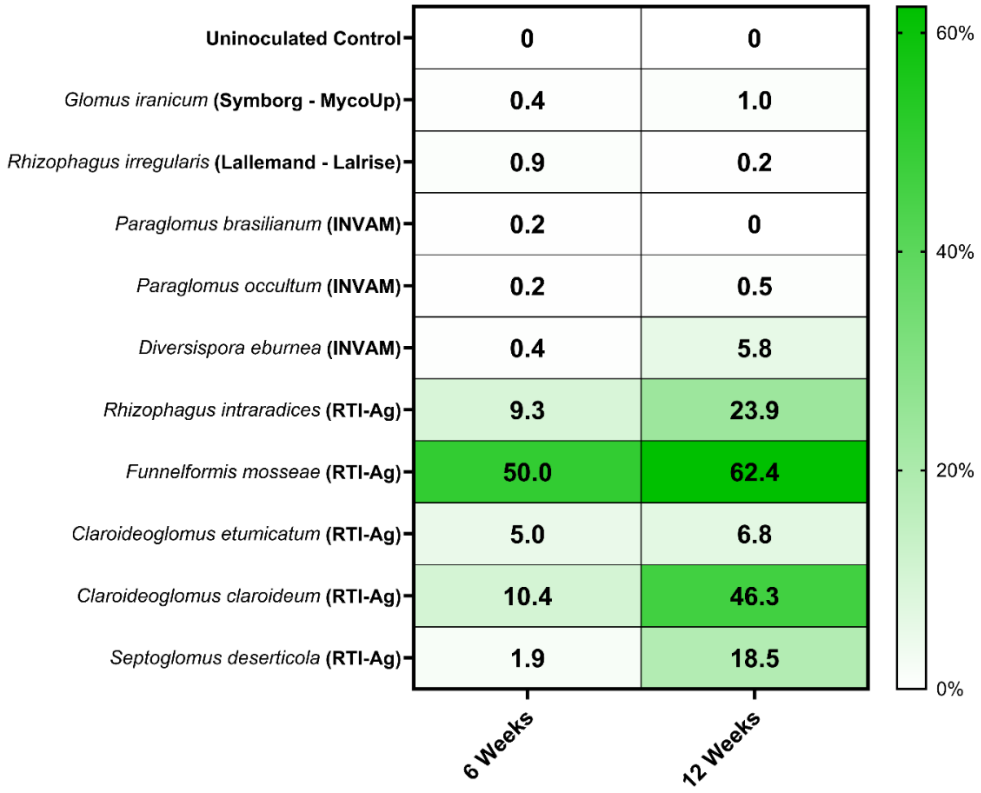

Figure 1: Heat map showing percentage of apple root colonized 6- and 12-weeks post-inoculation. Darker green indicates higher values, while lighter green to white indicates lower values. It should be noted that the mycorrhizal structures of *Paraglomus* spp. have been reported to stain weakly (or not at all) in trypan blue and other traditional stains (INVAM).

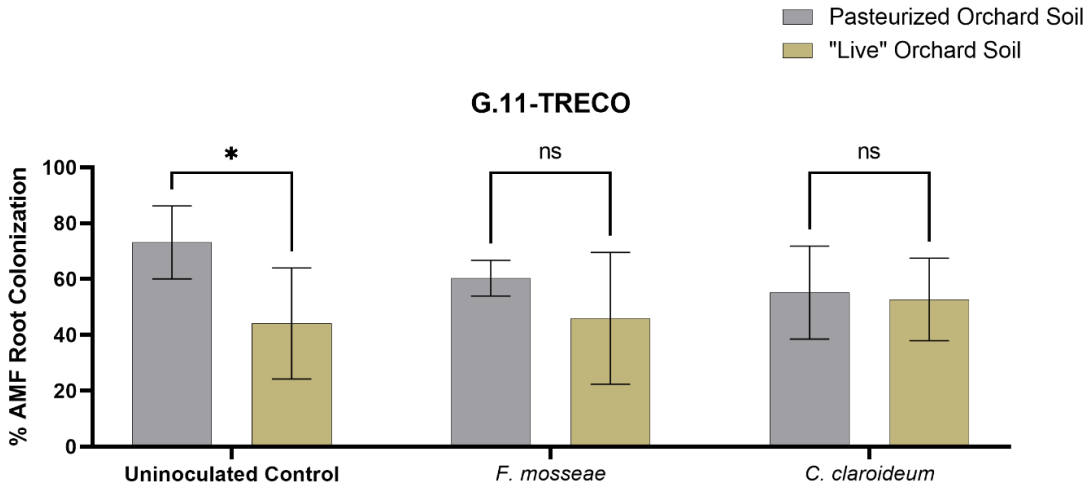

Figure 2. The percentage of root tissue colonized by AMF after 8-weeks when G.11 (TRECO) rootstocks were cultivated in pasteurized (grey bars) vs. "live" (tan bars) orchard soil with or without AMF inoculant. Mann-Whitney tests (with Holm-Sidak correction for multiple tests) were used to check for significant differences between soil treatments within each AMF treatment (Uninoculated control, *F. mosseae* and *C. claroideum*); * = significant, ns = not significant.

Figure 3: (A) Experiment designed to assess the ability of *R. irregularis* to improve tolerance to water stress in G.11. **(B)** G.11 rootstock in orchard soil with soil moisture sensor **(C)** Handheld device used to measure stomatal conductance non-destructively **(D)** Measuring stem water potential using a Scholander pressure chamber.

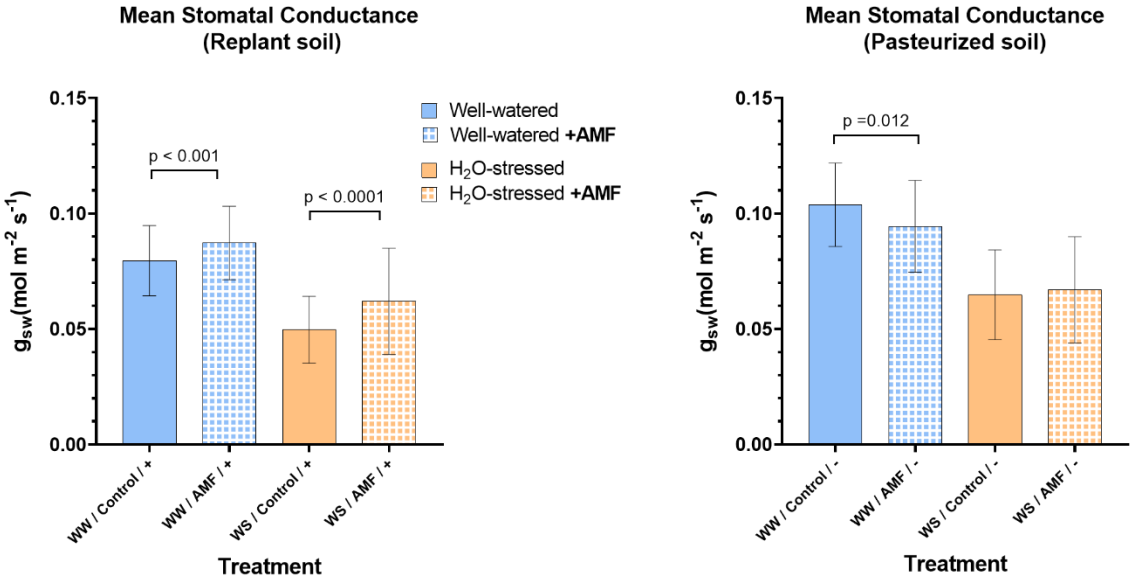

Figure 4: Mean stomatal conductance by G.11 rootstocks cultivated in "live" (left) and pasteurized (right) orchard soil with (orange bars) or without (blue bars) a water deficit (30-40% field capacity). Hatched bars represent treatments in which plants were colonized by *R. irregularis* prior to experiencing the water-stressed conditions.

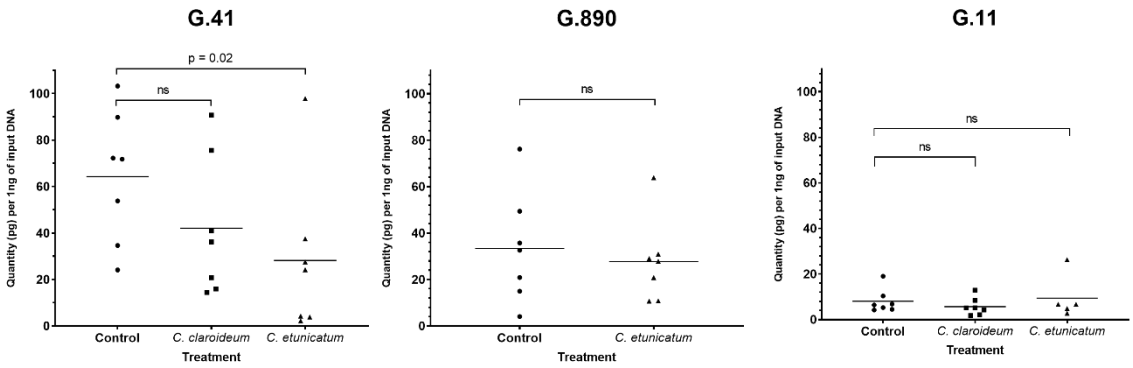

Figure 5: Amount of *R. solani* AG-5 DNA present in apple root tissue 4-weeks after transplantation into pasteurized orchard soil containing *R. solani* AG-5 inoculum. Prior to transplantation, micropropageted plantlets were either pre-colonized by *C. claroideum*, *C. etunicatum* or not colonized (Control). The experiment with G.11 was conducted separately from the experiment with G.41 and G.890 due to micropropagated plantlets arriving at different times. Statistical analysis of G.11 and G.41 data was performed via Ordinary 1-way ANOVA on ln transformed values followed by Dunnett's multiple comparisons test; data for G.890 was normal/non-transformed and was analyzed via an unpaired t test with Welch's correction.

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Project Title: Assessing refugia plantings for biocontrol services

Report Type: Continuing 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
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
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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

Objectives

1. Quantify natural enemy and plant communities in refugia plantings with weekly sampling at sites in the Columbia Basin across two growing seasons.
   Deviations: none.

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.
   Deviations: none.

3. Create a codling moth parasitoids reference collection housed at WSU-TFREC, to document and assist with identifications in Objective 2.
   Deviations: none.

4. Share findings, including practical advice on economics of plantings relative to biocontrol benefits.
   Deviations: none.

Significant Findings

- Refugia supported high numbers of beneficial 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. In addition, 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.

Methods

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

Monitoring for beneficial insects was conducted in flower plantings and varying distances into adjacent apple orchards. The flowers were previously planted by orchardists and have been studied for pollinator diversity starting in 2022 in association with the WSDA Specialty Crop Block Grant project entitled “Ensuring reliable pollination for Washington apples with cultural practices and conservation” led by the PI Dr. Orpet. The current 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. Similar sampling will continue in 2025.

Field procedures varied between years. In 2023, six sites were studied. In 2024, five of those were studied; one was excluded because the orchard was cut down. In both 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, 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 both 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.

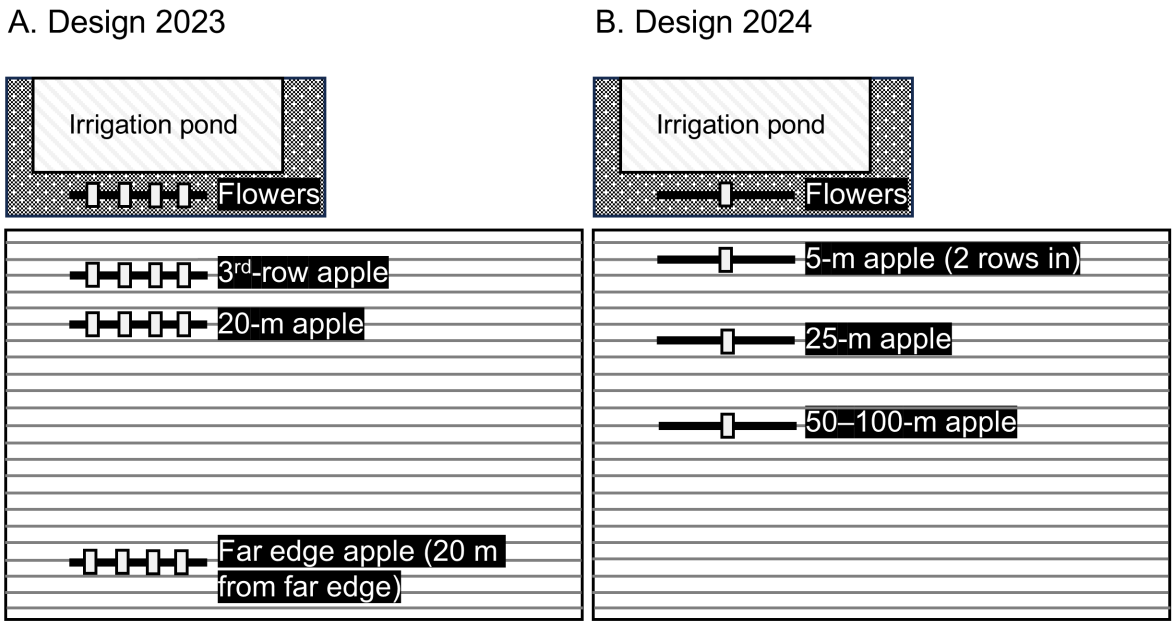

Figure 1. Diagram of sampling design in 2023 (A) and 2024 (B). An example is given where flowers were planted around an irrigation pond. Other plantings could be strips by roadsides or fields next to orchards. Orchard dimensions and row spacing varied between sites.

In 2023, four sticky cards were placed in each plot, and in 2024 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 both years, two blue vane traps, which are effective for collecting Hymenoptera (including parasitoids), were deployed in each plot for one 24-h interval every two weeks.

In 2024, a survey of flowering plants was made on each visit by walking through each plot and recording all flowering species.

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

In addition to the sticky card and blue vane trap sampling described in Objective 1 in refugia and orchards, additional pest sampling was done in the orchard plots. Twenty first-year shoots in each 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 several hymenopterans emerged from laboratory codling moth colonies. It is 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.

Objective 4. – Share findings.

A project website has been established by investigator M Luppino (https://cahnrs.wsu.edu/tfrec-orpet/insect-habitat-survey/) that includes photos of flower plots and some of the insects found. Luppino is scheduled to speak about the project at NCW Apple Day in 2025, and field day visit to one of the flower plantings will be planned.

Results and Discussion

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

Sticky card data from 2023 showed beneficial insects were found in variable numbers in flower refugia. The woolly apple aphid parasitoid *Aphelinus mali* was scarcely found in flower plots relative to orchard plots (Figure 2A). Syrphid flies appeared to make great use the refugia (Figure 2B), but green lacewings did not (Figure 2C). Bigeye bugs (*Geocoris)* were very abundant in most refugia, but scarcely found in orchards (Figure 2D). The opposite was true of small black ladybugs that eat mites (Figure 2E), whereas similar numbers of aphid-eating ladybugs (i.e., two-spotted ladybugs, transverse ladybugs, convergent ladybugs) were found in refugia as orchards (Figure 2F).

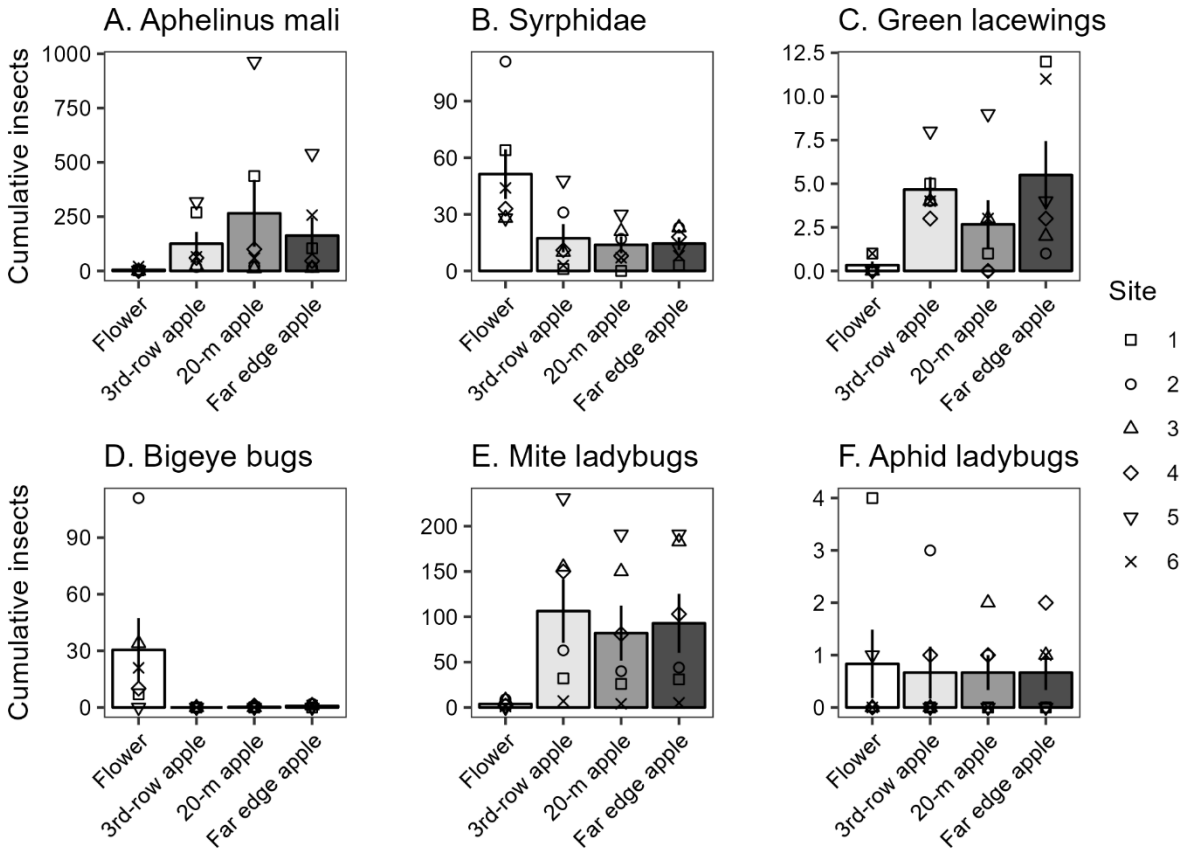

Figure 2. Cumulative number of beneficial insects found on sticky cards during 2023 at six sites. Individual points show data for one site, and bars show the mean of the six with standard error bars.

The monitoring showed that refugia likely improve the stability of syrphid populations and predatory bugs. Adult syrphids eat nectar from flowers. Orchards tended to have few flowers and low flower diversity, so orchards probably support a smaller syrphid community than would be possible if season-long flowers were available, like in refugia plantings. Bigeye bugs were surprisingly abundant in refugia. These are predatory bugs known to attack aphids in orchards.

Other beneficial insects seemed indifferent to refugia. *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 small black ladybugs that eat mites. Perhaps few mites occur in the refugia. On the other hand, green lacewings are highly mobile generalists and might have been expected to occur in refugia, but they were rare compared with orchards. Similarly, there were about as many aphid-eating ladybugs in refugia as orchards.

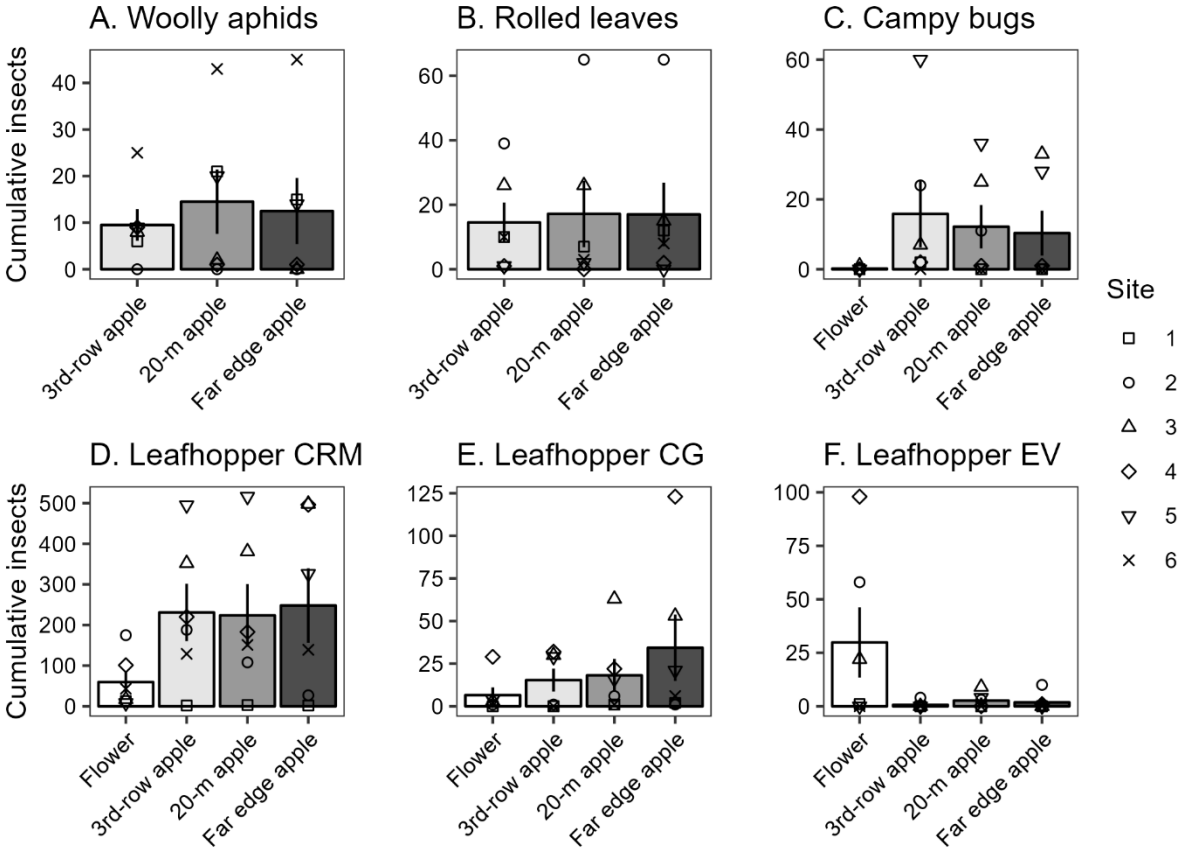

Figure 3. Cumulative number of pest insects found with apple tree sampling (panels A, B) or sticky cards (panels C–F) during 2023 at six sites. Individual points show data for one site, and bars show the mean of the six with standard error bars.

The sticky card method may be ineffective for monitoring some taxa like aphid-eating ladybugs. Anecdotally, many ladybugs and ladybug larvae were seen in some of the 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. Blue vane samples are still undergoing quantification for beneficial insects. Sticky cards from 2024 are also still undergoing quantification.

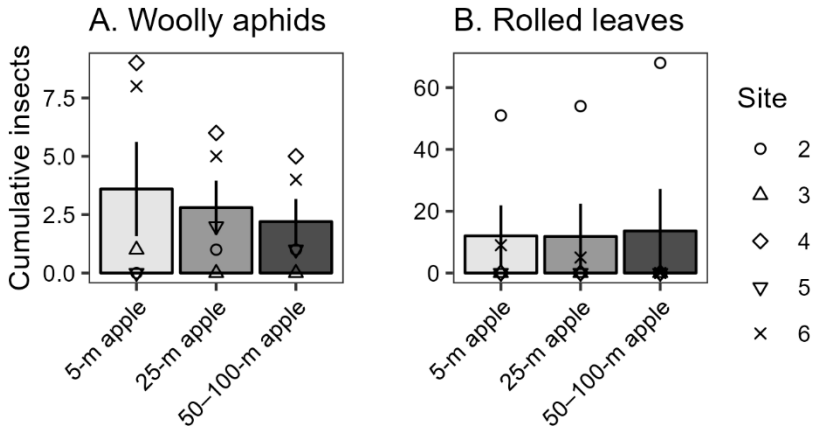

Figure 4. Cumulative number of pest insects found with apple tree sampling in 2024 at five sites. Individual points show data for one site, and bars show the mean of the five with standard error bars.

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

There was generally no clear signal of spillover of insects from refugia into orchards. However, spillover is difficult to infer with the study design. We might expect to find more of a given insect in the orchard in plots closer to refugia if the refugia is a source of that insect. However, if insect dispersal is great and is highly influenced orchard specific factors like prey density, signals may not be clear. A relatively small number of refugia-generated insects could colonize orchards, and from there a numerical response to high prey density could occur. This may result in more insects in the orchard (where there is food, e.g., woolly apple aphids) than in the refugia despite the possible importance of refugia as an annual source population of predators.

Despite the limitations of the study, it seems reasonable to suggest that the refugia studied are relatively unimportant for specialized insects that feed on apple pests because densities of a specialist's 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 specialist like *A. mali.* Generalists that require flowers during the adult stage and are highly mobile, e.g., syrphid flies, might benefit from refugia.

The direct effect of refugia on pests is also difficult to infer. Woolly apple aphids and rolled leaves in apple orchards were found in similar abundance within orchards regardless of distance to refugia in both years of study (Figures 2A,B and 3A,B). *Campylomma* bugs from sticky cards were also found in similar abundance regardless of distance to refugia (Figure 3). 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. Likewise, two of the leafhopper vectors of cherry X-disease (*Colladonus montanus reductus, Colladonus geminatus*) were uncommon in refugia relative to orchards (Figure 3). 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 do not risk being a major source of X-disease to neighboring cherry orchards; evidence from

movement studies in Oregon suggest the species is not very dispersive, which is consistent with our observations here.

Other pest insects sampled included green apple aphids, apple grain aphids, and rosy apple aphids. Very few of these were found in the orchards monitored.

Objective 3. – Create a codling moth parasitoid reference collection

Parasitoids and pathogens in codling moth colonies managed by RT Curtiss were collected during 2023 and 2024. Identification, quantification, and creation of a reference collection is ongoing.

Objective 4. – Share findings.

Development of educational materials to share is ongoing. Information for a field day in 2025 and other outputs will be shared on the project website ((https://cahnrs.wsu.edu/tfrec-orpet/insect-habitat-survey/) next 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 greatly produced or spilled into neighboring orchards. The same is true of pests; there was no evidence that these refugia are pest sources.

All objectives are on schedule for completion at the end of 2025. Two years of insect sampling have been conducted (2023 and 2024), and samples from 2023 have been quantified. Technical staff are now trained for sampling procedures and for identification of beneficial insects on sticky cards. Remaining work is to conduct one more season of insect sampling, create a beneficial insects reference collection, and share findings. The lead investigator, Orpet, has left Washington State University to Oregon State University starting January 1, 2025, so the lead role is being passed to RT Curtiss for the final year of the project. Curtiss will oversee reference collection creation and project administration. Sampling and insect quantification will be managed by Luppino, who filled this role in previous years. Orpet will stay as a co-PI to assist with administration and data analysis.

Project Title: Comprehensive monitoring and mapping antibiotics resistance in orchards

Report Type: Final

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Project Duration: 2-Year

Total Project Request for Year 1 Funding: $77,898
Total Project Request for Year 2 Funding: $80,235

Other Funding Sources: Awarded
Funding Duration: 2020-2025; 2023-2026; 2024
Amount: $443,707; $249,828; $25,886
Agency Name: USDA-NIFA-SCRI, WSDA-SCBG and WTFRC-Pear Research

WTFRC Collaborative Expenses: None

Budget

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Item	2022	2023	2024
Salaries[1]	52,262	54,352	0
Benefits[2]	10,044	10,446	0
Wages			
Benefits			
Equipment			
Supplies[3]	10,342	10,187	0
Travel	5,250	5,250	0
Miscellaneous			
Plot Fees			
Total	**77,898**	**80,235**	**0**

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

Objectives:

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

Significant Findings:

- No *Erwinia amylovora* isolates exhibited resistant to streptomycin and oxytetracycline in 2022, 2023 and 2024;
- 38 and 7 *Erwinia amylovora* isolates exhibited resistance/tolerance to kasugamycin in 2023 and 2024, respectively;
- Among 45 isolates, 73% were isolated from pear samples and 27% from apple samples;
- The resistant/tolerant isolates were isolated from orchards in 10 distinct locations, including Sunnyside, Mattawa, Prosser, Cashmere, Wenatchee, Malaga, and Entiat.
- Minimum inhibition concentration (MIC) against kasugamycin for 186 isolates isolated from different years in Washington state was determined and compared with 141 strains from other states.
- MIC_{50} was higher for WA isolates, and WA isolates from 2024 had the highest MIC_{50} as compared to previous years.
- No mutation was found in the kasugamycin target *ksgA* gene in most of the resistant/tolerant *E. amylovora* isolates except three.
- This is the first report of kasugamycin resistant/tolerant *E. amylovora* isolates in Washington or elsewhere.
- These results suggest that growers should take immediate actions in terms of how to and what antibiotic to use for controlling fire blight disease.
- Based on our findings, we recommended that growers should mix kasugamycin with oxytetracycline or be in rotation with streptomycin for fire blight control.

Significance to the industry and potential economic benefits. Since the identification of streptomycin-resistant strains of *E. amylovora* by Loper et al. in 1991, there has been limited data in evaluating the status of antibiotic resistance throughout the central Washington regions. The significance of this research to the industry lies in two aspects. First, this is the first report of kasugamycin resistant/tolerant *E. amylovora* isolates in Washington or elsewhere and the isolates were from orchards in 10 different locations, including Sunnyside, Mattawa, Prosser, Cashmere, Wenatchee, Quincy, Malaga, and Entiat. These results suggest that resistance may be more widespread than this study has indicated and resistance level has increased in 2024. Growers should take immediate actions in terms of how to and what antibiotic to use for controlling fire blight disease. Based on our findings, growers should take precautions in only applying kasugamycin to treat fire blight or should mix kasugamycin with oxytetracycline if still want to use kasugamycin for fire blight control or be in rotation with streptomycin. In summary, the findings of the current project directly benefit the growers of Washington state by providing instant feedback to growers in antibiotics resistance situation in orchards and growers should take immediate actions to avoid control failure.

Methods and Procedures:

In 2022 to 2024, we either collected symptomatic samples in central Washington by our own field trips to local area growers or samples were sent to us via mail by growers or consultants or extension specialists. We also collected asymptomatic blossom samples. Samples were placed in plastic bags and held on ice or in a refrigerator until they were processed. Samples were processed by cutting into small pieces with a sterile knife, washed briefly with sterile water, soaked in 900 µl 10 mM PBS, vortexed, and streaked for isolation onto five types of media: LB, CCT, LB + Sm 100 µg/mL, LB + Kg 100 µg/mL, LB + Tc 20 µg/mL and incubated at 82.5 Fº (28 °C) for 48 - 72 h. Colonies that appeared purple in color on CCT media, smooth, slightly raised and nonfluorescent were suspected to be *E. amylovora*. Screening for resistance was performed by observing the presence of individual colonies on antibiotic media. Isolates of known resistant *E. amylovora* strains were obtained from culture collections for use as positive controls. Isolates were then confirmed by PCR using *E. amylovora* specific primers G1-F and G2-R.

Spot dilution test was performed for selected resistant/tolerant strains (**Figure 1**). Bacteria were grown on LB plates and a single colony was inoculated in LB broth and grown for 24 hr with shaking at 250 rpm. Bacterial suspensions were adjusted to an absorbance of OD_{600} = 1 in PBS and 10-fold serial dilution was made in PBS. For each dilution, 5 µL was spotted onto plates: LB and LB + Kg 50, 75, 100 125, and 150 µg/mL and incubated at 82.5 Fº (28°C) for 48 - 72 h. Bacterial growth was visually observed on plates with or without antibiotics. Growth on plates without antibiotics was used as a control to compare to the plates with antibiotics.

In addition, the minimum inhibitory concentration (MIC) for a total of 327 isolates was determined (**Table 1**). Bacteria were grown on LB plates and a single colony was inoculated in LB broth with shaking at 250 rpm. Overnight bacterial suspensions were adjusted to an initial concentration of OD600 = 0.1 and 2-fold serial dilutions were performed, starting with LB + Kg1000 µg/mL and ending with LB + Kg 0.976 µg/mL. IC_{50} was defined as the concentration of antibiotics at which growth of the bacterium was 50 % less of that of the control without antibiotics. IC_{95} was defined as the concentration of antibiotics at which growth of the bacterium was 95 % less of that of the control without antibiotics.

Selected resistant/tolerant *E. amylovora* isolates were used to amplify the kasugamycin target *ksgA* gene by primers KsgA-F and KsgA-R. PCR products were then sequenced by Eton Biosciences Inc, San Diego, CA and compared to those of known sensitive strains.

Results and Discussion:

Samples were collected from more than 20 apple and pear varieties in central Washington, including 15 apple and 5 pear varieties, i.e. Gala, Jazz, Pink Lady, Fuji, Crips Pink, Granny Smith, Cosmic Crisp, Honey Crisp, Envy, Ambrosia, Golden Russet, Macintosh, and Sweet Tango; Bosc, Anjou, Bartlett and Star Krimson. A total of 186 *E. amylovora* isolates were obtained and confirmed by PCR. Among them, 38 and 7 isolates collected in 2023 and 2024 were shown to be resistant or tolerant to kasugamycin at 100/125 ppm, respectively, where 73% were isolated from pear samples and 27% from apple samples. Among the pear and apple samples, 58% were varieties Bosc or Bartlett and 58% were Pink Lady or Jazz, respectively. However, no isolates were found to be resistant to streptomycin or oxytetracycline.

Among the resistant/tolerant isolates, colony size was significantly smaller as compared to growth of the same isolate on LB medium and spot dilution assay showed similar growth for resistant/tolerant isolates at LB with antibiotics and without antibiotics (**Figure 1**). These

resistant/tolerant isolates were ablet to grow on plates with kasugamycin at 50, 75, 100, and 125 ppm. These findings indicated that these isolates from 2023 and 2024 were shown to be resistant/tolerant to kasugamycin.

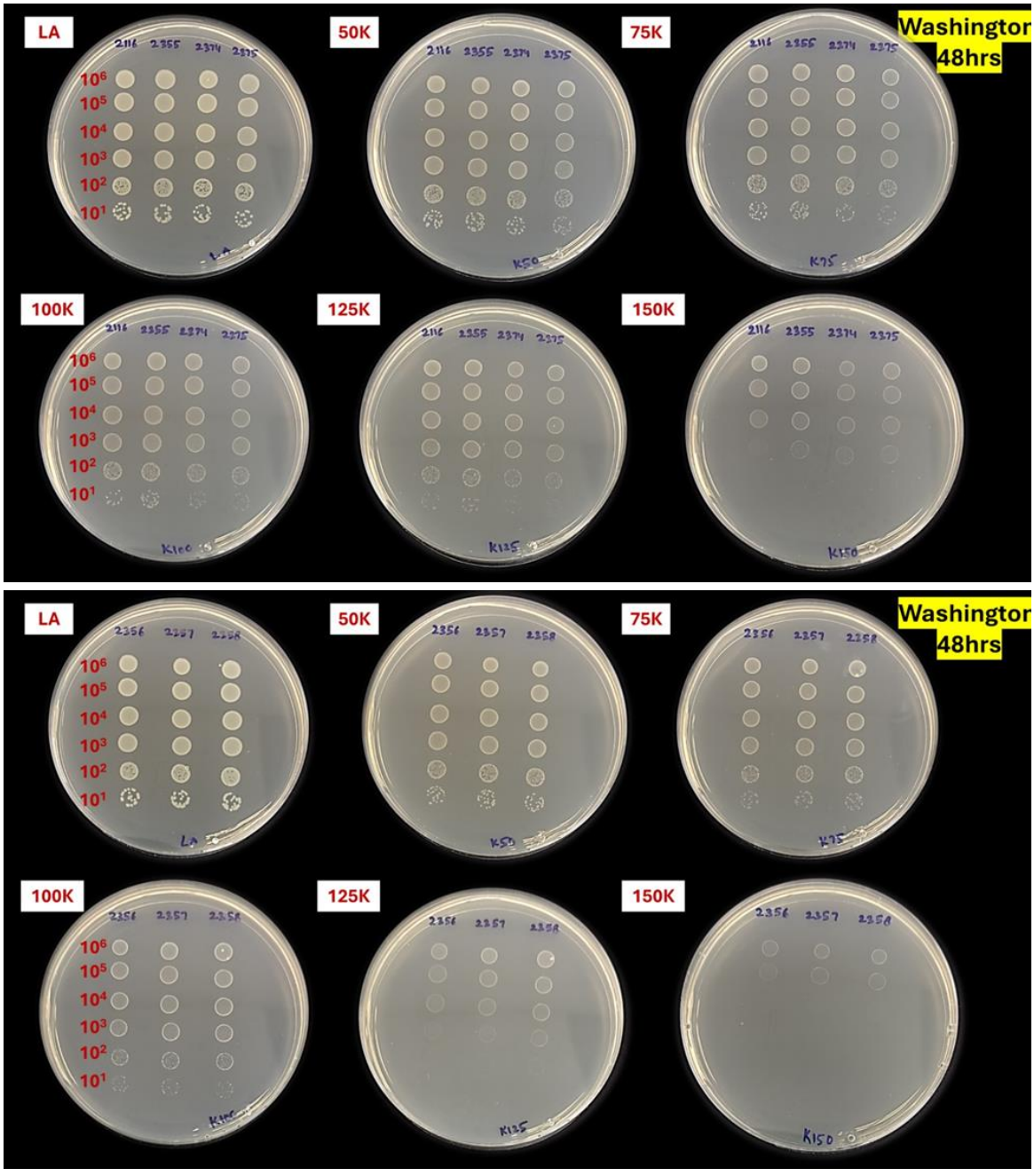

Figure 1. Spot dilution assay for representative resistant/tolerant isolates. Serial 10-fold dilutions were made in PBS. For each dilution, 5µL was spotted on LB plates containing no antibiotics or kasugamycin at 0, 50, 75, 100, 125, and 150 µg/ml. Pictures were taken 48 hours post inoculation.

Next, we determined MIC_{50} and MIC_{95} for 327 isolates from WA or other states. Among them, 186 isolates were collected in different years in WA and 141 isolates were isolated from other states or countries (**Table 1**). The MIC_{50} and MIC_{95} for selected isolates from WA isolated in recent years were shown in **Figure 2** and **Figure 3**, respectively. A few strains isolated from 2024 had the highest MIC_{50} of about 150 µg/ml and the MIC_{50} for most resistant/tolerant isolates was above 60 µg/ml (**Figure 2**). Similarly, a few strains isolated from 2024 had the highest MIC_{95} of about 400 µg/ml and the MIC_{95} for most resistant/tolerant isolates was above 200 µg/ml (**Figure 3**). For the rest of discussion, we only focused on MIC_{50}. By comparing to isolates from other regions or states, the average MIC_{50} for isolates from WA state was the highest as compared to other regions (**Figure 4**) and states/countries (**Figure 5**). In addition, the average MIC_{50} for isolates from WA state in 2024 was the highest as compared to other years (**Figure 6**). Surprisingly, a few strains collected in 2017 and 2018 also showed very high average MIC_{50} value (**Figure 6**) when fire blight epidemic occurred in those years, indicating that strains in WA may start to show resistance to kasugamycin earlier than this report has found.

Sequence comparison of the *ksgA* gene showed no difference for most resistant/tolerant isolates except for three, which had an IC_{50} value above 60 µg/mL, as compared to known type strains (data not shown). Based on previous studies, resistance to kasugamycin arises from mutations of its target gene *ksgA*, encoding an adenine demethylase, or present of a kasugamycin acetyltransferase gene aac(2′)-IIa, which acetylates kasugamycin. Our results indicate that resistance to kasugamycin of most *E. amylovora* isolates except three is not due to mutations in the *ksgA* gene.

Table 1 Numbers of *E. amylovora* isolates used to determine MIC_{50} to kasugamycin

Regions	State/Country	Number of isolates
Northwest	WA	186
West (Northwest)	CA, Utah, Idaho, Oregon, Montana	27
East	New York, Connecticut	88
Midwest	Michigan, Illinois	11
Southeast	Tennessee, Luisiana, Virginia	10
Others	New Zealand, Canada	5

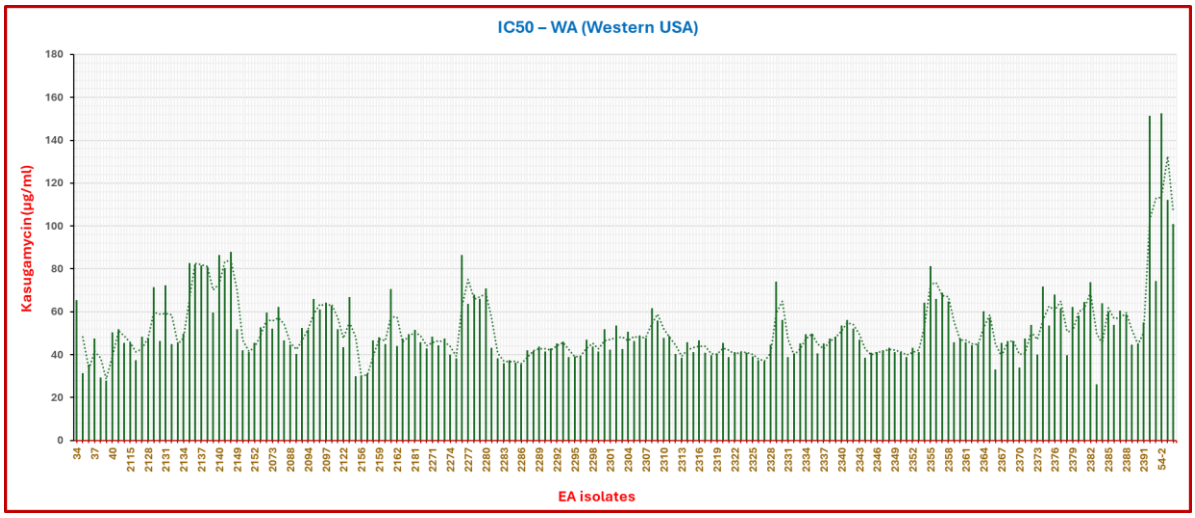

Figure 2 Minimum Inhibition Concentration (MIC_{50}) of selected *Erwinia amylovora* isolates from Washington state against kasugamycin. MIC_{50} was defined as the concentration of antibiotics at which growth of the bacterium was 50 % less of that of the control without antibiotics.

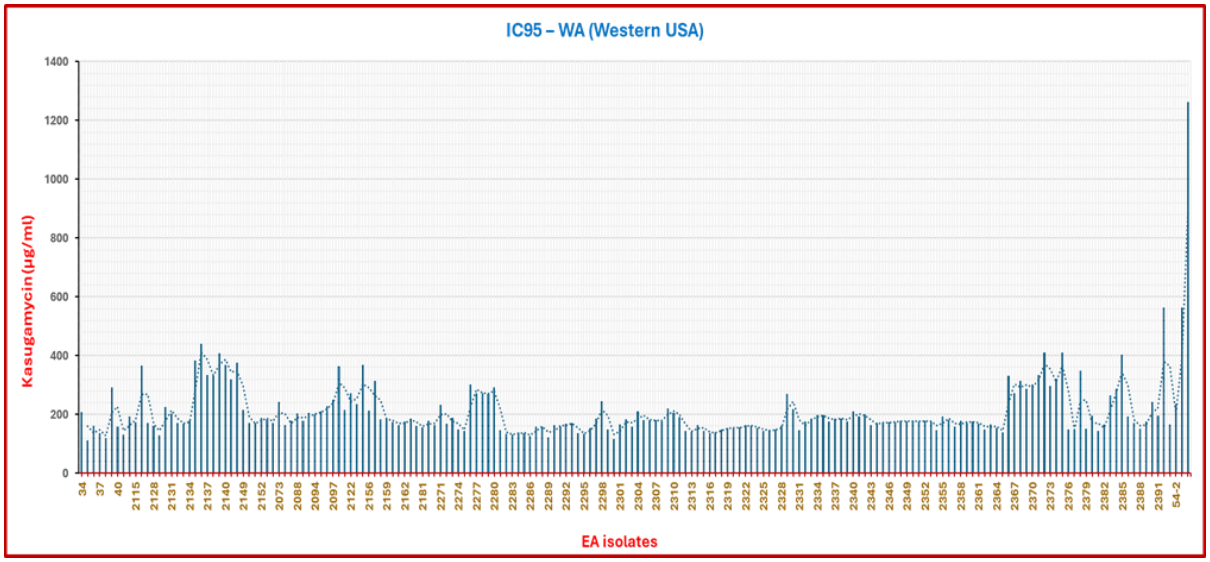

Figure 3 Minimum Inhibition Concentration (MIC_{95}) of selected *Erwinia amylovora* isolates from Washington state against kasugamycin. MIC_{95} was defined as the concentration of antibiotics at which growth of the bacterium was 95 % less of that of the control without antibiotics.

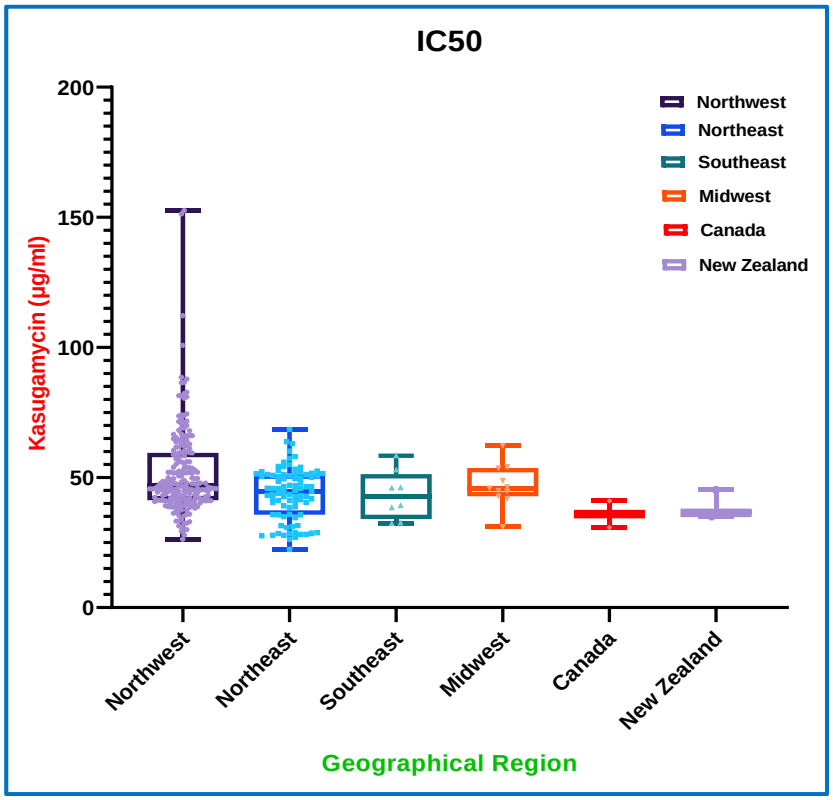

Figure 4 Distribution of minimum Inhibition Concentration (MIC_{50}) of *Erwinia amylovora* isolates from different regions against kasugamycin.

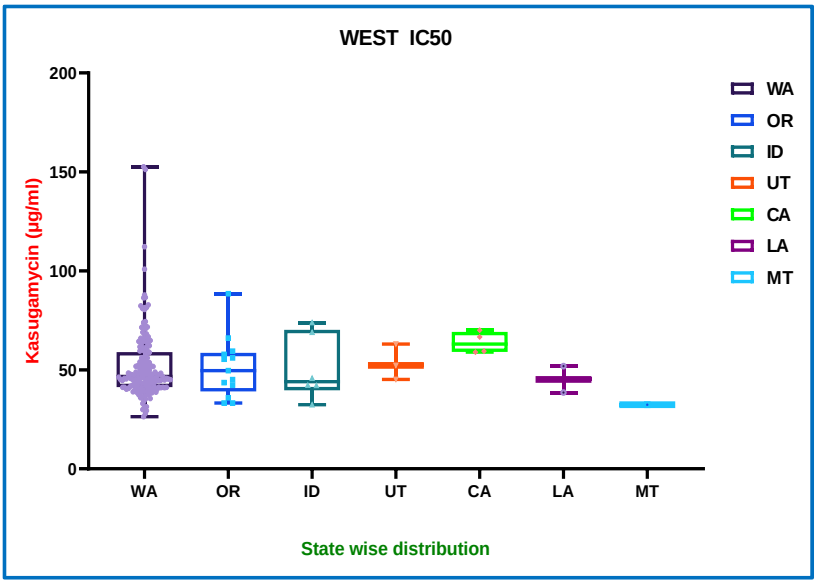

Figure 5 Distribution of minimum Inhibition Concentration (MIC_{50}) of *Erwinia amylovora* isolates from different states against kasugamycin.

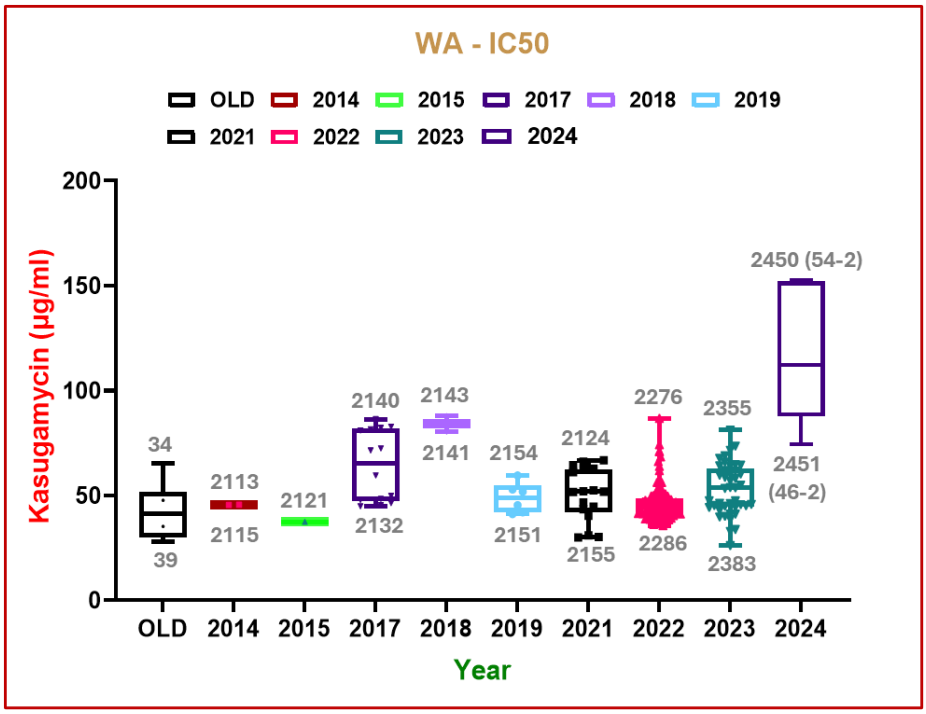

Figure 6 Distribution of minimum Inhibition Concentration (MIC_{50}) of *Erwinia amylovora* isolates from WA isolated in different years against kasugamycin.

Executive Summary

Project Title: Comprehensive monitoring and mapping antibiotics resistance in orchards

Key words: Fire blight, antibiotics resistance, streptomycin, tetracycline, kasugamycin

Abstract: Antibiotics remain one of the best tools for managing blossom blight of apple and streptomycin remains the better choice in terms of cost and efficacy in killing pathogens as compared to tetracycline and kasugamycin. The occurrence of streptomycin resistance of the fire blight pathogen in WA pear orchards in 1980s results in increased use of tetracycline and kasugamycin. However, there has been limited data evaluating the existence and extent of antibiotic resistance of *Erwinia amylovora* in central WA since then. The purpose of the current study was to comprehensively monitor and map antibiotics resistance in orchards in WA. During the 2022 to 2024 growing seasons, hundreds of diseased samples were collected from apple and pear orchards and 186 *E. amylovora* isolates were examined for their resistance to streptomycin, oxytetracycline and kasugamycin. Although no *E. amylovora* isolates exhibited resistance to streptomycin and oxytetracycline, 38 and 7 isolates exhibited resistance or tolerance to kasugamycin in 2023 and 2024, respectively. Among them, 73% and 27% were isolated from pear and apple samples, respectively. Minimum inhibition concentration (MIC) for these resistant/tolerant isolates was compared with 141 strains from other states and from different years. MIC_{50} was higher for WA isolates, and WA isolates from 2024 had the highest MIC_{50} as compared to previous years. No mutation was found in the kasugamycin target *ksgA* gene in most of the resistant/tolerant *E. amylovora* isolates except three. This is the first report of kasugamycin resistant/tolerant *E. amylovora* isolates in Washington or elsewhere and the isolates were from orchards in 10 distinct locations, including Sunnyside, Mattawa, Prosser, Cashmere, Wenatchee, Malaga, and Entiat. These results suggest that growers should take immediate actions in terms of how to and what antibiotic to use for controlling fire blight disease. Based on our findings, we recommended that growers should mix kasugamycin with oxytetracycline or be in rotation with streptomycin.

Project Title: Phase 3 New Biocontrol Strains Against Fire Blight

Report Type: Final Project Report

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Cooperators: None

Project Duration: 2-Year

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

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

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

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

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

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

Budget 2
Co PI 2: Tianna DuPont
Organization Name: Washington State University-Wenatchee
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Contract administrator email address: dewald@wsu.edu arcgrants@wsu.edu
Station Manager/Supervisor: Chad Kruger
Station manager/supervisor email address: cekruger@wsu.edu

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

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

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

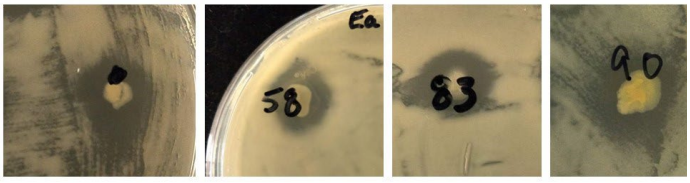
Significant Findings
UW- Dozens of bacterial strains with strong inhibition of *E. amylovora* growth *in vitro* were originally isolated in Phase 1 of this grant and confirmed in this Phase 3 grant.
Genomic analysis of the endophyte strains revealed a robust array of Biosynthetic Gene Clusters (BGCs). The presence of multiple antimicrobial compound BGCs suggests potential for the strains to be used in biological control of plant pathogens. PathogenFinder analysis predicted the selected isolates as not being human pathogens.

WSU 2023 field trial- A field trial was conducted in spring 2023 for the first three strains. The three UW treatments resulted in 39.5, 41.2 and 38 infections per 100 clusters, none of them significantly different than the water-treated control. The biological standard Blossom Protect + Buffer Protect, with 32 infections per 100 clusters, was also not significantly different than the water-treated control (19.2% relative control). The streptomycin standard provided significant control with 7.2 infections per 100 clusters (81.9% relative control). No treatments resulted in commercially important fruit skin marking of 3 or greater.

WSU 2024 field trial- The three UW treatments resulted in 11.7, 9.1 and 11.3 infections per 100 clusters, none of them significantly different than the water-treated control. The biological standard Blossom Protect + Buffer Protect, with 7.5 infections per 100 clusters, was significantly different than the water-treated control (41% relative control), as well as the streptomycin standard, which provided significant control with 1.1 infections per 100 clusters (91.5% relative control). No treatments resulted in commercially important fruit skin marking of 3 or greater. Fire blight pressure in 2024 was low due to cool temperatures during bloom.

Results and Discussion

Figure 1. Photos of in vitro screening results against *E. amylovora.* A lawn of the pathogen was spread onto agar plates, and candidate biocontrol strains (42, 58, 83, and 90) were spotted onto the lawn. Clearings around the candidate strains indicate inhibition of *E. amylovora* growth.



Strain Selection (UW): Four strains **(Figure 1)** were chosen for field trials. #UW 58 (4RDLA) had performed well in the Phase 2 trial in spring 2021 so it was tested again. #UW 42 (4RSC, a *Pseudomonas graminis* strain related to a Polish strain used for biocontrol of fireblight) and #UW 90 (3ThL1, *Pseudomonas fulva*) were also tested in Year 1 (spring 2023). Due to concern that the method used in the 2023 field trial included

surfactants (Regulaid) as though the treatments were chemicals, and this likely harmed the bacteria, we chose to repeat the trial in 2024 again with strains 58 and 90 and one new strain #UW 83 (3RF1, *Rouxiella aceris*) rather than select three new strains.

Genomic analysis (UW):
Three endophyte isolates (*Pseudomonas fulva* FB90-3ThL1, *Rouxiella aceris* FB83-3RF1, and *Erwinia* sp. FB58-4RDLA) were sequenced using either a NovaSeq or MiSeq System (Illumina). Raw sequence reads were uploaded to the Bacterial and Viral Bioinformatics Resource Center (BV-BRC), assembled using Unicycler v0.4.8, annotated with RASTtk v.1.073, and assessed for completeness using CheckM v1 (Table S1). Taxonomic classification employed the Type (Strain) Genome Server (TYGS), with species-level assignments made for strains showing genome distance scores (d4) ≥ 70%. PathogenFinder v1.1 analyzed genome assemblies (.fna) to predict human pathogenic potential, while antiSMASH v7 screened protein sequences (.faa) to identify biosynthetic gene clusters (BGCs) associated with plant growth promotion.

Power Analyses (UW):
We analyzed infections per 100 clusters across treatments, including an untreated control and experimental groups (e.g., UW58, UW83, UW90). Data were cleaned and grouped by treatment using the **dplyr** package in R (v4.4.1), and ranges were calculated to assess variability.

We conducted power analyses using the **pwr** package to evaluate the study's ability to detect treatment effects. First, we calculated the overall power of the study design to detect medium effects (f=0.25f = 0.25f=0.25) using Cohen's fff, which quantifies the proportion of variance explained by group differences in ANOVA. This analysis incorporated the harmonic mean sample size of 5.4 across groups. Next, we assessed pairwise power using Cohen's hhh, an effect size metric for differences in proportions, to evaluate the sensitivity of pairwise comparisons between treatments. Effect sizes were calculated using Cohen's fff for ANOVA and Cohen's hhh for pairwise comparisons of infection rates between groups. All analyses were conducted using R (version X.X.X), with the **dplyr**, **pwr**, and **effectsize** packages.

Results
Taxonomic analysis assigned two isolates to the species level: *Pseudomonas fulva* FB90-3ThL1 matched with *P. fulva* DSM 17717 (d_4 = 92.3) and *Rouxiella aceris* FB83-3RF1 matched with *R. aceris* SAP-1 (d_4 = 88.7). The third isolate, *Erwinia* sp. FB58-4RDLA, showed low sequence homology to its nearest type-strain, *Erwinia pyri* DE2 (d_4 = 25.8), suggesting it represents a novel species. *Erwinia* FB58-4RDLA exhibited low genomic similarity to any of the known plant pathogenic species within the genus, including *E. amylovora* CFBP 1232, *E. aphidicola* JCM 21238, and *E. pyrifoliae* DSM 12163. A search of LPSN and BacDive databases revealed no pathogenicity classification for *R. aceris*, whereas *P. fulva* was classified as Biosafety Level 1 and not listed as a plant pathogen. PathogenFinder analysis predicted all three isolates as non-human pathogens.

AntiSMASH analysis revealed that *P. fulva* FB90-3ThL1 contains five distinct biosynthetic gene clusters (BGCs): bicornutin A1 and A2, ririwpeptides A-C, techlisin, hydrogen cyanide, and carotenoids. *R. aceris* FB83-3RF1 and *Erwinia* FB58-4RDLA each contain BGCs for siderophores and aryl polyenes. *R. aceris* FB83-3RF1 contains genes for the siderophore frederiksenibactin and an aryl polyene cluster related to rhabdochromin from *Xenorhabdus doucetiae*, whereas *Erwinia* FB58-4RDLA contains genes for the aryl polyene APE Ec and the siderophore desferrioxamine. These BGCs correspond to compounds with potential plant growth promoting properties. The siderophore genes are associated with iron acquisition systems. The aryl polyene, bicornutin, ririwpeptide, and techlisin genes are associated with cyclic lipopeptide production with predicted antimicrobial and surfactant properties. The hydrogen cyanide BGC is associated with antimicrobial compound

production active in the rhizosphere, and the carotenoid genes are associated with pigment production linked to stress resistance and photoprotection.

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

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

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

[z] Inoculation was conducted on the evening of 3 May 2023 at full bloom (of king blooms) using a suspension of freeze-dried cells of *Erwinia amylovora* strain Ea153 (streptomycin and oxytetracycline sensitive strain) prepared at $5x10^6$ CFU ml^{-1} (verified at $4.2x10^6$ CFU ml^{-1}).

[y] Timings, 1: first bloom, 2: 70-90% bloom, 3: morning before evening inoculation (full bloom), 4: morning after inoculation, 5: 2 days after inoculation, 6: 4 days after inoculation (petal fall), 7: 7 days after inoculation

[x] Transformed sqrt(x + 1) prior to analysis of variance; non-transformed means are shown.

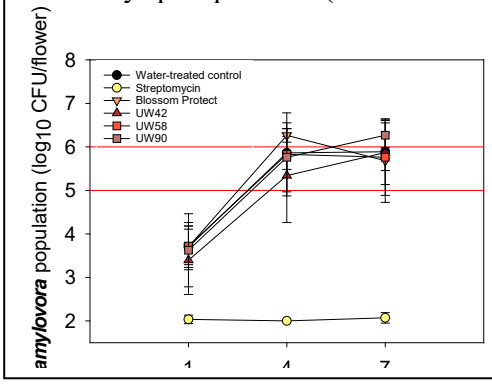
[w] Fruit skin marking is rated from an average of 25 fruit per tree. Rated on a 0 to 15 scale where ratings below 3 indicate no commercial downgrades.

[v] Amended with Regulaid: 16 fl oz per 100 gallons. Buffered to 5.6 pH.

[u] Treatments followed by the same letter are not significantly different at P=0.05 Fisher's T test (LSD).

[t] Amended with Regulaid: 16 fl oz per 100 gallons.

Figure 2. Effect of the biological products from the UW applied to Gala apple trees on the population size of *E. amylovora* strain 153N on flowers 1 day after inoculation (full bloom + 1 day), at petal fall (full bloom + 4 days) and 3 days post petal fall (full bloom + 7 days)



The UW42 treatment reduced *Erwinia amylovora* populations at 1 and 4 days after inoculation, although not significantly different than the water-treated control **(Figure 2)**. The other two UW treatments, as well as the biological standard Blossom Protect + Buffer Protect did not show a reduction of the population size of the pathogen compared to the water-treated control. The streptomycin standard significantly reduced *Erwinia amylovora* populations at all time points analyzed (1, 4 and 7 days after inoculation).

2024 field trial methods and results: A 0.25 ha research block of 6-yr-old WA 38 apples at Washington State University Columbia View Research Orchard East Wenatchee, WA, was used for this trial. The experiment was arranged in a randomized complete block with five single tree replicates. Products were applied to the whole tree according to manufacturer recommendations using a Stihl SR420 mist blower backpack sprayer. Products were applied to wet, near dripping at 0.1 to 0.2 gal/tree (100 gal/A). Application dates were: 10 Apr (1), 15 Apr (2), 17 Apr (3), 18 Apr (4, full bloom), 19 Apr (5), 20 Apr (6), 21 Apr (7), 22 Apr (8, petal fall), 30 Apr (9), 6 May (10). At 90-100% bloom (of the king blooms), on 18 Apr 2024, *Erwinia amylovora* was applied at $5x10^6$ CFU ml^{-1} (verified at $6.4x10^6$ CFU ml^{-1}) to lightly wet each cluster. Trees were visually evaluated for flower cluster infection weekly from when symptoms became visible, 22 days after inoculation, for 3 weeks and infection counts summed across all dates. Fruit were evaluated for fruit skin marking from an average of 25 fruit per tree on a 0 to 15 scale, where ratings below 3 indicate no commercial downgrades. Statistical analysis was performed with SAS v 9.4 using general linear mixed models (GLIMMIX) analysis of variance ANOVA and multiple means comparison (LSD) for infections (normal distribution of sqrt(x + 1) transformed).

Environmental conditions during bloom (10 Apr – 22 Apr 2024) were cool and ranged from a maximum average temperature of 66.4 °F to minimum average temperature of 39.9 °F with 38.6% average humidity. During petal fall (23 Apr – 6 May 2023) temperature ranged from an average maximum of 65.5 °F to a minimum of 41.7 °F with 49.5% average humidity. Two precipitation events occurred after the inoculation of *Erwinia amylovora*, one on 25 Apr (0.45 in), approximately 3 days after petal fall sprays, and on 4 May, 4 days after the petal fall + 7 day sprays (1.8 in). All applications were made under fast drying conditions.

The three UW treatments applied alone at tight cluster, the day before inoculation, the morning after inoculation, and 4 days after inoculation (petal fall) resulted in 11.7, 9.1 and 11.3 infections per 100 clusters, none of them significantly different than the water-treated control **(Table 2, Figure 3)**. The biological standard Blossom Protect + Buffer Protect, with 7.5 infections per 100 clusters, was significantly different than the water-treated control (41% relative control[1]), as well as the streptomycin standard, which provided significant control with 1.1 infections per 100 clusters (91.5% relative control). No treatments resulted in commercially important fruit skin marking of 3 or greater (Table 2). Fire blight pressure in 2024 was low due to cool temperatures during bloom.

Table 2. 2024 Field Trial Results. Effect of University of Washington and control treatments applied to apple, cv. WA 38, on the infection of *Erwinia amylovora* in apple blossoms in Wenatchee, WA, in 2024[*]

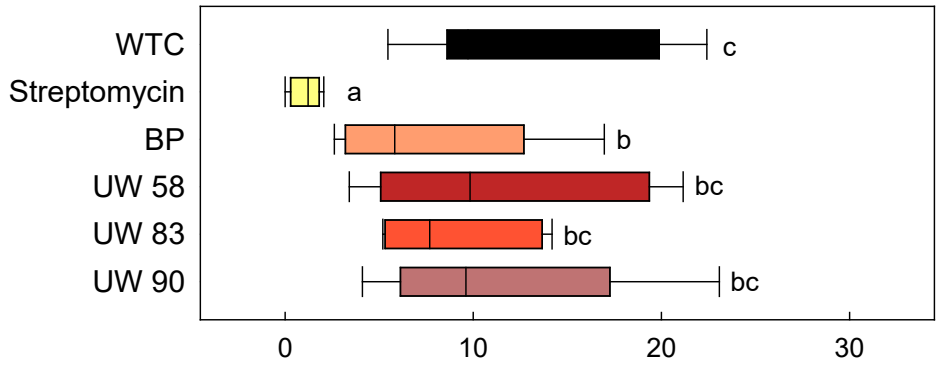
Treatment	Amount per 100 gal	Timing[z]	Infections per 100 clusters[y]				Fruit skin marking
Streptomycin standard (Firewall 50WP)[w]	8 oz	4,7	1.1	±	0.4	a[v]	0.05 ± 0.03
Blossom Protect + Buffer Protect	1.25 lb + 5 lb	2,3,6	7.5	±	2.6	b	0.06 ± 0.03
UW58	378.5 ml	1,3,5,8	11.7	±	3.3	bc	0.06 ± 0.02
UW83	378.5 ml	1,3,5,8	9.1	±	1.9	bc	0.08 ± 0.04
UW90	378.5 ml	1,3,5,8	11.3	±	3.2	bc	0.05 ± 0.04
Water-treated control	NA	4,5,8	12.8	±	2.1	c	0 ± 0

[*] Inoculation was conducted on the evening of 18 Apr 2024 at full bloom (of king blooms) using a suspension of freeze-dried cells of *Erwinia amylovora* strain Ea153 (streptomycin and oxytetracycline sensitive strain) prepared at $5x10^6$ CFU ml^{-1} (verified at $6.4x10^6$ CFU ml^{-1}).

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

[z] Timings, 1: 10 Apr (tight cluster), 2: 15 Apr (70-90% bloom), 3: 17 Apr, 4: 18 Apr (full bloom), 5: 19 Apr, 6: 20 Apr, 7: 21 Apr, 8: 22 Apr (petal fall), 9: 30 Apr, 10: 6 May. All applications were conducted in the morning, and inoculation was conducted on the evening of 18 Apr.
[y] Transformed sqrt(x + 1) prior to analysis of variance; non-transformed means are shown.
[x] Fruit skin marking is rated from an average of 25 fruit per tree. Rated on a 0 to 15 scale where ratings below 3 indicate no commercial downgrades.
[w] Amended with Regulaid: 16 fl oz per 100 gallons. Buffered to 5.6 pH.
[v] Treatments followed by the same letter are not significantly different at P=0.05 Fisher's T test (LSD).

Figure 3. Flower infections per 100 clusters in 2024 Field trial.



None of the University of Washington treatments significantly reduced *Erwinia amylovora* populations after inoculation compared to the water-treated control (**Figure 4**). The UW83 showed the highest reduction 4 days after inoculation, and this corresponds to the highest control observed with the

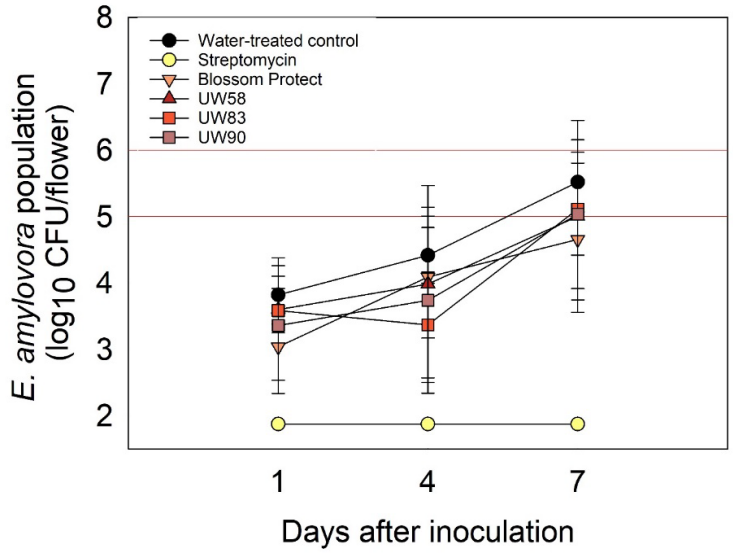


Figure 4. Effect of University of Washington treatments applied to WA 38 apple trees on the population size of *Erwinia amylovora* strain 153N on flowers 1 day after inoculation (full bloom + 1 day), at petal fall (full bloom + 4 days) and 3 days post petal fall (full bloom + 7 days).

University of Washington treatments. While the biological standard Blossom Protect + Buffer Protect showed a significant reduction of the population size of the pathogen compared to the water-treated control only 1 day after inoculation, the streptomycin standard significantly reduced *Erwinia amylovora* populations at all time points analyzed (1, 4 and 7 days after inoculation).

Additional Statistical Analysis (UW): The data showed considerable variability in infections per 100 clusters across treatments. The untreated, pathogen-inoculated control group exhibited a wide range of total infections (10–43), while experimental treatments showed narrower ranges (e.g., UW58: 6–26, UW83: 8–23, UW90: 6–33). To test the ability of the study to detect treatment effects, we conducted a power analysis, revealing that the study design provided only 13.6% power to detect medium effects (effect size for ANOVA, Cohen's $f = 0.25$). This greatly limited our ability to detect treatment effects, should they exist.

Next, we assessed pairwise comparisons of the effect size on infection rates (Cohen's h, effect size for proportions). For example, while the largest observed effect size, between Blossom Protect and the untreated control (Cohen's $h = 0.1733$), represented a 41% relative reduction in infection rate (12.75% to 7.52%), the low power of the study limits confidence in these findings and underscores the need for cautious interpretation.

Given these limitations, the findings should not be interpreted as evidence of either success or failure of the UW strains to suppress the pathogen. Future research with larger sample sizes and more robust experimental designs is needed to evaluate these treatments with greater confidence.

Further directions. With the large variation in the data, the study size should be substantially increased. It also may be necessary to apply the biocontrol strains sooner so they have more time to colonize plant tissue and express the antimicrobial compounds. Our new proposal that would have explored the hypothesis that endophyte strains will perform better if allowed to pre-colonize the plant was not funded. Since these strains are part of the natural plant microbiome, their advantage may be more from within than as a spray on flowers just before pathogen is applied. The strains were licensed to the endophyte company, IntrinsyxBio, that has global partnerships interested in further testing and ultimately commercializing the strains as biocontrol products.

Executive Summary

Project Title: Phase 3 New Biocontrol Strains Against Fire Blight

Key Words (3-5): fire blight, biocontrol, endophytes, Erwinia amylovora

Abstract

By tapping into the natural bacterial interactions of the plant microbiome, this project sought to develop biocontrol strains from within plants near apple-growing areas in Washington State. Earlier phases of this project yielded dozens of endophytic bacterial strains with strong inhibitory activity against *Erwinia amylovora*, the causal agent of fire blight. Phases 2 and 3 of the project included genomic analysis of the strains to eliminate those with potential harmful effects and field trials to test a select few strains for biocontrol of fire blight. The small size of the field trials, variable weather conditions between the years of the trials, and large variations in the infection numbers made it difficult to achieve statistical significance in biocontrol. However, some of the strains did result in infection ranges lower than the untreated controls. With larger trials and earlier inoculation of the trees with the biocontrol strains, a clearer indication of the impact of these Washington State-sourced endophyte strains against fire blight will be achieved.

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

Report Type: Final Project Report

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Cooperators: University of Girona (Girona, Spain)

Project Duration: 2-Year

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

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

WTFRC Collaborative Costs: None

Budget 1
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Contract administrator email address: dewald@wsu.edu | arcgrants@wsu.edu

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

Footnotes:

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

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

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

OBJECTIVES

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

SIGNIFICANT FINDINGS

Objective 1

- Peptides applied 4 times during bloom provided moderate disease control and may be best incorporated as rotational products in an integrated program during low-risk periods.
- In both years, peptides reduced *E. amylovora* populations. While in 2023 (high-risk scenario) the reduction was only significant 1 day after inoculation, in 2024 (moderate-risk scenario) *E. amylovora* populations were significantly lower than the water-treated control at 1, 4 and 7 days after inoculation.
- No interaction with surfactants allowed for use to improve efficacy.

Objective 2

- Peptides did not show fruit skin marking in any of the 25 fruit evaluated each year. None of the other treatments resulted in commercially important fruit skin marking of 3 or greater.

Objective 3

- Streptomycin (Firewall 50WP) and Actigard applied 3 times during the bloom period significantly reduced the number of infections compared to the peptides. Blossom Protect + Buffer Protect applied twice was not significantly different than the peptides in 2023, which was of high-risk for fire blight, but it significantly reduced infections in 2024.
- The reduction of *E. amylovora* population obtained with the peptides was comparable to the one obtained with streptomycin standard (antimicrobial control) at 1 day after inoculation in 2023 and at 1 and 4 days after inoculation in 2024.

RESULTS AND DISCUSSION

In vitro trials (2024)

Population levels of *E. amylovora* strain Ea153N were significantly reduced by both peptides, BP178 and BP100, showing logarithm reductions from 2 to 6 logarithms, depending on peptide type and concentration (Fig. 1). The peptide BP100 at the lower concentration (4.5 ppm) reduced *E. amylovora* populations by 2 logarithms, while applied at the highest concentration (18 ppm), the reduction was of 6 logarithms (reaching the detection limit of the plate counting technic). The peptide BP178 showed a strong reduction in *E. amylovora* population levels already at its lowest concentration (5.5 logarithm reduction after 3 h of contact test), and a 6-logarithm reduction (reaching the detection limit of the plate counting technic) when applied at the medium and high concentrations. BP100 at the highest concentration and BP178 at the medium and high concentrations were not significantly different than the streptomycin control (Firewall 50WP at 50 ppm equivalent to streptomycin at 25 ppm). Almost all reductions were observed 30 min after the contact test started, indicating the fast activity of these peptides.

The activity of the peptides was not affected by the addition of surfactants, neither Regulaid nor NuFilm P (Fig. 2). Both peptides tested at two of the three concentrations studied in the first experiment showed a reduction of 6 logarithms after a 3 h contact test, without differences between the presence or not of surfactant. For the water-treated control, the addition of surfactants significantly impacted (although slightly) *E. amylovora*, showing a small reduction in its population levels.

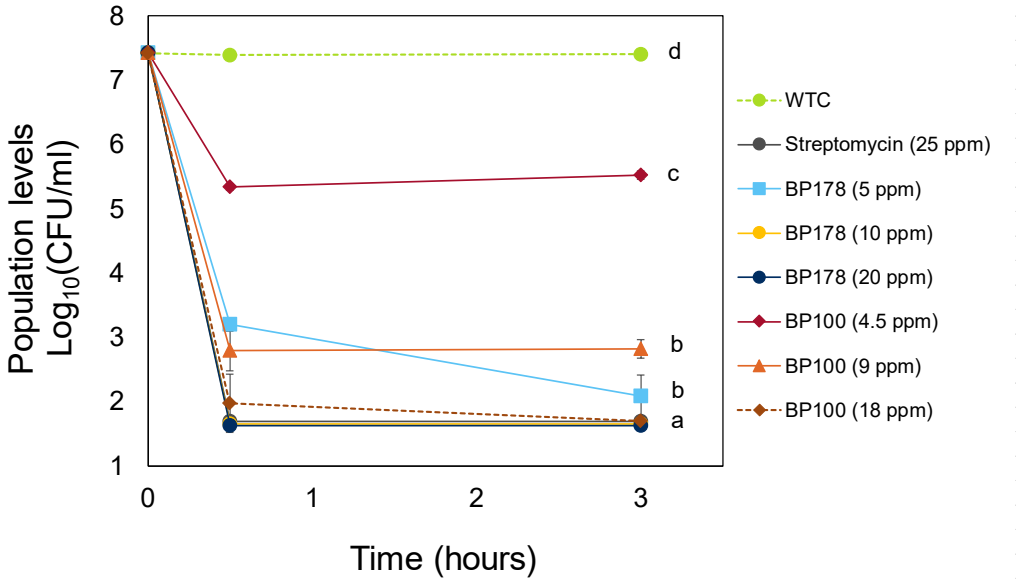

Figure 1. Effect of peptides at three different concentrations on the survival of *E. amylovora* strain Ea153N. A contact test was performed, and samples were taken after 30 min and 3 hours of incubation. Values are the means of three replicates and error bars represent the standard deviation of the mean. Different letters indicate significant differences between treatments and concentrations according to Tukey's test ($P < 0.05$).

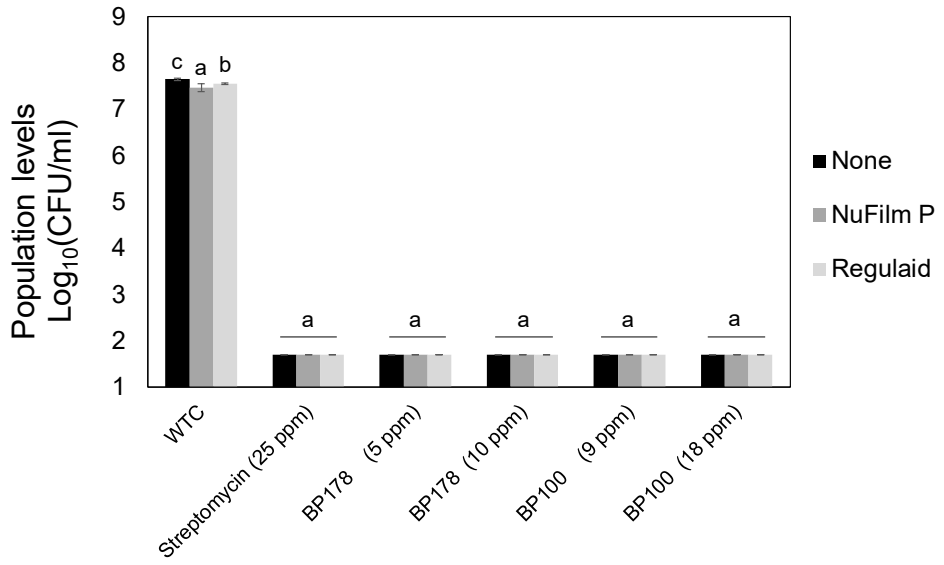

Figure 2. Effect of the surfactants on the activity of the peptides against *E. amylovora* strain Ea153N. Two different concentrations per peptide and two commonly used surfactants, Regulaid and Nufilm P, were tested. Values are the means of three replicates, and error bars represent the standard deviation of the mean. Different letters within a treatment indicate significant differences between the surfactants according to Tukey's test ($P < 0.05$).

Field trials (2023-2024)

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

The peptide BP178 applied alone on the flower clusters the day before inoculation, the morning after inoculation and 4 days after inoculation (petal fall) resulted in 40.4 infections per 100 clusters, not significantly different than the water-treated control in 2023 (Table 1). The biological standard Blossom Protect + Buffer Protect, with 32 infections per 100 clusters, was also not significantly different than the water-treated control (19.2% relative control[1]). The streptomycin standard applied the morning before evening inoculation (full bloom), the morning after inoculation and 4 days after inoculation (petal fall) provided significant control with 7.2 infections per 100 clusters (81.9% relative control). The systemic acquired resistance inducer Actigard applied at first bloom (3 days before inoculation), the morning before evening inoculation (full bloom) and 4 days after inoculation (petal fall) resulted in 16.4 infections per 100 clusters (58.7% relative control), significantly different than the water-treated control and not significantly different than the streptomycin standard. No treatments resulted in commercially important fruit skin marking of 3 or greater (Table 1).

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

Treatment	Amount per 100 gal	Timing [y]	Infections per 100 clusters [x]				Fruit skin marking [w]	
Streptomycin standard (Firewall 50WP) [v]	8 oz	3,4,6	7.2	±	1.6	a [u]	0.02	a
Blossom Protect + Buffer Protect	1.25 lb + 5 lb	2,3	32.0	±	2.0	b	0	a
Actigard [t]	2 oz	1,3,6	16.4	±	5.2	a	0	a
Bifunctional peptide BP178	4.3 oz (122 g)	2,4,6	40.4	±	3.8	b	0	a
Water-treated control	NA	3,4,6	39.6	±	4.9	b	0	a

[z] Inoculation was conducted on the evening of 3 May 2023 at full bloom (of king blooms) using a suspension of freeze-dried cells of *E. amylovora* strain Ea153N (streptomycin and oxytetracycline sensitive and nalidixic acid resistant strain) prepared at $5x10^6$ CFU ml^{-1} (verified at $4.2x10^6$ CFU ml^{-1}).

[y] Timings, 1: first bloom, 2: 70-90% bloom, 3: morning before evening inoculation (full bloom), 4: morning after inoculation, 5: 2 days after inoculation, 6: 4 days after inoculation (petal fall), 7: 7 days after inoculation

[x] Transformed sqrt(x + 1) prior to analysis of variance; non-transformed means are shown.

[w] Fruit skin marking is rated from an average of 25 fruit per tree. Rated on a 0 to 15 scale where ratings below 3 indicate no commercial downgrades.

[v] Amended with Regulaid: 16 fl oz per 100 gallons. Buffered to 5.6 pH.

[u] Treatments followed by the same letter are not significantly different at P=0.05 Fisher's T test (LSD).

[t] Amended with Regulaid: 16 fl oz per 100 gallons.

The peptide BP178 reduced *E. amylovora* populations at 1 and 4 days after inoculation, showing significant differences compared to the water-treated control 1 day after inoculation. The streptomycin

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

standard significantly reduced *E. amylovora* populations at all time points analyzed (1, 4 and 7 days after inoculation), while none of the other standards/treatments showed a reduction of the population size of the pathogen compared to the water-treated control (Fig. 3).

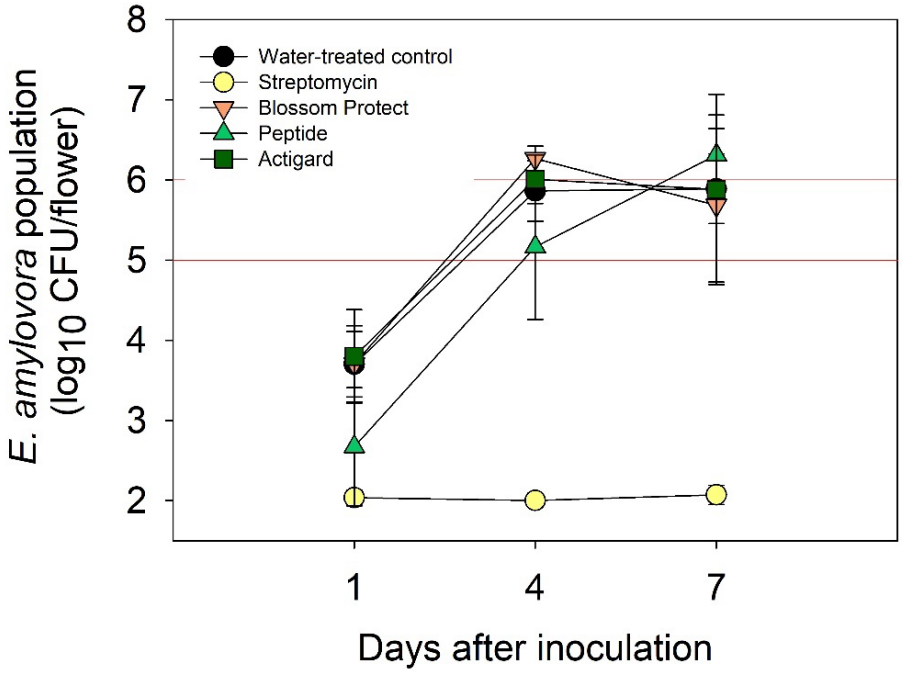

Figure 3. Effect of the peptide BP178 and controls applied to Gala apple trees on the population size of *E. amylovora* strain Ea153N on flowers 1 day after inoculation (full bloom + 1 day), at petal fall (full bloom + 4 days) and 3 days post petal fall (full bloom + 7 days).

In 2024, environmental conditions during bloom (15 Apr – 24 Apr, 2024) were cool and ranged from an average maximum temperature of 64.3 °F to minimum of 39.5 °F with 31.1% average humidity. During petal fall (April 25 – 1 May, 2024), temperature ranged from an average maximum of 63.0 °F to a minimum of 41.6 °F with 52.7% average humidity. One precipitation event occurred after the inoculation of *E. amylovora*, on 25 April (0.45 in), approximately 5 days after inoculation (26 to 30 h after the petal fall sprays) and another on 4 May (1.8 in) (Appendix 1 and 2). All applications were made under fast drying conditions.

The combination of both peptides, BP178 and BP100, resulted in 32.8 infections per 100 clusters, not significantly different than the water-treated control (38 infections per 100 clusters). The systemic acquired resistance inducer Actigard showed a significant reduction compared to the water-treated control, with 17.7 infections per 100 clusters (54% relative control). The antibiotic streptomycin significantly reduced infections (5.7 infections per 100 clusters, 81.5% relative control) compared to the water-treated control and both treatments tested. No treatments resulted in commercially important fruit marking of 3 or greater (Table 2).

The combination of both peptides significantly reduced *E. amylovora* populations at 1, 4 and 7 days after inoculation. The streptomycin standard significantly reduced *E. amylovora* populations at all time points analyzed (1, 4 and 7 days after inoculation), while none of the other standards/treatments, except Actigard at 4 days after inoculation, showed a significant reduction of the population size of the pathogen compared to the water-treated control (Fig. 4).

Table 2. Effect of the peptides and control treatments applied to apple, cv. Gala, on the infection of *E. amylovora* in apple blossoms in Wenatchee, WA, in 2024[*]

Treatment	Amount per 100 gal	Timing [z]	Infections per 100 clusters	Fruit skin marking [y]
Streptomycin standard (Firewall 50WP) [x]	8 oz	3,4,5	5.7 ± 1.3 a [w]	0.18 ± 0.05 a [v]
Blossom Protect + Buffer Protect	1.25 lb + 5 lb	1,3	21.9 ± 2.5 b	0.17 ± 0.04 a
Actigard [u]	2 oz	1,3,5	17.7 ± 2.8 b	0.27 ± 0.11 a
Peptide BP178 [t]	6 oz (170 g)	2,4		
Peptide BP100 [t]	2.8 oz (80 g)	5,6	32.8 ± 3.7 c	0.13 ± 0.03 a
Water-treated control	NA	3,4,5	38.0 ± 2.9 c	0.03 ± 0.01 a

[*] Inoculation was conducted on the evening of 20 Apr 2024 at full bloom (of king blooms) using a suspension of freeze-dried cells of *E. amylovora* strain Ea153N (streptomycin and oxytetracycline sensitive strain) prepared at $5x10^6$ CFU ml^{-1} (verified at $6.1x10^6$ CFU ml^{-1}).

[z] Timings 1: 15 Apr, 2: 17 Apr, 3: 20 Apr (full bloom), 4: 21 Apr, 5: 24 Apr (petal fall), 6: 27 Apr. All applications were conducted in the morning, and inoculation was conducted on the evening of 20 Apr.

[y] Fruit skin marking is rated from an average of 25 fruit per tree. Rated on a 0 to 15 scale where ratings below 3 indicate no commercial downgrades.

[x] Amended with Regulaid: 16 fl oz per 100 gallons. Buffered to 5.6 pH.

[w] Treatments followed by the same letter are not significantly different at P=0.05 Fisher's T test (LSD).

[v] Treatments followed by the same letter are not significantly different at P=0.05 Fisher's T test (LSD).

[u] Amended with Regulaid: 16 fl oz per 100 gallons.

[t] Amended with NuFilm P: 8 fl oz per 100 gallons. Buffered to 6.5 pH.

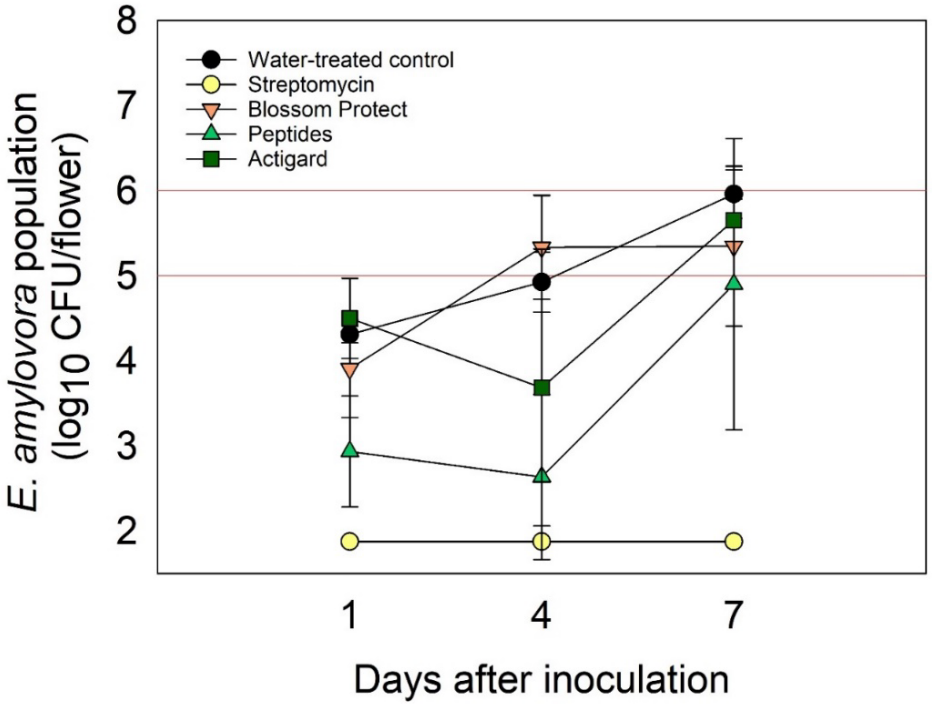

Figure 4. Effect of the peptides and control treatments applied to Gala apple trees on the population size of *E. amylovora* strain Ea153N on flowers 1 day after inoculation (full bloom + 1 day), at petal fall (full bloom + 4 days) and 3 days post petal fall (full bloom + 7 days).

Over the past decades, functional peptides have continuously been proposed as a potential tool to create both, more sustainable pesticides and disease resistant crops (Montesinos E. et al., 2012; Van Esse et al., 2020; Montesinos E., 2023), but more work is needed to study their efficacy in controlling diseases in the field. In the present grant, we studied the application of functional peptides for the control of fire blight in apple trees in Wenatchee, WA. Results from 2023, which was of exceptionally high-risk regarding fire blight conducive weather conditions, showed no control of fire blight with one of the experimental peptides applied on the flower clusters of young susceptible Gala apple trees the day before inoculation, the morning after inoculation, and 4 days after inoculation (petal fall) at a concentration of 4.3 oz/100 gal (1.22 g/3.785 L). The application of the peptide only to the area of the tree to be inoculated (flower clusters) using a manual pump 4-gallon backpack sprayer, as well as its application at a lower concentration than the one initially proposed due to reduced peptide availability for 2023 season could have been some of the reasons why we did not see control. Also, since peptides are experimental products, in 2023 the peptide was applied in the field by itself since no combinations with acidifiers or surfactants were tested.

In 2024, a couple of *in vitro* experiments were conducted prior to start the fire blight season in order to (i) confirm the bactericidal activity of the peptides against the *E. amylovora* strain Ea153N and determine their minimal bactericidal concentration, and (ii) test the effect of two commonly used surfactants on the bactericidal activity of the peptides to understand if we can combine them to obtain better results in the field. Results showed that both peptides tested have a high bactericidal activity against *E. amylovora* strain Ea153N and that their activity is mainly observed within the first 30 min of contact. Additionally, no negative effects were observed when peptides were mixed with Regulaid or NuFilm P at 8 fl oz/100 gal, indicating the possibility to include any of these surfactants in the treatment.

Results from the field trial in 2024 showed an improvement of the peptide's performance, probably due to an increase in the concentrations used, the addition of a fourth application, the application of the peptides to the whole tree using a Stihl SR420 mist blower backpack sprayer, and the combination of the peptides with the surfactant NuFilm P. Even though fire blight risk was moderate during 2024 (Appendix 2), a very similar percentage of infection was observed in the water-treated controls for both years, indicating that environmental conditions did not play a significant role in the increased performance of the peptides.

Reduction of *E. amylovora* populations was observed both years using the peptides; in 2024 the reduction was significant at all time points analyzed compared to the water-treated control, but only significant at 1 day after pathogen inoculation in 2023. This correlates with the increased control of the peptides observed in 2024, indicating that the bactericidal activity of the peptides is their main mechanism of action. The fact that in 2024 we applied the peptides to the whole tree, and not only to the flower clusters, could also have increased the activation of the defense system of apple trees by these peptides, especially the BP178 (Moll et al., 2022). However, a more in-depth study should be carried out in order to understand this mechanism, especially in the field.

Recent research suggests that the delivery of peptides using other application methods such as endotherapy are more effective in activating the plant defense system (Moll et al., 2024), and this could be an interesting idea to explore in order to achieve higher control. However, further advancement and assessment of endotherapy, considering technological aspects and social acceptance, are necessary. Another viable approach that has been proved more reliable than topical treatment could be the heterologous expression of peptides in plant crops, but its use is more restricted (genetic modified organisms are vaned in several countries), and it requires additional research in terms of food safety and environmental impact (Montesinos E., 2023).

Even though we obtained better results in 2024, the reduction in infections obtained with the peptides was not statistically significant compared to the water-treated control. Seeing that peptides are very active *in vitro*, and that the concentration used in the field is 15 to 50 times higher than their minimal bactericidal concentration, we hypothesize that clear and sunny spraying days could have impacted the activity of these peptides since proteins and peptides are generally known for being vulnerable to UV light (Gammelgaard et al., 2019). In 2024, all applications were done 1 to 2 h before sunrise, and this should have been enough time for the peptides to act against the pathogen, but would be interesting to study if evening applications improve their performance, as they are already recommended for other radiation sensitive products like the antibiotic kasugamycin (Slack S.M. et al., 2021).

Other mechanisms that could have affected the activity of the peptides against the target pathogen are (i) limited access of the peptide to the pathogen due to adsorption by envelopes or external structures (e.g., biofilm barriers, exopolysaccharides, capsules), (ii) active elimination from cells (e.g., efflux pumps, outer membrane vesicles secretion), (iii) degradation by proteases, (iv) or enzymatic chemical modification (Lima et al., 2021). To overcome these obstacles, the use of mixtures of peptides with different physicochemical characteristics and/or mechanisms of action could be of interest. Several physicochemical conditions and compounds can also reduce peptide activity (e.g., cations, pH, phenolics), especially from cationic amphipathic peptides like BP178 and BP100. Modifications of the peptide sequence with non-natural amino acids (e.g., including D-amino acids) (Ng-Choi et al., 2014), or adequate formulations (e.g., nanoencapsulation) could also be studied to minimize their impact.

METHODS

Synthesis of the bifunctional peptide (2023-2024, University of Girona)

Peptides were synthesized manually by the solid-phase procedure as previously described (Badosa et al., 2013). Briefly, a PAC-ChemMatrix resin (0.66 mmol/g) was used, and once the peptidyl sequence was completed, the resulting resins were treated with trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (TIS) (95:2.5:2.5) for 2 h at room temperature. Following TFA evaporation and diethyl ether extraction, the crude peptide was dissolved in H_2O, lyophilized, analyzed by HPLC, and characterized by mass spectrometry to determine its purity.

The bifunctional peptide BP178 is a long chain peptide (29 amino acids), and its manual synthesis is still expensive, especially the large amounts needed for field trials. In 2023, only 2 g of the peptide were provided, and adjustments to the protocol were made in order to reduce the amount of peptide needed. In 2024, and with the aim of increasing the number of applications in the field and the dose of the peptide, the synthesis of 3.5 g of BP178 was combined with the synthesis of 2 g of BP100, an antimicrobial peptide that has proven to be effective against fire blight (from *in vitro* to the field). The synthesis of BP100 is most cost effective due to its shorter length (11 amino acids).

In vitro trials (2024, Washington State University)

To confirm the minimal bactericidal concentration of the peptides against *E. amylovora* strain Ea153N and assess the effect of two different surfactants on the activity of the peptides, two *in vitro* experiments were conducted. In both experiments, three replicates for each concentration, peptide, and surfactant were used, and a water-treated control and an antibiotic control (streptomycin at 25 ppm) were included.

Peptide and antibiotic preparation: Lyophilized peptides were solubilized in sterile distilled water buffered with phosphate buffered saline at 6.5 pH to a stock concentration of 1 mM. For the second

experiment, sterile distilled water buffered with phosphate buffered saline at 6.5 pH was amended with the corresponding surfactant at a concentration of 0.6 ml/L (equal 8 fl oz/100 gal). Dilutions of the stock solutions were made to obtain the desired final peptide concentrations. The antibiotic control (Firewall 50WP) was prepared in sterile deionization water and filter sterilized inside the hood using a 0.2 µmm filter.

Bactericidal activity of the peptides: The bactericidal activity of the peptides was tested against a cell suspension of *E. amylovora* strain Ea153N (nalidixic acid resistant), adjusted to 10^7 CFU/ml. A contact test was performed at final peptide concentrations of 5, 10, and 20 ppm (1.5, 3.1, and 6.25 µM) for BP178 and 4.5, 9, and 18 ppm (3.1, 6.25, and 12.5 µM) for BP100. The multi-well plate was incubated at room temperature under low shaking conditions (90 rpm) and samples were taken after 30 min and 3 h. Culturable cells were quantified using the plate counting method, consisting of plating 20 µl of 10-fold dilutions on nutrient agar amended with nalidixic acid, and incubating plates at 28°C for 2 to 3 days to determine the CFU/ml.

Effect of two surfactants on the activity of the peptides: The effect of two commonly used surfactants, Regulaid and Nufilm P, on the activity of both peptides was studied against a cell suspension of *E. amylovora* strain Ea153N (nalidixic acid resistant), adjusted to 10^7 CFU/ml. A 3 h contact test was performed at final peptide concentrations of 5 and 10 ppm for BP178 and 9 and 18 ppm for BP100 and CFU/ml were quantified using the plate counting method, as described above.

Field trials (2023-2024, Washington State University)

Site and plots: A 0.6 acre research block of 250 third to forth leaf apple trees cultivar Gala rootstock G41 planted on a 4 ft spacing at the WSU Columbia View Orchard 48 Longview Rd. East Wenatchee, WA 98802-8283 was used for the trials. Soils are Cashmont Gravely Sandy Loam with a 3-8% slope. The site has good air drainage and some wind protection.
The experiment was arranged in a randomized complete block with five single tree replicates (100+ flower clusters). Experimental blocks were spaced from one another by two buffer trees.

Treatments and inoculation: Treatments included the peptides, as well as positive and negative controls. Positive controls included the antibiotic streptomycin as a control for bactericidal activity, the systemic acquired resistance inducer acibenzolar-S-methyl (Actigard) as a control for plant defense activation, and Blossom Protect + Buffer Protect as an organic control. A water-treated control was applied as a negative control treatment. Treatments were applied to the whole tree according to manufacturer recommendations using a Stihl SR420 mist blower backpack sprayer. Products were applied to wet, near dripping previously calibrated to equal 100 gal/A. In 2023, the peptide was applied by tree to the area of the tree to be inoculated (flower clusters) using a manual pump 4-gallon backpack sprayer. Application dates in 2023 were: 30 Apr (1), 2 May (2), 3 May (3, full bloom), 4 May (4), 5 May (5), 7 May (6, petal fall), 10 May (7); in 2024 were: 15 Apr (1), 17 Apr (2), 20 Apr (3, full bloom), 21 Apr (4), 24 Apr (5, petal fall), 27 Apr (6). All applications were made under fast drying conditions. At 80-90% bloom (of the king blooms), on 3 May 2023 and 20 April 2024, *E. amylovora* strain Ea153N (streptomycin and oxytetracycline sensitive and nalidixic acid resistant strain) was applied at $5x10^6$ CFU ml^{-1} (verified at $4.2x10^6$ CFU ml^{-1} and $6.1x10^6$ CFU ml^{-1}) to lightly wet each cluster using freeze dried inoculum. A 4-gallon backpack sprayer (solo) will be used to lightly wet clusters.

Evaluation: Trees were visually evaluated for flower cluster infection weekly from when symptoms became visible, 9 to 16 days after inoculation, for 2 weeks and infection counts summed across all dates. Infected flower clusters were removed in each evaluation. *E. amylovora* was enumerated 1, 4, and 7 days after inoculation from a bulk sample of 10 flowers per replicate (2 flowers from 5 different clusters). Flowers were sonicated in sterile DI water for 3 minutes and a 10-µl sample of the wash and

two 1:100 dilutions were spread on nutrient agar amended with nalidixic acid (50 µg/ml) and cycloheximide (50 µg/ml) to selectively enumerate *E. amylovora* (Ea153N). Fruit was evaluated for fruit skin marking before fruit colored over. 25 fruit per replicate were rated. Russet ratings are on a 1 to 15 scale with individual values lower than 3 considered insignificant for commercial packing. Environmental conditions were tracked on an hourly basis including temperature, humidity, leaf wetness, solar radiation and windspeed.

Statistical analysis

Statistical analysis was performed with SAS v 9.4. For the first *in vitro* experiment, general linear mixed models (MIXED) analysis of variance ANOVA with time as repeated measure was used. General linear mixed models (GLIMMIX) analysis of variance ANOVA was used for the second *in vitro* experiment. For both experiments, multiple means comparisons were performed according to the Tukey's honestly significant difference (HSD) test at a P value ≤ 0.05. For the field trials, general linear mixed models (GLIMMIX) analysis of variance ANOVA and multiple means comparison (LSD) for infections (normal distribution) and fruit marking (negative binomial distribution) were used.

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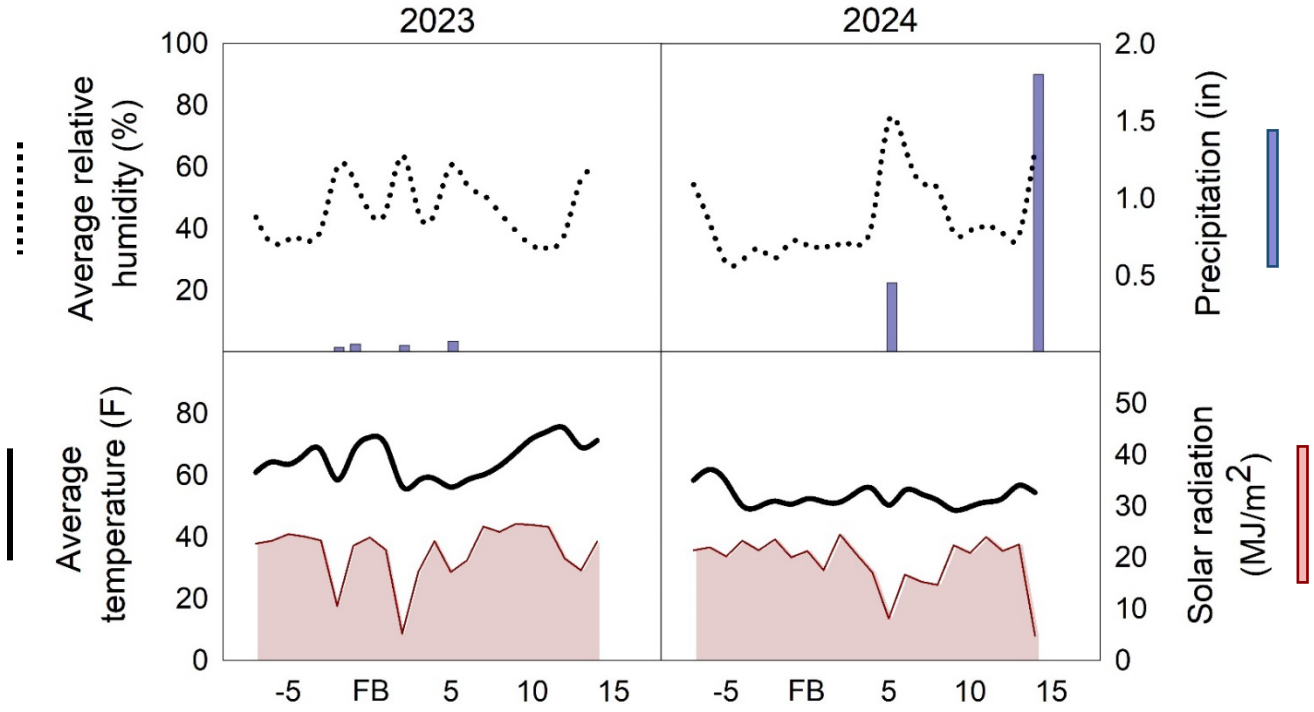
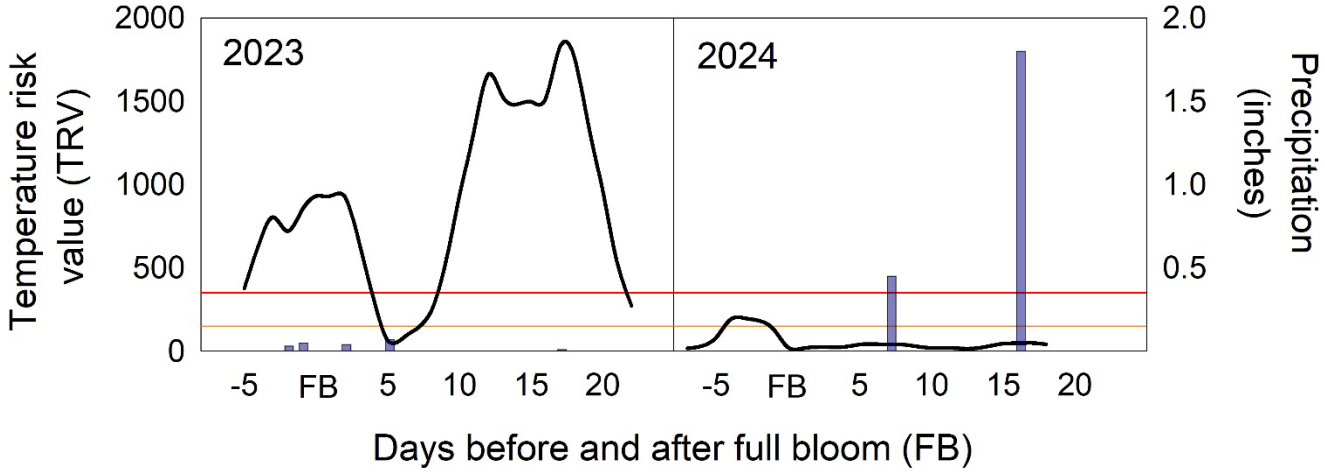
Appendix 1. Environmental conditions (temperature, humidity, precipitation and solar radiation) during bloom.



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



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

Key words: Fire blight, functional peptides, control

Abstract:

Functional peptides have been proposed as sustainable tools for controlling plant diseases, including fire blight in apple trees, but field efficacy remains underexplored. This study evaluated the application of peptides to manage fire blight caused by *Erwinia amylovora* in Wenatchee, WA. In 2023, under high disease-risk conditions, peptide applications on apple cv. Gala trees did not significantly reduce infection rates. Factors such as localized application (on the flower clusters), suboptimal concentrations, and the absence of surfactants likely influenced the outcome. In contrast, 2024 field trials demonstrated improved peptide performance, likely due to increased concentration, whole-tree application, and co-application with the surfactant NuFilm P. Peptide activity correlated with a reduction in bacterial populations, suggesting a primarily bactericidal mechanism of action. Laboratory assays confirmed the peptides' bactericidal activity against *E. amylovora* and compatibility with surfactants.

Despite enhanced field performance in 2024, the peptides did not achieve statistically significant infection reduction compared to the water-treated control. Environmental factors, such as UV exposure or proteolytic degradation, likely contributed to the moderate efficacy observed. Increasing peptide concentration through the use of novel methods with higher delivery efficiency or the optimization of more cost-efficient production systems, and the adoption of strategies to mitigate environmental challenges (e.g., evening applications, peptide modifications, or advanced formulations like encapsulation) could improve field performance. So overall, while peptides show promise, their successful application requires further research and optimization to address these critical challenges.

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

Report Type: Final Project Report

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Project Duration: 2-Year

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

Other related/associated funding sources: None

Budget 1

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Item	3/1/2023	3/1/2024
Salaries	$20,064.00	$20,867.00
Benefits	$9,000.00	$9,361.00
Wages		
Benefits		
RCA Room Rental		
Shipping		
Supplies	$10,328.00	$10,749.00
Travel	$250.00	$250.00
Plot Fees		
Miscellaneous		
Total	$39,642.00	$41,227.00

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

Objectives:

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

Significant Findings

- Chitosan lactate demonstrated the strongest in vitro inhibitory activity among all tested products.
- A reproducible wounding and inoculation method to mimic trauma blight symptoms in crabapple and apple was developed.
- Sodium caprylate exhibited significant phytotoxicity on apple trees when applied at its effective in vitro bactericidal concentration (0.4%). The maximum safe concentration of sodium caprylate for apple trees was determined to be 0.08%, based on dose-response testing assays with 'Fuji' seedlings.
- Among tested commercial chitosan-based products, TidalGrow SPECTRA used at concentrations above the recommended by the manufacturers, was the only one providing significant disease control and reducing disease severity in both greenhouse and field trials. The other commercial products, and also chitosan lactate alone, had reduced or no activity controlling shoot blight.
- Rhamnolipids also reduced symptom severity and provided effective disease control consistently among trials, particularly in the days immediately following infection.
- There were important differences in the efficacy of rhamnolipids and the chitosan formulation of TidalGrow SPECTRA between greenhouse and field trials, with fire blight symptoms developing faster and more aggressively in the field. Factors that could explain some of these differences are:
    - Greenhouse and field trials were performed with trees of different host cultivars, 'Pink Lady' and 'Gala', respectively, with differing susceptibilities to fire blight.
    - Greenhouse trials were performed during mid and late summer, with higher top temperatures recorded than during field trials, which were carried out at the beginning of summer.
    - The pH of rhamnolipids in field trials was adjusted to 5.5 instead of 4.0, in order to compare results with mixed treatments with Cueva adjusted to the same pH to avoid phytotoxicity issues with copper.
- None of the products tested in the field caused russeting
- Single spray applications of both rhamnolipids and chitosan from TidalGrow SPECTRA induced plant defense-related genes, with peak expression observed at 3-7 days post-spray. This suggests that their efficacy in greenhouse and field trials may be partially attributed to the activation of plant's natural defenses. These results indicate that treatment efficacy might be improved by spacing sprays more than 3 days apart.
- In vitro tests showed rhamnolipids synergistically enhanced antibiotic activity when co-applied with streptomycin and especially kasugamycin. This means that rhamnolipids could be used as co-adjuvants to increase antibiotic efficacy. However, apple seedling assays revealed incompatibilities between rhamnolipids and both Actigard and chitosan-based products, combined treatments with other plant defense elicitors are discommended.
- Chitosan formulation in TidalGrow SPECTRA enhanced the activity of other plant defense inducers like Actigard when combined. These combinations worked at lower product concentrations while maintaining or improving efficacy compared to individual treatments at full concentration.
- Overall, while rhamnolipids and chitosan in TidalGrow SPECTRA provided consistent protection against shoot blight and/or significantly reduced symptom severity in both greenhouse and field trials. Although the observed protection could be considered intermediate-low, the trials were performed under very high infection pressure. Their consistent activity suggests these treatments may be viable eco-friendly alternatives to other agrochemicals that can be integrated into existing management strategies, particularly under lower infection pressure conditions.

Results and Discussion

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

As part of **Objective 1**, we tested the in vitro antimicrobial activity of different products with potential use in the orchard against shoot blight. Of all the tested products, chitosan lactate demonstrated the strongest in vitro activity against *E. amylovora*. It significantly reduced *E. amylovora* populations by 50% and 95% at very low concentrations of 0.03% and 0.05%, respectively (**Figure 1C**). The next most effective products were sodium caprylate, with IC_{50} and IC_{95} values of 0.17% and 0.45%, respectively (**Figure 1A**), and rhamnolipids (**Figure 1B**). Rhamnolipids exhibited a biphasic growth inhibition curve, with two distinct concentrations (IC_{50} at 0.10% and IC_{50} at 0.40%) causing a 50% reduction in populations during each phase.

Chitosan oligosaccharides derived from mushrooms and crabs showed some bactericidal activity but did not completely inhibit *E. amylovora* growth under the test conditions, indicating low effectiveness against the pathogen (**Figure 1D** and **1E**, respectively).

Raw chitosan could not be included in this assay due to incompatibilities with the experimental conditions. Chitosan solutions are highly viscous and require acidic conditions for solubilization. Sodium lactate was soluble but precipitated during incubation, making it impossible to measure bacterial growth (**Table 1**).

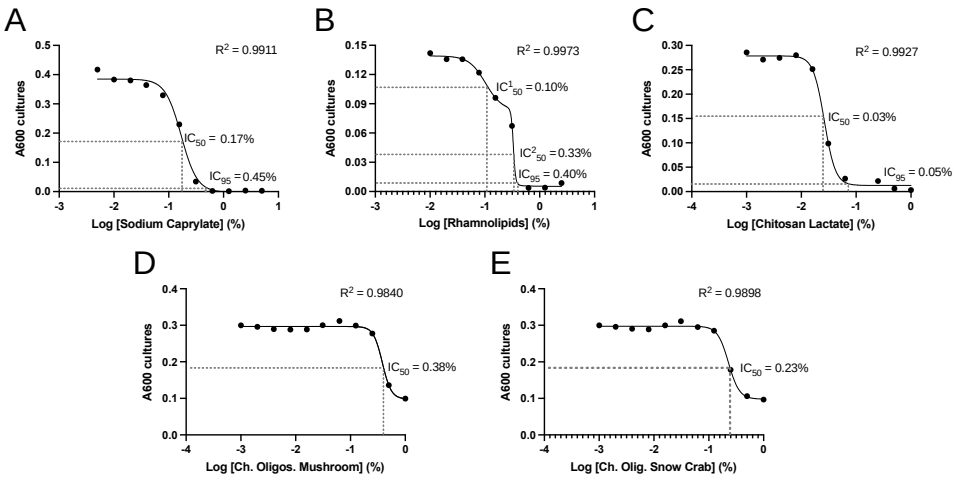


Figure 1. Effect of sodium caprylate (A), rhamnolipids (B), chitosan lactate (C), chitosan oligosaccharides from mushroom (D), and chitosan oligosaccharides from snow crab (E) on *E. amylovora* growth. Each chart shows the IC_{50} and IC_{95} values obtained in triplicate assays. Curves were adjusted by non-linear regression after log-transformation of the assayed compound concentrations. The R^2 values indicate the goodness of fit of the curves to the obtained data.

Table 1. Performance of different compounds inhibiting *E. amylovora* growth.

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

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

[b] The effect of rhamnolipids on *E. amylovora* growth fitted a biphasic curve with two significant decreases of bacterial growth, defined by the first and second IC_{50} values (0.10 % and 0.33%). In this case, the IC_{95} value was calculated manually, interpolating the value directly from the chart.

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

[d] The assayed concentrations did not completely inhibited *E. amylovora* growth, and higher concentrations were difficult to dissolve. NA, not applicable.

In summary, all the tested substances demonstrated antimicrobial properties that could be useful for managing fire blight in the field. A key aspect of this assay was its design to determine whether the compound itself, rather than just its pH, had inhibitory effects on *E. amylovora*. The test was conducted under controlled pH conditions in a medium that supports pathogen growth. This is significant because many agricultural products are prepared at low pH levels before application, and this acidity may also contribute to killing pathogen cells in the field. Notably, the activity of caprylic acid, rhamnolipids, and chitosan has been reported to persist or even increase at low pH levels, such as pH 4.0 or 5.0.

2. Optimization of treatment conditions using apple seedlings and crabapples.

Key findings from 'Fuji' seedling assays revealed:

1. Caprylic acid showed significant phytotoxicity at 0.4% (concentration selected based on in vitro assays). All treated plantlets developed extensive necrotic lesions across their leaves within 24 hours of initial application. Follow-up experiments confirmed this effect, showing necrotic spots on leaf surfaces even at 2-fold dilutions (0.4% to 0.16%). Concentrations of 0.08% and lower did not cause phytotoxicity under experimental conditions, leading us to adopt this concentration for subsequent potted plant experiments.
2. Some plants treated with Armour-Zen 15% (8 qt/50 gal) and chitosan oligosaccharides from mushroom (1%) showed isolated necrotic spots on some leaves in one out of three/six plants at the end of the experiment. As a precautionary measure, we reduced these product concentrations in subsequent assays (**Table 2**).
3. Commercial chitosan-based products contained acetic acid with pH ranges from 3.77 to 5.22. Control tests using water buffered to pH 3.2-3.7 (with glacial acetic acid and K_2HPO_4) showed no effect on *E. amylovora* infections compared to distilled water controls, ruling out potential bactericidal effects of acetic acid alone.
4. Plantlets treated with Armour-Zen 15% and inoculated with *E. amylovora* developed significantly larger necrotic lesions than untreated controls at 10 days post-inoculation.
5. Plants treated with rhamnolipids and caprylic acid showed smaller necrotic lesions compared to untreated controls sprayed with water or water buffered to pH 3.2, although these differences were not statistically significant (data not shown).

3. Development and optimization of an inoculation method mimicking trauma blight conditions

As part of **Objective 2**, we evaluated two methods to replicate trauma blight symptoms: (1) cutting with sterile scissors followed by spray inoculation, and (2) spray inoculation followed by wounding with scissors. The first method simulates plant tissue damage from strong winds or hail, where *E. amylovora* cells subsequently reach wounds through biological vectors (e.g., insects) or physical vectors (e.g., wind-driven rain). The second method represents a less common scenario where *E. amylovora* is present on plant surfaces before wind or hail damage creates entry points into plant tissues. Generally, *E. amylovora* is considered a poor colonizer of leaves and other plant organs besides flowers. Abundance of *E. amylovora* cells on other plant surfaces than flowers are more likely in heavily damaged orchards with abundant ooze, necrotic lesions, or infected flowers.

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

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

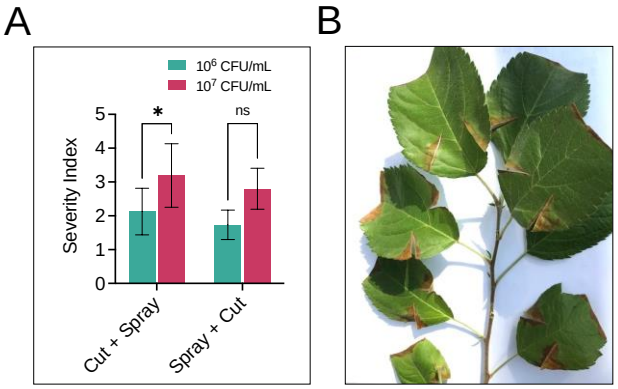


Figure 2. Optimization of an inoculation method to recreate trauma blight-like symptoms using white crabapple plants. Two inoculation methods, cutting leaves plus spray inoculation with *E. amylovora* (Cut + Spray), and spray inoculation followed by cutting with sterile scissors (Spray + Cut), were used. Results were average values of 5-8 replicate leaves per shoot in two different shoots. The asterisk indicates significant differences associated with inoculum concentration when sprayed after cutting ($P \leq 0.05$) (A). Cut positions and representative fire blight symptoms 7 days after inoculation (B).

3. Evaluation of treatments for trauma blight control in greenhouse trials

Based on results from **Sections 1, 2**, and **3**, we optimized treatments with different compounds as well as an inoculation method mimicking trauma blight conditions (**Table 2**) for testing on two-year-old 'Pink Lady' apple trees. The treatments included Actigard as a plant defense elicitor control, along with copper (Cueva), streptomycin (FireWall), and oxytetracycline (FireLine) as bactericide controls. Disease control efficacy was calculated as the percentage reduction in disease incidence in inoculated wounds compared to the untreated control. Treatments performed during the first greenhouse trial in 2023 are summarized in **Table 2**, with results reported in **Figure 3**.

In this first trial, among the 10 treatments tested, only FireWall, TidalGrow SPECTRA, and FireLine provided significant fire blight disease control throughout the experiment, with median efficacy values of 76.8%, 63.9%, and 56.6%, respectively, at 33 days post-inoculation ($P \leq 0.0117$) (**Figure 3A**). Rhamnolipids showed relatively good disease control of 46.7% at 11 dpi ($P = 0.0120$) but declined to 12.4% by the end of the experiment ($P = 0.0021$). Other treatments, such as Cueva and ChitoAg-80, provided similarly low but statistically significant disease control at 33 dpi, with values of 12.4% and 13%, respectively ($P \leq 0.0482$) (**Figure 3A**). However, these values, based on infection

incidence, do not fully capture the activity of the tested products. **Figure 3B** shows that although Actigard, Cueva, and rhamnolipids provided low disease control efficacy percentages, they reduced lesion size by 30-60% compared to untreated controls during the same period ($P \leq 0.0356$). Conversely, the other commercial chitosan-based compounds, Armour-Zen 15% and ChitoAg-80, performed poorly in reducing disease symptoms (**Figure 3B**) and controlling the disease, particularly Armour-Zen 15% (**Figure 3A**).

Table 2. Spray treatments applied on 2-year-old potted 'Pink Lady' trees in the 2023 greenhouse trial.

Treatment	No. Trees	Active compound	Application concentration	% Active compound	Application Pattern [d]
Actigard 50 WG	5	Acibenzolar-S-methyl (50%)	2 oz/100 ga	0.007	A
FireWall 50 WP	4	Streptomycin sulfate (65.8%)	8 oz/100 ga	0.039	B
Fire Line 45 WP	4	Oxytetracycline hydrochloride (48.8%)	9 oz/100 ga	0.033	B
Cueva	5	Copper octanoate (10%)	4 qt/100 ga	0.100	B
Armour-Zen 15%	4	Chitosan (15%)	4 qt/100 ga [a]	0.150	A
ChitoAg-80	5	Chitosan (4%)	2 qt/100 ga	0.020	A
TidalGrow SPECTRA	4	Chitosan (5.75%)	22 fl oz/ 2 ga	0.494	A
Rhamnolipids [b]	5	Rhamnolipids (90%)	0.40%	0.36	A
Sodium caprylate [b]	5	Sodium caprylate (98%)	0.08% [c]	0.076	A
Chitosan Oligos. [b]	5	98.2% Deacetylated chitin (mushroom)	0.20%	0.200	A
Untreated control	5	NA	NA	NA	A

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

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

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

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

Comparisons among the three commercial chitosan-based products indicated that high chitosan concentrations might be important for significant protection against fire blight, although other factors probably contribute to chitosan efficacy. Treatments with TidalGrow SPECTRA, Armour-Zen 15% and ChitoAg-80 were designed to achieve different final chitosan concentrations of around 0.5%, 0.15% and 0.02%, respectively (**Table 2**). The most effective protection was observed with TidalGrow SPECTRA prepared with the highest chitosan concentration. Interestingly, despite its lower chitosan concentration, ChitoAg-80 provided better disease control than Armour-Zen 15%, which contained 7.5 times more chitosan. Similarly, symptom severity was slightly reduced in plants treated with ChitoAg-80 compared to those treated with Armour-Zen 15% (**Figure 3B**). These findings suggest that additional ingredients within the commertial product composition may contribute to enhance ChitoAg-80 activity at lower doses or decrease Armour-Zen 15% efficacy despite its higher chitosan dosage.

For treatments with sodium caprylate, none of the trees sprayed at a 0.08% concentration exhibited signs of phytotoxicity on the leaves. While the analysis did not show statistically significant differences compared to the control, it is notable that, except for one tree, symptom severity was generally reduced relative to the control.

Based on these results, we designed the treatments for the 2024 greenhouse trial, which included Actigard (2 oz/100 gal), FireWall 50 WP (8 oz/100 gal), and Cueva (4 qt/100 gal) as controls for plant defense induction and bactericides, respectively. In this trial, we reduced the number of treatments, focusing only on those that showed better results in the previous trial, specifically rhamnolipids (0.4%) and TidalGrow SPECTRA (1100 fl oz/100 gal). For comparison, we also

included a treatment with pure chitosan lactate derived from snow crab, prepared at a 0.2% concentration (**Table 3**).

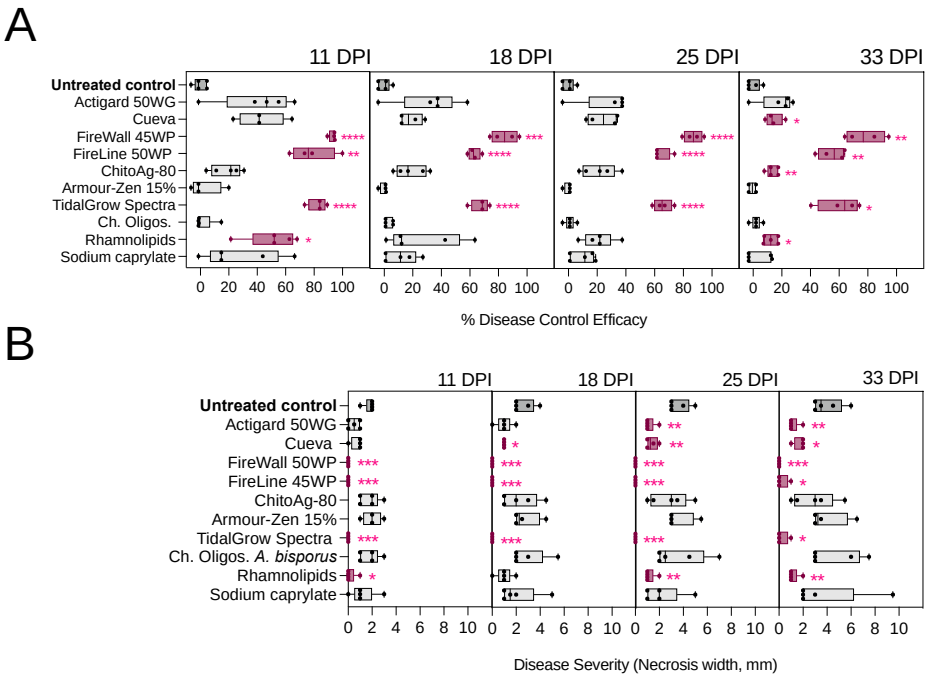


Figure 3. Efficacy of different products in trauma blight control and symptom severity reduction throughout time in potted 'Pink Lady' trees, in a first greenhouse trial in 2023. The **%** disease control was calculated as the % disease incidence reduction with respect to the % disease in the untreated control, multiplied by 100. Disease severity was measured as the longest distance between the cut and the necrotic lessions in the inoculated leaf. Asterisks show statistically significant differences between the indicated treatment and the untreated control ($\alpha = 0.05$). Treatments providing significant disease control and/or symptom reduction are highlighted in pink.

Table 3. Spray treatments applied on potted 'Pink Lady' trees in the 2024 greenhouse trial.

Treatment	No. Trees	Active compound	Application concentration	% Active compound	Application Pattern[b]
Actigard 50 WG	5	Acibenzolar-S-methyl (50%)	2 oz/100 ga	0.007	A
FireWall 50 WP	4	Streptomycin sulfate (65.8%)	8 oz/100 ga	0.039	B
Cueva	4	Copper octanoate (10%)	4 qt/100 ga	0.1	B
TidalGrow SPECTRA	6	Chitosan (5.75%)	1100 fl oz/ 100 ga	0.494	A
Rhamnolipids [a]	7	Rhamnolipids (90%)	0.40%	0.36	A
Chitosan lactate. [a]	6	Deacetylated chitin (snow crab)	0.20%	0.2	A
Untreated control	9	NA	NA	NA	A

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

[b] **A**, for products with recognized or potential plant-defense inducers: treatment application on 6.20.24, 6.23.24; tree wounding and inoculation on 6.25.24; treatment application 6.26.24 (9-11h post-inoculation) and 6.29.24. **B**, for products with bactericidal activity only: treatment application 6.24.24; tree wounding and inoculation on 6.25.24; treatment application on 6.26.24 (9-11h post-inoculation) and 6.29.24.

In the 2024 trial, all tested products, including pure chitosan lactate, initially demonstrated strong trauma blight control, with median control efficacy percentages ranging from 56.1% to 92.6% (**Figure 3**). However, none of the products prevented wound infections completely, and the

differences compared to untreated controls diminished over time. By the end of the experiment (36 dpi), only TidalGrow SPECTRA (12.5%) and FireWall (15%) maintained statistically significant control against shoot blight infections ($P \leq 0.0081$), although at low levels (**Figure 3A**). Similar to the 2023 greenhouse trial, while most tested products could not prevent infections (**Figure 3A**), many significantly reduced symptom severity ($P \leq 0.0067$) (**Figure 3B**). Chitosan lactate was the only tested product that showed no clear effects on symptom severity, with treated trees developing lesions slightly smaller in size but statistically undistinguishable from to those of untreated controls (**Figure 3B**). These results together with the ones from 2023 trial indicate that chitosan derivatives like chitosan lactate and chitosan oligosaccharides alone are not good options for shoot blight control. Even among the chitosan-based commercial products, only the chitosan formulation in TidalGrow SPECTRA showed significant differences from the controls among trials.

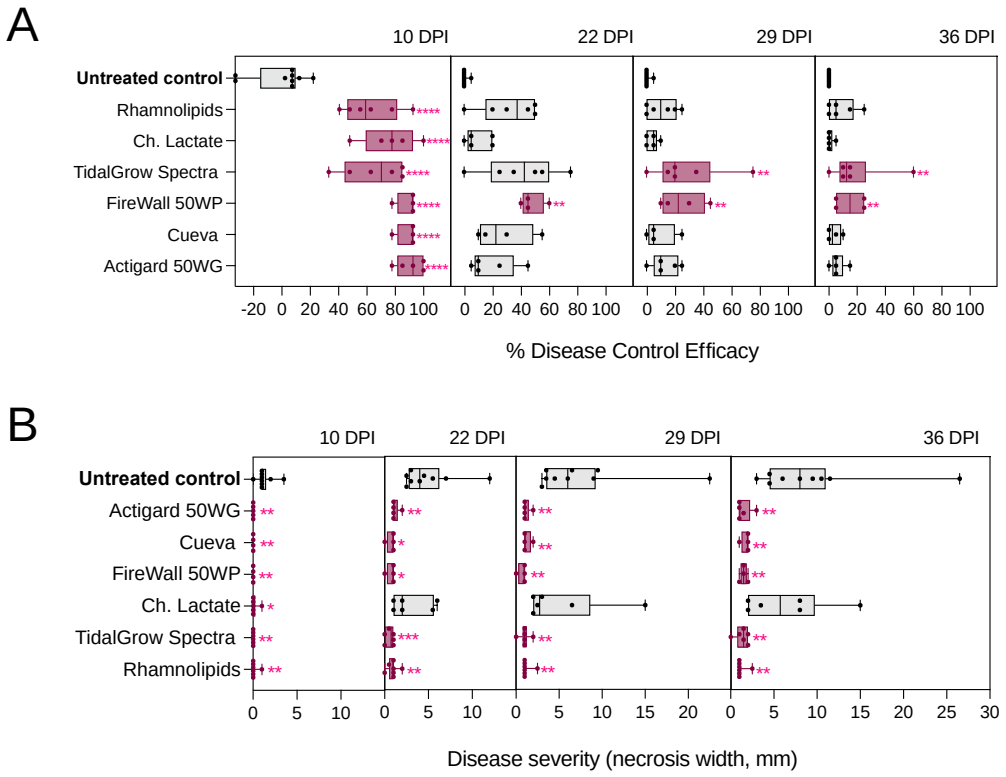


Figure 4. Efficacy of different products in trauma blight control and symptom severity reduction throughout time in potted 'Pink Lady' trees, in a second greenhouse trial carried out in summer of 2024. Asterisks show statistically significant differences between the indicated treatment and the untreated control ($\alpha = 0.05$). Treatments providing significant disease control and/or symptom reduction are highlighted in pink.

In summary, analysis of both disease control and severity measurements from the 2023 and 2024 greenhouse trials showed that TidalGrow SPECTRA consistently reduced disease incidence and severity across both trials. Its performance often matched that of conventional chemical treatments, suggesting its potential as a sustainable disease management option. Rhamnolipids demonstrated consistent efficacy in controlling shoot infections, particularly during the initial days following shoot inoculations, despite showing reduced efficacy at later time points. This product effectively reduced symptom severity in both trials, achieving results comparable to Actigard and even copper or antibiotic treatments. Both TidalGrow SPECTRA and rhamnolipids maintained consistent performance patterns across trials, although with different efficacies (**Figures 2 and 3**). These results

were achieved under high infection pressure conditions (with 100% wound infection in control trees by trial end). This suggests that these products may demonstrate even better performance under less infection-conducive conditions.

4. Rhamnolipid and chitosan performance assessment under field conditions

Based on results from the 2023, we designed treatments for a field trial using 'Gala' apple trees, at WSU Columbia View Research Farm in Wenatchee, WA. While we standardized conditions in greenhouse trials by pruning trees and applying treatments when new shoots reached 5-10 inches in length, field assays were timed using petal fall as a reference point. Products with potential plant defense-activating properties were applied days before and after *E. amylovora* inoculation, following the greenhouse trial protocol (**Table 4**). For field applications, we modified the pH of rhamnolipids to 5.5 instead of 4.0. This pH more closely aligns with standard agrochemical buffering practices and prevents copper phytotoxicity when used in combined treatments with copper (Cueva) (**Table 4**).

Table 4. Spray treatments applied on 'Gala' trees in a field trial carried out in spring of 2024.

Treatment	Rate per 100 gallons water	pH	Surfactant	Timing*
Untreated, Inoculated Check	na	na	na	PF, PF+3, PF+6, PF+9
Firewall 50WP	8 oz	5.6	Regulaid @16 oz	PF+6
Cueva	4 qt		na	PF, PF+6
Rhamnolipids	0.4 %	5.5	na	PF, PF+3, PF+6, PF+9
Cueva + Rhamnolipids	3 qt + 0.4 %	5.5	na	PF, PF+6
Chitosan (TidalGrow SPECTRA)	1100 fl oz	na	na	PF, PF+3, PF+6, PF+9

*PF, petal fall; PF+3, petal fall + 3 days; PF+6, petal fall + 6 days (morning after evening inoculation); PF+9, petal fall + 9 days.

The results from the field trial are summarized in **Figure 4**. Disease symptoms developed and progressed more rapidly and aggressively in field trials compared to greenhouse trials. These differences may be attributed to both cultivar variation ('Gala' versus 'Pink Lady') and environmental conditions. The spring to early summer field conditions proved more conducive to disease development than the greenhouse environment, where higher peak temperatures were recorded during mid to late summer trials.

0.5% Chitosan (TidalGrow SPECTRA) and 0.4% rhamnolipids demonstrated low but statistically significant efficacy in controlling shoot infections in the field ($P \leq 0.0282$). TidalGrow SPECTRA achieved 32.3% and 8.5% control at 7 and 12 dpi respectively, while rhamnolipids showed 17.3% and 12.0% control at the same time points (**Figure 4A**). As observed in greenhouse trials, control plants exhibited 90-100% wound infection by the experiment's end, indicating very high infection pressure. This extreme disease pressure may explain why even FireWall, the most effective treatment, provided low protection (median of 16.1%) against shoot blight by the end of the experiment (**Figure 4A**). Neither Cueva alone nor its combination with rhamnolipids provided any protection against shoot infections, even during early post-inoculation periods.

Interestingly, treatments with rhamnolipids, but not chitosan (TidalGrow SPECTRA), significantly reduced symptom severity, with lesions being 92.5% smaller than those in untreated control trees at

the final experimental time point (23 dpi) (**Figure 4B**). However, the combination of rhamnolipids with copper provided less protection than rhamnolipids alone. These findings suggest a potential negative interaction between copper and rhamnolipids, which may reduce the latter's efficacy in controlling infections.

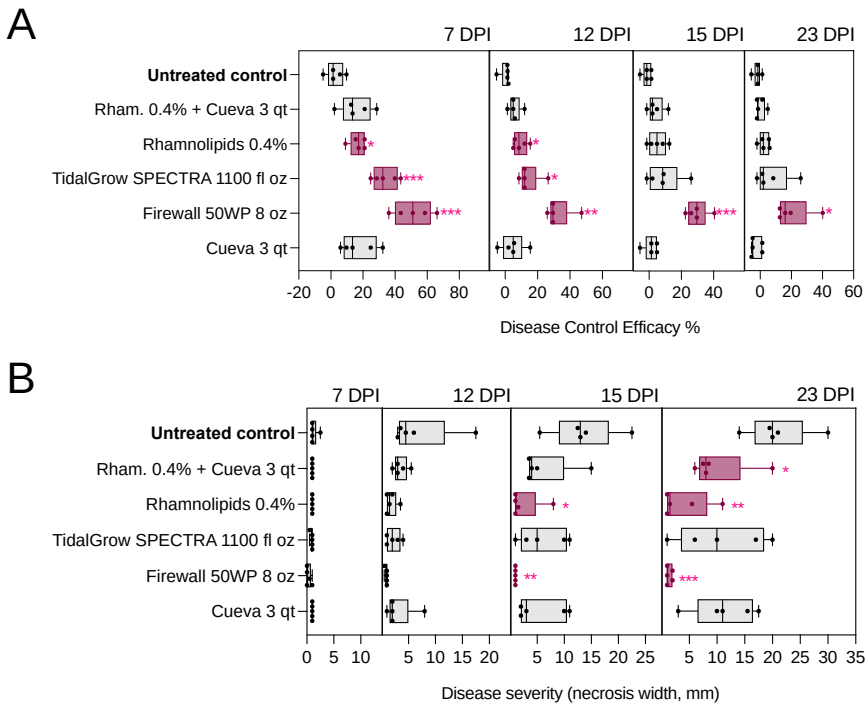


Figure 5. Efficacy of different products in trauma blight control and symptom severity reduction throughout time in a field trial on 'Gala' trees, carried out in 2024. Asterisks show statistically significant differences between the indicated treatment and the untreated control ($\alpha = 0.05$). Treatments providing significant disease control and/or symptom reduction are highlighted in pink.

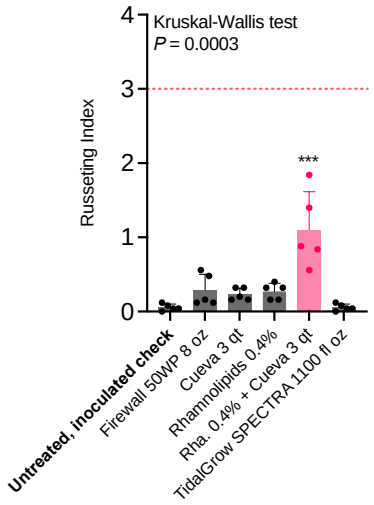


Figure 6. Russeting marks in fruit after treatments. Each dot in the graph is an average value of 25 fruit per tree (5 trees/treatment), which were monitored for the presence of russeting marks, and recorded based on a standard russeting index ranging from 0 to 5, where values above 3 (dotted line in the graph) are considered detrimental for the fruit marketability. Asterisks denote statistically significant differences with the untreated control.

Although the combination of Cueva + rhamnolipids increased the russeting marks with respect to the control ($P = 0.0003$), none of the applied treatments induced significant fruit russeting above the threshold of 3 (**Figure 6**), demonstrating that both rhamnolipids and chitosan are bioactive

compounds that can be safely used without compromising fruit marketability. While these treatments did not completely prevent infections under high infection pressure conditions, our results indicate that both rhamnolipids and the chitosan formulation from TidalGrow SPECTRA show promise as sustainable alternatives to conventional chemical treatments. These products could be valuable components of integrated pest management strategies, particularly for controlling shoot blight under low to intermediate infection pressure conditions.

5. A single spray treatment with rhamnolipids or chitosan (TidalGrow SPECTRA) induces plant defense-related genes

Results from greenhouse and field trials suggested that rhamnolipids and chitosan (TidalGrow SPECTRA) may induce plant defense responses. To verify this hypothesis, we monitored the expression of key defense-related genes: pathogenesis related proteins 1 and 2 (*PR1*, *PR2*), which are associated with salicylic acid-mediated defense responses and systemic acquired resistance; Plant Defensin 1 (*PDF1*), a marker for jasmonic acid and ethylene-mediated defenses; and phenylalanine ammonia-lyase (*PAL*), a key enzyme in the phenylpropanoid pathway involved in both basal and induced resistance.

The analysis revealed that rhamnolipid treatments significantly affected the expression of all four analyzed genes ($P \leq 0.0476$). *PR1*, *PR2*, and *PAL* reached peak expression 7 days after spray application, while *PDF1* showed maximum expression levels 3 days after spray (**Figure 7A**). Similarly, TidalGrow SPECTRA treatments significantly increased expression levels of *PR1*, *PR2*, and *PAL* ($P \leq 0.0314$) (**Figure 7B**). These results support our observations from plant assays, indicating that while chitosan and rhamnolipids possess in vitro bactericidal activity, their efficacy in greenhouse and field trials can be partially attributed to the activation of multiple plant defense pathways.

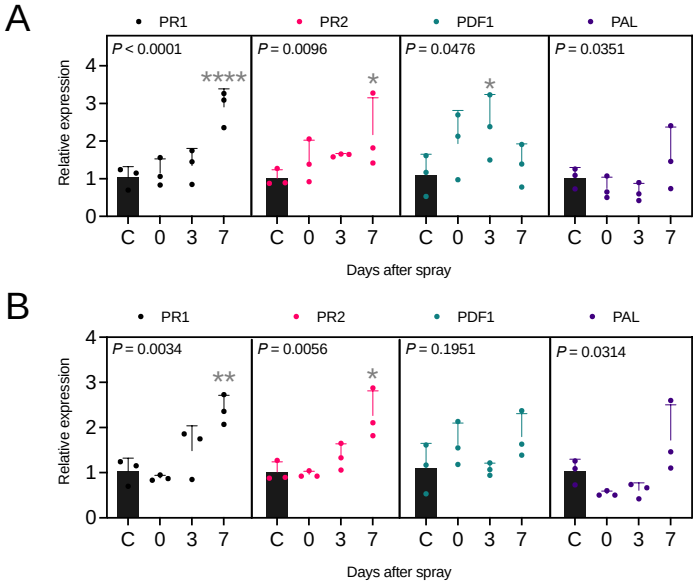


Figure 7. Relative expression of *PR1, PR2, PDF1* and *PAL* genes in potted 'Pink Lady' trees after one spray treatment with 0.4% rhamnolipids (A) and TidalGrow SPECTRA at 1100 fl oz/100 ga (0.49% chitosan) (B). Each dot corresponds to a pooled sample of three different trees. Columns show average relative expression values of 3 pooled samples (i.e., 9 trees) and the error bars indicate the SD. Untreated trees were used to normalize the relative expression of target genes across different samples, and the elongation factor 1 alpha gene (*EF1α*) was used as endogenous control. The *P* values indicate the significance of differences between the compared groups (untreated control trees, C, and sprayed trees at times 0, 3 and 7 days post-spray), assessed by one-way ANOVA analysis ($\alpha = 0.05$). Asterisks denote significant differences between control trees and sprayed trees at the specified time point, assessed by post hoc multiple comparisons tests.

6. Characterization of product interactions with antibiotics and plant defense inducers

As part of **Objective 1**, we also explored interactions between products. We conducted an in vitro assay to evaluate interactions between rhamnolipids (Rha) and three antibiotics commonly used against *E. amylovora* in the field: streptomycin (Sm), kasugamycin (Kg), and oxytetracycline (OTc). This assay relied on turbidimetric measurements, which prevented testing the effects of TidalGrow SPECTRA due to its dark color, and turned the culture medium black. Accordingly, this experiment was performed only with rhamnolipids.

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

For all tested compounds, the concentrations required to reduce *E. amylovora* populations by 50% were higher when used alone compared to when combined (**Figure 8**). Combinations of rhamnolipids with streptomycin and especially, kasugamycin, demonstrated strong synergistic interactions (FIC values of 0.4 and 0.29, respectively) (**Figure 8A,B**). This means that mixtures of antibiotics with rhamnolipids enhanced the bactericidal activity above the values expected in an additive model. In fact, mixtures of Rha+Sm and Rha+Kg at concentrations equal to the IC_{50} reduced *E. amylovora* populations below 95%, in comparison to the around 50% reduction observed with the products applied separately (**Figure 8A,B**). Combinations of rhamnolipids with oxytetracycline resulted in FIC values close to 1 (**Figure 8C**), meaning that rhamnolipids had no clear effect enhancing this antibiotic activity against *E. amylovora*. Overall, our findings suggest that rhamnolipids could serve as effective coadjuvants, enhancing the action of antibiotics in the field in a cost-effective manner.

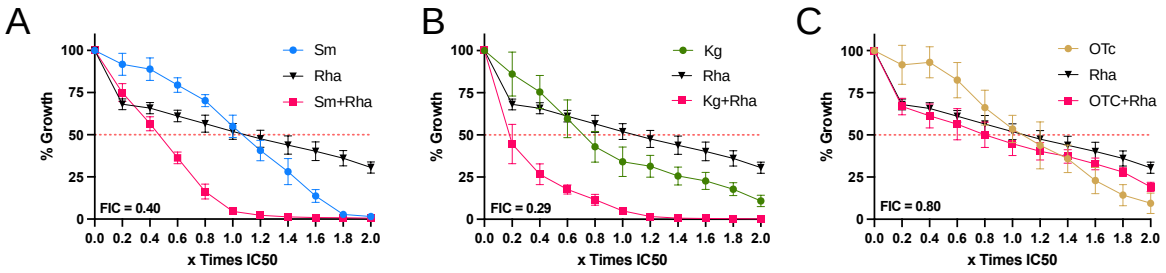


Figure 8. Interaction between rhamnolipids and antibiotics in inhibiting *E. amylovora* growth. Graphs show the percentage of *E. amylovora* growth inhibition by streptomycin (Sm), rhamnolipids (Rha) and their combination (Sm+Rha) (A), kasugamycin (Kg), Rha and their combination (Kg+Rha) (B), and oxytetracycline (OTc), Rha, and their combination (OTC+Rha) (C), at concentrations ranging from 0 to 2× their IC_{50}. The IC_{50} represents the concentration at which 50% growth inhibition is observed. Synergistic effects are indicated when the concentration of the products in the mixture required to reduce growth below 50% is lower than the IC_{50} of the individual products. The dotted red line marks the 50% growth inhibition threshold. Error bars represent the SD from three replicates in two independent assays.

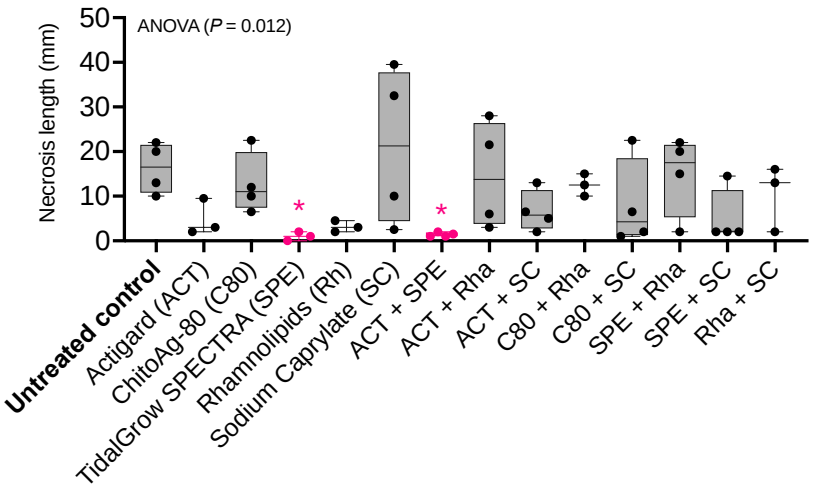
In a separate assay using 'Gala' seedlings, we tested plant defense inducers alone and in combination with rhamnolipids or chitosan-based products to evaluate potential enhancement of their activity. All products were tested at half the concentrations used in greenhouse trials to better detect positive interactions between product combinations. The tested treatments included Actigard (1 oz), rhamnolipids (0.2%), and the two chitosan-based products that performed best in greenhouse trials: ChitoAg-80 (1 qt) and TidalGrow SPECTRA (550 fl oz). Treatments and inoculations followed the greenhouse trial protocol, with *E. amylovora* sprayed after cutting five leaves twice at the base (10 wounds/plant). For comparison, treatments with sodium caprylate (0.04%) were also included. Each treatment was applied to 4-5 seedlings. Results showed that treatments significantly affected the

extension of necrotic lesions (ANOVA, $P = 0.012$). Multiple comparison tests indicated that TidalGrow SPECTRA (550 fl oz) and its combination with Actigard (1 oz) effectively reduced symptom severity (**Figure 9A**).

We additionally employed multivariable logistic regression analysis to determine whether the presence of rhamnolipids or chitosan in combined treatments had positive or negative effects on necrotic lesion extension. The analysis revealed that rhamnolipids in mixed treatments had a detrimental effect on the activity of other plant defense inducers, enhancing disease development (**Figure 9B**). The odds ratio for mixtures containing rhamnolipids was 3.656, indicating that the likelihood of observing necrotic lesions larger than those in control trees increased 3.7-fold when rhamnolipids were present in the mixture ($P < 0.0001$). Conversely, the presence of TidalGrow SPECTRA in mixtures reduced the probability of observing necrotic lesions larger than those in control plants by almost 50% ($P = 0.028$) (**Figure 9B**).

While further studies are needed to draw robust conclusions about the interactions of rhamnolipids and chitosan with other products, overall, our results suggest that rhamnolipids could serve as effective coadjuvants to enhance the efficacy of antibiotics in the field. Meanwhile, TidalGrow SPECTRA may positively impact the control of shoot blight infections and/or symptom development when combined with Actigard or other chitosan-based products. These findings also indicate that such effects and activity peaks might be achieved while reducing the concentrations of all active compounds in the mixture.

A



B

Variable	Odds ratio[a]	95% CI (profile likelihood)	*P* value[b]
ACT in MIX	0.646	0.3447 to 1.148	0.152
Rha in MIX	3.656	1.951 to 7.139	**<0.0001 (****)**
SPE in MIX	0.5085	0.2692 to 0.9071	**0.0281(*)**
Ch in MIX	0.6046	0.2767 to 1.273	0.1946
SC in MIX	1.064	0.5823 to 1.941	0.8403

[a] Values above 1 indicate increase of the likelihood to observe worsen symptoms. Values below 1 indicate the opposite.

[b] *P* values below 0.05 indicate statistically significant effects

Figure 9. Interaction between Actigard (ACT), rhamnolipids (Rh), ChitoAg-80 (C80), and TidalGrow SPECTRA (SPE) and their effects on fire blight symptom development. Effect of treatments, applied alone or in combination, on fire blight symptom severity. Asterisks indicate significant protection with respect to the untreated control (A). Multivariable logistic regression analysis showing the effect of the presence of specific plant defense elicitors in mixtures with other plant defense elicitors, on the extension of necrotic lesions (B).

Executive summary

Project title: New products for the prevention and control of shoot trauma blight

Keywords: Fire blight, rhamnolipids, chitosan, plant defense elicitors, sustainable disease management

Abstract

Extensive work on the prevention of blossom infection in Washington has been critical to reduce the impact of fire blight, which can cause severe infections in warm wet springs. However, few tools are available to prevent shoot blight infections, especially after hail and wind damage (trauma blight). This study investigated the potential of sustainable products, including chitosan-based formulations and rhamnolipids, for managing shoot blight. Initial in vitro screening revealed that all tested products had bactericidal activity against *Erwinia amylovora*, suggesting their potential to reduce pathogen populations on plant surfaces. Among these, two treatments showed promise: the chitosan formulation in TidalGrow SPECTRA, applied at 1100 fl oz/100 ga, and rhamnolipids applied at 0.4%. These products consistently affected disease development across two greenhouse trials and one field trial. While the level of shoot blight control varied from moderate to low depending on the trial, it is important to note that these results were obtained under extremely high infection pressure conditions. Under these challenging conditions, treatments with chitosan from TidalGrow SPECTRA and rhamnolipids provided protection levels and/or disease severity reduction comparable to conventional treatments such as copper (Cueva), Actigard, and antibiotics (FireWall and FireLine). This suggests these products might serve as potential alternatives to less environmentally friendly agrochemicals commonly used in fire blight management. Molecular analyses indicated that both chitosan from TidalGrow SPECTRA and rhamnolipids activated plant defense-related genes, including those associated with salicylic acid and jasmonic acid pathways. This suggests a dual mode of action: direct antimicrobial activity and stimulation of the plant's natural defenses. Additional in vitro studies revealed that rhamnolipids strongly enhanced the activity of streptomycin and kasugamycin, although they showed negative interactions with certain plant defense inducers, including Actigard. Chitosan in TidalGrow SPECTRA enhanced the activity of other chitosan-based products and Actigard when mixed together. Further research is required to refine application protocols, optimize product combinations, and evaluate long-term impacts on tree health and fruit quality. While the studied products show promise for reducing reliance on conventional chemicals, more extensive field trials are necessary before making definitive recommendations for commercial use.

Proposal Title: New organic products and timings: tools for season long integrated fire blight management
Report Type: Continuing report

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Cooperators: George Sundin, Kerik Cox, Sara Villani, Jim Adaskaveg

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $24,311
Total Project Request for Year 2 Funding: $27,297
Total Project Request for Year 3 Funding: $26,085

Other related/associated funding sources:

Comprehensive Fire Blight Management Systems for the United States. PD: Sundin, G., PI: Adaskaveg, J., Cox, K., **DuPont, S.T.**, Gallardo, K., Johnson, K., Kon, T., Khan, A., Rothwell, N., Villani, S., Youfu, Z.
Funding Duration: 2020 - 2024
Amount: $418,722
Agency Name: National Institute of Food and Agriculture (NIFA)
Notes: Project number MICL08595
Notes2: 2024 final year of project. Products included in table 2.

New Biocontrol Strains from Washington Native Plants, Phase 3. PI. Doty, S. CO-PI: **DuPont, S.T.**
Washington Tree Fruit Research Commission.
Funding Duration: 2023-2024
Amount: $7,549

Industry Gift Grants
Funding Duration: 2024 to 2026
Amount: $72,750 in MOAs 2024

Agency: Industry companies testing products (Gowan, SAN Group Biotech USA, ProFarm, AgroVentures, GroPro, SymAgro)
Notes: New product testing gift grants cover the cost of individual product review as prioritized by company partners. Core funding for project staff and plot fees is necessary to provide ongoing capacity to test new products and to allow testing of products prioritized by apple and pear grower stakeholders but not private companies.

WTFRC Collaborative Costs: None

Budget 1
Primary PI: Tianna DuPont
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Station manager/supervisor email address:

Item	1/1/2024	1/1/2025	1/1/2026
Salaries	$12,500.00	$13,000.00	$13,520.00
Benefits	$5,720.00	$5,949.00	$6,187.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$1,540.00	$3,657.00	$1,540.00
Travel	$1,755.00	$1,755.00	$1,755.00
Plot Fees	$2,796.00	$2,936.00	$3,083.00
Miscellaneous			
Total	$24,311.00	$27,297.00	$26,085.00

Footnotes:
Salaries: 3 months full time of Classified Staff at $4,167 pay rate.
Benefits: @ 45.8%
Supplies: $400 for Personal Protective Equipment and spray supplies, $1,000 for laboratory supplies (petri dishes, media, gloves, pipet tips, autoclave bags, analytical materials, etc.), $140 for respiratory fit tests. Year 2: participant coffee, snack and lunch for intensive workshop $17 + $14 ea x 150 = $2117..
Travel: Travel to Columbia View Research Orchard 26 mi x 64 trips at $0.655 per mile = $1100 ea year. Travel to give presentations to stakeholder groups and meet with growers who have questions 1000 mi at $0.655 per mile = $655 ea year.
Plot fees: 1 acre at $2,796 per acre 2024, at $2,936 per acre 2025, and at $3,083 per acre 2026. Note 1 of 3.5 acres designated for fire blight program.

ORIGINAL PROJECT OBJECTIVES

This grant aims to improve organic fire blight management by finding effective, new, and organic alternatives for the control of blossom blight, in order to avoid depending on the use of antibiotics, achieving a reduction in antibiotic resistance in the environment.

The specific goals are:

1. Test new organic fire blight products of interest for the growers for efficacy and fruit safety.
2. Improve organic product timings by identifying relationships between product efficacy and environmental conditions (e.g. UV, humidity).
3. Provide research-based recommendations to growers through web-based platforms (Crop Protection Guide, Decision Aid System), news alerts, field days and consultations.

SIGNIFICANT FINDINGS

Objective 1

- Seven out of the twenty-one organic products tested in WA 38 for fire blight control significantly reduced the number of infections compared to the water-treated control. They include four biological control agents, a program with a biological control agent followed by an organic acid, and two plant extracts.
- Four out of eight organic products tested in Gala for fire blight control significantly reduced the number of infections compared to the water-treated control. They include two biological control agents, a mineral based product, and an oxidizer.
- The experimental product GWN-10474 did best at the 30 oz rate showing a rate effect.
- Experimental endophytes applied at tight cluster, full bloom, day after full bloom and petal fall did not suppress *E. amylovora* populations or reduced infections compared to the water treated control in 2023 in a cool year similar to no significant reductions in 2024 in a warm/high pressure year.
- Two PSU experimental biologicals provided significant control compared to the water treated check (40 to 47% relative control[1]) and not significantly different than the oxytetracycline standard. Formulation PSU2 also provided significant control (42%) in a previous trial in 2023. New formulations PS4,5,6 were not better than PSU2.
- Alternative *bacillus* products performed as well as Serenade Aso but require a second year of testing.
- No treatments resulted in commercially important fruit marking of 3 or greater.

Objective 2

- Multiple years of data collection are required to draw conclusions on this objective.

METHODS

Site: For experiment 1 a 1.5-acre research block sixth leaf ‘WA 38’ apple trees at the WSU Columbia View Orchard 48 Longview Rd. East Wenatchee, WA 98802-8283 was used. For experiment 2 a 0.25 ha research block of 4-yr-old Gala apples was used for this trial. Soils are a Cashmont Gravely Sandy Loam with a 3-8% slope. The site has good air drainage and some wind protection.

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

Experimental design: Five blocks of trees were designated in each location. Individual trees were marked as plots in a randomized complete block where suitable trees were selected based on sufficient bloom (100+ flower clusters).

Treatments: Experimental treatments were compared to FireLine 45 WP (oxytetracycline) and FireWall 50 WP (streptomycin sulfate) as a comparison and as "treated controls". An untreated-inoculated control treatment (treated with water) and an untreated non-inoculated control treatment were included. Products were applied by tree to the area of the tree to be inoculated (whole tree) according to manufacturer recommendations using a Stihl SR420 mist blower backpack sprayer with a wetting agent. Products were applied to wet, near dripping previously calibrated to equal 100 gal/A (approx. 0.1 to 0.2 gal/tree). Application dates for experiment 1 (Wa38) were: 10 Apr (1), 15 Apr (2), 17 Apr (3), 18 Apr (4, full bloom), 19 Apr (5), 20 Apr (6), 21 Apr (7), 22 Apr (8, petal fall), 30 Apr (9), 6 May (10). Application dates were: 15 Apr (1), 17 Apr (2), 20 Apr (3, full bloom), 21 Apr (4), 24 Apr (5, petal fall), 27 Apr (6). At 80-90% bloom (of the king blooms).

Inoculation: At 90-100% bloom (of the king blooms), on 18 Apr 2024 (WA38 trial) and 20 April (Gala trial), *Erwinia amylovora* strain 153N (streptomycin and oxytetracycline sensitive and nalidixic acid resistant strain) was applied at $5x10^6$ CFU ml^{-1} (verified at $6.4x10^6$ CFU ml^{-1}) to lightly wet each cluster using freeze dried inoculum. A 4-gallon backpack sprayer (solo) was used to lightly wet clusters. Whole trees (100+ flower clusters) were inoculated.

Evaluation and statistical analysis: Trees were visually evaluated for flower cluster infection from when symptoms became visible, 22 days after inoculation, for 3 weeks and infection counts summed across all dates. Fruit were evaluated for fruit skin marking from an average of 25 fruit per tree on a 0 to 15 scale, where ratings below 3 indicate no commercial downgrades. A qualitative simplified scale (clean, slight marking, moderate and severe marking) is added to provide clear results to stakeholders.

For some treatments, pathogen populations on flowers were also evaluated. Ten flowers from five flower clusters were bulk sampled from each replicate tree on three dates corresponding to full bloom + 1 day, petal fall and petal fall + 4 days. Each sample was immersed in sterile phosphate buffer and sonicated for 3 min. After sonication, appropriate dilutions were spread on nutrient agar medium amended with cycloheximide (50 µg ml^{-1}) to inhibit contaminating fungi and nalidixic acid (50 µg ml^{-1}) to specifically detect our strain; detection limit is 10^2 CFU/flower. Colonies were counted after 3 days.

Statistical analysis was performed with SAS v 9.4 using general linear mixed models (GLIMMIX) analysis of variance ANOVA and multiple means comparison (LSD) for infections (normal distribution of sqrt(x + 1) transformed for WA38 trial and normal distribution Gala trial).

Environmental variables (e.g. temperature, RH, moisture, etc.) were monitored using the Ag Weathernet site at the location.

RESULTS AND DISCUSSION

Environmental conditions during bloom (10 Apr – 22 Apr 2024) were cool and ranged from a maximum average temperature of 66.4 °F to minimum average temperature of 39.9 °F with 38.6% average humidity. During petal fall (23 Apr – 6 May 2023) temperature ranged from an average maximum of 65.5 °F to a minimum of 41.7 °F with 49.5% average humidity. Two precipitation events occurred after the inoculation of *Erwinia amylovora*, one on 25 Apr (0.45 in), approximately 3 days after petal fall sprays, and on 4 May, 4 days after the petal fall + 7 day sprays (1.8 in) (Appendix 1 and 2). All applications were made under fast drying conditions.

Experiment1 WA 38: Of biological products, Blossom Protect, the high dose of GWN-10474, and two of the PSU products resulted in 7.5, 7.2, and 6.7 and 6.7 infections per 100 clusters respectively (40 to 47% relative control[2]), all significantly different than the water-treated control and not significantly different than the oxytetracycline standard. Serenade ASO, Stargus, the other two PSU products, YSY, the three UW strains, and the medium dose of GWN-10474 were not significantly different than the water-treated control. Of plant extracts, the high dose of Problad Verde and the low dose of Reckoning resulted in 4.5 and 8.4 infections per 100 clusters respectively (65 and 34% relative control), both comparable to the oxytetracycline standard and significantly different than the water-treated control. The high dose of Reckoning and the low dose of Problad Verde were not significantly different than the water-treated control. The organic acid Dart applied alone was not different than the water-treated control, but when applied following Blossom Protect, the program resulted in 4.2 infections per 100 clusters (67% relative control), significantly better than the water-treated control and comparable to the oxytetracycline standard. The streptomycin standard had significantly fewer infections than the water-treated control and all the treatments tested, with 1.1 infections per 100 clusters (91% relative control). No treatments resulted in commercially important fruit marking of 3 or greater (Table 1, Appendix 3). Reckoning, Problad Verde, and Dart trials currently with only one year's data will need another year's data to confirm if results are consistent in a warm year.

Table 1. Biologicals, mineral based biopesticides, plant extracts, and organic acid treatments for control of fire blight of apple (WA 38)

Treatment	Amount per 100 gal	Timing [z]	Infections per 100 clusters [y]		Fruit skin marking [x]
Streptomycin standard (Firewall 50WP) [w]	8 oz	4,7	1.1 ± 0.4	a[v]	0.05 ± 0.03
Aureobasidium pullulans (Blossom Protect) + citric acid and calcium carbonate (Buffer Protect),	1.25 lb + 5 lb	2,3,6			
Caprylic acid (41.7%), capric acid (28.3%) (Dart) [t]	1 qt	9,10	4.2 ± 0.8	b	0.31 ± 0.09
Banda de Lupinus albus doce (20%) (Problad Verde) [s]	90 fl oz	4,5,8	4.5 ± 0.9	b	0.02 ± 0.02
Oxytetracycline standard (Fireline 45WP) [w]	9 oz	4,7	5.4 ± 2.0	bc	0.1 ± 0.05
PSU2	2 x 10^{10} CFU/ml	2,3,6	6.7 ± 1.9	bcd	0.02 ± 0.01
PSU5/PSU6	10^{10} CFU/ml	2,3,6	6.8 ± 1.7	bcde	0.03 ± 0.02
Bacillus amyloliquefaciens (GWN-10474) [s]	30 oz	3,5,8	7.2 ± 1.4	bcde	0.01 ± 0.01
Aureobasidium pullulans (Blossom Protect) + citric acid and calcium carbonate (Buffer Protect)	1.25 lb + 5 lb	2,3,6	7.5 ± 2.6	bcde	0.06 ± 0.03
PSU4	2 x 10^{10} CFU/ml	2,3,6	7.8 ± 1.0	bcdef	0.02 ± 0.02
Papiliotrema terrestris (AgroventuresY)	1.25 lb	2,3,6	7.9 ± 1.2	bcdef	0.1 ± 0.07
Thyme oil (2%) (R) [r]	44 fl oz	4,5,8	8.4 ± 3.5	bcde	0.06 ± 0.03
Bacillus amyloliquefaciens (GWN-10474) [s]	20 oz	3,5,8	8.6 ± 2.4	bcdefg	0.04 ± 0.02
Endophyte (UW83)	378.5 ml	1,3,5,8	9.1 ± 1.9	cdefg	0.08 ± 0.04
Thyme oil (2%) (R) [r]	64 fl oz	4,5,8	10.7 ± 3.2	defg	0.12 ± 0.04

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

Bacillus amyloliquefaciens (Stargus) [s]	2 qt	1,2,4,8, 9	10.8	±	1.7	defg	0.1 ± 0.05
PSU5	10^9 CFU/ml	2,3,6	11.0	±	1.4	defg	0.14 ± 0.04
Banda de Lupinus albus doce (Problad Verde) [s]	45 fl oz	4,5,8	11.0	±	4.4	defg	0.04 ± 0.03
Endophyte (UW90)	378.5 ml	1,3,5,8	11.3	±	3.2	defg	0.05 ± 0.04
Caprylic acid (41.7%), capric acid (28.3%) (D) [t]	1 qt	4,5,8	11.5	±	2.0	efg	0.24 ± 0.08
Endophyte (UW58)	378.5 ml	1,3,5,8	11.7	±	3.3	defg	0.06 ± 0.02
Bacillus subtilis (Serenade ASO) [s]	96 fl oz	1,2,4,8, 9	12.3	±	4.3	efg	0.09 ± 0.06
Water-treated control	NA	4,5,8	12.8	±	2.1	fg	0 ± 0
Papiliotrema terrestris (AgroventuresY) + activator	1.25 lb + 0.3 lb	2,3,6	13.2	±	3.1	fg	0.03 ± 0.01
Bacillus amyloliquefaciens (GWN-10474) [s]	10 oz	3,5,8	13.8	±	3.3	g	0.04 ± 0.03

[z] Timings, 1: 10 Apr (tight cluster), 2: 15 Apr (70-90% bloom), 3: 17 Apr, 4: 18 Apr (full bloom), 5: 19 Apr, 6: 20 Apr, 7: 21 Apr, 8: 22 Apr (petal fall), 9: 30 Apr, 10: 6 May. All applications were conducted in the morning, and inoculation was conducted on the evening of 18 Apr.

[y] Transformed sqrt(x + 1) prior to analysis of variance; non-transformed means are shown.

[x] Fruit skin marking is rated from an average of 25 fruit per tree. Rated on a 0 to 15 scale where ratings below 3 indicate no commercial downgrades. All fruit were clear.

[w] Amended with Regulaid: 16 fl oz per 100 gallons. Buffered to 5.6 pH.

[v] Treatments followed by the same letter are not significantly different at P=0.05 Fisher's T test (LSD).

[u] Treatments followed by the same letter are not significantly different at P=0.05 Fisher's T test (LSD).

[t] Amended with Xena Spreader-Sticker: 4 fl oz per 100 gallons. Buffered to 6.0 pH.

[s] Amended with NuFilm P: 16 fl oz per 100 gallons.

[r] Amended with Regulaid: 16 fl oz per 100 gallons.

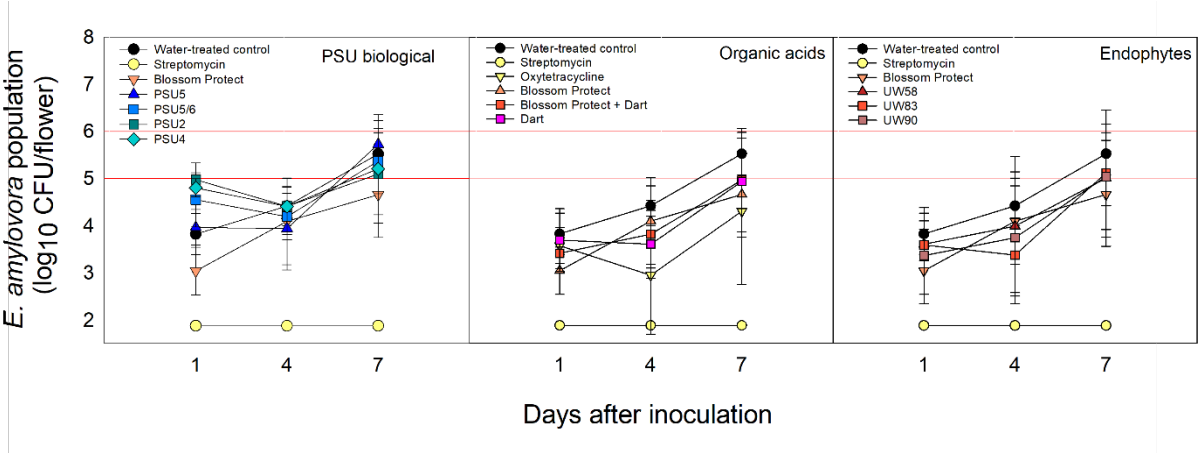


Figure 1. Effect of PSU biological, UW endophytes, and organic acid treatments applied to WA 38 apple trees on the population size of *Erwinia amylovora* strain 153N on flowers 1 day after inoculation (full bloom + 1 day), at petal fall (full bloom + 4 days) and 3 days post petal fall (full bloom + 7 days).

Experiment2 Gala: Of biological products (Blossom Protect, Serenade ASO, Agriphage), Blossom Protect and Agriphage had significantly fewer infections compared to the water-treated control (42.4% and 41.8% relative control[3]). The mineral-based product Alum significantly reduced infections compared to the water-treated control, with 11.3 infections per 100 clusters (70.3% relative control). Plant extracts, Cinnerate and Thyme Guard with three applications resulted in 33.5 and 23.8 infections per 100 clusters (11.9% and 37.4% relative control) not significantly different than the water-treated control. The peracetic acid and hydrogen peroxide treatments (Oxidate 5.0) applied the morning before evening inoculation, the day after inoculation, and at petal fall had a slight numerical reduction of blossom infections to 27.0 infections per 100 clusters, which was not significantly different from the water-treated control. However, Oxidate 5.0 applied the day after inoculation, at petal fall, and 3 days after petal fall showed a significant reduction in infections (21.9 infections per 100 clusters, 42% relative control) compared to the water-treated control. The antibiotic streptomycin significantly reduced infections (5.7 infections per 100 clusters, 81.5% relative control) compared to the water-treated control. No treatments resulted in commercially important fruit marking of 3 or greater (Table 2).

Consideration of product relative efficacy should include multiple trials over multiple years. In comparison across 11 trials conducted between 2022 and 2023 Alum and Blossom Protect + Buffer have had the highest control (of the above suite of products) with moderate efficacy from essential oils and peracedic acid products (WA,CA, NY, MI, NC 2022-2023 SCRI, unpublished). These trends were comparable to a recent review of antibiotic alternatives (DuPont et al. 2024).

Table 2. Treatments for control of fire blight of apple (Gala)

Treatment	Amount per 100 gal	Timing[z]	Infections per 100 clusters			Fruit skin marking[y]	
Streptomycin standard (Firewall 50WP)[x]	8 oz	3,4,5	5.7	± 1.3	a[w]	0.18 ± 0.05	a[v]
Alum[s]	8 lb	3,4,5	11.3	± 1.9	ab	0.21 ± 0.04	a
Blossom Protect + Buffer Protect	1.25 lb + 5 lb	1,3	21.9	± 2.5	abc	0.17 ± 0.04	a
Oxidate 5.0	128 fl oz	4,5,6	21.9	± 2.8	abc	0.75 ± 0.04	a
Agriphage[r]	2 qt	3,4,5	22.1	± 2.3	abc	0.19 ± 0.06	a
Thyme Guard[u]	2 qt	3,4,5	23.8	± 2.5	bcd	0.6 ± 0.14	a
Oxidate 5.0	128 fl oz	3,4,5	27.0	± 4.5	bcd	0.59 ± 0.07	a
Serenade ASO[t]	96 fl oz	3,4,5	31.3	± 6.5	cd	0.1 ± 0.05	a
Cinnerate	32 fl oz	3,4,5	33.5	± 8.0	cd	0.13 ± 0.03	a
Water-treated control	NA	3,4,5	38.0	± 2.9	d	0.03 ± 0.01	a

[z] Timings 1: 15 Apr, 2: 17 Apr, 3: 20 Apr (full bloom), 4: 21 Apr, 5: 24 Apr (petal fall), 6: 27 Apr. All applications were conducted in the morning, and inoculation was conducted on the evening of 20 Apr.
[y] Fruit skin marking is rated from an average of 25 fruit per tree. Rated on a 0 to 15 scale where ratings below 3 indicate no commercial downgrades.
[x] Amended with Regulaid: 16 fl oz per 100 gallons. Buffered to 5.6 pH.
[w] Treatments followed by the same letter are not significantly different at P=0.05 Tukey Honest Significant Differences test.
[v] Treatments followed by the same letter are not significantly different at P=0.05 Fisher's T test (LSD).
[u] Acidified to pH 4.
[t] Amended with NuFilm P: 16 fl oz per 100 gallons.
[s] Amended with Regulaid: 16 fl oz per 100 gallons.
[r] Amended with sodium thiosulfate to inactivate chlorine contents in the water.

Oxidate 5.0 applied at both timing combinations, the mineral-based product Alum, and streptomycin significantly reduced *Erwinia amylovora* populations 1 day after inoculation. Alum and streptomycin

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

also showed significant differences compared to the water-treated control at 4 and 7 days after inoculation (Fig. 2).

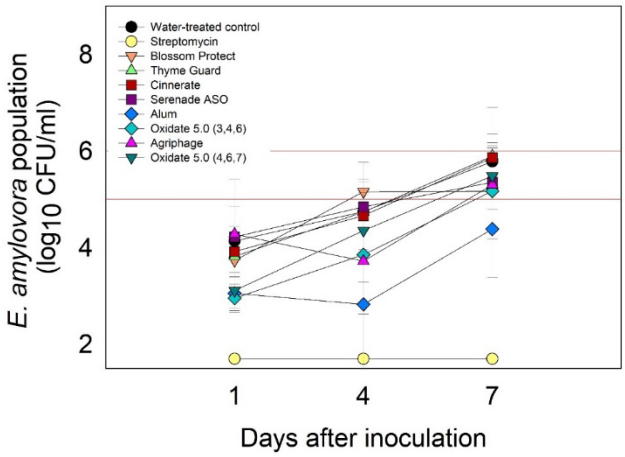


Figure 2. Effect of the treatments applied to Gala apple trees on the population size of *Erwinia amylovora* strain 153N on flowers 1 day after inoculation (full bloom + 1 day), at petal fall (full bloom + 4 days) and 3 days post petal fall (full bloom + 7 days).

Objective 3: Provide research-based recommendations to growers through web-based platforms (Crop Protection Guide, Decision Aid System), news alerts, field days and consultations.

In 2024 Extension of fire blight management best management practices included:

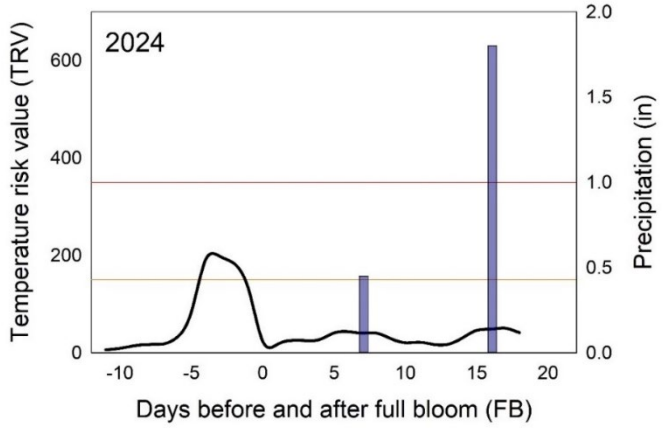
- A field day in June 2024 focused on removal strategies for fire blight in season (20 participants)
- A fire blight webinar series (SCRI funded) including webinars April 4, April 18 and November 6.
- Completion of a new fire blight animation tutorial explaining the life cycle of fire blight.
- Presentations to industry groups Mar 18, Feb 29, Feb 15, Feb 14, Feb 12, Feb 7, Jan 25, Jan 16 (762 participants total).
- Updates to the Tree Fruit Crop Protection Guide including addition of prohexodine calcium to reduce fire blight severity.

Appendix 1. Environmental conditions during bloom.

Date	Min Air Temp (°F)	Max Air Temp (°F)	Humidity (%)	Leaf wetness (u)	Precipitation (in)	Wind speed (mph)	Total Solar Radiation (MJ/m²)
4/10/2024	35.6	63.4	41.3	0	0	3.5	19.42
4/11/2024	39.6	61.6	52.9	0	0	2.9	12.63
4/12/2024	43.1	70.4	48.7	0	0	3.2	18.36
4/13/2024	42.1	75.1	54.4	0.02	0	3.2	21.36
4/14/2024	46.1	80.6	42	0	0	4	21.93
4/15/2024	47.9	66.5	29.1	0	0	6.2	20.16

4/16/2024	40.4	58.4	29.8	0	0	5.3	23.18
4/17/2024	34.8	62.9	33.7	0	0	4.3	21.35
4/18/2024	39.3	63.6	30.5	0	0	5.2	23.47
4/19/2024	38.2	63	36	0	0	5.1	19.96
4/20/2024	35.1	69.3	34.8	0	0	4.1	21.19
4/21/2024	41.6	62.8	34.1	0	0	4.6	17.47
4/22/2024	35.3	66.2	35	0	0	3.6	24.42
4/23/2024	38.3	70.2	35.1	0	0	3.3	20.62
4/24/2024	43.7	68.9	42.4	0	0	3.4	17.01
4/25/2024	44.2	55.1	75.1	0.17	0.45	3.2	8.08
4/26/2024	47.4	67.6	66.7	0.15	0	2.9	16.6
4/27/2024	42.3	64.6	54.9	0.02	0	4.1	15.25
4/28/2024	42.9	65.6	53.3	0	0	4.3	14.63
4/29/2024	37.3	60.3	38.8	0	0	5.2	22.33
4/30/2024	41.1	61.6	39.5	0	0	3.5	20.8
5/1/2024	36.2	66.2	40.5	0	0	3.8	23.9
5/2/2024	34.8	71.4	38.7	0	0	2.9	21.19
5/3/2024	39.8	70.4	37.7	0	0	4.6	22.48
5/4/2024	48.3	61.1	65.4	0.33	1.8	5.1	4.7
5/5/2024	44.1	67.5	62.9	0.28	0	4.5	13.32
5/6/2024	43.6	66.2	41.4	0	0	4.4	24.15

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



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DuPont, S. T., Cox, K., Johnson, K., Peter, K., Smith, T., Munir, M., and Baro, A. 2024. Evaluation of biopesticides for the control of Erwinia amylovora in apple and pear. Journal of Plant Pathology 106:889-901.